THE ACTIONS OF THYROXINE ON THE UTERUS DURING PERI-IMPLANTATION AND IMPLANTATION PERIODS IN RATS

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ABSTRACT

Thyroid hormone plays important role in modulating the uterine reproductive events. However, the mechanisms underlying the action of this hormone in the uterus particularly during embryo implantation have not been fully identified. In order to understand these, in this study, effects of thyroxine on the uterus during peri-implantation and implantation periods were investigated. These include identifying changes in the uterine expression of the receptors related to thyroid hormone functions i.e. thyroid hormone receptor (TR-α and β), thyroid stimulating hormone receptor (TSHR), retinoic acid receptor (RAR), retinoic X receptor (RXR), vitamin D receptor (VDR) and extracellular signal-regulated kinase (ERK1/2). Further, changes in expression of these proteins under the influence of sex-steroids were also identified. In addition, effects of thyroxine on expression of aquaporins (AQP) protein which is important in uterine fluid homeostasis and the expression of the proteins related to uterine receptivity development such as integrin αvβ3, E-cadherin, Msx-1 and Ihh in the uterus during peri-implantation periods were analyzed. Studies were conducted using two models: (i) methimazole-induced hypothyroid pregnant rats treated with different doses of thyroxine (20, 40 and 80 μg/kg/day) and (ii) rats under different sex-steroid influence (both ovariectomised, sex-steroid replaced and intact at different phases of the oestrous cycle). Finally, the effects of thyroxine treatment on embryo implantation rate were determined. **Results:** In model (i), expression of TRα-1, TRβ-1, RXR and ERK1/2 proteins in the uterus during peri-implantation period increased with increasing doses of thyroxine. These proteins were found to be distributed in the stroma at a relatively higher amount following thyroxine treatment. Meanwhile, thyroxine treatment causes increased in expression of TRα-1, TRβ-1, TSHR, RAR and ERK1/2 proteins and mRNAs in the uterus during implantation period. Peri-implantation uterus had its luminal size decrease following treatment with thyroxine with an associated decrease in

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expression of AQP-1, 5 and 7. Thyroxine treatment increases expression of the uterine receptivity proteins i.e. integrin αvβ3, E-cadherin, Msx-1 and Ihh during peri-implantation period. The numbers of embryo implantation were also found to increase by thyroxine. In the meantime, treatment of ovariectomised rats with estradiol (E2) increases the expression of TRα-1, TRβ-1, TSHR, VDR, RAR and ERK1/2 with expression of these proteins were increased at estrus phase of the oestrous cycle, under E2 dominance. These proteins were found to be distributed in the uterine luminal and glandular epithelia and stroma. Following progesterone (P) treatment and at diestrus phases of the oestrous cycle (under P dominance), expression of these proteins was lesser as compared to under E2 influence and were found to be distributed only in the uterine stroma. **Conclusions:** The changes as documented above could explain the important role of thyroxine on the uterus during peri-implantation and implantation periods as well as help to explain the role of sex-steroids in regulating thyroxine function in the uterus. Dysregulations in these parameters could potentially lead to infertility.

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ABSTRAK

Hormon tiroid memainkan peranan penting dalam memodulasi kejadian yang berkait dengan pembiakan di dalam rahim. Namun, mekanisme yang mendasari tindakan hormon ini di dalam rahim terutama semasa implantasi embrio belum sepenuhnya dikenalpasti. Untuk memahami hal ini, tindakan hormon tiroksin pada rahim semasa tempoh peri-implantasi dan implantasi, diselidiki. Ini termasuklah mengenalpasti perubahan ekspresi reseptor yang berkaitan dengan fungsi hormon tiroid termasuklan reseptor hormon tiroid (TR-α dan β), reseptor hormon tiroid (TSHR), reseptor retinoat asid (RAR), reseptor retinoik X (RXR) reseptor vitamin D (VDR) dan kinase pengatur siknal ekstraselular (ERK1 / 2) di dalam rahim. Selanjutnya, perubahan ekspresi protein ini di bawah pengaruh seks steroid juga diidentifikasikan. Selain itu, tindakan tiroksin pada ekspresi protein aquaporins (AQP) yang penting di dalam homeostasis bendalir rahim dan ekspresi protein yang terlibat dengan reseptiviti uterus seperti integrin αvβ3, E-cadherin, Msx-1 dan Ihh semasa tempoh peri-implantasi turut dianalisis. Penelitian dilakukan dengan menggunakan dua model: (i) tikus hamil hipotiroid yang diinduksi menggunakan methimazole dan diikuti dengan pengubatan menggunakan dos tiroksin yang berbeza (20, 40 dan 80 μg / kg / hari) dan (ii) tikus dibawah pengaruh steroid seks yang berbeza (di ovariektomi, – diikuti dengan penggantaian seks steroid) serta tikus yang utuh pada fase oestrous yang berbeza. Akhirnya, tindakan tiroksin pada implantasi embrio juga ditentukan. Hasil penemuan: Pada model (i), ekspresi protein TRα-1, TRβ-1, RXR dan ERK1 / 2 di dalam rahim selama tempoh implantasi meningkat dengan peningkatan dos tiroksin. Protein ini didapati di stroma pada jumlah yang secara relatif lebih tinggi setelah pengubatan dengan tiroksin. Sementara itu, pengubatan tiroksin turut menyebabkan peningkatan ekspresi protein TRα-1, TRβ-1, TSHR, RAR dan ERK1 / 2 dan mRNA di dalam rahim selama implantasi embrio. Ukuran luminal rahim peri implantasi turun setelah pengubatan dengan tiroksin beserta dengan penurunan

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ekspresi AQP-1, 5 dan 7. Pengubatan tiroksin turut meningkatkan ekspresi protein reseptor di dalam rahim uterine termasuklah intergrin αvβ3, E-cadherin, Msx-1 dan Ihh. semasa tempoh implantasi. Jumlah implantasi embrio juga didapati meningkat berikutan pengubatan tiroksin. Sementara itu, pengubatan tikus ovariektomi dengan estradiol (E2) meningkatkan ekspresi TRα-1, TRβ-1, TSHR, VDR, RAR dan ERK1/2 serta ekspresi protein-protein ini meningkat pada fase estrus kitar oestrous, di bawah dominasi E2 Protein ini didapati di epitelia lumen dan glandular serta stroma. Setelah perawatan dengan progesteron (P) dan semasa fasa diestrus kitar oestrous (di bawah dominasi P), ekspresi protein ini lebih rendah dibandingkan dengan pengaruh E2 dan hanya didapati didistribusi pada stroma sahaja. Kesimpulan: Perubahan seperti yang didokumentasi di atas dapat menjelaskan peranan penting tiroksin didalam rahim selama tempoh peri-implantasi dan implantasi dan juga membantu menjelaskan peranan seks steroid di dalam mengatur fungsi tiroksin di dalam rahim. Penyalahaturan parameter-parameter ini berpotensi menyebabkan kemandulan.

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LIST OF SYMBOLS AND ABBREVIATIONS

ACTH	:	Adrenocorticotropic Hormone
APS	:	Ammonium Persulfate
AQP	:	Aquaporin
ATP	:	Adenosine-triphosphate
BSA	:	Bovine Serum Albumin
cAMP	:	Cylic AMP
cDNA	:	Complementary DNA
CG	:	Chorionic gonadotrophins
DAB	:	Diaminobenzidine
ddH_2O	:	Double-distilled water
dH_2O	:	Distilled water
DNA	:	Deoxyribonucleic acid
E2	:	17β-estradiol
EGF	:	Epidermal Growth Factor
ELISA	:	Enzyme-linked immunosorbent assay
ENaC	:	Epithelial Sodium Channel
ER	:	Estrogen receptor
FGF	:	Fibroblast growth f ctors
FSH	:	Follicle-stimulating hormone
GAPDH	:	Glyceral ehyde-3-Phosphate Dehydrogenase
GDP	:	Guinine Diphosphate
GnRH	:	Gonadotrophin-releasing hormone
GPCR	:	G-protein coupled receptor
GTP	:	Guinine trophosphate

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H&E	:	Hematoxylin and eosin
HRP	:	Horseradish peroxidase
IF	:	Immunofluorescence
IGF	:	Insulin-like growth factor
IHC	:	Immunohistochemistry
IP	:	Intraperitoneal
IVF	:	In-vitro Fertilization
LH	:	Luteinizing hormone
LIF	:	Leukaemia Inhibitory Factor
mRNA	:	Messenger RNA
Muc-1	:	Mucin-1
P	:	Progesterone
PBS	:	Phosphate-buffered saline
PBST	:	Phosphate-buffered saline/Tween
PG	:	Prostaglandins
PR	:	Progesterone receptor
PVDF	:	Polyvinylidene diflouride
qPCR	:	Quantitative real-time PCR
RNA	:	Ribonucleic acid
SDS	:	Sodium dodecyl sulfate
SDS-PAGE	:	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHBG	:	Sex hormone-binding globulin
T4	:	Thyroxine
TEMED	:	Tetramethylethylenediamine
TR	:	Thyroid Hormone Receptor
TSHR	:	Thyroid Stimulating Hormone Receptor

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CHAPTER 1: INTRODUCTION

1.1 Research Background

Uterus is a highly dynamic tissue that is controlled by the sex-steroids levels changes throughout the female reproductive cycle. Estradiol (E2) and progesterone (P) are the primary sex-steroid hormones that are involved in the cyclical changes in endometrial morphology and function during the female reproductive cycle. Morphological changes include increased proliferation under the influence of E2 (Knobil, 2013). In humans, thickness of the endometrial luminal and glandular epithelia reaches the maximum at mid-cycle (Knobil, 2013). Similarly, in rodents, thickness of this layer increases prior to ovulation (Westwood, 2008) and is maintained following ovulation (Senturk & Erel, 2008). In addition endometrial stroma was also reported to change throughout the female reproductive cycle under the influence of sex-steroids. In humans and primates, endometrium undergoes shedding at menstruation, followed by glands and basal layer proliferation.

Apart from the sex-steroids, other hormones and paracrine factors are also reported to influence endometrial morphology and functions. This include the thyroid hormone (TH) (Choksi *et al.*, 2003; Poppe Kris & Brigitte Velkeniers, 2004). The important role TH can be seen in a condition associated with deficiency in TH which results in menstrual irregularities (Poppe & Velkeniers, 2002). Besides its effect on the uterus, TH has also been found to influence the secretion of gonadotropin-releasing hormone (GnRH) and the synthesis of sex steroid-binding globulin (SSBG) (Krassas *et al.*, 2010). TH actions are known to be mediated by thyroxine (T4) and triiodothyronine (T3), which are the two biologically active form of TH where the latter is formed from the conversion of T4 in the peripheral tissues (Bianco, 2013). T4 and T3 bind to thyroid hormone receptors (TR) which exist as isoforms: TRα and TRβ (Flamant *et al.*, 2006). TRs form heterodimers with nuclear retinoic acid receptors (RARs) to interact with

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specific DNA sequences, denoted as thyroid hormone responsive elements (TREs), which then activate gene expression and proteins synthesis (Lee S. & Privalsky, 2005). TH also transduces signals at downstream via activating extracellular signalling kinase (ERK1/2) or MAPK intracellular signaling cascade (Davis *et al.*, 2000). ERK1/2 signaling in the uterus is important for multiple endometrial events (Klemmt *et al.*, 2006; Velarde *et al.*, 2009). Studies have demonstrated a potential link between decreased level of ERK1/2 signaling protein and endometriosis (Mormile & Vittori, 2013; Velarde *et al.*, 2009; Yotova *et al.*, 2011).

The synthesis of TH is controlled by thyroid stimulating hormone (TSH), which is also found to play a crucial role in uterus (Mizukami *et al.*, 1994). TSH has been found to be involved in uterine glucose transport, as study has shown that TSH administration up-regulated the expression of *Glut-1* mRNA in both the human endometrial stromal cells and Ishikawa (uterine adenocarcinoma) cells *in-vitro* (Aghajanova L. *et al.*, 2011). TSH acts via binding to a membrane-bound thyroid stimulating hormone receptor (TSHR) (Davies *et al.*, 2010; Mizukami *et al.*, 1994). It was reported that TR and TSHR are expressed in human and rodent uterus (Aghajanova L. *et al.*, 2011; Oner & Oner, 2007). Uterine expression of TR and TSHR was found to be influence by sex-steroids. This was supported by studies which documented that chronic administration of conjugated equine estrogen and medroxyprogesterone acetate to ovariectomised macaques resulted in up-regulation of TR and TSHR levels in the uterine compartments (Hulchiy Mariana *et al.*, 2012).

In addition, retinoic acid (RA) is crucial to maintain uterine functions (Grenier *et al.*, 2007). Being a lipophilic metabolite in terms of Vitamin A, RA acts via binding to retinoic acid receptor (RAR) or retinoic X receptor (RXR) isomers which are nuclear receptors (Chambon, 1996). The expression of RAR has been found in the uterine

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stroma of mice (Nakajima *et al.*, 2016), uterine epithelium of rats (Boehm *et al.*, 1997) and in human endometrium (Kumarendran *et al.*, 1996).Vitamin D has also been found to play a significant role in multiple uterine functions for example in regulating uterine smooth muscle contraction (Thota *et al.*, 2014) and uterine cell proliferation (Yoshizawa *et al.*, 1997). The receptor for Vitamin D (VDR) was reported to be expressed in the uterus in the follicular and luteal phases of the cycle in buffalos, which indicated the influence of sex-steroids on uterine VDR expression (Emam M. A. *et al.*, 2016).

TH plays a crucial role during early pregnancy period i.e. peri-implantation and implantation periods. Successful implantation is the outcome of the reciprocal interactions between competent blastocyst and the receptive endometrium (Altmae *et al.*, 2012; Franchi *et al.*, 2008). The free-floating blastocyst interacts with the receptive endometrium through the trophoblast cell layer and then becomes intimately attached to the endometrial tissue and later invades the stroma (Kaneko *et al.*, 2013). Implantation depends on an organized cellular and molecular interaction between the blastocyst and the receptive endometrium. These involve spatiotemporally regulated endocrine, paracrine and juxtacrine modulators (Sharkey & Macklon, 2013; Sharkey & Smith, 2003). Implantation begins with apposition, continues through attachment and ends with invasion of the blastocyst into the stroma of the endometrium.

The embryo with its own molecular processes regulating cellular growth and differentiation interacts with the uterus during a specific period known as 'implantation window' period. In humans, implantation occur in the mid-secretory phase between day 20 to 24 (Degrelle *et al.*, 2013), while in mice, implantation occurs at day 4.5 (Loubiere *et al.*, 2012). This implantation window period is under control by sex-steroids (Sharkey & Smith, 2003). Additionally, TH has also been found to influence the implantation-

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related events (Ashkar *et al.*, 2010). It was reported that *in-vitro* TH supplementation could help increases the hatching rate of bovine blastocysts (Costa *et al.*, 2013). It has also been demonstrated that low level of TH could cause implantation failure that leads to infertility (Priya *et al.*, 2015).

The phase prior to implantation that is refractory to blastocyst adhesion is known as pre-implantation period (Dey *et al.*, 2004; Sharkey & Smith, 2003). During this transient period, endometrium undergoes transformation to a receptive state by acquiring morphological and functional changes under the influence of sex-steroids (Makker & Singh, 2006). In humans, the importance of sex-steroids in controlling uterine receptivity development can be seen from studies on women with ovarian failure who underwent oocyte IVF where exogenous E2 and P administration were sufficient to induce a receptive endometrium (de Ziegler *et al.*, 1998). Disturbances in the regulation and development of uterine receptivity can be adversely affected in condition such as overproduction of endogenous androgens in females in polycystic ovarian syndrome (PCOS) (Giudice, 2006).There are several molecules that are exclusively expressed during the peri-implantation period. Expressions of these molecules indicate transformation of the uterus into the receptive state. Among the well-established molecules include integrin αvβ3, E-cadherin, msx-1 and Ihh (Zhang S. *et al.*, 2013). Integrin αvβ3 is one of the uterine receptivity molecules and is expressed in the epithelium at the time of implantation under the influence of progesterone (Dekel *et al.*, 2014). E-cadherin mediates the compaction process of pre-implantation embryos and is important for the maintenance and function of epithelial cell layer during receptivity (Chakravarty, 2014; Sayem A. S. M. *et al.*, 2017). Besides, several intracellular proteins including muscle segment (msx) and Indian hedgehog (ihh) are also crucial for the development of endometrial receptivity (Nallasamy *et al.*, 2012). The effect of TH on uterine receptivity development however has not been fully elucidated.

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During the peri-implantation period, several changes also occur in the uterus, which include a reduction in the uterine fluid volume. Changes in uterine fluid volume occurs due to the movement of H_2O between different uterine compartments (Sharkey & Smith, 2003) via the water channels, Aquaporins (AQPs) (Sharkey & Macklon, 2013). AQPs are porous water channels within the cell membranes which selectively allows movements of water while restricting movements of ions (Verkman, 2005). Expression of AQPs in uterus is regulated by sex-steroids (Huang H.-F. *et al.*, 2006; Lindsay Laura A & Christopher R Murphy, 2006). AQP-1 is reported to be expressed in the uterine vasculature and uterine smooth muscles under the influence of E2 and E2 and/or P influence (Lindsay Laura A & Christopher R Murphy, 2006). AQP-5 is reported to be expressed in the peri-implantation uterus and involved in implantation process, as the isoform was redistributed to the apical membrane of the luminal and glandular epithelia under influence of sex-steroids (Lindsay Laura A & Christopher R Murphy, 2007). In addition, AQP-7, a non-selective H_2O channel which mediates the transport of H_2O, glycerol, urea and other small non-electrolytes was reported to be expressed in the uterus (Laforenza *et al.*, 2016). Recently, expression of AQP-7 in the uterus was found to be influenced by testosterone (Salleh Naguib, Helmy Mohd Mokhtar, Normadiah M. Kassim, *et al.*, 2015). The effect of TH on fluid regulation during the peri-implantation period has never been identified.

As mentioned, TH plays a critical role in successful implantation processes. In hypothyroidism, establishment of pregnancy is impaired which contributes towards female infertility. A study has documented that hypothyroidism was prevalent in 53.7% of infertile females, and thyroxine treatment in has resulted in pregnancy within 6 weeks to 2-year period (Priya *et al.*, 2015). Although clinical associations between the complications of pregnancy and thyroid disorders have been extensively reported

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(Granfors *et al.*, 2013; Kilic *et al.*, 2008; Yalamanchi & Cooper, 2015) molecular mechanisms underlying these are widely unknown.

1.2 Hypothesis

Deficiency of TH could affect expression of the proteins (TR-α, TR-β, TSHR, RXR/RAR and ERK1/2) in uterus related to TH action, water channel (AQP), receptivity molecules (Integrin αvβ3, E-cadherin, Msx-1 and Ihh) and ultimately embryo implantation. We further hypothesized that thyroxine treatment could restore the mechanisms of TH action in the uterus during early pregnancy period.

Alongside of TH action in uterus, it is also hypothesized that sex-steroids could affect the expression of the proteins (TR-α, TR-β, TSHR, RAR, VDR and ERK1/2) in uterus related to thyroid action which could provide a fundamental understanding on the action of sex-steroids in relation to thyroid hormone function in uterus during the early pregnancy period.

1.3 Research Objectives

The objectives of this study are to:

- Investigate the effects of thyroxine on expression of proteins related to thyroid hormone functions (TR-α, TR-β, RXR and ERK1/2) in uterus during peri-implantation period.
- Investigate the effects of hypothyroidism and thyroxine treatment on thyroid hormone receptor, thyroid stimulating hormone receptor, retinoic acid receptor and extracellular kinase signaling protein expression in the uterus at the day of embryo implantation in rats.

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- ❖ Investigate the effects of thyroid hormone on uterine fluid volume and aquaporin (AQP) subunits (AQP-1, 5 and 7) expression in the peri-implantation period.
- ❖ Investigate the effects of thyroid hormone on the expression of uterine receptivity proteins (Integrin αV/β3, E-cadherin, Msx-1 and Ihh) in rat during peri-implantation period.
- ❖ Investigate differential expression of the receptors for thyroid hormone, thyroid stimulating hormone, vitamin D and retinoic acid and extracellular signal-regulated kinase in uterus of rats under influence of sex-steroids

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CHAPTER 2: LITERATURE REVIEW

2.1 Female Reproductive System

The female reproductive system is composed of two ovaries, fallopian tubes and uterus which connect to cervix and vagina. In rodents, there are two uterine horns, which are capable of holding multiple embryos (Hofstetter *et al.*, 2006). Ovaries are important to produce ovum and gonadal steroids, namely estradiol and progesterone (Knobil, 2013).

The follicles develop in ovaries in each reproductive cycle which then mature into a Graffian folliclethat will release ovum following fertilization, embryo moves towards the uterus with the help of ciliary beats (Moore *et al.*, 2015). The period by which the fertilized oocyte moves to the uterus, prior to implantation is known as pre-implantation period. The length of pre-implantation period is 4 to 4.5 days in rats (Sayem A. S. M. *et al.*, 2017) and 7 days in humans (Li Q. H. *et al.*, 2011). Implantation takes place in uterus.

Figure 2.1 shows anatomy of female reproductive organs including uterus, ovary and fallopian tube. Uterus consists of three layers, namely, endometrium, myometrium and perimetrium. Since uterus has smooth muscles which are arranged in longitudinal, spiral and circular manners with connective tissue interception, it is also capable of stretch and expand (Hartshorne & Gorecka, 2011). Uterus undergoes preparatory changes for the events in each reproductive cycle, known in both humans and primates as the menstrual cycle (Martin, 2007). During each reproductive cycle, the endometrium layer undergoes extensive proliferative and secretory changes to be receptive to the fertilized oocyte (Banker & Gupte-Shah, 2015). Once fertilized oocyte develops into a blastocyst, it will implant into the receptive endometrium and then starts developmental process of embryogenesis. If embryo implantation fail to occur, endometrium will be slough off due to changing levels of sex-steroids, particularly due

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to a falling level of progesterone, and this event is as menstruation in primates and humans (Knobil, 2013).

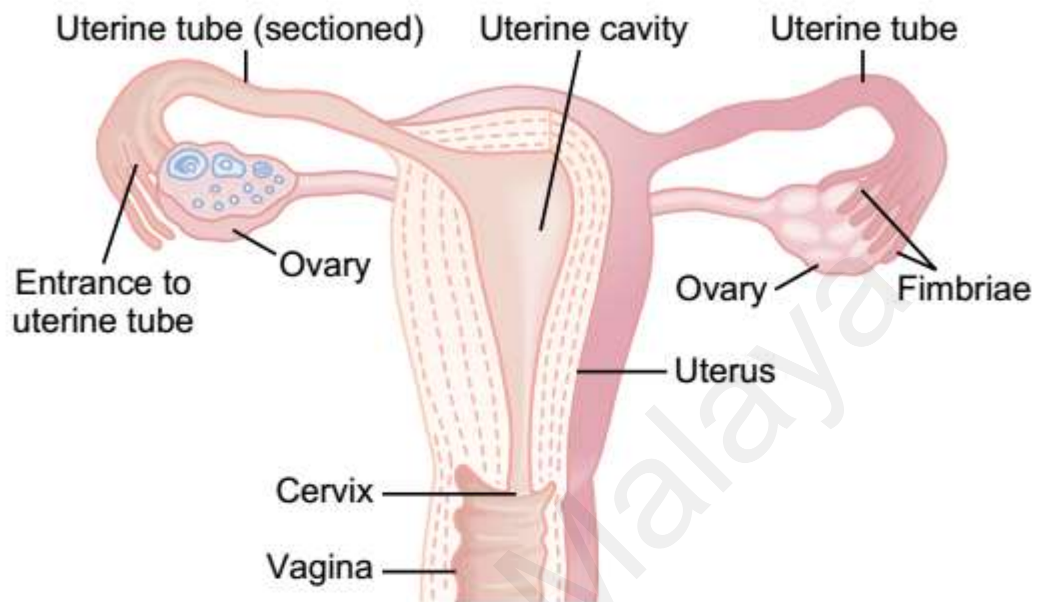

Figure 2.1 Anterior view of female reproductive organs.

(Adapted from Text Book of Medical Physiology by Guyton & Hall, 11th Ed. 2006)

2.2 Menstrual Cycle

Menstrual cycle is an event that occurs monthly, due to changes in the female sex hormones secretion and corresponding changes in the ovaries and other female reproductive organs (Knobil, 2013). the rhythmic changes in the cycle is intended to prepare the endometrium for implantation (Talbi *et al.*, 2006). Menstrual cycle can be sub-divided into two; the ovarian cycle and the uterine cycle. The ovarian cycle is comprised of follicular, ovulation, and luteal phases and the uterine cycle is characterized by menstruation, proliferative and secretory phases (Vegetti & Alagna, 2006). The anterior pituitary hormones, namely, follicular stimulating hormones (FSH) and luteinizing hormones (LH) regulates the ovarian events. Under the influence of

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FSH, follicles start to develop and release E_2 simultaneously (Knobil, 2013). Eventually one follicles will become and oocyte will be released following a surge in luteinizing hormone (LH) (Stouffer, 2003).Elevated E_2 levels will enhance endometrial growth through increasing proliferation of the endometrial layers (Munro *et al.*, 2010).

Ovulation will take place at the end of proliferative phase following the LH surge in which ovum will be released from ovary into fallopian tube (Knobil, 2013). Following ovulation, preovulatory follicle will transform into corpus luteum which secrets progesterone(Moore *et al.*, 2015). This corpus luteum will release amount of progesterone which will increase the endometrial vascularity (Munro *et al.*, 2010).

If implantation occurs, the implanted embryo will support the corpus luteum to continue releasing progesterone for maintaining the uterine layers via producing chorionic gonadotropin (CG) (Banerjee & Fazleabas, 2010). High levels of progesterone will also provide inhibitory feedback to the anterior pituitary to inhibit the release of FSH (Knobil, 2013). If fertilization does not occur, in humans and primates, menstruation will initiate due to a falling progesterone level as a consequence of degeneration of the corpus luteum and shredding the lining of the endometrium (Banerjee & Fazleabas, 2010). Table 2.1 shows the roles of different hormones during the menstrual cycle and Figure 2.2 shows the changes in ovary and uterus.

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Table 2.1 Role of different hormones during menstrual cycle

Hormone	Producing Organ	Role
GnRH	Hypothalamus	Acts on anterior pituitary to release FSH and LH.
FSH	Anterior pituitary	Acts on ovarian follicles, to mature and produce estrogens.
LH	Anterior pituitary	LH surge will cause discharge of ovum from graafian follicles, and turn it into corpus luteum which secretes estrogen and increasing levels of progesterone.
Estrogen	Preovulatory follicles (Ovary)	❖ Acts on uterine endometrium growth. ❖ High levels of estrogen (gradually) will cause negative feedback inhibition of FSH and LH. ❖ Very high levels of estrogen will act on GnRH and reverse the effects causing to release sudden surge of LH and FSH.
Progesterone	Corpus luteum (Ovary)	❖ Acts on uterine endometrium to become spongy and highly vascular. ❖ Causes negative feedback effect on anterior pituitary to inhibit LH release. ❖ Drop in LH will cause degeneration of corpus luteum and decreasing levels of sex-steroid. ❖ Decrease levels of progesterone will cause menstrual flow.

Adapted and modified from dimensions of Human Sexuality, 5e, (1999)

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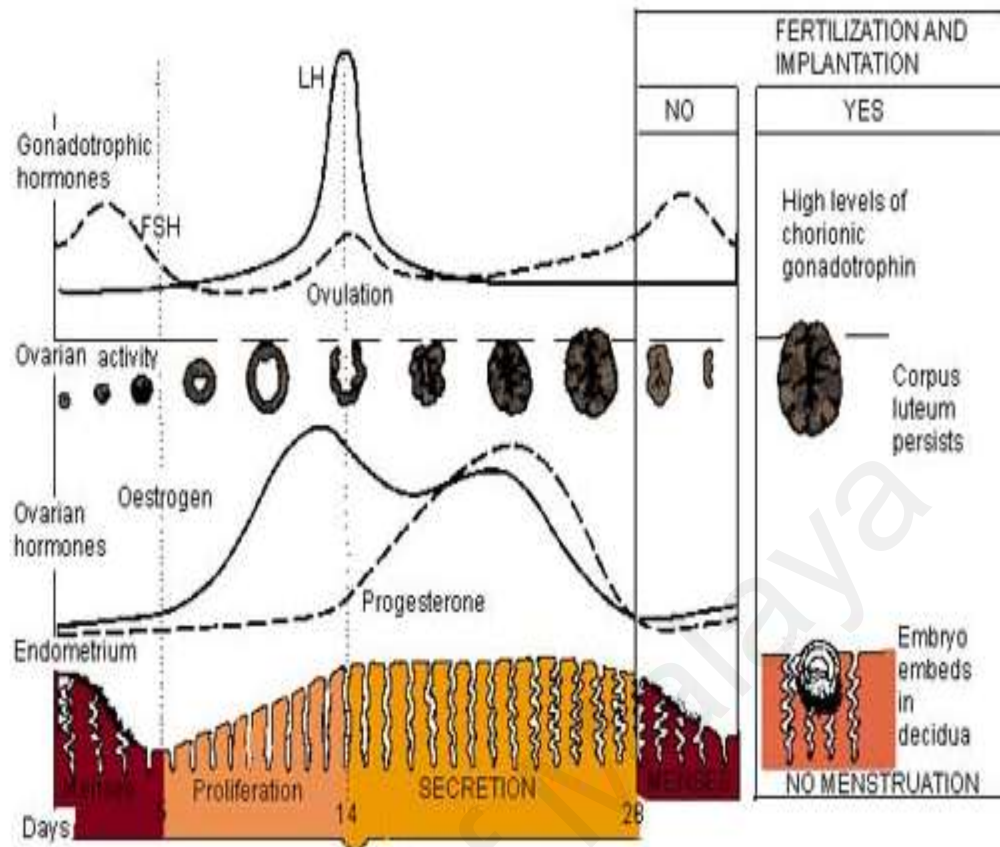

Figure 2.2 Changes occurred during the menstrual cycle.

(Adapted from: http://patient.info/)

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2.3 Estrus Cycle in rats

Reproductive cycle in rodents is called estrous cycle, which can be further divided into four phases: proestrus, estrus, metestrus and diestrus. The phases changing gradually under the control of ovarian steroids (Marcondes *et al.*, 2002). The cycle last for 5-6 days (Zhang S. *et al.*, 2013). E2 levels become high during proestrous and estrous phases. On the hand, level of progesterone becomes high during metestrous and diestrous phases (Wang Haibin & Sudhansu K Dey, 2006). Interchanging of these phases correlates with changes in steroid hormone levels progesterone dominated diestrous phase which then changes into proestrous phase under E2 dominance (Zhang S. *et al.*, 2013). It has been reported that ovulation occurs at 4th to 5th day of the estrous cycle (Croy *et al.*, 2013). Table 2.2 shows how different phases of estrous cycle can be detected by a vaginal smear and Figure 2.3 shows changes in hormone levels during the oestrous cycle.

After mid-luteal phase in unmated rat, the elevated levels of progesterone inhibit LH release from anterior pituitary which consequently causes the degradation of corpus luteum (table 2.2). However in mated rats, embryo implantation supports the corpus luteum to continue releasing progesterone and maintains the endometrium through chorionic gonadotropin (CG) (Banerjee & Fazleabas, 2010). In addition, in mated rats, decidualization does not occur spontaneously. .

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Table 2.2 Characteristics of different phases of estrous cycle in rats

Phases	Predominant sex-steroid hormone	Duration of phase	Effects	Phase Detection; based on cell types present in vaginal smear
Proestrus	Estradiol	12-24 hours	Hypothalamus would get positive feedback by the increased estradiol levels. Therefore estradiol and LH peaks will increase more.	Epithelial cells that are round and nucleated
Estrus	Estradiol	25-27 hours	Ovulation occurs due to LH surge.	Cornified cells that are irregular in shape without nucleus
Metestrus	Progesterone	6-8 hours	Causes LH secretion inhibition and prevention of ovulation due to endogenous shift from estradiol to progesterone (luteal phase).	Presence of the same proportion among epithelial, cornified, and leukocytes cells
Diestrus	Progesterone	55- 57 hours	Ovulation is inhibited.	Leukocytes cells that are little round in shape

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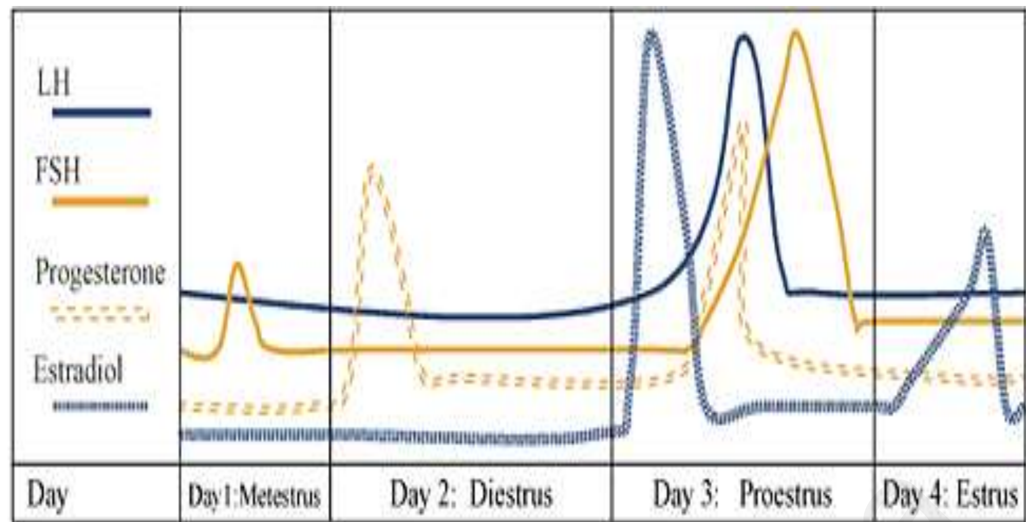

Figure 2.3 Hormonal changes throughout different phases of estrous cycle in rats
(Figure was adopted from Mary, Frederick & Nicholas 2002)

2.4 Events during pre-implantation period

Pre-implantation period is crucial for a successful implantation. During this period, embryo develops into blastocyst that is ready for implantation (Yuan *et al.*, 2018). *Milieu* within the uterine lumen is optimized with precise regulation of fluid, electrolytes and nutrients content (Satterfield *et al.*, 2010). Additionally, the endometrium acquires a receptive state, a state that is ready to accept the implanting blastocyst (Zhao H. *et al.*, 2015) which occurs just prior to the embryo implantation, specifically known as peri-implantation period. In this literature review, changes in uterine luminal fluid environment and development of endometrial receptivity during the peri-implantation period are discussed.

2.4.1 Uterine luminal fluid environment

Uterine luminal fluid is an ultra-filtrate of the plasma. Its formation and secretion is regulated by sex-steroid hormones (Morris *et al.*, 2010). Multiple ion channels in the endometrium are involve in mediating the movement of the fluid in uterus (Ruan *et al.*, 2014). The ion channels allow movement of electrolytes and water

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between different uterine compartments, i.e. the lumen, stroma and blood vessels. Uterine fluid volume, electrolyte compositions and pH can change throughout the reproductive cycle (Chan H. C. *et al.*, 2013). The mechanisms underlying these changes are mainly due to differential expression of epithelial ion channels under the influence of different sex-steroids (Ruan *et al.*, 2014). Precise regulation of uterine fluid volume, its electrolytes content and pH is crucial for successful reproductive events, such as sperm transport, fertilization, embryo transport, embryo development and embryo implantation (Chan H. *et al.*, 2007). Disturbances in uterine fluid volume, its electrolytes and pH can be the result of dysregulated expression of epithelial ion channels thus can adversely affect the normal uterine reproductive processes.

It was reported that uterine fluid sodium, potassium and fluid secretion into the uterine lumen increased following 17 β-estradiol treatment to the ovariectomised rats while fluid and electrolyte reabsorption occurred following progesterone treatment (Salleh N. *et al.*, 2005). Analysis of potassium concentration in the uterine luminal fluid collected from ovariectomised rats treated with E2 showed ten times higher than plasma concentration of potassium (Chinigarzadeh *et al.*, 2015; Tantayaporn *et al.*, 1974). The compositions of potassium and Cl^- in human luminal fluid is consistently higher than plasma (Deachapunya & O'Grady, 1998). Similar changes in the electrolyte composition of the uterine luminal fluid have been reported in other species, such as cow where potassium concentration was found higher than the plasma during estrus phase of the reproductive cycle (Carlson *et al.*, 1970; Lippes *et al.*, 1972). Besides, sodium and calcium concentrations in the uterine fluid of human and cow were reported to be lower when compared to plasma (Gerena & Killian, 1990; Leese, 1988). However, ions concentrations of the uterine fluid in the are almost similar to serum with some exceptions (Gholami *et al.*, 2013).

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Uterine fluid potassium plays crucial role in fertilization. Reduced number of pregnancies were observed in mice with low uterine fluid potassium (Quinn *et al.*, 1985). Uterine luminal fluid also assists sperm to migrate towards the utero-tubular junction (Kim N. *et al.*, 1996). In addition, it also plays important role in the implantation process. During implantation, uterine fluid reabsorption could cause uterine luminal closure, where in rodents assists the blastocyst to come in contact with the opposing uterine walls which then start the adhesion phase of implantation (Salleh N *et al.*, 2005). In this literature, focus is given to the mechanisms underlying uterine fluid volume changes which involve the water channel, the aquaporin (AQP), as widely reported (Richard *et al.*, 2003; Shahzad H., N. Giribabu, K. Karim, S. Muniandy*, et al.*, 2017).

2.4.2 Epithelial water channels

Specific water channel namely the AQP is located at the apical membrane of the uterine epithelial cells, stromal cells and blood vessels, which is involve in controlling the fluid secretion and absorption. (Salleh Naguib, Helmy Mohd Mokhtar, Normadiah M Kassim*, et al.*, 2015). The differential expression of this channel under the influence of hormone in particular sex-steroids have been found to be crucial in determining the changes of the uterine luminal fluid volume.

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2.4.2.1 Aquaporin (AQP) water channels

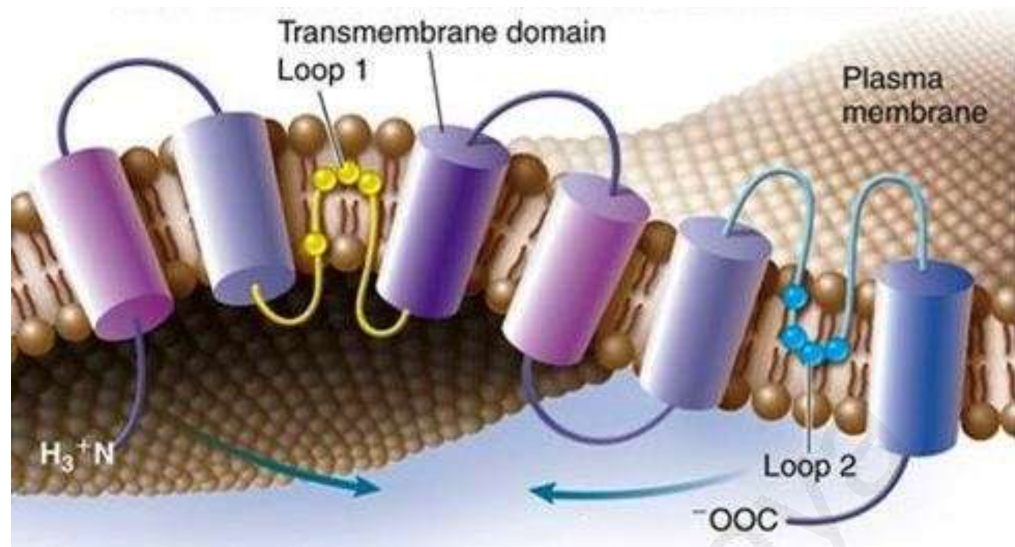

Figure 2.4 Structure of Aquaporin

(Adapted from Biology, Brooker, Widmaier and Graham; Chapter 49, slideplayer.com. this representation highlights the AQP's two separate regions which combine to form the water pore)

AQP is an intrinsic membrane protein with low molecular weight (26–34 kDa) that facilitate a rapid and passive movement of H_2O across the secretory epithelia, along with small molecules such as urea and glycerol (Papadopoulos & Verkman, 2013). In addition, AQP is also found to be involved in transporting gases, i.e., oxygen, carbon dioxide, nitric oxide and small solutes. This channel is also involved in non-transporting functions of K^+ and Cl^-, cell to cell adhesions, membrane polarization and regulating activity of other ion channels (Verkman, 2013).

AQP allows the cell to maintain its fluid volume and internal osmotic pressure depending on the hydrostatic and/or osmotic pressure differences across the cell membrane (Shatil-Cohen *et al.*, 2011). AQP can be regulated by the factors which affect permeability and subcellular localization of these channels (Carbrey & Agre, 2009).

To date, thirteen AQP isoforms have been identified; AQP-0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and12 (Day *et al.*, 2014). They are further classified into three sub-categories: (i) water selective AQPs: 0, 1, 2, 4, 5, 6, 8 that primarily transport water

across plasma membrane, (ii) Aquaglyceroporins AQPs: 3, 7, 9, 10 that transport water and small neutral solutes urea and glycerol and (iii) super-aquaporins AQPs: 11, 12 – the functional data is limited (Ishibashi *et al.*, 2009).

AQPs are widely distributed in tissues that are involved in water transport. For example, AQP-1 and AQP-2 facilitate rapid water transport in the kidneys where approximately 150-200 liters of water needed to be reabsorbed from the primary filtrate each day (Nejsum, 2005). Expressions of AQPs have also been reported in the brain (Tait *et al.*, 2008), lungs, (Zhang Z. *et al.*, 2010) eyes, (Tradtrantip *et al.*, 2009), guts (Zhao K. & Wu, 2012) and skin (Hara-Chikuma & Verkman, 2005).

AQP-1 is the first and most widely expressed subunit. In the female reproductive tract, AQP-1 is expressed in the uterine vasculature (Denker *et al.*, 1988) and in the uterine smooth muscles, mainly under the effects of sex-steroids (Lindsay Laura A & Christopher R Murphy, 2006). AQP-1 plays a crucial role in regulating the uterine H_2O movement (Sales *et al.*, 2013) and has been proposed to be involved in the development of stromal edema in uterus during peri-implantation period (Huang H.-F. *et al.*, 2006).

Expression of AQP-5 was reported in the epithelium of the uterine glands in rats at the time of embryo implantation (Lindsay L. A. & C. R. Murphy, 2007). The shifting of AQP-5 expression to the apical membrane of the uterine luminal epithelium was also observed under progesterone influence in rats (Lindsay L. A. & C. R. Murphy, 2006). AQP5 was also reported to be expressed at the apical membrane of uterine epithelial cells following treatment with progesterone in rats (Lindsay Laura A & Christopher R Murphy, 2006). AQP-7 is a non-selective water channel which mediates the transport of H_2O, glycerol, urea and other small non-electrolytes. It is reported to be expressed in the uterus (Zhang D. *et al.*, 2012) and is involved in decidualization (Huang H. F. *et al.*,

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2006). It has also been found to be involved in the efflux of glycerol and triglycerides (Wakayama *et al.*, 2014).

Other isoforms of AQP has also been reported to be expressed in the female reproductive tract (Huang H.-F. *et al.*, 2006) under sex-steroid influence (Lindsay L. A. & C. R. Murphy, 2006). AQP-2 expression in the endometrium of humans w was found high at the time of implantation (He *et al.*, 2006). AQP-2 expression was found to increase in the endometrium and myometrium following treatment with E2 in ovariectomised rats (Jablonski *et al.*, 2003).

2.4.3 Uterine Receptivity development

Uterine receptivity is a physiological process by which the endometrial epithelium acquires morphological and functional changes for blastocyst acceptance and implantation and is influence by ovarian steroid hormones (Makker & Singh, 2006). Receptivity development involves multiple and complex mechanisms (Tranguch *et al.*, 2005). The endometrium becomes receptive to blastocyst during a well-defined period, known as "window of implantation". Normally the endometrium is refractory to implantation outside of this window period (Foulk, 2012). Endometrial receptivity reaches highest peak at mid luteal phase in humans. Coordinated signal transduction between blastocyst and endometrium, and endometrium itself assist in receptivity development.

E2 and progesterone are crucial for cyclical development of endometrium and preparation of endometrium into the receptive state (Trolice & Amyradakis, 2012). Well-sequenced events have been identified for secretory transformation of endometrium under progesterone influence. Development of the uterine receptivity is also dependent on estradiol for inducing rapid proliferation of the endometrial tissue followed by progesterone for enhancing secretory changes (Foulk, 2012). Imbalance in

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the ratio of E2 and progesterone is likely to affect the process of endometrial secretory transformation which could lead to luteal phase defects and alteration of uterine receptivity (de Ziegler *et al.*, 1998). Figure 2.5 shows the uterine receptivity relating to ovarian hormones and phases of reproductive cycles in mice and humans.

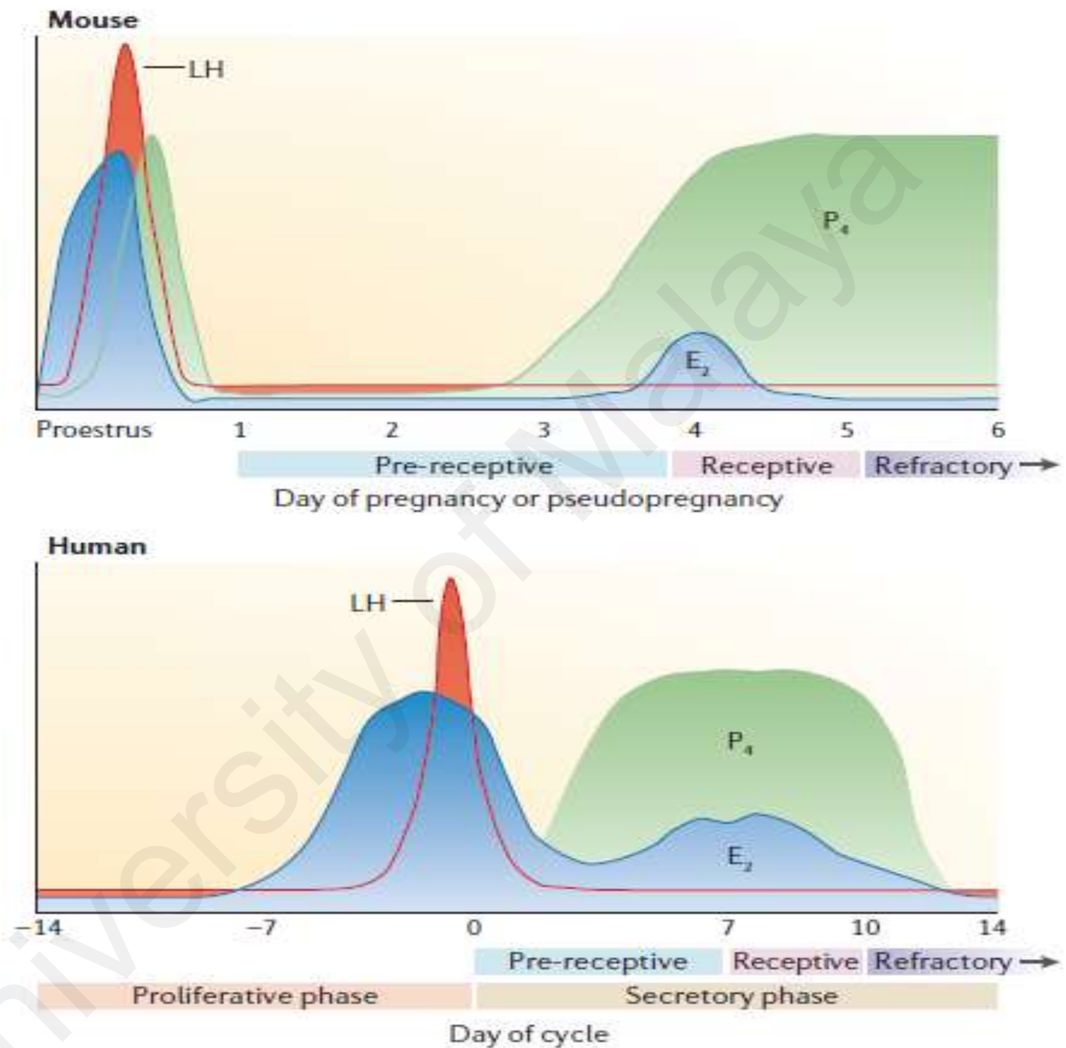

Figure 2.5 The window of uterine receptivity in mice and humans

Adopted from: (Wang H. & S. K. Dey, 2006)

2.5 Blastocyst events during implantation period

Successful embryo implantation involves the physical and physiological contact between an implantation competent blastocyst and a receptive endometrium which involve three main processes: apposition, adhesion and invasion (Dey *et al.*, 2004). After fertilization, embryos can reach to the uterine cavity at approximately 4-5 days in

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humans and move within the uterine lumen by a rhythmic myometrial contraction until implantation begins at the endometrial wall. Embryo implantation occurs by day 6 to 7 in human (Foulk, 2012). However, in rats and mice, fertilized embryo enters the uterine cavity by 3 to 4 days and implantation takes place at approximately day 5 of pregnancy (Zhang S. *et al.*, 2013).

Embryo implantation begins with initial contact of blastocyst to endometrial wall of uterus, a process which is known as "apposition". This process is assisted by closure of the uterine lumen due to reduction of the uterine fluid volume (Salleh N *et al.*, 2005). At this stage, blastocyst still remains floating in the uterine cavity despite of being in close contact with the endometrium and could be flushed out. Therefore, blastocyst must adhere to the uterine luminal epithelium a process called "adhesion" (Kimber & Spanswick, 2002).

Adhesion of blastocyst to the endometrium is established, when surface projections of blastocyst interlink with apical protrusions on the endometrial epithelium (Singh *et al.*, 2011). Adhesion between blastocyst and endometrium is mediated by surface adhesion molecules which are expressed equally on surface of the blastocyst and endometrium (Kimber & Spanswick, 2002). This adhesion mediated by surface molecules stabilizes the blastocyst onto endometrial epithelium (Foulk, 2012).

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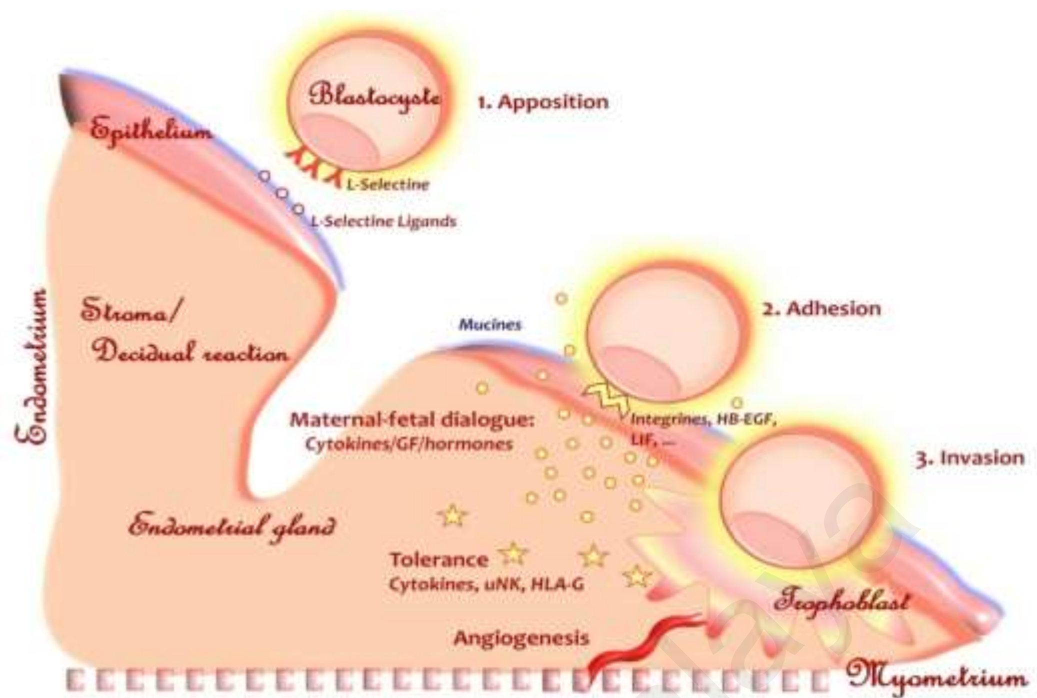

Figure 2.6 Three stages of embryo implantation

(Adapted from: Endometrial receptivity Report; http://cpma-ulg.be/consultations-et-examens/femme/bilan-de-receptivite-endometriale/)

Final stage of embryo implantation is invasion of blastocyst via trophoblastic cells. Trophoblastic cells tunnel into the basal lamina by proliferation and invade the stromal tissues (Wang Haibin & Sudhansu K Dey, 2006). This invasion of trophoblast cells occur at day 7 of pregnancy in rats (Enders & Schlafke, 1967). For achieving successful invasion, endometrial stromal cells undergo remolding and transformation into large decidual cells. These decidual cells also assist to hold further deep invasion of trophoblastic cells into the endometrium (Zhou *et al.*, 1997). Throughout the whole embryo implantation process and pregnancy, sex-steroids is essential in all mammals (Wetendorf & DeMayo, 2012). However, effects of other hormones on implantation are widely studied. Figure 2.6 depicts the three stages of embryo implantation process.

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2.6 Brief introduction on thyroid hormone, its receptor and mechanisms of action

Thyroid hormones (TH) are vital for normal growth and development and influence metabolism in most tissues. The thyroid gland secrets two major hormones: thyroxine (T4) and triiodothyronine (T3) (Kirsten, 2000; Medici *et al.*, 2017). Both hormones modulate energy utilization and heat production and facilitate growth. Thyroid gland produces T4 largely, although T3 is more potent (approximately 90%). However most T3 (more than 80%) is derived from the conversion of T4 by deiodination in peripheral tissues (Stathatos, 2012).

The synthesis of thyroid hormones requires iodine, which can be found in diet such as, milk, dairy products, eggs, fish and iodized table salt. After digestion, iodine is converted to iodide and transported to the thyroid gland. Iodide is then being oxidized by thyroid peroxidase (TPO) enzyme and incorporated to tyrosine residues of thyroglobulin (TG), stimulating 3-monoiodotyrosine (MIT) and 3, 5-diiodotyrosine (DIT) productions in the thyroid follicles. MIT and DIT form the T3 and T4 by coupling. Then the hormones are stored as part of the TG in the colloid and excrete through the circulation once required (Abdalla & Bianco, 2014; Mullur *et al.*, 2014).

The functions of thyroid hormone are mediated by specific nuclear thyroid hormone receptors that act as modulators of gene expression. Thyroid hormone receptors (TR) belong to the superfamily of nuclear receptors which are encoded by the TRα and TRβ genes (Visser *et al.*, 2011). TRα gene encodes TRα1 that is mainly expressed in brain, heart, skeletal muscle and female reproductive tract, uterus. TRα2 and TRα3 are truncated isoforms which are not active (figure 2.7). In addition, TRβ gene encodes TRβ1 which is expressed wildly including uterus and TRβ2 is expressed primarily in the brain, retina, and inner ear; and TRβ3 is expressed in kidney, liver and

lung. The TRα1, TRβ1 and TRβ2 are the main hormone-binding isoforms (Cheng S. Y. *et al.*, 2010; Rosen *et al.*, 2011). Besides the genomic pathway, thyroid hormone also acts via a non-genomic pathway (Davis P. J. *et al.*, 2016).

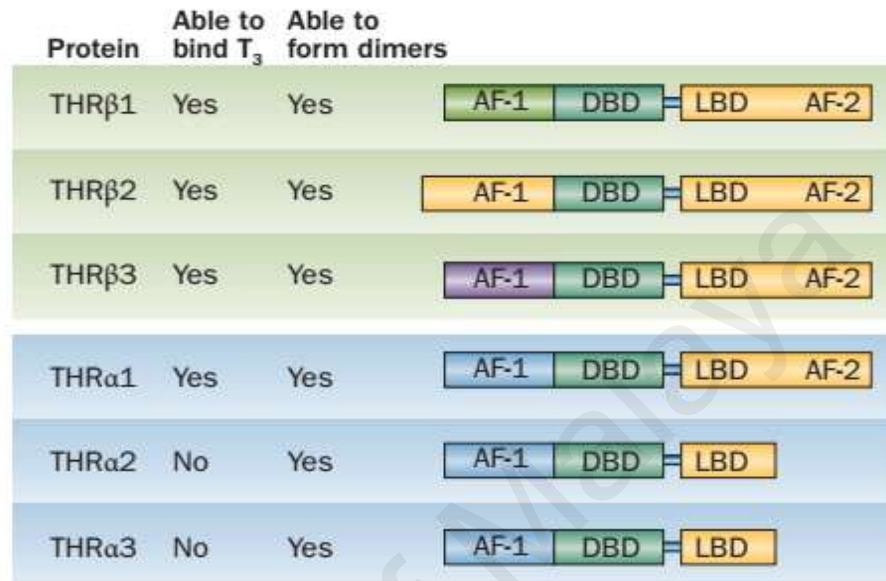

Figure 2.7 The considerable homology of TRα and TRβ isoforms.
Abbreviations: AF-1, activation function-1; AF-2, activation function-2; DBD, DNA-binding domain; LBD, ligand-binding domain. Source: (Ortiga-Carvalho *et al.*, 2014).

TH (T4 or T3) enter into the target cell from circulation and then T4 gets converted to T3 by deiodinase enzyme in cytoplasm, which then binds to TR in the nucleus of the cell. The activated TR forms heterodimer with a second transcription factor, 9-*cis* retinoic acid receptor (RXR) (see figure 2.8). This heterodimer binds with the thyroid hormone response elements (TREs) in the DNA.

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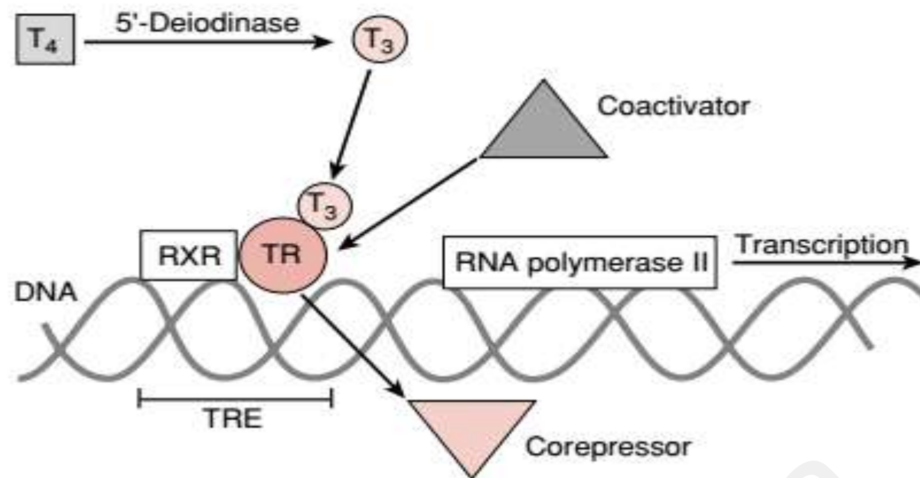

Figure 2.8 Mechanisms of thyroid hormone action at cellular level

(Adopted from Medical Physiology, by Rodney A. Rhoades PhD, George A. 2nd Ed., 2003)

Binding of heterodimer (T3, TR and RXR) to TREs displaces repressors of transcription and recruits additional co-activators. Consequently RNA polymerase II becomes activated and the transcription of the target gene occur resulting associated protein expression (Brent, 2012; Yen *et al.*, 2006).

Non-genomic actions of thyroid hormone have also been increasingly reported (Davis *et al.*, 2010). Thyroid hormone begins its nongenomic actions at the receptors in the plasma membrane, mitochondria or cytoplasm. These receptors can mediate transcriptional actions of T3/T4 by sharing structural homologies with nuclear thyroid hormone receptors (TR), or without homologies with TR, such as the integrin αvβ3 receptor on the plasma membrane. Nongenomic actions of thyroid hormone via integrin αvβ3 by T4 can stimulate gene expression that affects angiogenesis and cell proliferation (Davis P. J. *et al.*, 2016). Therefore, the effects of both non-genomic and genomic can overlap in the cell nucleus. These plasma membrane receptors, integrin αv/β3, have been found (Bergh *et al.*, 2005) and reported to mediate actions in multiple tissues, including bone (Davis P. J. *et al.*, 2016), blood vessels and the heart (Davis *et al.*, 2011).

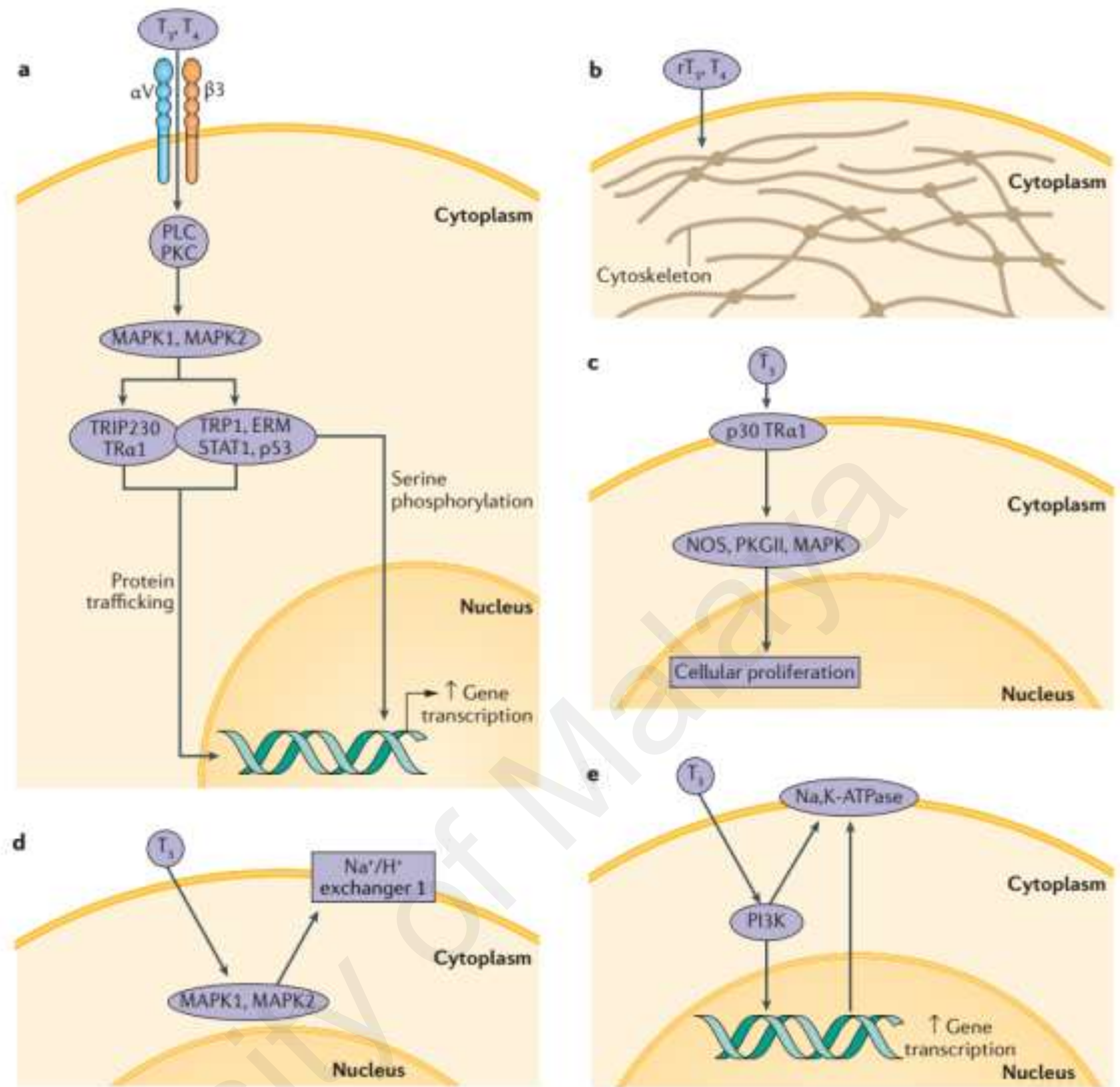

Figure 2.9 The non-genomic actions of thyroid hormone.

(a) Nongenomic actions of the hormone that begin at integrin αvβ3 include regulation of intracellular trafficking of specific proteins to the nucleus and serine phosphorylation of some of these proteins in the course of nuclear entry. Directed to the nucleus from the cytoplasm, some of these proteins might be involved in modulation of transcription of specific genes (TABLE 1) and in cell proliferation. These pathways depend on activation of phospholipase C (PLC), protein kinase C (PKC), mitogen activated protein kinase (MAPK)1 and MAPK2. **(b)** The cytoskeleton is regulated by T4 and rT3. **(c)** Cell proliferation, for example, osteocytes, initiated at the plasma membrane by the binding of T3 to a truncated TRα1 (p30 TRα1) that is transcriptionally inactive. The TR–T3 complex activates a series of signal-transducing proteins (PKGII and ERK) and nitric oxide synthase (NOS) to increase cell proliferation. **(d)** The sodium–proton exchanger (Na+/H+ exchanger) in the plasma membrane can be actived by T3 via MAPK1 and MAPK2. **(e)** Local activation of the plasma membrane sodium pump (Na,K-ATPase) by T3can occur via phosphatidylinositol 3-kinase (PI3K)/AKT. Transcription of the gene encoding this pump might also be affected by this mechanism. PKGII, protein kinase G II; TRα1, thyroid hormone receptor-α. Source: (Davis P. J. *et al.*, 2016)

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2.6.1 Thyroid Hormone Action in Female Reproductive System

Thyroid hormones have several effects in the female reproductive system. Both hypothyroidism and hyperthyroidism are known to affect the sex steroids and ovarian functions (Andreeva, 2014). Lack of thyroid hormone or hypothyroidism is found to cause a number of reproductive disorders, from menstrual irregularities to various adverse effects during peri-implantation and implantation which result in infertility (Poppe K. & B. Velkeniers, 2004). It has been reported that prolonged hypothyroidism severely reduces ovarian follicular reserve in adult rats (Meng *et al.*, 2017). Furthermore ovarian follicular development and granulosa cell proliferation are found to be interrupted due to inadequate thyroid hormone supply in prepubertal rats (Dijkstra *et al.*, 1996). Thyroid hormones are reported to act in the female reproductive tract through the receptors. Aghajanova et al., 2011 demonstrated the expression and distribution of the receptors for thyroid hormone (TR), thyroid stimulating hormone (TSH) and iodothyronine deiodinase (DIO) enzyme in the endometrium of human uterus during the menstrual cycle (Aghajanova L. *et al.*, 2011). It was also reported that the expression of TRα1, TRα2, TRβ1 and TSHR in endometrium increased during uterine receptivity in peri-implantation period (Aghajanova L. *et al.*, 2011). TH may interfere with activity of sex-steroids in its target tissues, including the reproductive tract. It has been demonstrated that lack of TH is known to reduce estrogenic response of the endometrial cells, resulting in reduced endometrial thickness (Inuwa & Williams, 1996b). Some reported effects of TH in the female reproductive system have been listed in table 2.3.

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Table 2.3 Evidences of TH Action and related Molecules in the Female Reproductive System

Localization	Molecules	Evidence and possible role	Species	References
Endometrial Stroma	TR-α, TR-β, RXR and ERK1/2 (Expression and distribution)	Thyroxine increases expression and distribution of TR-α, TR-β, RXR and ERK1/2 during peri-implantation period	Rat	(Sayem A. S. M. *et al.*, 2017)
Endometrium	TRα1, TRβ1 (mRNA and proteins levels)	Mifepristone: down-regulation of TRα1 and TRβ2; up-regulation of TRβ1 and DIO2 mRNA	Human	(Catalano *et al.*, 2007; Li C. *et al.*, 2014; Scoccia *et al.*, 2012)
Endometrium	Thyroglobulin (TG) and thyroid peroxidase (TPO) protein levels	TG and TPO are expressed by TH	Human	(Catalano *et al.*, 2007)
Endometrium	Estrogen receptor proteins	Lack of TH reduces uterine cells' estrogenic response	Rat	(Inuwa & Williams, 1996b)
Glandular and Luminal epithelium of uterus	TSHR, TRα1, TRβ1 (mRNA and proteins levels)	TSHR in the luminal epithelium and TRα1 and TRβ1 in the glandular and luminal epithelium increase on luteinizing hormone (LH)	Human	(Aghajanova L. *et al.*, 2011)

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Blastocyst (in vitro)	TH receptors mRNA and proteins; iodothyronine deiodinase (DIO2 and DIO3) mRNAs (T3 produced in the bovine follicular fluid is an indirect indicator of DIO1 mRNA). TRα expressed in oocyte, zygote and all the cleavage stages from blastocyst stage embryos in different culture.	TH on Early Embryo development increase blastocyst formation and hatching rate; improve embryo quality (greater total cell counts and reduced proportions of apoptotic cells); improve post-cryopreservation viability.	Bovine Human	(Ashkar *et al.*, 2010; Costa *et al.*, 2013; Degrelle *et al.*, 2013; Loubiere *et al.*, 2012)
Ovary	TRα1, TRα2, TSHR, TRβ1, DIO2, DIO3	T3 dose-dependent mRNA expression of inflammation-associated genes; COX-2, MMP9, 11bHSD1	Human & rat	(Du & Li, 2013; Fedail *et al.*, 2014)
Oocyte and Granulosa Cells (GC)	TRα1, TRβ1 and TRβ2 mRNA	Expression has been found	Human	(Zhang S. S. *et al.*, 1997)
Follicular fluid	T3 hormone	T3 hormone present in follicular fluid	Human	(Zhang S. S. *et al.*, 1997)

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2.6.2 TSH role in Uterus

Thyroid stimulating hormone (TSH) controls the synthesis of TH which acts via thyroid stimulating hormone receptor (TSHR). TSHR is a transmembrane receptor and a prime regulator for TSH functions (Davies *et al.*, 2010; Mizukami *et al.*, 1994). TSHR is reported to be expressed in thyroid glands, kidneys, thymus, heart, adipose tissue (Dutton *et al.*, 1997). Expression of TSHR in human endometrium was reported to be increased at the time of endometrial receptivity (Aghajanova L. *et al.*, 2011).

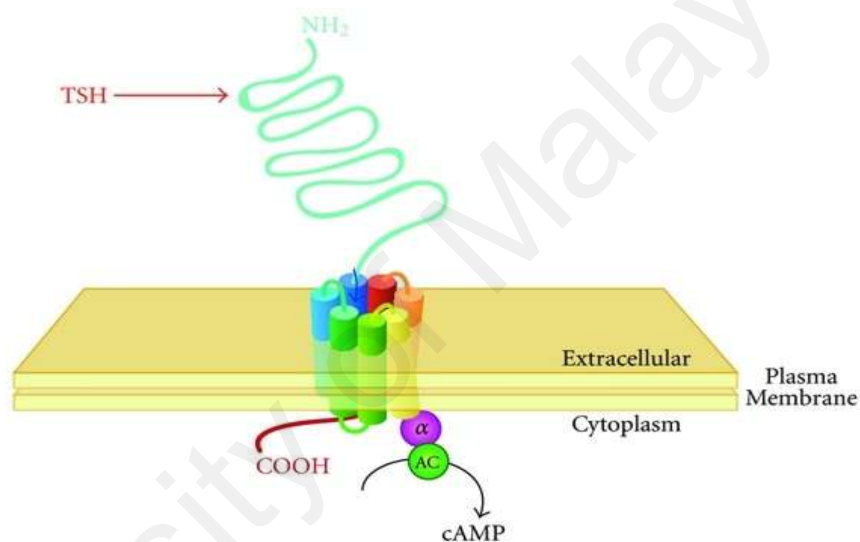

Figure 2.10 Structure of TSH receptor (TSHR) showing interaction of TSH
(Adopted from; https://www.hindawi.com/journals/jtr/2012/351864/fig8/)

Expression of TSHR in uterus has been found to be influence by ovarian steroids. It has been reported that TSHR and TR expression in uterus were up-regulated following chronic administration of conjugated equine estrogen and medroxyprogesterone acetate to ovariectomized cynomolgus macaques (Hulchiy M. *et al.*, 2012). Figure 2.9 shows the interaction of TSH with its receptor.

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2.7 Vitamin D Role in Uterus

Vitamin D plays crucial role in many uterine functions, such as uterine smooth muscle contraction (Thota *et al.*, 2014) and regulation of uterine cell proliferation (Yoshizawa *et al.*, 1997). It has been reported that vitamin D3 has vital role in regulation of immunity in the endometrial stroma via antigen presenting cells, assisting in the implantation process in rats (Jeddi-Tehrani *et al.*, 2009; Zarnani A. H. *et al.*, 2010). It has also been suggested that Vitamin D3 regulate calcium and phosphorus metabolism in uterus via vitamin D receptor (VDR) (Kinuta *et al.*, 2000; Shahbazi *et al.*, 2011).

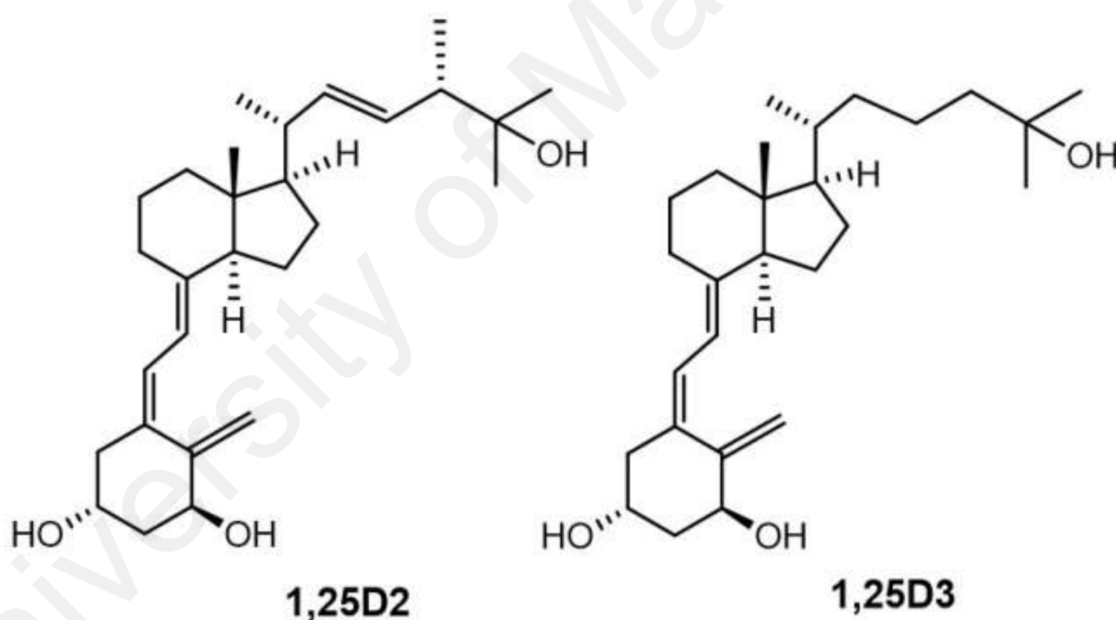

Figure 2.11 The Structure of Vitamin D2 (1,25D2) and Vitamin D3 (1,25D3). Source: (Kutner & Brown, 2018)

Vitamin D receptor (VDR) has been found to be expressed in the uterus in buffalos during the follicular and luteal phases of the reproductive cycle, implicating the eminent role of this vitamin in the process of reproduction (Emam M. A. *et al.*, 2016). Reduced VDR expression in thyroid disorders has been reported to enhance infertility and pregnancy loss in humans, suggesting that thyroid hormone and VDR have important roles in fertility (Twig *et al.*, 2012).

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2.8 Retinoic Acid and its Role in Uterus

Retinoic acid (RA) is a low molecular weight acid. RA is vital for the maintenance of female reproductive system functions, as RA controls numerous physiological functions such as cell proliferation and differentiation (Grenier *et al.*, 2007). RA is also a lipophilic metabolite in terms of Vitamin A. Retinoic acid or vitamin A deficiency results in morphological changes of uterine luminal and glandular epithelia, menstrual irregularities, pregnancy loss and fetal malformations (Zheng *et al.*, 2000). To gain normal features of uterus morphology and fertility, it is necessary to recover the deficiency of this metabolite (Li X. H. *et al.*, 2004). RA has been found to regulate the expression of matrix metalloproteinases (MMP) which is locally produced in the endometrial stromal cells of the rat uterus during decidualization (Osteen *et al.*, 2003; Zheng *et al.*, 2000).

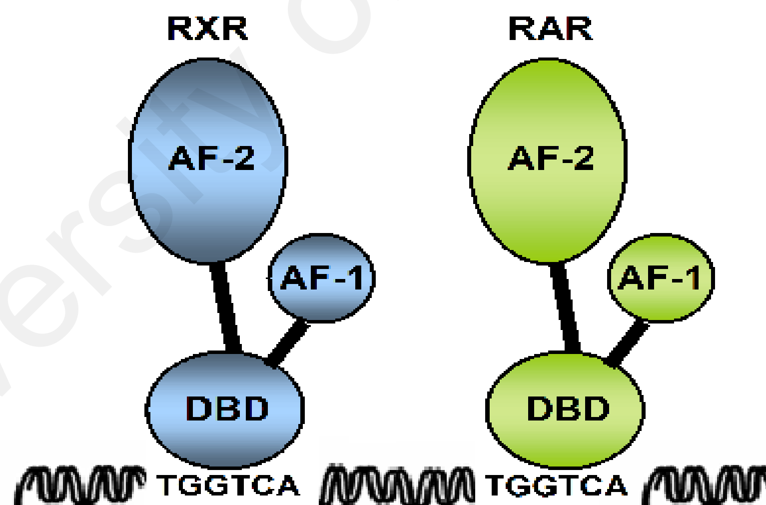

Figure 2.12 Structure of Retinoic acid isoforms, shows binding sites

Abbreviation; AF-1: activation function-1, AF-2: activation function-2, DBD: DNA-binding domain (Le May & Li, 2012).

Retinoic acid acts via retinoic acid receptor (RAR) or retinoic X receptor (RXR) isomer (figure 2.11), nuclear receptor (Chambon, 1996). RAR expression has been found in the uterine stroma of mice (Nakajima *et al.*, 2016), uterine epithelia of rat

(Boehm *et al.*, 1997) and human uterus (Kumarendran *et al.*, 1996). In addition, expression of RAR in rat uterus has been stimulated by ovarian steroids (Boehm *et al.*, 1997) and in humans (Kumarendran *et al.*, 1996). RAR or RXR involves in the formation of functional heterodimer complex with TR, which interact with specific thyroid hormone responsive element (TRE) on DNA, initiating protein synthesis (Liu Y. Y. & Brent, 2010).

2.9 ERK Role in Uterus

Extracellular signal-regulated kinase 1/2 (ERK1/2), a member of well-known mitogen-activated protein kinase (MAPK) pathway, was reported to be involved in the regulation of cellular proliferation and differentiation in several organs including uterus (Paul *et al.*, 2011; Shan *et al.*, 2013). Aberrant decidualization in the stromal cells of endometrium has been linked with unexplained endometrial pathologies such as endometriosis (Karpovich *et al.*, 2005; Klemmt *et al.*, 2006), as endometriosis condition results in reduced decidualization capacity. A potential link between ERK1/2 signaling protein level and endometriosis have been demonstrated in many studies (Mormile & Vittori, 2013; Velarde *et al.*, 2009; Yotova *et al.*, 2011). ERK1/2 stimulation to cAMP-dependent cell cycle regulation in cultured human endometrial stromal cells (hESCs) and abnormal level of phospho-ERK1/2 in hESCs derived from uterus with endometriosis, indicate the importance of ERK1/2 signaling in the mouse uterus during different endometrial events (Klemmt *et al.*, 2006; Velarde *et al.*, 2009).

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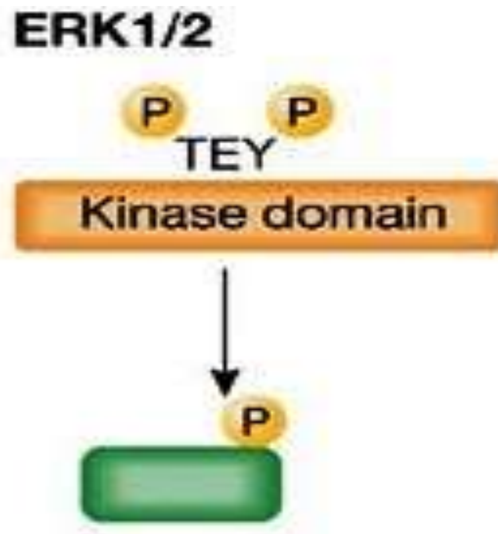

Figure 2.13 Hypothetical ERK1/2, transmitting signals to downstream

The activating phosphorylation of ERK1/2 results in the activation of the kinase activity, and ERK1/2 phosphorylates both downstream target molecules (Nishimoto & Nishida, 2006).

ERK1/2 signaling is crucial for embryo implantation processes, as reported in mice and humans (Lee C. H. *et al.*, 2013). It has also been reported to mediate non-genomic action of thyroid hormone (Chen *et al.*, 2014; Deb & Das, 2011; Iordanidou *et al.*, 2010). Female sex hormones i.e. Estrogen and Progesterone have been found to regulate ERK1/2 protein expression in uterine artery smooth muscle in rats (Xiao *et al.*, 2009). Figure 2.12 shows a hypothetical ERK1/2.

2.10 Hypothyroidism and female fertility

TH deficient disorder, known as hypothyroidism, has been found to influence female fertility negatively (Mintziori *et al.*, 2016). Hypothyroidism has been reported to contribute to female infertility (Priya *et al.*, 2015). There is a report that In a population of infertile women, hypothyroidism was observed to be prevalent in 23.9% of these women (Verma I. *et al.*, 2012). It has also been observed that thyroxine treatment in hypothyroid female improves the fertility and increase rate of successful pregnancy (Yoshioka Waka *et al.*, 2015). Chronic hypothyroidism reduces the ovarian follicular reserve and the size of the growing follicle in female rats, which has been demonstrated

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to impact on fertility (Meng *et al.*, 2017). Moreover, hypothyroidism decreases the level of estradiol and estrone, and increase conversion rate of testosterone to androstenedione in women (De Groot *et al.*, 2012). However, underlying pathophysiological mechanisms to explain infertility in hyperthyroidism are largely unknown.

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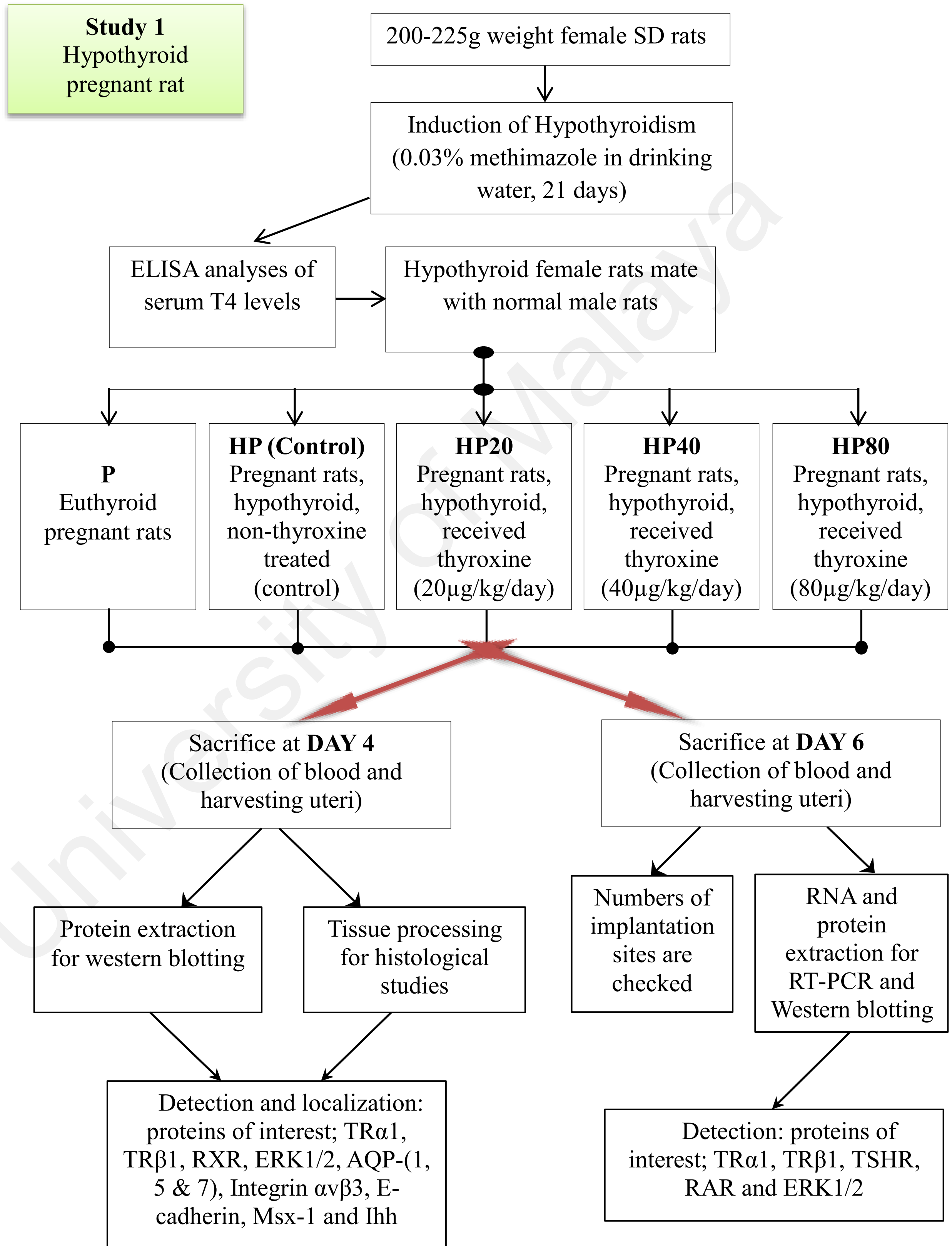
Study 1: Investigating the effect of thyroxine on expression of the proteins of interest in the peri-implantation period and implantation outcomes



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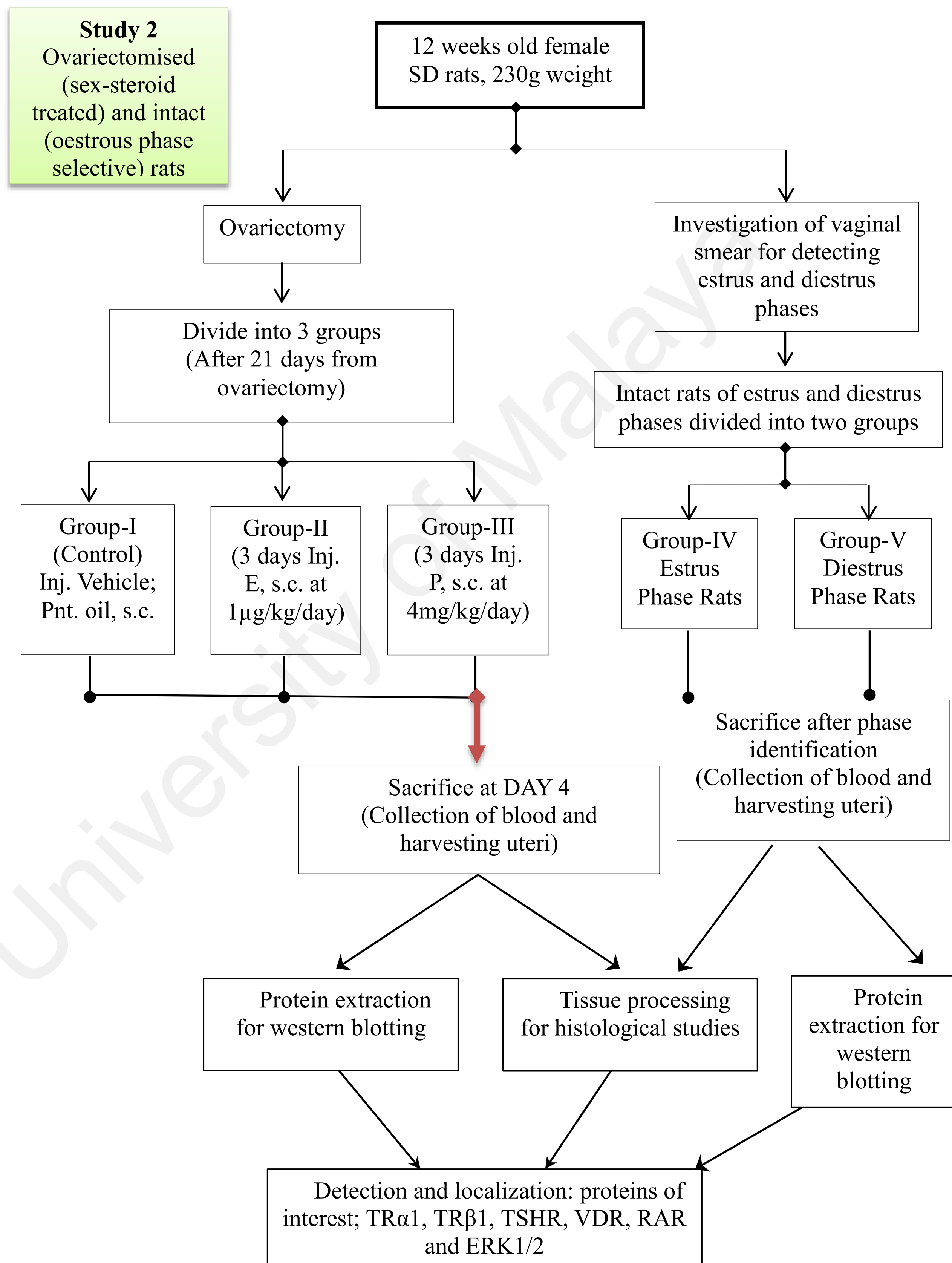
Study 2: Investigating the effect of sex-steroids and oestrous cycle phases on expression of the proteins of interest



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3.1 Materials

3.1.1 Animals

Three month-old adult female Sprague-Dawley (SD) rats, weighted 200-230g, were purchased from Animal Experimental Unit (AEU), UM. Animals were transported by transporting cages from AEU to the Animal house at the Department of Physiology, UM. Then the rats were housed in a clean and well ventilated animal room with standardized conditions (lights on 12 hr from 06:00 hours to 18:00 hours: room temperature 24°C; with 3-4 animals per cage). They were fed with rat chow diet (Harlan, Germany) and tap water *ad libitum*. Food and water were changed at two days interval. All experimental procedures were approved by the Faculty of Medicine, Animal Care and Use Committee (ACUC), UM with ethics no: 2015-181201/MOL/R/SM. Figure 3.1 shows the housing and caging of the rats.

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Figure 3.1 Housing and caging of the rats

3.1.2 Chemicals and consumables

Table 3.1 List of chemicals and consumables that were used in this study

	Chemicals	Source
1.	Methimazole	Sigma-Aldrich, St. Louis, USA
2	Thyroxine	Sigma-Aldrich, St. Louis, USA
3.	T4 Rat ELISA kit	Cusabio Biotech Co., LTD, USA
4.	17 β-estradiol, Progesterone	Sigma-Aldrich, St. Louis.
5.	Paraformaldehyde, Ethanol, Methanol, Xylene	Sigma-Aldrich, St. Louis
6.	NaCl, $NaHCO_3$, $NaHPO_4$, KCL, $MgSO_4$,CaCl, Glucose-D, HEPES. Trinatrium Citrate	Merck KGaA, Darmstadt, Germany
7.	Parrafin Wax	Tyco Healthcare Group MA, USA
8.	ABC staining Kit	Santa Cruz, CA, USA
9.	Ultra Cruz mounting media	Santa Cruz, CA, USA
10	4CN optic substrate kit	Bio Rad, CA, USA
11.	Spectra multicolor broad range	Fermentas, USA
12.	Bovine Serum Albumin (BSA)	Innovative, Peary Court Novi, Michigan, USA
13.	Tetramethylenediamine (TEMED)	Sigma-Aldrich, St. Louis
14.	RNA extraction kit	Qiagen, Germany
15.	RNAs free water, RNA Later	Ambion, Foster city, USA
16.	cDNA Conversion kit	PCR Biosystems, London, UK
17.	ViPrimePlus qPCR Green Master Mix with ROX	Vivantis Technologies , USA
18.	Pro-Prep protein extraction solution	Intron Biotechnology, Korea
19.	Micro BCA protein assay Kit	Thermo Scientific
20.	Membrane (PVDF) Poly Vinylidene fluoride	Bio Rad, CA, USA
21.	Multicolor broad range Ladder	Fermentas, USA

3.1.3 Sterilization

Proper sterilization of equipments, plastic tips, disposable glass and collection tubes were done by autoclaving for 20 min at 1.05 kg/cm² on liquid cycle. Double distilled water (ddH_2O) was used for the required experiments.

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3.2 Methods

3.2.1 Methods for Study 1

3.2.1.1 Induction of Hypothyroidism in intact female rats

Hypothyroidism was induced in female SD rats via oral administration of 0.03% methimazole (MMI) (M8506, Sigma-aldrich, St. Louis, USA) dissolved in drinking water. This method of hypothyroidism induction has been used largely in research (Cooper *et al.*, 1984; O'Hare *et al.*, 2015). The other drug, 6-propyl-2-thiouracil (PTU) has also been used to induce hypothyroidism. Both MMI and PTU induce hypothyroidism by inhibiting thyroid iodide peroxidase (TPO) enzyme. But MMI is a more potent inhibitor of iodination than PTU. One comparative study on the mechanism of TPO inhibition by MMI and PTU in human reported that MMI inhibition of TPO was irreversible in both in-vitro and in-vivo study, whereas PTU inhibition was found to be reversible either by dialysis or dilution (Nagasaka & Hidaka, 1976).

(a) **Preparation of MMI and Treatment**

300mg MMI was dissolved in 1 liter drinking water in order to make-up to 1 liter of 0.03% MMI solution. 0.03% MMI solution was then given to the rats for 21 days. Figure 3.2 shows administration of MMI via drinking water to the rats. The MMI dissolved drinking water was changed at every two days interval. Each rat was found to consume 30ml MMI dissolved drinking water per day. After 14 days of MMI treatment, all the rats were found less active, a signs which indicate the possibility of hypothyroidism development.

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Figure 3.2 The treatment of MMI via drinking water

(b) **Measurement of serum thyroxine level prior to mating**

Following completion of MMI treatment, blood was collected from tail vein in serum separator tube and allowed samples to clot two hours at room temperature before centrifugation for 15 minutes at 1000×g. Serum was then collected and serum thyroxine levels were measured by using a rat thyroxine ELISA kit (CSB-E05082r, Cusabio Biotech Co., LTD, USA) in order to confirm development of hypothyroidism. MMI administration causes serum thyroxine levels in euthyroid female rats to decrease (Table 3.2), by approximately three fold as compared to control (non-methimazole treated euthyroid female rats).

Table 3.2 Serum levels of thyroxine hormone following MMI treatment

Treated samples	Groups	Thyroxine levels (Mean ± SD)
MMI*	Control (no treatment)	32.37±0.52 ng/ml
	0.03% (in drinking water, 21 days)	12.27±0.45 ng/ml

*MMI- Methimazole.

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3.2.1.2 Animals and hormone treatment

Following hypothyroidism induction, oestrous cycle phase of the female rats were identified (following the methods as outlined above). Only rats with three consecutive regular cycles were used. Rats at proestrus phase were cohabited with healthy adult fertile male rats of the same strain at 1:1 ratio. Vaginal smear was performed the following morning in order to detect the presence of sperm, which was denoted as pregnancy day-1 (GD-1). GD-1 rats were divided into five (5) groups with six (6) animals per group as below. Thyroxine (T2376, Sigma, Aldrich, St. Louis, USA), dissolved in corn oil (Sigma-aldrich, Germany) was injected subcutaneously at the neck scruff of these rats as shown in Figure 3.3.

P : Euthyroid pregnant rats

HP : Hypothyroid pregnant rats, non-thyroxine treated (control)

HP20 : Hypothyroid pregnant rats, receiving thyroxine at 20µg/kg/day

HP40 : Hypothyroid pregnant rats, receiving thyroxine at 40µg/kg/day

HP80 : Hypothyroid, pregnant rats received thyroxine at 80µg/kg/day

Thyroxine doses were selected based on the previous reported doses (Michael *et al.*, 1991).

For peri-implantation study, treatment was given for three (3) consecutive days (day 1 till day 3) and the rats were sacrificed at day 4 (GD-4). Treatment was continued until day 5 for implantation study and rats were sacrificed at day 6 (GD-6).

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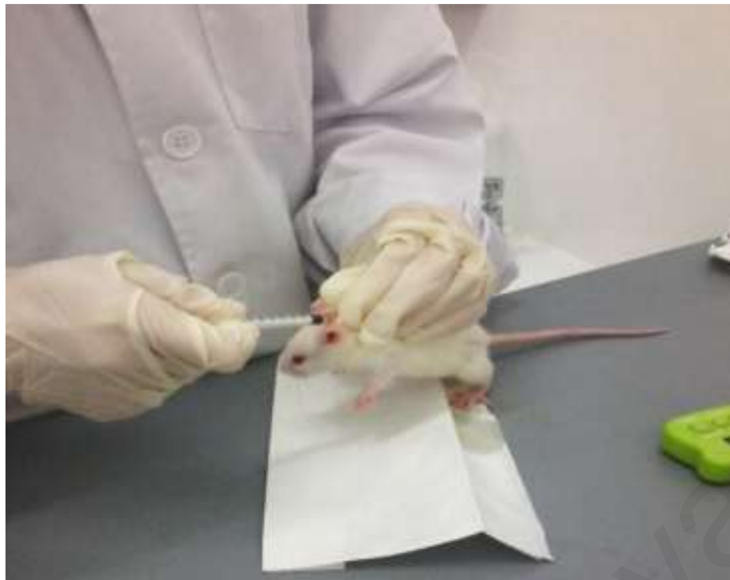

Figure 3.3 Drugs were administered subcutaneously behind the neck scruff

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(a) **Preparation of Different Thyroxine doses**

First stock solution (ST) of thyroxine was prepared by using corn oil as solvent. Then the stock solution was diluted with different volumes of corn oil to prepare three doses of thyroxine in three injection bulks (see figure 3.4). From the injection bulks, 0.1 cc was injected with 1cc syringe once a day.

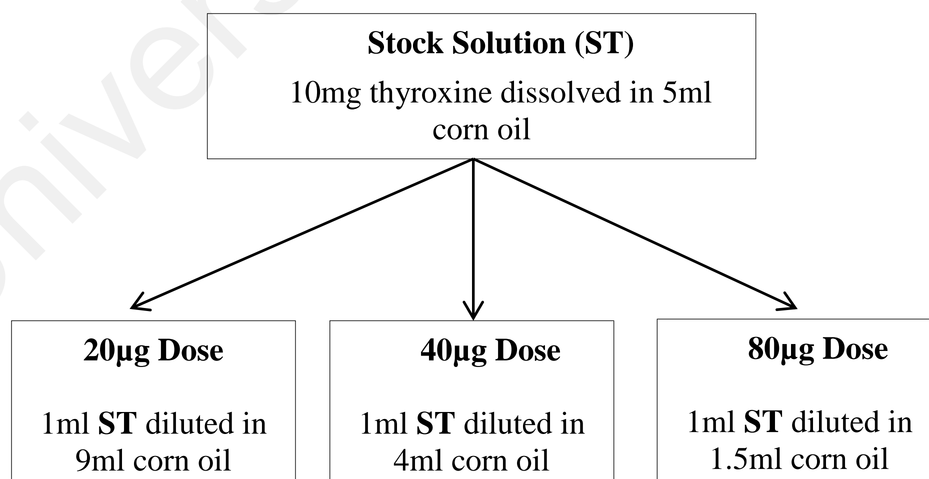

Figure 3.4 Preparation of thyroxine doses

3.2.1.3 Detection and counting of embryo implantation sites

Rats on day 6th of pregnancy were anesthetized and injected with 1 ml Chicago Sky Blue Dye (Sigma Aldrich, Dorset, UK) intravenously via tail vein. Sodium chloride solution (0.85%) was used to dissolve the dye. After 20 minutes, rats were sacrificed by cervical dislocation and the uteri were collected. The dye turned areas of high vascularity into blue stained areas including the embryo implantation sites (figure 3.5).

Figure 3.5 Chicago Sky Blue Dye staining of implantation sites in uterus

Images of implantation sites (blue-stained spots) and intermediate spaces between implantation sites (unstained) in uterus were captured and number of implantation sites was counted.

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3.2.1.4 Hematoxylin and eosin staining of the uterus

To study the uterine morphology during peri-implantation and implantation, staining by hematoxylin and eosin (H&E) was done. In brief, the prepared polylysine slides containing uterine sections from each experimental group were selected and dewaxed by three changes of xylene. Sections were then hydrated by serial changes of ethanol in decreasing concentrations prior to immersion in distilled water and stained with hematoxylin. Sections were washed with tap water to remove excess hematoxylin and then briefly with acid alcohol for few seconds to remove excess background staining.

Bluing of sections was done by using tap water. This changes the color of the nucleus from reddish purple to crisp blue/purple before being stained with eosin. Eosin stains the cytoplasm and connective tissues while hematoxylin stains the nucleus. Sections were again dehydrated by using increasing concentrations of ethanol and further cleared from any water by xylene. Slides were left to dry prior to being mounted and covered with a mounting media followed by a coverslip. H&E was performed to evaluate the uterus for further immunofluorescence study in peri-implantation rats (Figure 3.6). H&E staining follows the methods as described by (Giribabu *et al.*, 2016).

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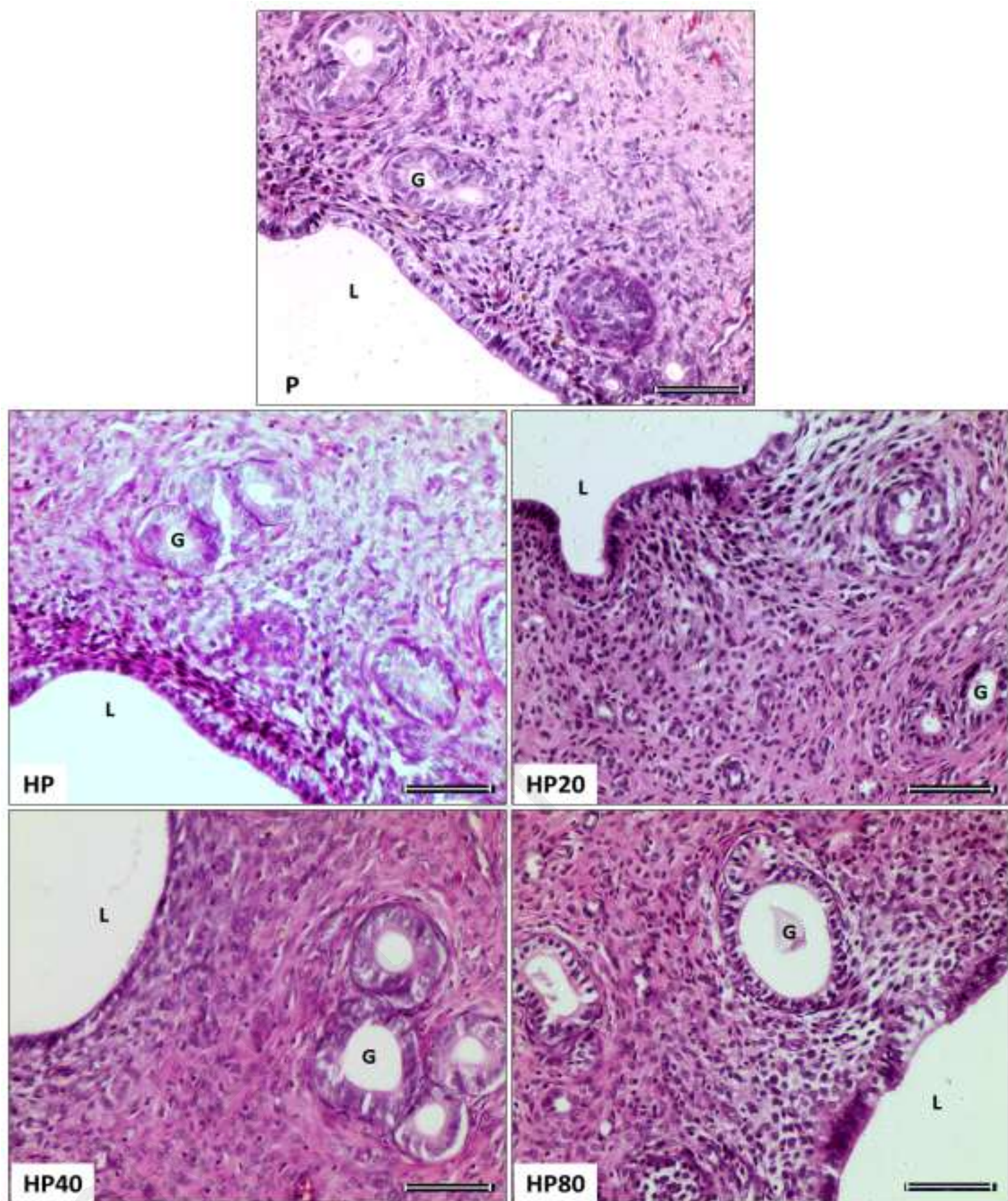

Figure 3.6 Representative H&E images for immunofluorescence analysis in peri-implantation rats.

Scale bars represent 50 µm.

3.2.2 Methods for Study 2

3.2.2.1 Anesthesia

Animals were given intramuscular injection of Ketamine 80mg/kg in combination with xylazine (a sedative) 8mg/kg. Weight of each rat was measured prior to anesthesia. After anesthetic injections, animals were placed immediately on a heat pad to avoid a fall in the body temperature.

3.2.2.2 Bilateral Ovariectomy to eliminate endogenous sex-steroids

Bilateral ovariectomy is a procedure where both ovaries are removed from pelvis. This procedure is performed to eliminate the effect of endogenous sex steroids, prior to specific treatment regimes. Shortly after anesthesia, animals were assessed for anesthetic effects by checking the plantar reflexes. When animals were confirmed to be under complete anesthesia with the breathing and body temperature maintained, the procedures were started immediately. Firstly, area near to the both side of the flanks were shaved under strict sterile measures. A small dorsal midline incision was made on the side of flanks, using sharp scissor. After incising the skin, connective tissues and muscles were exposed. Muscles were cut open, peritoneal cavity was exposed by cutting through the peritoneum. Ovaries were located by identifying the distribution of retroperitoneal fat pads. Ovaries with the retroperitoneal fat were grasped by using blunt forceps. In brief, surgical procedures during ovariectomy are shown in Figure 3.6.

Before removing the ovaries, a hemostat was placed between the oviduct and the uterus to prevent excessive bleeding. Ovaries were removed by using cutting the end of oviduct with a sharp scissor. Then hemostat was removed and uterus was returned into the abdominal cavity. Muscles and skin incisions were sutured by using curved needles of with absorbable polyglactin (Vicryl 910) sutures (muscles) and non-absorbable

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sutures (skin) respectively. After surgery, animals were given intramuscular injection of 0.1 ml of Kombitrim antibiotic to prevent post-surgical wound infection.

Figure 3.7 Surgical procedures during ovariectomy

(A) Skin area over flank area was shaved. (B) Area over shaved skin surface was approached. (C) Incision through the muscle layers was made to reach the retroperitoneal fat. (D) Ovary was pulled through the muscle incision and ligature was released.

3.2.2.3 Identification of oestrous cycle

Oestrous cycle comprises of four phases which are proestrous, estrous, metestrous and diestrous which occur at particular interval of time which can be identified by microscopic analysis of the vaginal smear. In this study, identification of oestrous cycle was done to select rats at estrous and diestrous phases. Vaginal secretion was collected every morning by using a pre-filled plastic pipette with 10μL of normal saline. The pipette was inserted into rat's vagina and the saline was flushed. Immediately following flushing, saline plus secretion was retrieved by pipetting. The fluid retrieved was then placed on glass slides and kept for drying at 37ºC in the

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incubator for 15 min. After drying, slides were stained with eosin and hematoxylin stain to view the different cell types. Proportion and orientation of different cell types were used to identify the different phases of oestrous cycle as follows:

(i) Round and nucleated cells which are epithelial cells along with some irregular shaped cells without nuclei called cornified cells were present in proestrous phase,

(ii) Cornified cells were only present in estrous phase of cycle

(iii) Metestrous comprised of same proportion of three different kinds of cells i.e epithelial cells, cornified cells and leukocyte cells.

(iv) Dark blue stained leukocyte cells with round shape were predominantly seen in diestrous phase of the cycle.

Figure 3.8 shows different cell types in the vaginal smear which define different phases of the cycle

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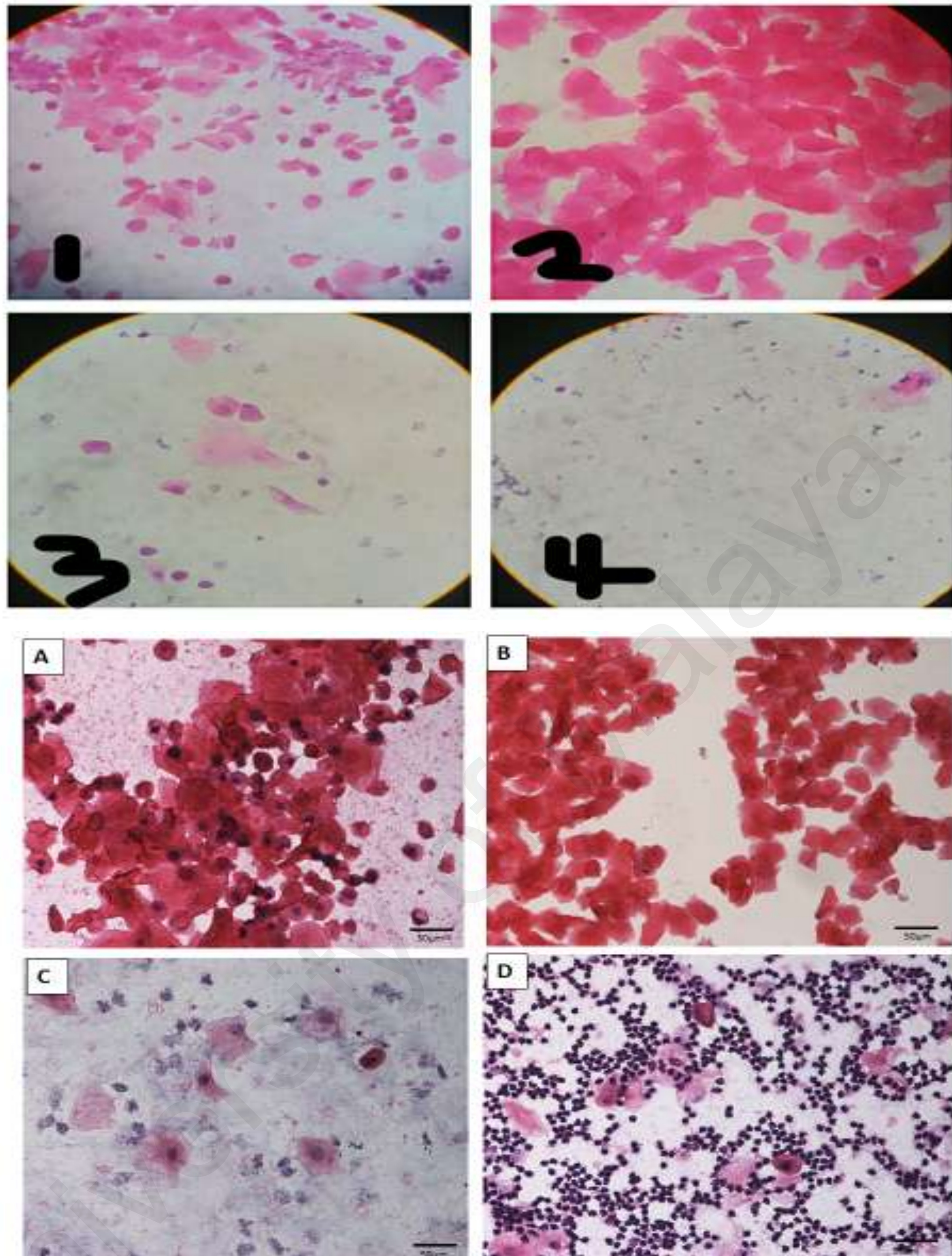

Figure 3.8 Estrous cycle identification based on cell appearance

(1 to 4 and A to D: Different phases of oestrous cycle in rat. 1 A corresponds to proestrous, 2 B= estrous, 3 C = metestrous, 4 D = diestrous. Magnification at 10x (1 to 4) and 40x (A to D), scale bar =50µm. n=48).

3.2.2.4 Animals and hormone treatment

In the second study model, animals were divided into the following groups and received subcutaneous (s.c.) injections for 3 days as follows:

C : Peanut oil only (control)

E2 : s.c. at 1 μg/kg/day

P : s.c. at 4 mg/kg/day

Doses of E2 and P were selected based on the previous reported doses (Salleh N. *et al.*, 2015).

In another cohort of rats, estrus and diestrus phases were identified to study the effects of endogenous estradiol and progesterone respectively. In this study, only rats found to have a three consecutive regular cycle based on the appearance of the cells in the vaginal smear were used. Rats were divided into the following groups following estrous cycle phase identification:

Es : Estrus

Ds : Diestrus

3.2.3 Measurement of serum sex-steroid levels

Serum sex-steroids levels were measured in both study 1 and study 2 prior to sacrifice. Blood samples were collected through cardiac puncture by using 1ml syringe 22G needles. Collected blood was transferred to new clean tube and kept at room temperature for 15 min to allow clot. The collected clotted blood was centrifuged for 15 min at 5000 rpm to separate clear fluid (serum) from blood. Clear serum was gently separated and collected into a new labeled autoclaved sterilized tube by careful pipetting in order to prevent mixing with RBCs. Samples were kept and stored at -20ºC. Levels of estradiol and progesterone were measured by using enzyme-linked immunoassay

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(ELISA) technique. ELISA procedures follow the guidelines as stated by the product sheet from the manufacturer (Cayman Chemical-USA, Estradiol ELISA kit-582251 and Progesterone ELISA kit-582601).

Table 3.3 shows the measured levels of serum sex-steroids following thyroxine treatment at Day 4 pregnancy during peri-implantation period from the study 1. Serum estradiol levels were not significantly changed following thyroxine treatment in hypothyroid pregnant rats (table 3.3). However, the levels of progesterone were reduced following thyroxine treatment, as compared to the non-treated hypothyroid pregnant rats ($p<0.05$).

Table 3.3 Serum levels of estradiol and progesterone following thyroxine treatment at Day 4 pregnancy (Peri-implantation period)

Treatment	Estradiol (pmol/L)	Progesterone (nmol/L)
P	79.67±9.02	182.83±2.12
HP	71.50±16.2	197.85±1.91*
HP20	65.00±5.66	155.30±9.48**
HP40	61.50±12.73	163.50±3.54**
HP80	59.00±11.31	170.80±12.45**

Abbreviation- HP: *$p < 0.05$ compared to P. **$p < 0.05$ compared to HP. Data were expressed as mean ± S.D. with n = 6 rats per group. P- Intact euthyroid pregnant rats, HP- hypothyroid pregnant rats, HP20- hypothyroid pregnant rats receiving 20µg/kg/day thyroxine, HP40- hypothyroid pregnant rats receiving 40µg/kg/day thyroxine, HP80- hypothyroid pregnant rats receiving 80µg/kg/day thyroxine.

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Table 3.4 shows the measured levels of serum sex-steroids following thyroxine treatment at Day 6 pregnancy during implantation period from the study 1. In hypothyroid pregnant rats, serum estradiol levels were not significantly change following thyroxine treatment (table 3.4). However, in these rats, the levels of progesterone were reduced following thyroxine treatment ($p<0.05$).

Table 3.4 Serum levels of estradiol and progesterone following thyroxine treatment at Day 6 pregnancy (Implantation period)

Treatment	Estradiol (pmol/L)	Progesterone (nmol/L)
P	75.52 ± 8.05	151.28 ± 11.18
HP	68.67 ± 8.02	250.50 ± 12.16*
HP20	74.67 ± 6.03	200.95 ± 9.23**
HP40	66.83 ± 8.25	219.82 ± 10.12**
HP80	72.93 ± 9.10	218.87 ± 8.27**

Abbreviation- *$p < 0.05$ compared to P. **$p < 0.05$ compared to HP. Data were expressed as mean ± S.D. with $n = 6$ rats per group. P- Intact euthyroid pregnant rats, HP- hypothyroid pregnant rats, HP20- hypothyroid pregnant rats receiving 20µg/kg/day thyroxine, HP40- hypothyroid pregnant rats receiving 40µg/kg/day thyroxine, HP80- hypothyroid pregnant rats receiving 80µg/kg/day thyroxine.

Table 3.5 shows the measured levels of serum sex-steroids following subcutaneous injection from the study 2. Serum levels of E2 were increased by approximately two fold, three days after E2 treatment (table 3.5). Serum levels of P increased by three folds following three days of 4 mg/kg/day P treatment.

Table 3.5 Serum levels of sex-steroids following subcutaneous injection

Treated samples	Groups	Hormone levels (Mean ± SD)
17-β estradiol (E2)	Control	37.6±0.12 pg/ml
	1 µg/kg/day	68.5±0.14 pg/ml
Progesterone (P)	Control	3.97±0.13 ng/ml
	4 mg/kg/day	21.86±0.11 ng/ml

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Table 3.6 shows the measured levels of serum sex-steroids at different phases of oestrous cycle from the study 2. E2 levels were high at Es phase and P levels were high at Ds phase (Table 3.6).

Table 3.6 Serum levels of sex-steroids at different phases of oestrous cycle

Treated samples	Groups	Hormone levels (Mean ± SD)
17-β estradiol (E2)	Estrus phase (Es)	17.71±2.28 pg/ml
	Diestrus phase (Ds)	13.47±2.56 pg/ml
Progesterone (P)	Estrus phase (Es)	12.98±2.53 ng/ml
	Diestrus phase (Ds)	23.46±2.81 ng/ml

3.2.3.1 Principle of the ELISA

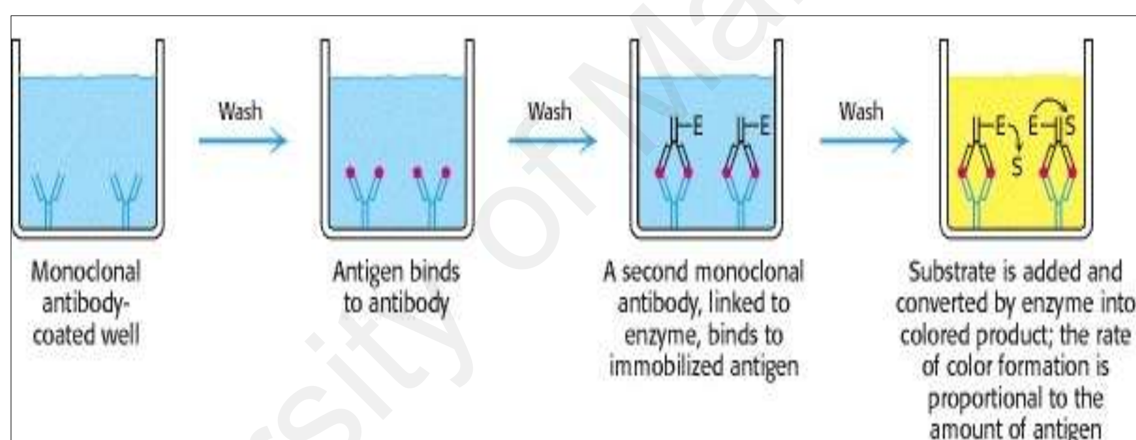

Figure 3.9 Principle of ELISA

Target antigen can be detected by this ELISA technique. In this technique, antibody is coated on the microtiter well. A sample containing antigen is added to the well and allowed to react with the antibody attached to the well, forming antigen-antibody complex (see figure 3.9). After the well is washed, a second enzyme-linked antibody specific for a different epitope on the antigen is added and allowed to react with the bound antigen. After that, unbound secondary antibody is removed by washing. Finally substrate is added to the plate which is hydrolyzed by enzyme to form colored products.

3.2.4 Uterine histological procedures

3.2.4.1 Preparation of paraffin (wax) block and morphological analysis

A day after the last drug treatment, rats were humanely sacrificed and uterine horns were removed and fixed in 4% paraformaldehyde (PFD), at 4°C for 4 to 5 hrs. Tissues were then processed through different grades of ethanol, incubated overnight in chloroform, transferred into paraffin wax for 3 hrs, placed into molds prefilled with melted wax and cooled immediately at -60 ^{o} C to harden the wax. Tissues were cut into 5 µm sections and mounted onto glass slides. The slides were then stained with hematoxylin and eosin (H&E), visualized under a light microscope under magnifications of 4×, 20 × and 40 ×. The circumferences of uterine lumen were measured by using NIS-Elements AR program. All images were captured by using a Nikon Eclipse 80i that was attached to a light microscope (Olympus, Japan). Histological procedures follow the methods as described by (Giribabu *et al.*, 2016).

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(a) ***Basic steps involved in the preparation of histological specimen***

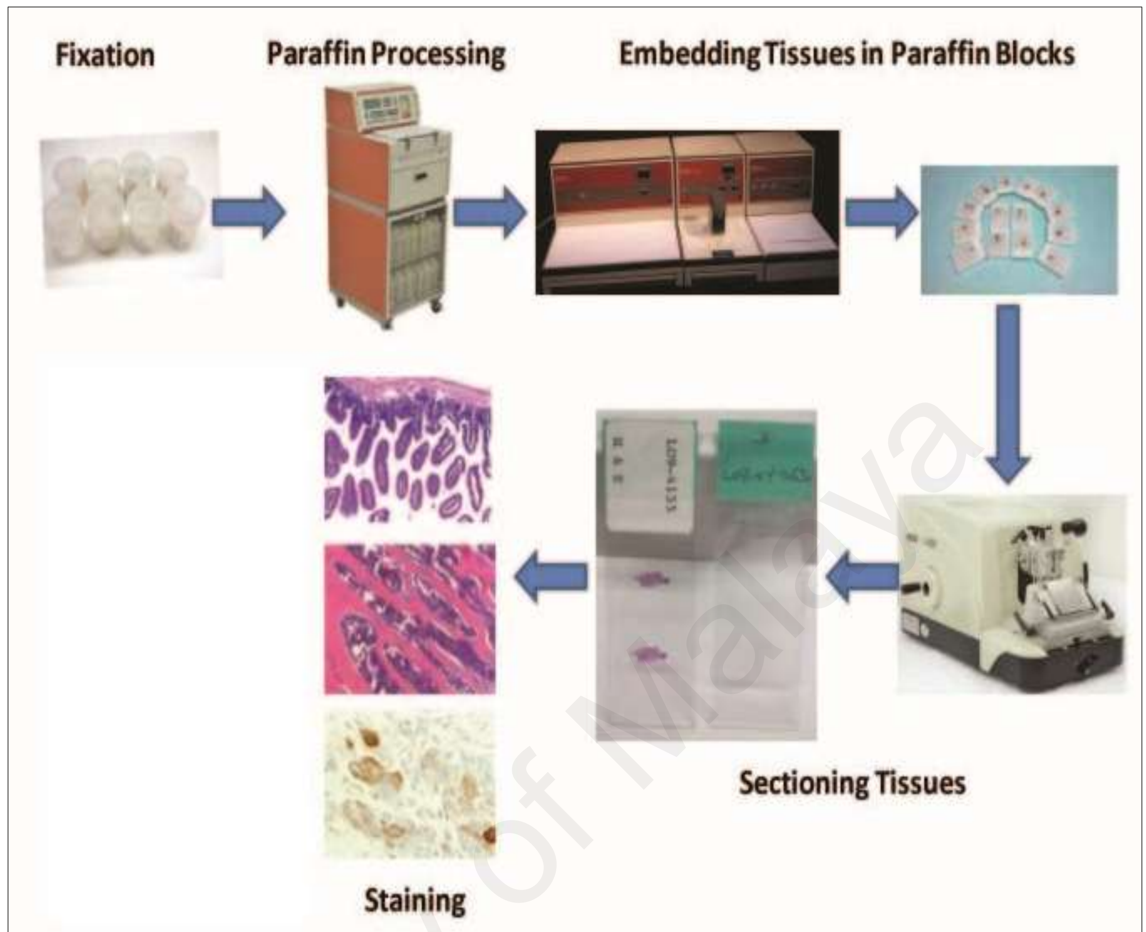

Figure 3.10 Flow chart of tissue processing & sectioning

Preparation of histological specimen starts with tissue processing. Tissue processing describes the steps required to take animal tissue from fixation to the state where it is completely infiltrated with a suitable histological wax and can be embedded ready for section cutting on the microtome. Tissue processing involves following steps before going to tissue sectioning;

- Obtaining a fresh specimen
- Fixation
- Paraffin Processing (Dehydration, Clearing and Wax infiltration)
- Embedding or blocking out

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After the preparation of blocks or embedding units, microtome machine is used for sectioning the tissue blocks prior to perform immunohistochemistry or immunofluorescence (see figure 3.10).

3.2.4.2 Immunohistochemistry

Tissue was embedded by formalin fixed paraffin and sections were made at 5µm thickness. Before the slides were stained, hydration was carried out by using decreasing grades of ethanol. Antigen retrieval was performed by boiling the slides into antigen retrieval solution for 20 minutes and cooling for 20-30 minutes. After antigen retrieval, sections were incubated with primary antibody diluted in 1.5% blocking serum for one hr at room temperature. Sections were then incubated with biotinylated secondary antibody for 30 min at approx. 1µg/ml. After washing with PBS, slides were incubated with AB Enzymes for 30 min. The sites of antibody binding were visualized by DAB (DiaminobenzidineHCl) (Santa Cruz, US) staining, which gave dark-brown stains. Stained sections were washed in de-ionized water for five min and were counter-stained with haematoxyllin for 5-10 sec. After staining, paraffin embedded sections were dehydrated in decreasing grades of ethanol 10 sec each. The stained slides were then mounted with 1-2 drops of DPX mounting media with a glass cover slip. Immunohistochemical procedures were shown in table 3.7. Immunohistochemistry follows the methods as described by (Giribabu *et al.*, 2016).

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Table 3.7 Immunohistochemistry staining procedure

PROCESS STEPS	PROCEDURES
Deparaffination	3 changes in xylene for 5 minutes each.
Rehydration	Tissue rehydration with decreasing ethanol grades 100% and 95% twice each for 10 minutes.
Antigen retrieval	Preparing 10mM sodium citrate buffer,pH 6.0 Slides immersed in sodium citrate buffer container and heated for 10minutes at 50ºC.
Blocking of endogenous peroxidase	Sections were incubated with 0.1% hydrogen peroxidase diluted in PBS for 30minutes.
Blocking serum	Sections were incubated in 1.5% blocking serum in PBS for 1 hour.
Primary antibody	Sections were incubated with primary antibody of interest diluted in 1.5 % blocking serum overnight.
Secondary antibody	After washing with PBS, sections were incubated with biotinylated secondary antibody for 30 minutes.
AB enzyme reagent	After washing sections were incubated with AB enzyme reagent for 30 minutes
Peroxidase substrate staining	Sections were incubated with 1-3 drops of peroxidase substrate until suitable staining developed.
Counter staining	Sections were counter stained with hematoxylin for 10 seconds
Dehydration	Sections were dehydrated with increasing grades of ethanol 95% and 100% twice each for 10 seconds.
Mounting	1-2 drops of permanent mounting media was applied and covered with glass cover slips.

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(a) **Basic procedures and principles of Immunohistochemistry**

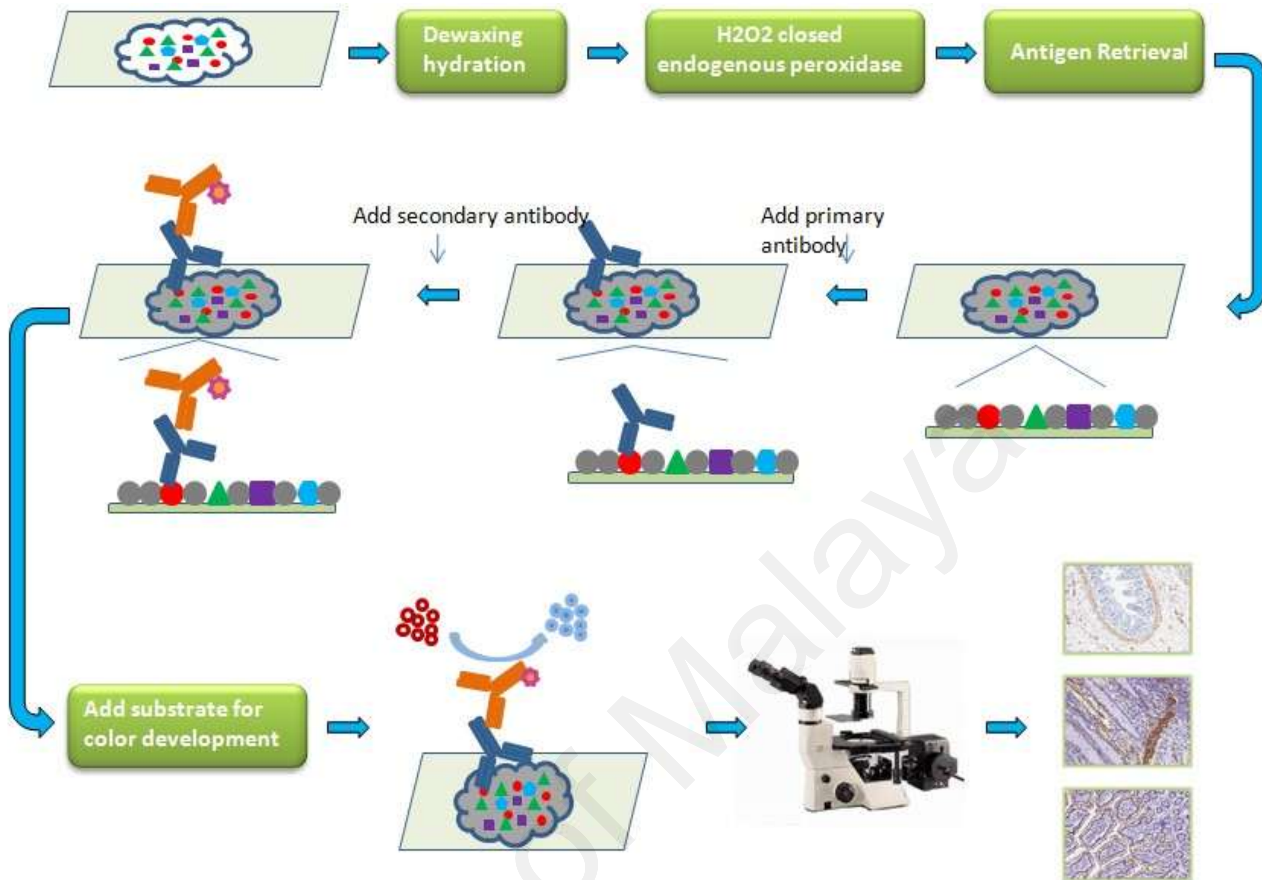

Figure 3.11 Principle of immunohistochemistry

Immunohistochemistry (IHC) is a widely used biological technique that combines anatomy, physiology, immunology and biochemistry. Based on the antigen-antibody binding reaction, IHC can be considered as a method that visualizes distribution and localization of specific antigen or cellular components in tissue sections. Figure 3.11 shows the principle involved in the immunohistochemical procedures. Major components in a complete immunohistochemistry experiment:

i) Primary antibody binds to specific antigen in the tissue embedded on the slides,

ii) The antibody-antigen complex is formed by incubation with a secondary antibody which is enzyme-conjugated antibody,

iii) With presence of substrate, the enzyme catalyzes to generate colored deposits at the sites of antibody-antigen binding,

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iv) Color detection of the targeted antigen or protein distribution is done by the microscopic observation.

3.2.4.3 Immunofluorescence

Blocking was done with 10% blocking serum diluted In PBS for one hour to suppress non-specific binding. Blocking serum is ideally derived from the same species in which secondary antibody is raised. Sections were then incubated with primary antibody diluted in 10% blocking serum diluted in PBS for one hr. Washing was done to remove the unbound excess labeled antibodies and incubated with florochrome conjugated secondary antibody diluted in 1-5 µg/ml PBS with 10% normal blocking serum for 45 min in dark chamber. After washing, Ultra-cruz mounting media was applied and signals were visualized under florescent microscope. Sites of antibody bindings appeared fluoresce green. Table 3.8 shows the immunofluorescence procedures. Immunofluorescence follows the methods as described by (Giribabu *et al.*, 2016).

Table 3.8 Immunoflorescence standard staining procedure

PROCESS STEPS	PROCEDURE
Deparaffination	3 changes of xylene for 5 minutes each.
Rehydration	Washing sections in 100% ethanol 10 minutes each twice. Washing in 95% ethanol 10 minutes each twice.
Antigen unmasking	Slides immersed in a container containing sodium citrate buffer with 6.0 pH. Heating for 10 minutes at 50ºC for 10 minutes.
Blocking serum	Sections incubated with 10% blocking serum diluted in PBS for 1 hour. Blocking serum was used from same species from which secondary antibody is derived.
Primary antibody	Sections incubated with primary antibody diluted in 10 % serum for overnight.
Florochrome conjugated Secondary antibody	After washing with PBS, sections were incubated with florochrome conjugated secondary antibody for 1 hour in dark chamber.
Mounting media	After washing with PBS, sections were immediately mounted using ultra cruz mounting media and covered with cover slips.

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3.2.5 Quantitative Real time qPCR (qPCR)

Specific fragments of DNA can be amplified through polymerase chain reaction (PCR). Real time PCR is a sensitive technique that can detect gene expressions in the real time during amplification process as it occurs. Fluorescent probes are used to measure amplification of DNA in real time PCR. In this study, real Time PCR system was used to assess gene expression with application of highly sensitive ViPrimePlus qPCR Green Master Mix with ROX (Oceanside, CA, USA).

3.2.5.1 Sample collection and RNA extraction

Whole uterine tissues were washed with PBS and kept in five volume of RNA Later (Ambion, USA) solution prior to RNA extraction. Tissues were kept and stored (-20ºC) in RNA Later solution for preventing damage to tissue cellular RNA. Total RNA extraction was done using RNeasy Fibrous tissue Mini kit QIAGEN, Germany. Uterine tissues were retrieved from RNA Later solution and were soaked in lysis buffer RLT Plus with added β-mercaptoethanol. Uterine tissues in buffer solution were homogenized using a rotor-stator TissueRuptor (230V, 60Hz QIAGEN, Germany). Tissue and buffer homogenate was transferred to new 1.5 ml eppendoff tube after centrifuge at 20ºC for 3 minutes.450 µl of pure ethanol was then added to clear lysate by mixing through pipette gently. After gentle mixing 700µl of homogenate was transferred to RNeasy Mini spin column placed in a new 2ml collection tube provided. This step is usually repeated two times for each sample of ethanol and RLT plus homogenizer until all volume is transferred to RNeasy Mini spin column. Flow through was discarded each time. Mixture of 10µl DNase I stock solution with 70µl RDD buffer was then added directly to RNeasy spin column after washing with RW1 buffer, to remove DNA residuals. RNeasy Mini spin column was then incubated at room temperature for 20 minutes.500µl of RPE washing buffer was used for washing membrane twice. After washing RNeasy spin column was placed in a new 1.5ml

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collection tubes and 30µl RNase free water was added. Total RNA was collected by centrifuging RNeasy Mini spin column for one min. RNeasy Mini spin column was discarded and flow through was saved.

3.2.5.2 Quality control of extracted RNA

Isolated RNA can be checked for quality control by determining the quantity, purity and integrity of the RNA. Quantification of nucleic acid is traditionally done using UV absorption a spectrophotometer. The absorbance is measured at 260 and 280 nm in simplest form. An A260 reading of 1.0 is equivalent to about 40 µg/ml of RNA and the OD at 260 nm is used to determine the RNA concentration in a solution. Ratio of the absorbance at 260/280nm were used to asses RNA purity. RNA concentration and purity was measured using Nano Drop 2000, UV-vis Spectrophotometer, UK by 260/280 UV absorption ratios. Pure RNA has an A260/A280 of 2.1.Value of 1.8-2.0 ate also acceptable and indicates that the RNA is pure.

Integrity of RNA can be affected either by insufficient nucleic acid extraction or exogenous source. Assessment of each isolated RNA sample integrity was done using Ethidium bromide 1% standard agarose gel electrophoresis in TBE buffer. Gel electrophoresis was ran at 90 V for 45 minutes and was observed in gel documentation system for its 18S and 28S ribosomal RNA bands. Figure 3.12 shows agarose gel electrophoresis

Figure 3.12 RNA Agarose gel electrophoresis

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3.2.5.3 Conversion of RNA to cDNA

After extraction and isolation of total RNA, RNA was converted to cDNA prior to running Real time PCR. Reverse transcription to cDNA was done by using High Capacity qPCRBIO cDNA synthesis kit (PCR Biosystems, London, UK). A 20µl reaction generated single stranded cDNA by adding up to 9µl of RNA (1000ng), 10 µl of RT buffer and 1µl of RT enzyme. Reaction was ran in thermal cycler program which included incubation for 37°C for 60 min, heating to 95°C for 5 min and hold in 4°C for conversion to reverse transcriptase (Kwok & Higuchi, 1989).

3.2.5.4 Running the Real time PCR

Real time PCR experiment was performed to observe the mRNA gene expression levels of the target proteins. In real-time PCR, the amplified region of cDNA was probed with a fluorescence-labeled probe. Expressions of target DNA was ensured using highly specific primers and probes. All probes and primers including reagents were purchased from Applied Biosystem USA. GAPDH was used as housekeeping genes. Target assay was validated *in-silico* by using whole rat genome and *in-vitro* by using whole rat cDNA to ensure that target sequences were detected (Applied Biosystems, USA). All experiments were carried out in triplicates and were pipetted in reaction plate with each reaction combination of 5µl of master mix buffer, 1µl of target primer, 3.5µl of RNeas free water and 1µl (50ng) of cDNA. Reaction plate for running Real time PCR was loaded in Applied Biosystem Step One Plus thermal cycler and was set according to manufacturer guidelines which included 50ºC reverse transcriptase for 2 min, 95°C activation of DNA polymerase for 15 sec, 95ºC denaturation for 1 sec and 60 ºC annealing for 20 sec. Data was analyzed according to the Comparative Ct (2- ΔΔ Ct) method to calculate the fold changes. (Wong & Medrano, 2005). Δ Ct =Ct of target gene- Ct of housekeeping genes.

ΔΔ Ct = Δ Ct of treated samples- Δ Ct of non-treated samples.

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$\Delta\Delta$ Ct = Δ Ct of treated samples- Δ Ct of non-treated samples.

Fold change = Ct (2- $\Delta\Delta$ Ct).

All measurements were normalized using GenEx software (MultiD, Sweden) followed by Data Assist v3 software from Applied Biosystems (USA) which was used to evaluate RNA fold changes. Table 3.9 shows primers used in this study.

Table 3.9 List of primers used in this study

Accession number	Gene	Primer Sequences
NM_001017960.1	TRα1 F TRα1 R	GGCAAGTCACTCTCTGCCTT CCAGAGCGGTCTGTTGACAT
NM_012672.3	TRβ1 F TRβ1 R	CAGGATCCGTGGTTTCCCTC AGCTCTGGCATTCCCTTATTCA
NM_012888.1	TSHR F TSHR R	CCAGCACCCAGACTCTGAAG AGGCTCGAAAAGGCAAGACT
NM_053842.2	ERK F ERK R	GGACGACTTACCTAAGGAGAAGC GAGCCCTTGTCCTGACCAAT
NM_031528.2	RAR F RAR R	CTGTTTGCTCCAGGAGAAGGGG TCTCGATGGAGTGGTTTGAGCC

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3.2.5.5 Principles of Real-Time PCR using SYBR Green chemistries

(a) **Basic principles of PCR**

PCR is a method to amplify, or increase, the amount of a specific DNA sequence. Typically, the target DNA sequence is amplified using a solution containing DNA polymerase and nucleotides, and primers that are complementary to the target DNA sequence. When this solution is heated, the double-stranded DNA (dsDNA) denatures, separating into two separate strands. As the solution cools, the primers anneal to the target sequences in the separated DNA strands. The DNA polymerase then forms a new strand by extending the primers with nucleotides, creating a complimentary copy of the target DNA sequence. When repeated, this cycle of denaturing, annealing, and extending increases exponentially the number of target DNA sequences (see figure 3.13). Ideally, no amplification occurs if the target DNA sequence is not present.

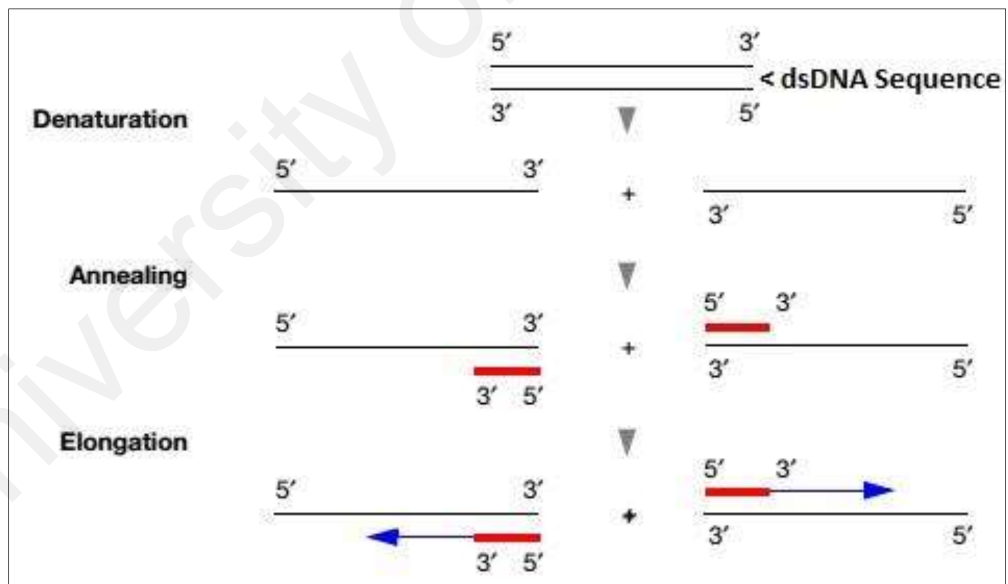

Figure 3.13 Amplification of a specific DNA sequence in PCR

(b) **SYBR Green dye fluorescence detection of PCR amplification**

The SYBR Green chemistry is a method for performing real-time PCR analysis.

The dye present in SYBR Green master mix binds the minor groove of double-stranded

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DNA (dsDNA) during the elongation step of PCR system (see figure 3.14). When the SYBR Green dye binds to dsDNA, then the intensity of the fluorescence increases. As more double-stranded amplicons are produced by repeating the PCR cycle, SYBR Green dye fluorescence increases more. SYBR Green dye binds to any double-stranded DNA molecule. Detection is monitored by measuring the increase in fluorescence throughout the cycle.

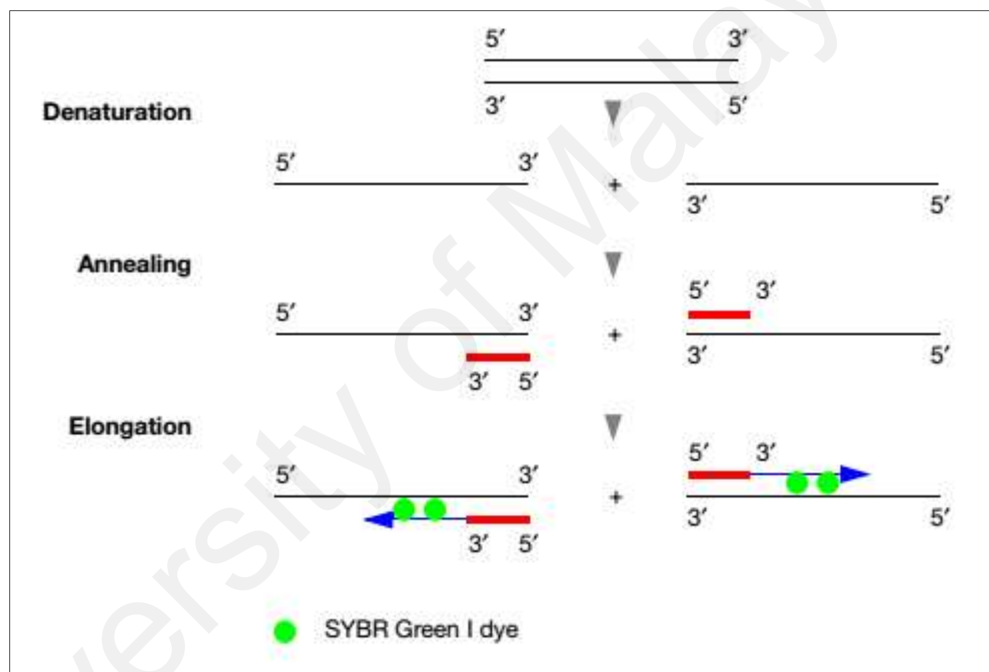

Figure 3.14 Principles of PCR using SYBR Green chemistries

3.2.6 Western Blotting

3.2.6.1 Sample collection and protein extraction

Uterine tissues were exposed and immediately kept in liquid nitrogen -196 ºC and then stored at -80ºC for protein extraction step. Proteins from all kind of cells and tissues can be extracted by PRO-PREP solution kit with very high yield purified proteins. Total proteins from uterine tissue were isolated by using PRO-PREP (Intron, UK) solution kit. It is a simple method in which uterine tissues of 30mg (wet weight)

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were loaded into appropriate tube containing 400µl of PRO-PREP solution. Uterine tissue with PRO-PREP solution was then homogenized with ultrasonic cell disruptor (Branson) for 5-6 min. After homogenization, samples were incubated for 20 mins in ice for induction of cell lysis. Tissue homogenate were then centrifuged for 15 min at 4ºC at 13000 rpm. Supernatant was collected and stored in -20ºC.

3.2.6.2 Protein quantification

Extracted protein samples were quantified for protein concentration by using Micro BCA Protein Assay kit (Thermo Scientific-USA). BCA assay kit which is sensitive and not affected by compositional protein differences as compared to other dye binding methods. BCA kit formulation is detergent compatible bicinchoninic acid, which is sensitive for colorimetric detection. BCA kit contains, Micro BCA reagent A (MA), reagent B (MB), reagent C (MC) along with bovine serum albumin in standard ampules of 2.0mg/ml. 40, 20, 10, 5, 2.5, 1, 0.5 and 0 µg/ml of standard BSA concentrations were prepared and extracted protein samples were diluted 1 in 50 with PBS buffer. Standard serial dilutions 50µl, protein sample dilution in PBS 50µl and BSA reagents 50µl were loaded inGreiner UV 49 transparent 96 well plate (Thermo ScientificTM-US) as a serial dilution. All reaction for standard dilution and samples were loaded in triplicates. Loaded 96 well plates were sealed and incubated at 37ºC for 2 hours. Protein quantification was measured with intensity of color changed using Multi Mode plate reader at 570nm wavelength. Standard curve was obtained and sample concentrations were calculated from a linear plot of BCA standard concentration (X-Axis) with that of intensity of color changed in absorbance. Figure 3.15 show the standard curve for protein quantifications.

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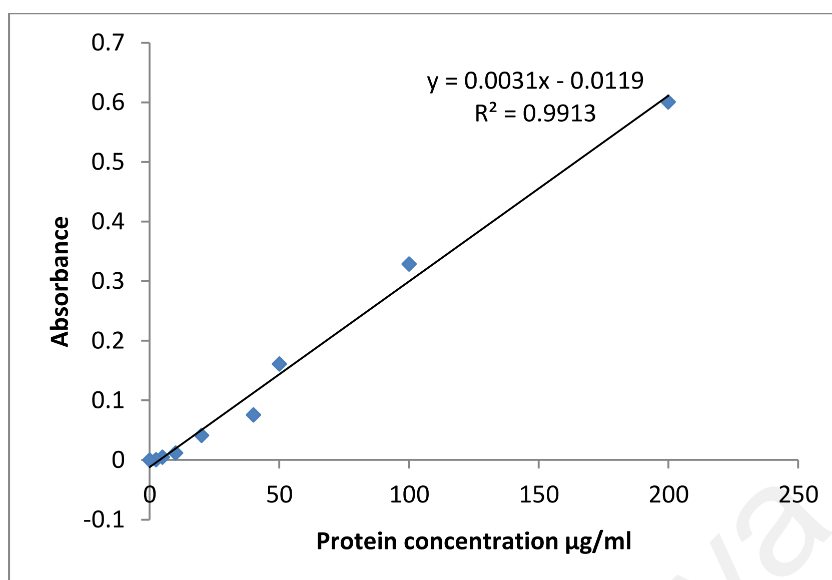

Figure 3.15 Standard curve for protein quantification

3.2.6.3 Running the Western blot

(a) **SDS gel preparation**

SDS –PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis, is a technique for separation and detection of proteins. Within the polyacrylamide gel, proteins move towards negative anode in an electric field. Determination of electrophoretic protein mobility is made by proteins heating step and detergent SDS. Samples were separated within the gel according to their molecular weight. The SDS PAGE gel run can be divided into staking gel and resolving gel**.** Stacking gel with pH 6.8 was poured on top of the resolving gel of pH 8.8 (after solidification) and a gel comb was inserted in the stacking gel. For this study two types of gel % were used i.e 10% and 12%. Acrylamide concentration in SDS PAGE determines different gel percentage and used for target proteins of various molecular weight in the sample. For determination of high molecular weight proteins low percentage SDS PAGE gel (10%) were used and for low molecular weight high percentage gel (12%) were used. Table 3.10 shows the different gel compositions.

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Table 3.10 Table shows composition of SDS PAGE Resolving and Stacking gel

Chemicals	Resolving gel 10% (µl) 10ml	Resolving gel 12% (µl) 10ml	Stacking gel 4% (µl) 5ml
ddH_2O	3800	3200	2975
30% Acrylamide	3400	4150	670
1.5M Tris (pH8.8)	2600	2600	-
0.5M Tris (pH6.8)	-	-	1250
10% SDS	100	100	50
10% APS	100	100	50
TEMED	10	10	5

After gel preparation, samples were prepared with loading dye and were heated for 5 minutes at 95ºC. Prepared samples were loaded into the wells of stacking gel after loading of prestained protein ladder in first lane. Gel cast was covered and connected to anodes. Gel caster was then run at appropriate voltage for recommended time until dye reached at the bottom of gel.

(b) **Protein transfer**

After SDS PAGE electrophoresis, proteins are transferred from SDS PAGE to PVDF membrane (Biorad, UK). Gel caster was opened and stacking gel were removed. Resolving gel was removed carefully from glass plates and were placed in a container containing distilled water for 5 minutes in a shaker. PVDF membrane were soaked in pure methanol for 2 min for activation and then were soaked in two buffer for 5 min. To prepare gel for blotting, activated membrane were sandwiched between gel and absorbent Whatman filter paper on blotting cassette. Blotting cassette were clamped along with membrane sandwiched, loaded in wet transfer tank for electro blotting at 100V for 120 minutes.

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(c) **Membrane blocking**

After completion of electro blotting, membrane was removed from gel sandwich and washed with PBST buffer for 5 minutes. Membrane was then incubated with 5% BSA Bovine serum albumin for one hour at 4ºC.

(d) **Antibodies incubation**

Membrane was incubated with primary antibody at 4ºC overnight. Dilution of primary antibody was done in PBST buffer with 1:1000 ratio. Next day, membrane was incubated with secondary antibody horseradish peroxidase HRP diluted in 1:2000 for one hour after washing with PBST buffer, thrice.

(e) **Visualization**

Clorometric (HRP) substrate Opti- 4CN Kit is used for detection and visualization of target proteins. Appearance of proteins bands were viewed as it appears on the membrane due to colorimetric substrate in the kit solution.

(f) **Western blot analysis**

Target protein bands were captured by gel documentation system (Vilber Lourmat, from Fisher Scientific, USA) and density of each band was measured by Image J software (National Institutes of Health, Bethesda, MD, USA). Expression levels of target proteins with different treatment groups were calculated over housekeeping protein bands. Table 3.11 shows the primary and secondary antibodies.

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Table 3.11 Primary and secondary antibodies in Western blot and Histology

Target gene	Primary antibody	Secondary antibody
TRα1	Goat polyclonal IgG	Donkey anri-goat IgG HRP, FITC
TRβ1	Goat polyclonal IgG	Donkey anri-goat IgG HRP, FITC
TSHR	Rabbit polyclonal IgG	Donkey anti-rabbit IgG-HRP,FITC
VDR	Mouse monoclonal	Goat anti-mouse IgG- HRP,FITC
RAR	Rabbit polyclonal IgG	Donkey anti-rabbit IgG-HRP,FITC
RXR	Rabbit polyclonal IgG	Donkey anti-rabbit IgG-HRP,FITC
ERK 1/2	Mouse monoclonal	Goat anti-mouse IgG- HRP,FITC
AQP1	Rabbit polyclonal IgG	Donkey anti-rabbit IgG-HRP,FITC
AQP5	Goat polyclonal IgG	Donkey anti-goat IgG-HRP,FITC
AQP7	Rabbit polyclonal IgG	Donkey anti-rabbit IgG-HRP,FITC
Integrinαvβ3	Mouse monoclonal	Goat anti-mouse IgG- HRP,FITC
E-cadherin	Mouse Monoclonal	Goat anti-mouse IgG- HRP,FITC
Msx-1	Goat polyclonal IgG	Donkey anti-goat IgG-HRP,FITC
Ihh	Goat polyclonal IgG	Donkey anti-goat IgG-HRP,FITC
GAPDH	Rabbit polyclonal IgG	Goat anti-rabbit IgG-HRP,FITC
βeta Actin	Mouse Monoclonal	Goat anti-mouse IgG- HRP,FITC

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3.2.6.4 Basic procedures and principles of Western Blot

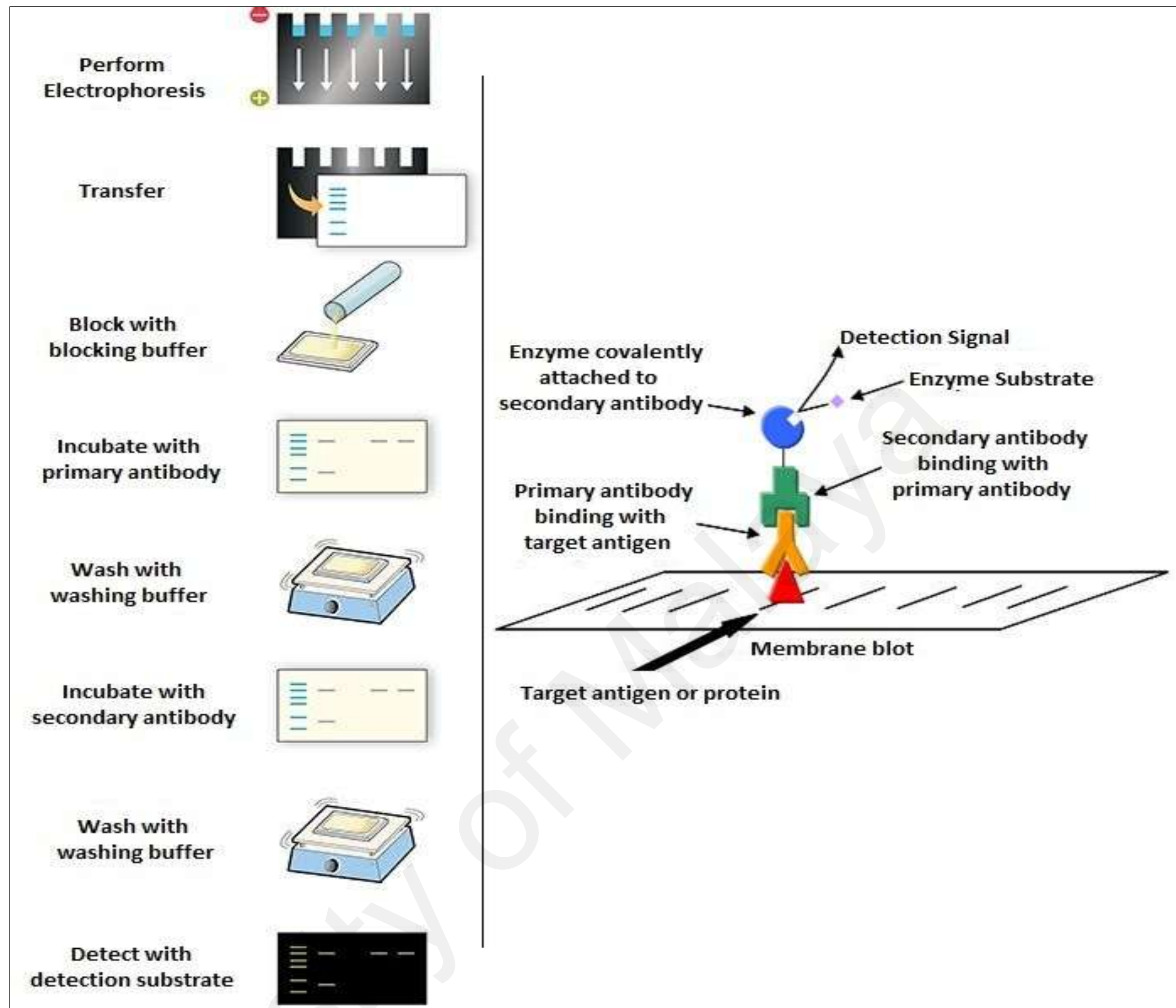

Figure 3.16 Flow chart of basic procedures and principles of western blot

The amount of protein expressed in tissues or cells was quantified by using Western blot, also known as immunoblot or protein blot. This technique gives relative quantity of the protein of interest in the sample and allows the relative quantity of protein expression to be determined, provided that similar quantity of protein lysate in each sample was used for comparison. Western blot comprises of several steps as summarized in figure 3.16. Firstly, protein lysate obtained from cells or tissues were separated by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the protein molecular weight, protein ionic charges and gel concentration (pore size). The separated proteins were then transferred onto a

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Polyvinylidene fluoride (PVDF) membrane and the membrane was consequently blocked to reduce the non-specific bands. Subsequently, the target proteins were incubated with primary and IgG- HRP conjugated secondary antibody and the bands were detected by using a substrate detection reagent. Western blot procedures follow the methods as described by (Giribabu *et al.*, 2016).

3.2.7 Statistical analysis

Well design and presented as the results were presented as mean ± SEM (n=6) expect the histological studied n=4. The statistical method used to analyze the data was unpaired t-test using SPSS statistic 18.0 software. The data were considered statistically significant when $p < 0.05$ with ANOVA one-way to determine effect of each treatment.

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CHAPTER 4: INVESTIGATING THE EFFECTS OF THYROXINE ON EXPRESSION OF PROTEINS RELATED TO THYROID HORMONE FUNCTIONS (TR-A, TR-B, RXR AND ERK1/2) IN UTERUS DURING PERI-IMPLANTATION PERIOD

4.1 Introduction

Successful implantation requires coordinated development of the blastocyst and the uterus which occur during the peri-implantation period. Changes in blastocyst and uterus involve series of cellular and molecular events. During the peri-implantation period, uterus undergoes transformation into a receptive state (Turnbull *et al.*, 1995). It has been known that uterine receptivity development requires coordination by the sex-steroids, particularly progesterone (Okada *et al.*, 2003). Besides, several paracrine factors such as leukaemia inhibitory factors (LIF), prostaglandins and cytokines are also involved (Gao *et al.*, 2015; Morawska-Pucinska *et al.*, 2014).

There have been reports that thyroid hormone also plays an important role in the development of uterine receptivity (Aghajanova L. *et al.*, 2011). Thyroid hormone enhances the expansion rate of the blastocoel cavity of cryopreserved bovine embryos in-vitro (Ashkar *et al.*, 2010). Together with the appearance of pinopodes, which are the markers of endometrial receptivity, it has been found that expression of TRα-1, TRα-2, TRβ-1 and TSHR in endometrial cells also increased (Aghajanova L. *et al.*, 2011). Moreover, TRα-1 and TRβ-1 are expressed in glandular and luminal epithelium during mid-luteal phase and secretary phase with increased expression (Aghajanova L. *et al.*, 2011). The mechanisms that lead to increase expression of these proteins in uterus during the uterine receptivity period are currently not fully understood. Down-regulation of TRα-1 and TRα-2, and up-regulation of TRβ-1 in response to the treatment

of anti-progestin RU486 depict the role of sex-steroids, particularly progesterone (Catalano *et al.*, 2007). The role of progesterone on peri-implantation stage is essential for the endometrium-embryo interaction in the primate (Sengupta & Ghosh, 2000). It has been shown that thyroid hormone promotes the growth of trophoblast by stimulating the secretion of progesterone and estrogen during endometrial receptivity (Ohara *et al.*, 2004). Lack of thyroid hormone consequents in reduction of the uterine cells' estrogenic response, resulting in development of reduced endometrial thickness (Inuwa & Williams, 1996b).

We hypothesized that thyroid hormones could also influence the expression levels of thyroid hormone receptors, RXR and ERK1/2 signaling pathway that is involved in thyroid function based on the observations that thyroid hormone up-regulates these receptors in *Xenopus laevis* (Puzianowska-Kuznicka *et al.*, 1997; Scarlett *et al.*, 2008). Therefore this study aims to identify the levels of TRα-1, TRβ-1 and, ERK1/2 and RXR in the absence of thyroid hormone and how these changes following administration of thyroxine during the peri-implantation.

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4.2 Results

4.2.1 Levels of TRα-1 and RXR proteins expression in uterus

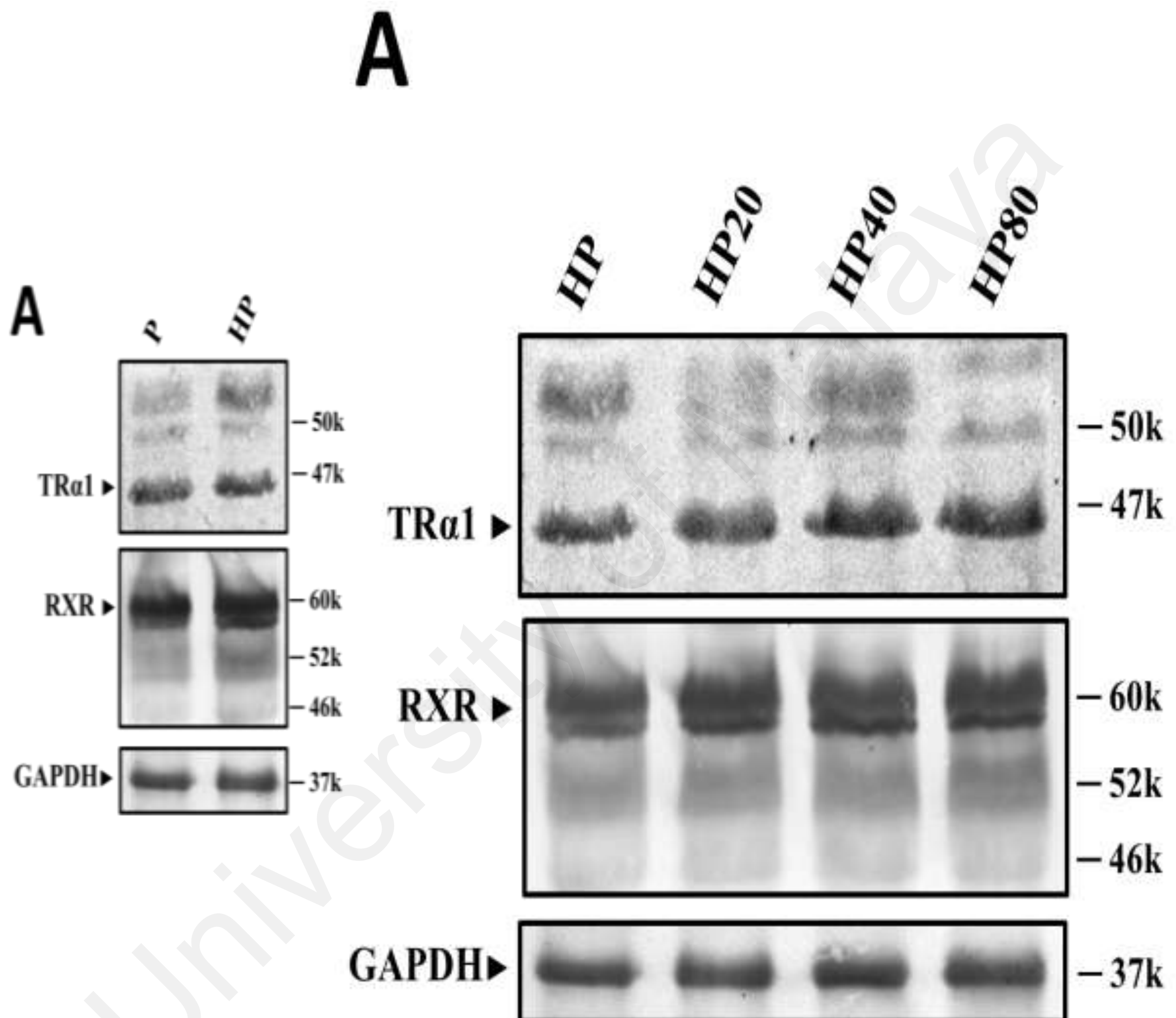

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Figure 4.1 Expression level of TRα-1 and RXR proteins in uterus.

(A) Representative immunoblot images of TRα-1 and RXR in uterus. (In next page) (B) Ratio of band intensity of TRα-1 and RXR proteins versus endogenous GAPDH protein. Data were presented as mean ± SEM; n = 6 per group; $^{*, Ø}$ $p < 0.01$ compared to HP.

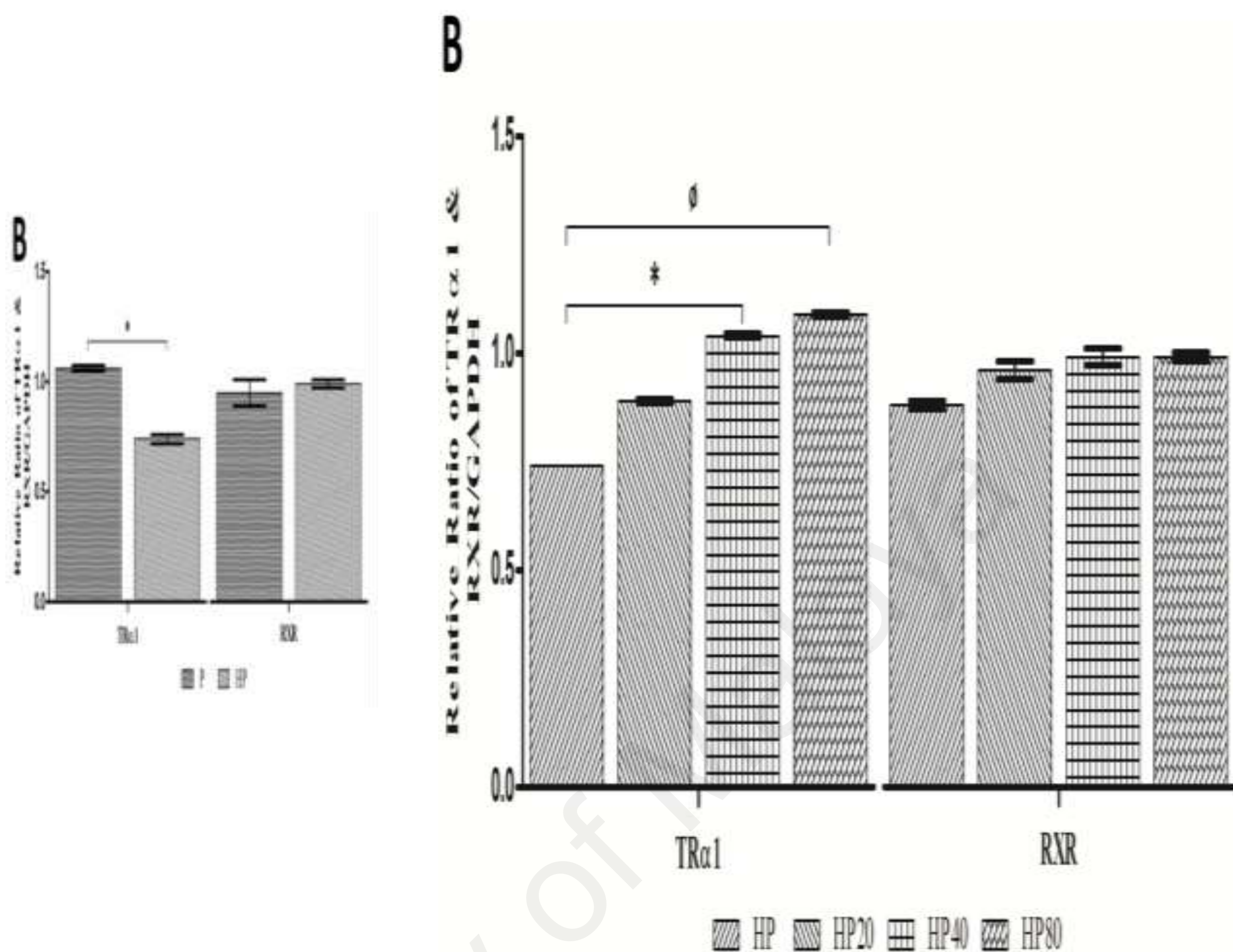

Figure 4.1 Expression level of TRα-1 and RXR proteins in uterus.

(A) Representative immunoblot images of TRα-1 and RXR in uterus. (B) Ratio of band intensity of TRα-1 and RXR proteins versus endogenous GAPDH protein. Data were presented as mean ± SEM; n = 6 per group; $^{*, Ø}$ $p< 0.01$ compared to HP.

Levels of TRα-1 protein expression in hypothyroid pregnant rats were slightly increased when 20µg/kg/day thyroxine was given. The expression levels of TRα-1 protein were significantly increased following treatment with 40 and 80µg/kg/day thyroxine (P < 0.01) (Fig. 4.1A and 4.1B). However, expression levels of RXR protein in hypothyroid pregnant rats were not significantly different following thyroxine treatment (Fig. 4.1A & 4.1B). In addition, presence of two bands of RXR (Fig 4.1A) could be due to an increase in protein mass as a result of glycosylation or multimer formation.

4.2.2 Levels of TRβ-1 and ERK1/2 proteins expression in uterus

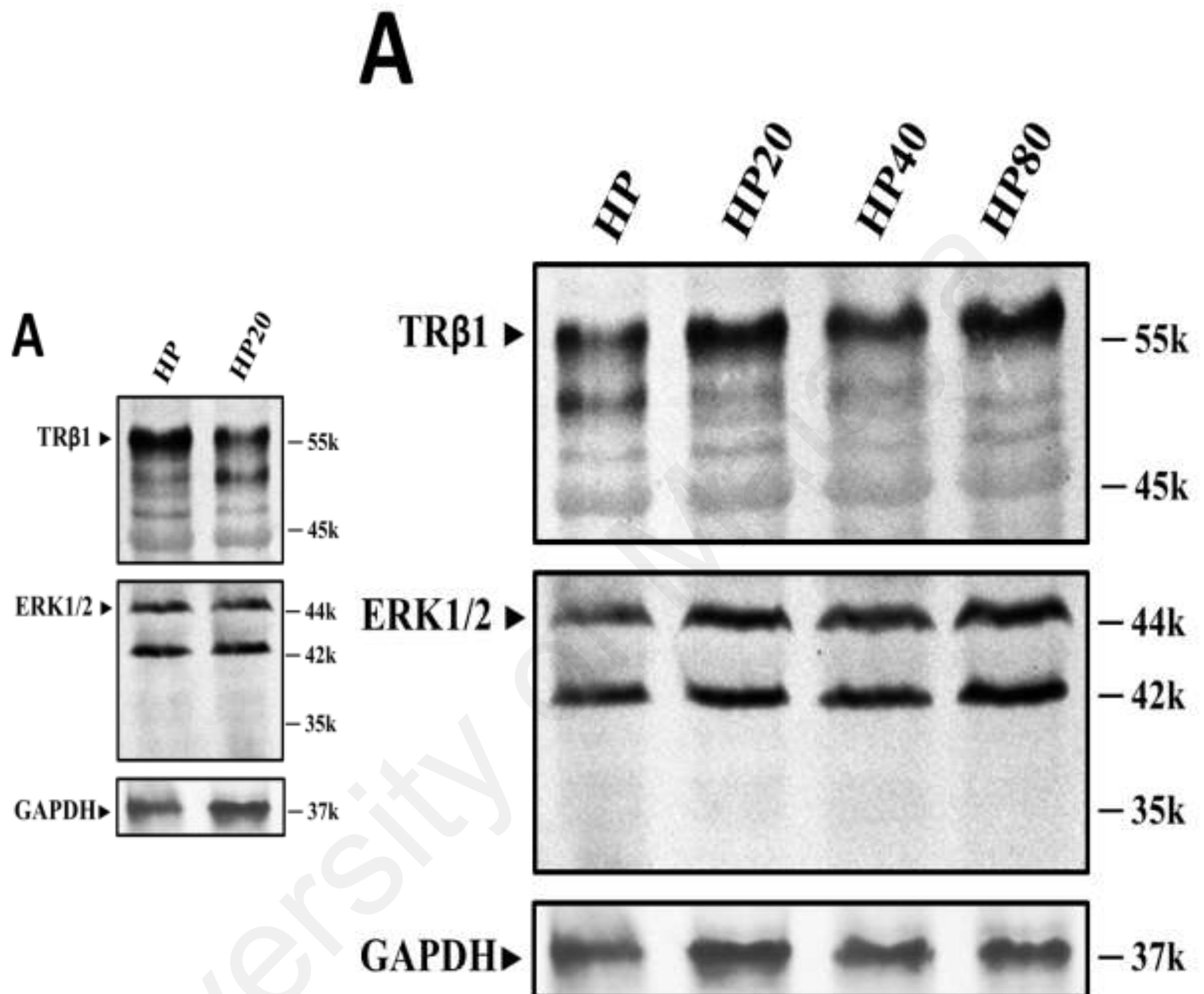

Figure 4.2 Expression level of TRβ-1 and ERK1/2 proteins in uterus.

(A) Representative immunoblot images of TRβ1 and ERK1/2 in uterus. (In next page) (B) Ratio of band intensity of TRβ1 and ERK1/2 proteins versus endogenous GAPDH protein. Data were presented as mean ± SEM; n = 6 per group; $^{\#,}$ * $p< 0.01$ compared to HP; ^{Ø} $p< 0.001$ compared to HP

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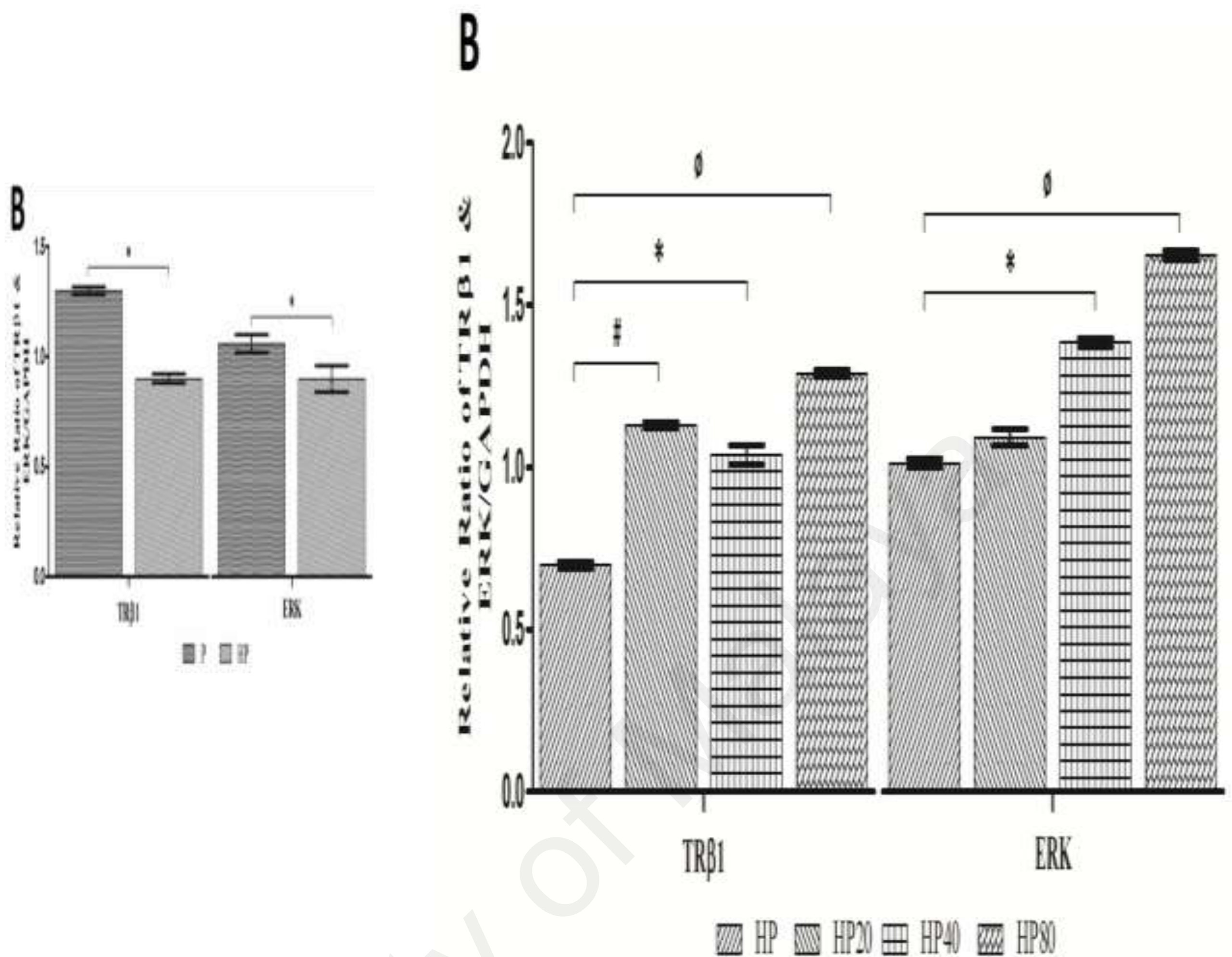

Figure 4.2 Expression level of TRβ-1 and ERK1/2 proteins in uterus.

(A) Representative immunoblot images of TRβ1 and ERK1/2 in uterus. (B) Ratio of band intensity of TRβ1 and ERK1/2 proteins versus endogenous GAPDH protein. Data were presented as mean ± SEM; n = 6 per group; $^{\#,}$ * $p< 0.01$ compared to HP; ^{Ø} $p< 0.001$ compared to HP

Expression levels of TRβ1 protein in hypothyroid pregnant rats were increased when 20 and 40μg/kg/day thyroxine were given. The levels of TRα-1 protein expression was significantly increased following treatment with 40 and 80μg/kg/day thyroxine ($P < 0.01$) (Fig. 4.2A and 4.2B). However, the presence of two bands of TRβ1 could be due to an increase in protein mass as a result of glycosylation or multimer formation. In hypothyroid pregnant rats, slightly increased in expression of ERK1/2 protein was observed following treatment with 20μg/kg/day thyroxine. The expression levels of this protein were significantly increased following treatment with 40 and 80μg/kg/day thyroxine ($P < 0.05$) (Fig. 4.2A and 4.2B).

4.2.3 Distribution of TRα-1 and RXR proteins in uterus

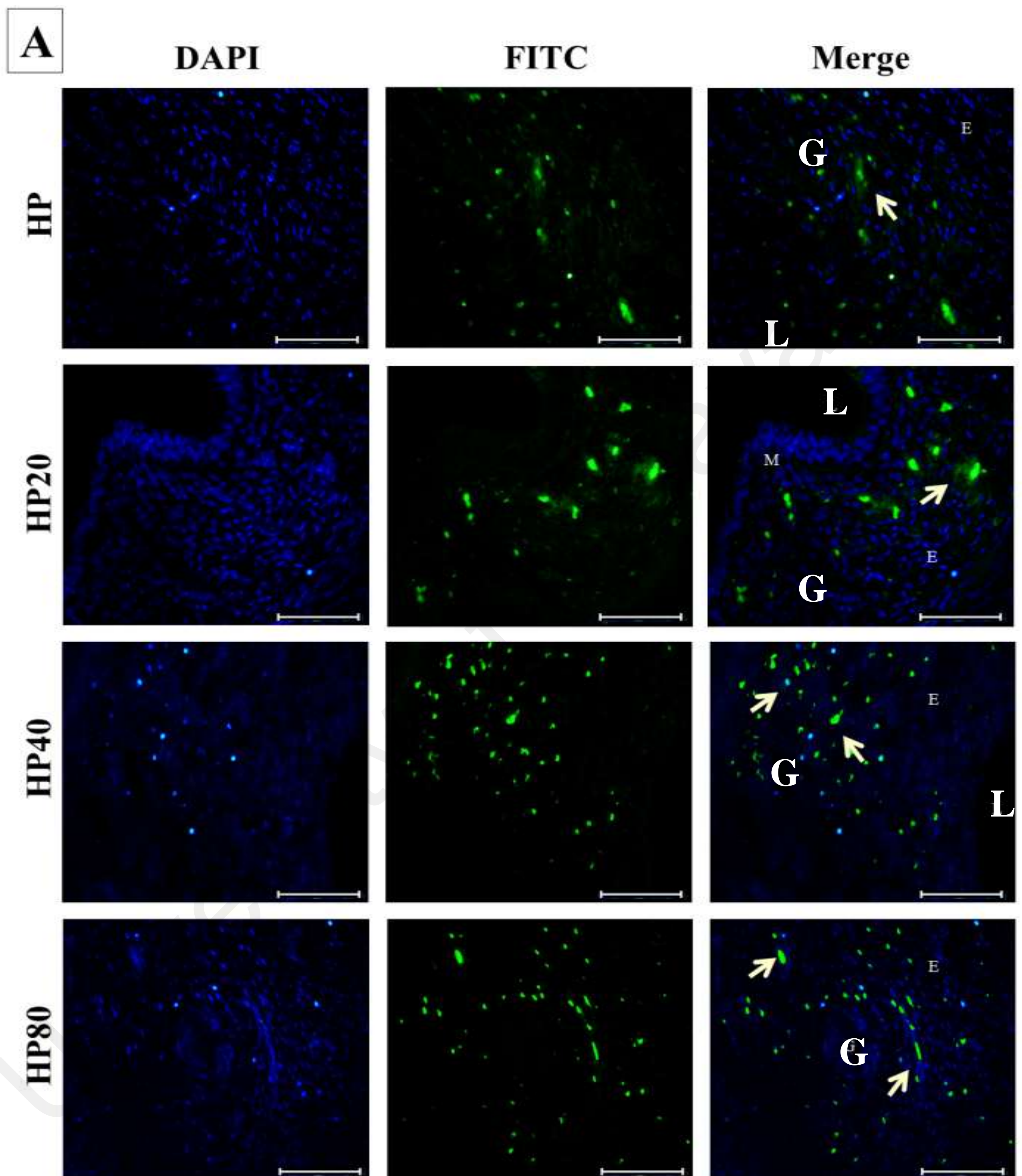

Figure 4.3 Distribution of (A) TRα1 and (In next page) (B) RXR in uterus.

Green fluorescence signals indicate sites where TRα1 was distributed and red fluorescence signals indicate sites where RXR was distributed. Data were presented as mean ± SEM from 4rats of each group. Scale bar represents 50 µm. Arrows show protein distribution sites. *DAPI*, 4′,6-diamidino-2-phenylindole dihydrochloride; *FITC*, fluorescein isothiocyanate; *PE*, phycoerythrin; E, endometrium; L, lumen; G, gland; M, endometrial muscle. HP= non-treated hypothyroid; HP20- hypothyroid, treated with 20µg/kg/day thyroxine, HP40= hypothyroid, treated with 40µg/kg/day thyroxine , HP80= hypothyroid, treated with 80µg/kg/day thyroxine.

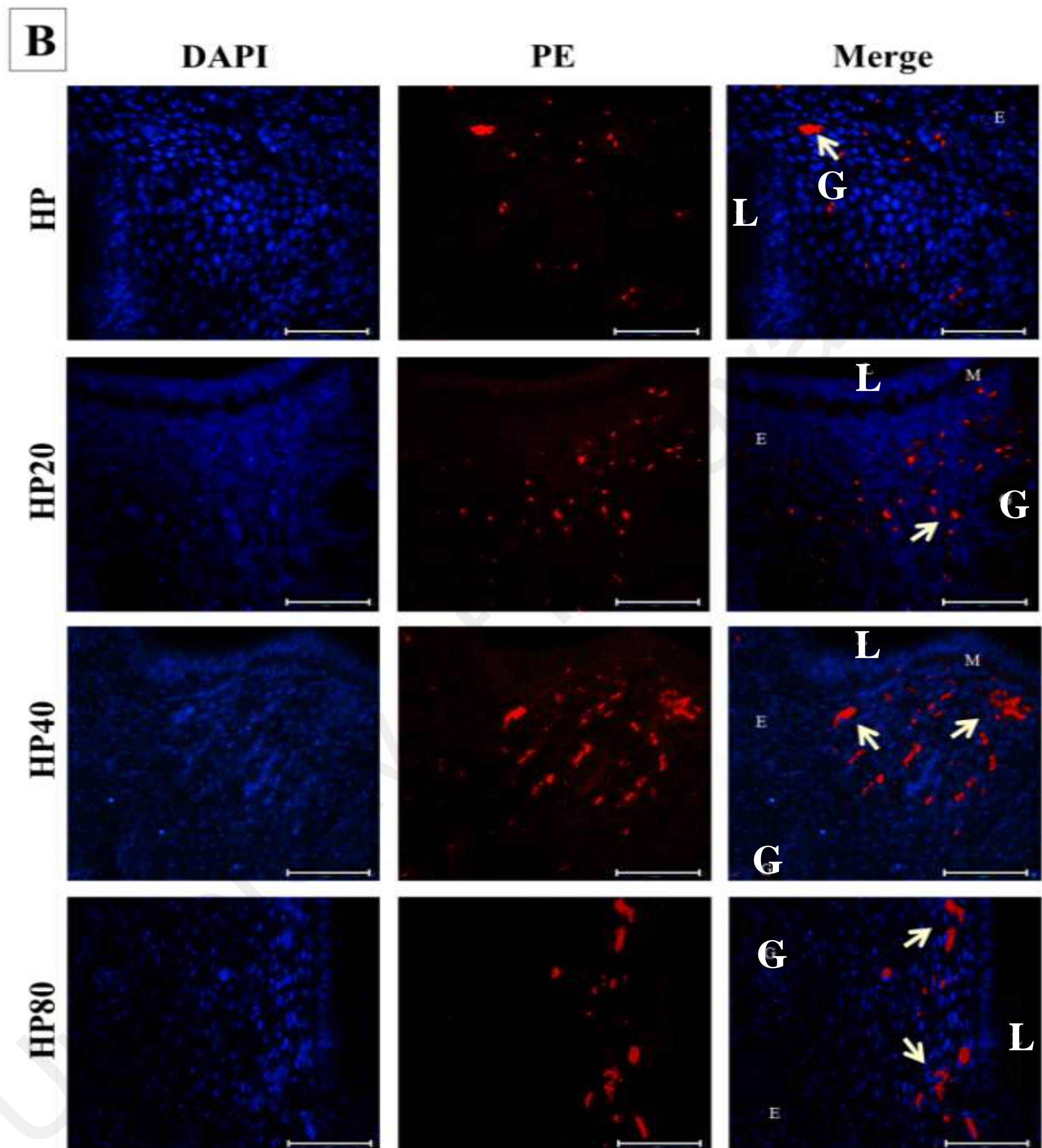

Figure 4.3 Distribution of (A) TRα1 and (B) RXR in uterus.

Green fluorescence signals indicate sites where TRα1 was distributed and red fluorescence signals indicate sites where RXR was distributed. Data were presented as mean ± SEM from 4rats of each group. Scale bar represents 50 µm. Arrows show protein distribution sites. *DAPI*, 4′,6-diamidino-2-phenylindole dihydrochloride; *FITC*, fluorescein isothiocyanate; *PE*, phycoerythrin; E, endometrium; L, lumen; G, gland; M, endometrial muscle. HP= non-treated hypothyroid; HP20- hypothyroid, treated with 20µg/kg/day thyroxine, HP40= hypothyroid, treated with 40µg/kg/day thyroxine , HP80= hypothyroid, treated with 80µg/kg/day thyroxine.

In fig. 4.3A, immunofluorescence images showed that TRα-1 protein distribution in hypothyroid pregnant rats receiving 20µg/kg/day thyroxine was relatively higher when compared to non-treated pregnant hypothyroid rats as indicated by higher fluorescence signals. This protein was found to be distributed mainly in the stroma. Following treatment with 40µg/kg/day and 80µg/kg/day thyroxine, relatively higher TRα-1 distribution was observed (fig. 4.3A). Meanwhile, distribution of RXR was of no different either with or without thyroxine treatment (fig.4.3B).

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4.2.4 Distribution of TRβ1 and ERK1/2 proteins expression in uterus

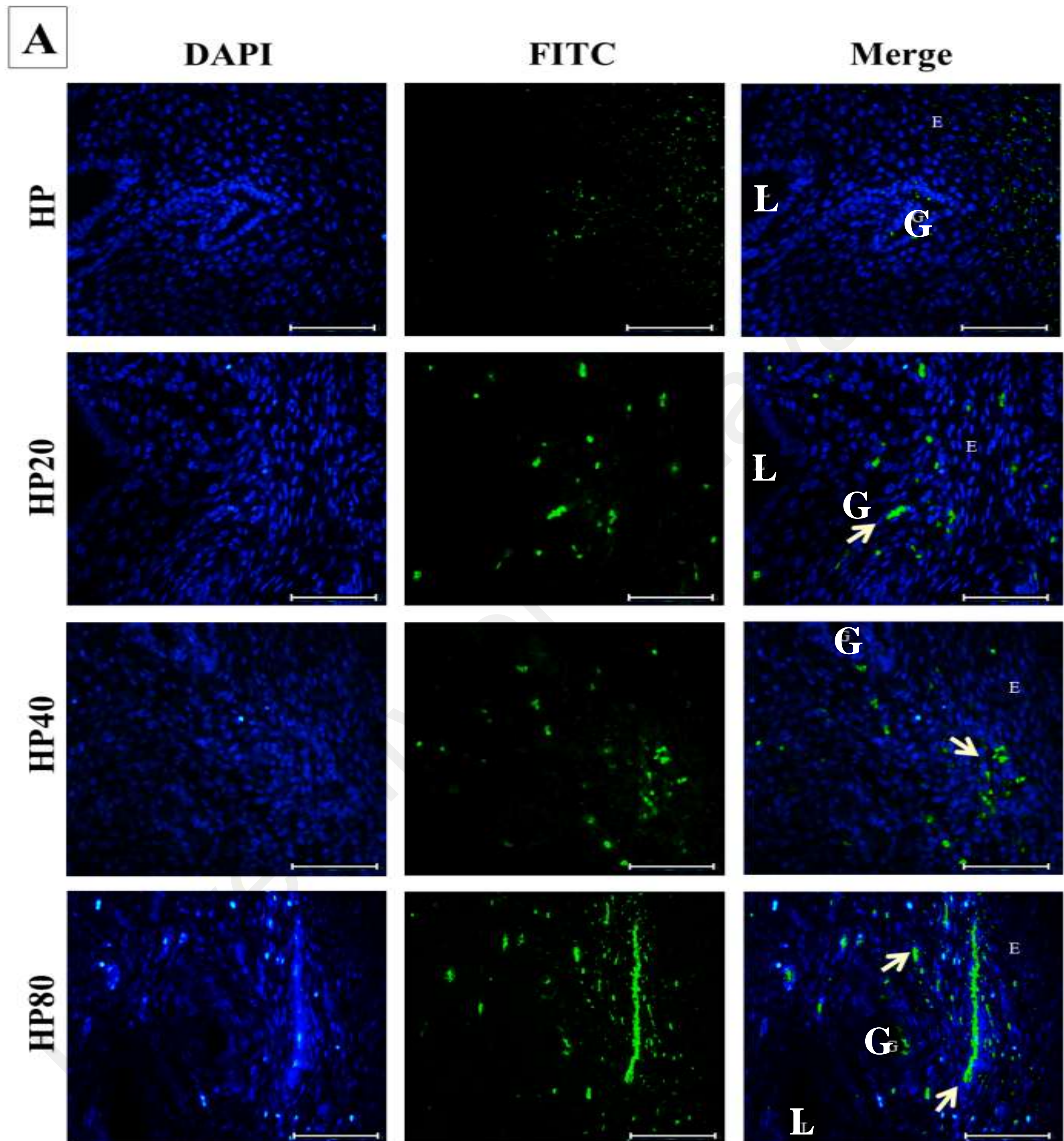

Figure 4.4 Distribution of (A) TRβ1 and (In next page) (B) ERK1/2 in uterus.

Green fluorescence signals indicate sites where TRβ1 was distributed and red fluorescence signals indicate sites where ERK1/2 was distributed. Analysis was done qualitatively. Scale bar represents 50 µm. Arrows show protein distribution sites. *DAPI*, 4′,6-diamidino-2-phenylindole dihydrochloride; *FITC*, fluorescein isothiocyanate; *PE*, phycoerythrin; E, endometrium; L, lumen; G, gland; M, endometrial muscle. HP= non-treated hypothyroid; HP20- hypothyroid, treated with 20µg/kg/day thyroxine, HP40= hypothyroid, treated with 40µg/kg/day thyroxine , HP80= hypothyroid, treated with 80µg/kg/day thyroxine.

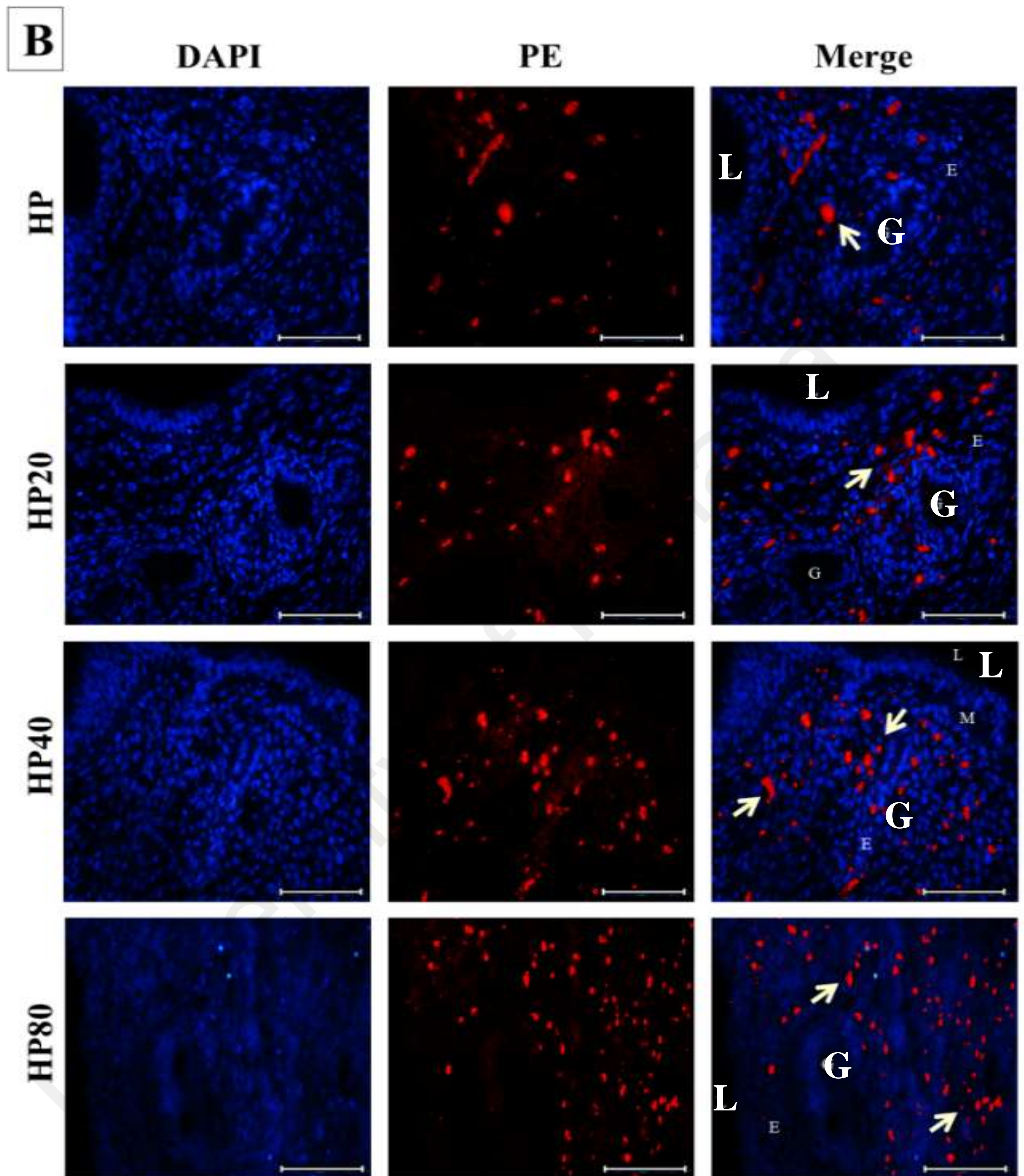

Figure 4.4 Distribution of (A) TRβ1 and (B) ERK1/2 in uterus.

Green fluorescence signals indicate sites where TRβ1 was distributed and red fluorescence signals indicate sites where ERK1/2 was distributed. Analysis was done qualitatively. Scale bar represents 50 µm. Arrows show protein distribution sites. *DAPI*, 4′,6-diamidino-2-phenylindole dihydrochloride; *FITC*, fluorescein isothiocyanate; *PE*, phycoerythrin; E, endometrium; L, lumen; G, gland; M, endometrial muscle. HP= non-treated hypothyroid; HP20- hypothyroid, treated with 20µg/kg/day thyroxine, HP40= hypothyroid, treated with 40µg/kg/day thyroxine , HP80= hypothyroid, treated with 80µg/kg/day thyroxine.

In fig. 4.4A, immunofluorescence images showed high distribution of TRβ-1 in uterine stroma of hypothyroid pregnant rats receiving 80μg/kg/day thyroxine treatment. In hypothyroid pregnant rats that were treated with 20μg/kg/day and 40μg/kg/day thyroxine, relatively higher TRβ-1 distribution was observed when compared to non-treated hypothyroid pregnant rats (fig. 4.4A). Immunofluorescence images show distribution of ERK1/2 protein increased with increasing doses of thyroxine (fig. 4.4B).

4.3 Discussion

In this study, it was found that thyroxine can up-regulates thyroid hormone receptors (TRα-1 and TRβ-1) and signaling protein ERK1/2 in the uterus during peri-implantation period. Up-regulation of these proteins could be important in enhancing thyroid hormone action in the uterus during this period. Although this study has shown that thyroxine was able to up-regulate its own receptor expression in uterus, detail mechanisms underlying its action have yet to be revealed. It is possible that thyroxine could stimulate transcription of the genes that encode TR-α1 and TR-β1, subsequently resulted in increased expression of the related proteins. There were findings which showed that thyroid hormone could activate transcription of the gene that encode mRNA for TR-α1 and TR-β1 in the liver of *Rana catesbeiana* and Senegalese sole (Helbing *et al.*, 1992; Manchado *et al.*, 2009), and these observations might support our hypothesis. It has been reported in tadpoles that the two promoters in the genes encoding TR-α, one located in the upstream of exon a and another in the upstream of exon b are responsive to thyroid hormone stimulation. Thyroid hormone also induced activation of TR-β gene by specifically activate exon b promoters rather than exon a which resulted in up-regulated TR-β mRNA expression (Kanamori & Brown, 1992). As thyroid hormone could induce transcription of exon b, therefore there is a possibility that this hormone could also induce activation of the gene that encode mRNA for TR-α1 (Kanamori & Brown, 1992; Yaoita & Brown, 1990). Beside thyroid hormone, other

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hormones including sex-steroids have also been shown able to influence transcription of the gene that encodes its own receptor expression. For example, estrogen could up-regulate estrogen receptor mRNA via inducing gene transcription in the liver of *Xenopus laevis* (Barton & Shapiro, 1988).

Enhanced thyroid hormone action in uterus during peri-implantation period as a consequence of increased expression of thyroid hormone receptors and ERK1/2 signaling pathway could have implication on uterine energy homeostasis. It is known that the events that occur in uterus during this period require increased energy levels such as development of uterine receptivity as well as blastocyst (Shirane *et al.*, 2012). Thyroid hormone plays important role in glucose homeostasis (Chidakel *et al.*, 2005) as evidence by its action in regulating transcription of several genes that encodes proteins for glucose metabolism in the liver, skeletal muscle and adipose tissue (Bianco *et al.*, 2002). Therefore, increased thyroid hormone action during this period is needed to enhance endometrial glucose homeostasis that is crucial for development of uterine receptivity.

It has been reported that both receptors for thyroid hormone and RXR are required for thyroid hormone action during development of the embryos of *Xenopus laevis* (Puzianowska-Kuznicka *et al.*, 1997). The heterodimer complex that exist between thyroid hormone receptors and RXR that is needed to recognize the specific DNA sequence are crucial for the biological effects of thyroid hormone (Kim S. W. *et al.*, 1996; Kurokawa *et al.*, 1993). However, our data showed that thyroid hormone did not have any effects on expression of RXR. It is plausible that RXR expression could be stimulated by other hormones such as sex-steroids. A study by Cai et al. showed that sex-steroids, in particular dihydrotestosterone can interact with RXR in hepatocytes (Cai *et al.*, 2003). Meanwhile RXR expression was found to correlate with

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estrogen/progesterone receptor expression in human breast carcinoma cells which further support our speculation (Ditsch *et al.*, 2012).

In this study, it was found that thyroxine treatment to hypothyroid rats increases the expression of ERK1/2 protein. It is known that the signals from thyroid hormone are transduced within the cell via intracellular ERK1/2 signaling, and activation of this signaling pathway could lead to transcription activation of several genes encoding the proteins that are involve in angiogenesis which include basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) (Scarlett *et al.*, 2008). In addition, thyroid hormone was also reported to stimulate serine phosphorylation of TR-β1 via mitogen-activated protein kinase MAPK or ERK1/2 intracellular signaling *in-vitro* (Davis *et al.*, 2000).

In this study, levels of progesterone in hypothyroid pregnant rats was found to decrease following administration of thyroxine. The reasons for the decrease could be due to the consequence of inhibitory effect of thyroxine on ovarian steroidogenesis. Our findings comply with other report which showed that administration of thyroid hormone decreases the weight of ovary in chickens as this hormone could induce atresia of the pre-ovulatory follicles, that ultimately reduces levels of progesterone (Sechman, 2013). A negative relationship between thyroid hormone concentration in the blood and ovarian function has also been observed during chicken sexual maturation (Altmae *et al.*, 2012; Kaneko *et al.*, 2013).

In conclusion, the study outcomes suggested that thyroid hormone could play a role in peri-implantation period via up-regulating the receptors related to its functions as well as up-regulating the ERK1/2 signaling pathway. These changes might have implications on embryo-endometrial interactions, uterine receptivity development and subsequently, the female fertility.

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CHAPTER 5: EFFECTS OF HYPOTHYROIDISM AND THYROXINE TREATMENT ON THYROID HORMONE RECEPTOR (TR), THYROID STIMULATING HORMONE RECEPTOR (TSHR), RETINOIC ACID RECEPTOR (RAR) AND EXTRACELLULAR KINASE SIGNALING PROTEIN (ERK1/2) EXPRESSION IN THE UTERUS AT THE DAY OF EMBRYO IMPLANTATION IN RATS

5.1 Introduction

Thyroid hormone (TH) plays important role in embryo implantation (Ashkar *et al.*, 2010), an event which depends on three factors i.e. viability of the blastocyst, development of endometrial receptivity and establishment of close contact between blastocyst and endometrium (Koot & Macklon, 2013). Implantation is known to be under hormonal control (Sharkey & Smith, 2003), mainly by sex-steroids, however TH has also been reported to be involved in implantation-related events(Ashkar *et al.*, 2010). It was shown that *in-vitro* TH supplementation could help increase hatching rate of bovine blastocysts (Costa *et al.*, 2013). It was also reported that TH help to regulate the response of the uterus to estrogen via modulating estrogen receptor and estrogen-induced peroxidase activity in rats (Keeping *et al.*, 1982), which could have implications on implantation. Lack been found to cause infertility (Priya *et al.*, 2015).

Hypothyroidism is one of the conditions that contribute to female infertility. In a population study, 53.7% of infertile female were found hypothyroid, and treatment with thyroxine has resulted in 33.3% of subclinical hypothyroid women conceived within 6 weeks to 2-year period (Verma Indu *et al.*, 2012). Another study showed that high successful pregnancy rate and shorter duration of infertility until pregnancy were observed after thyroxine treatment in infertile patients with sub-clinical hypothyroidism (Yoshioka Waka *et al.*, 2015). The mechanisms in which deficiency in TH can cause

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infertility are largely unexplored. There was a study in rats which showed that uterine epithelial cell height and their nuclear volume was decreased with significant increase in basement membrane thickness in hypothyroidism (Inuwa I. M. & M. A. Williams, 2006). In was earlier found in rats that the absolute volume of endometrium and thickness of myometrium in hypothyroid rats was significantly reduced but was significantly increased following thyroxine treatment (Inuwa & Williams, 1996a).

TRα and TRβ are two isoforms of TH that have been reported to be expressed in uterus in rodents (Sayem Abu Sadat Md *et al.*, 2017) and humans (Aghajanova Lusine *et al.*, 2011). TRs form heterodimers with retinoic acid receptors (RARs), which then interact with specific DNA sequences, denoted as thyroid hormone responsive elements (TREs), leading to initiation of gene and protein synthesis (Lee S. & Privalsky, 2005). RAR expression has also been reported in uterus of humans (Cheng Y.-H. *et al.*, 2008) and rodents (Sayem Abu Sadat Md *et al.*, 2017). In addition to acting through the nuclear receptor, TH also transduces signals at downstream via activating ERK1/2 signaling cascade, which is an important signaling pathway in uterus (Liu J. *et al.*, 2017). ERK1/2 is reported to be involved in decidualization (Liu J. *et al.*, 2017) and implantation (Fluhr *et al.*, 2013). Besides TR, TSHR has also been reported to be expressed in the endometrium of humans (Colicchia *et al.*, 2014) and rodents (Sayem Abu Sadat Md *et al.*, 2017). TSHR expression is reported to increase at the time of embryto implantation (Aghajanova L. *et al.*, 2011).

Despite of the important role of TR TSHR, RAR and ERK1/2 in uterine physiology particularly during implantation, effect of hypothyroidism and consequently thyroxine treatment on expression of these proteins during this period remains unknown. Therefore, the aims of this study were to investigate changes in expression of these proteins in uterus in both clinical conditions.

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5.2 Results

5.2.1 Numbers of embryo implantation sites at day 6 pregnancy

Figure 5.1 Representative images showing embryo implantation sites in hypothyroid rats receiving thyroxine treatment

Blue-stained granules indicate sites of embryo implantation. Arrows indicate implantation sites.

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In hypothyroid pregnant rats, the number of implantation sites markedly reduced as compared to euthyroid rats (Fig. 5.1 and Table 5.1). Thyroxine treatment to hypothyroid rats caused increase in the number of embryo implantation sites, in a dose-dependent manner.

Table 5.1 Effects of thyroxine treatment on embryo implantation

Treated groups	No. of implantation sites (mean ± sd)
P	11.50±0.57
HP	3.25±0.57*
HP20	5.75±0.50
HP40	10.25±0.95
HP80	11.33±0.95**

*$p < 0.05$ compared to P. **$p < 0.05$ compared to HP. Data were expressed as mean ± S.D. with n = 6 rats per group.

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5.2.2 Expression levels of TRα-1, TRβ-1, TSHR, ERK1/2 and RAR proteins in uterus at the day of implantation (GD-6)

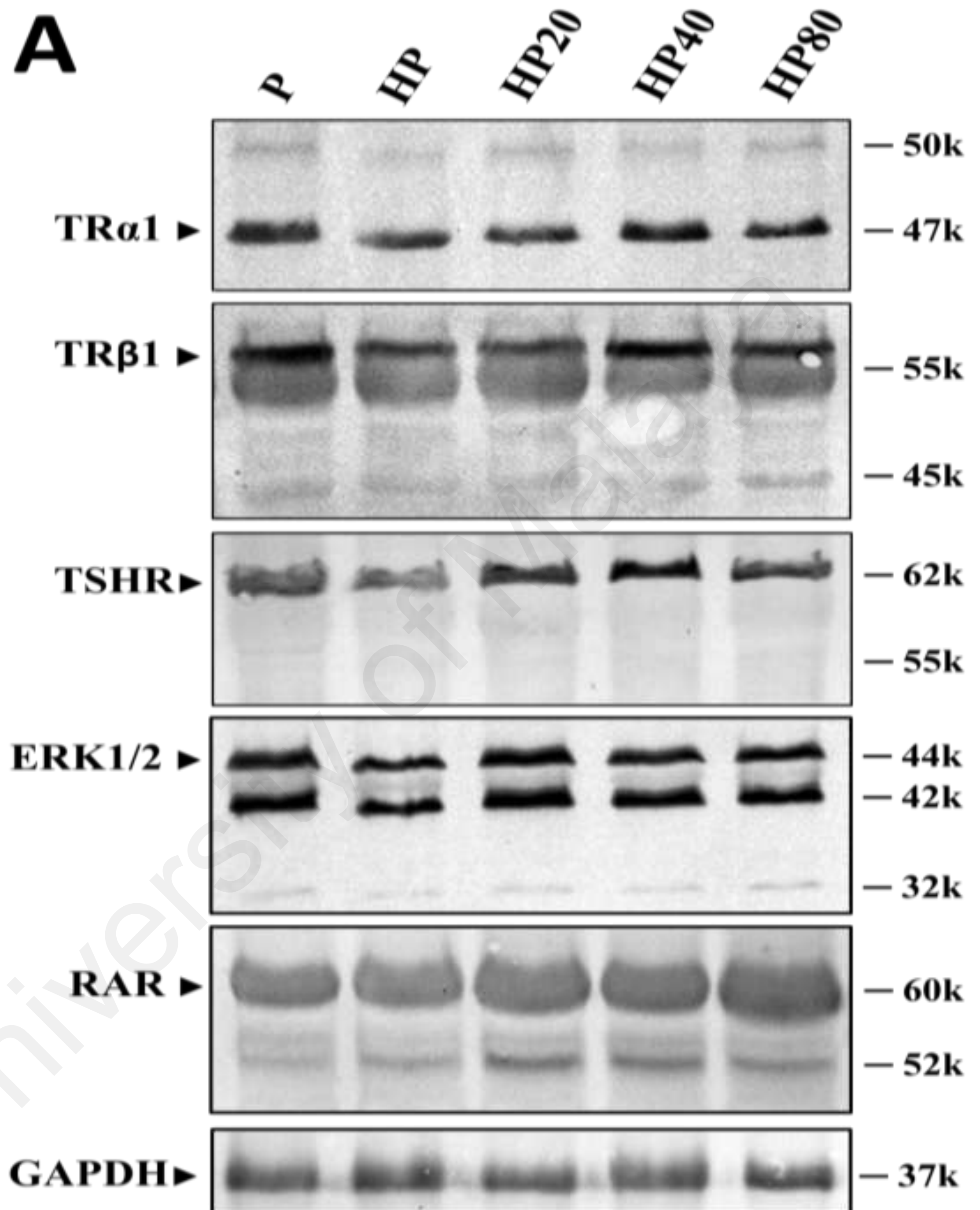

Figure 5.2 Expression levels of TRα-1, TRβ-1, TSHR, ERK1/2 and RAR proteins in uterus

(A) Representative immunoblot images of TRα-1, TRβ-1, TSHR, ERK1/2 and RAR in uterus, (In next page) (B) Ratio of band intensity of TRα-1, TRβ-1 and TSHR proteins versus endogenous GAPDH protein, (C) Ratio of band intensity of ERK1/2 and RAR proteins versus endogenous GAPDH protein. Data were presented as mean ± SEM; n = 6 per group. Bars that do not share the same symbols differ significantly at $P<0.05$.

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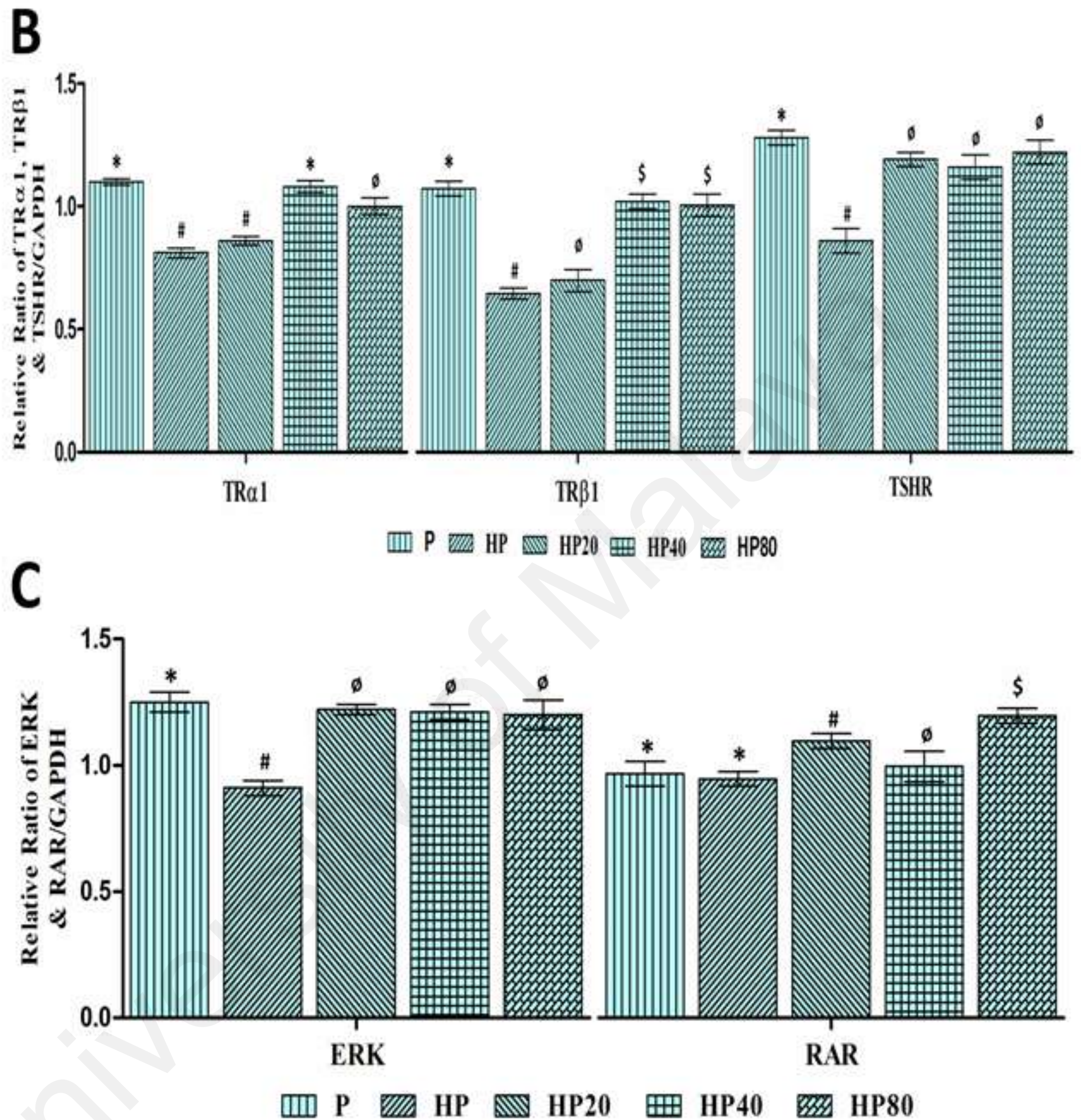

Figure 5.2 Expression levels of TRα-1, TRβ-1, TSHR, ERK1/2 and RAR proteins in uterus

(A) Representative immunoblot images of TRα-1, TRβ-1, TSHR, ERK1/2 and RAR in uterus, (B) Ratio of band intensity of TRα-1, TRβ-1 and TSHR proteins versus endogenous GAPDH protein, (C) Ratio of band intensity of ERK1/2 and RAR proteins versus endogenous GAPDH protein. Data were presented as mean ± SEM; n = 6 per group. Bars that do not share the same symbols differ significantly at *P*<0.05.

Expression of TRα-1 protein markedly reduced in hypothyroid pregnant rats as compared to euthyroid pregnant rats (Fig. 5.2A and 5.2B). In hypothyroid pregnant rats,

slight increase in expression of TRα-1 protein was observed following treatment with 20 μg/kg/day thyroxine. The levels of this protein further increased following treatment with 40 and 80μg/kg/day thyroxine, achieving the levels observed in euthyroid pregnant rats.

Similarly, in hypothyroid pregnant rats, expression of TRβ1 protein was significantly lower than euthyroid pregnant rats (Fig. 5.2A and 5.2B). In hypothyroid pregnant rats, the levels of TRβ-1 protein slightly increased following treatment with 20 μg/kg/day thyroxine, and further increased following treatment with 40 and 80 μg/kg/day thyroxine.

In the meantime, levels of expression of TSHR protein markedly decreased in hypothyroid pregnant rats as compared to euthyroid pregnant rats (Fig 5.2A and 5.2B). In hypothyroid pregnant rats, TSHR levels significantly increased following treatment with 20μg/kg/day thyroxine and further increased following treatment with 40 and 80μg/kg/day thyroxine.

Levels of ERK1/2 protein in hypothyroid pregnant rats were markedly lower when compared to euthyroid pregnant rats (Fig. 5.2A & 5.2C). In hypothyroid pregnant rats, ERK1/2 levels increase with increasing dose of thyroxine treatment and increase with the levels following treatment with 80μg/kg/day thyroxine approaching those in euthyroid pregnant rats.

Expression levels of RAR protein were slightly reduced in hypothyroid pregnant rats ($p<0.05$) as compared to euthyroid pregnant rats (Fig. 5.2A & 5.2C). RAR levels in hypothyroid pregnant rats increased following thyroxine treatment and were not significantly different between different doses of thyroxine.

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5.2.3 Expression level of *Trα1*, *Trβ1*, *Tshr, Erk1/2* and *Rar* mRNAs in the uterus at the day of implantation (GD-6)

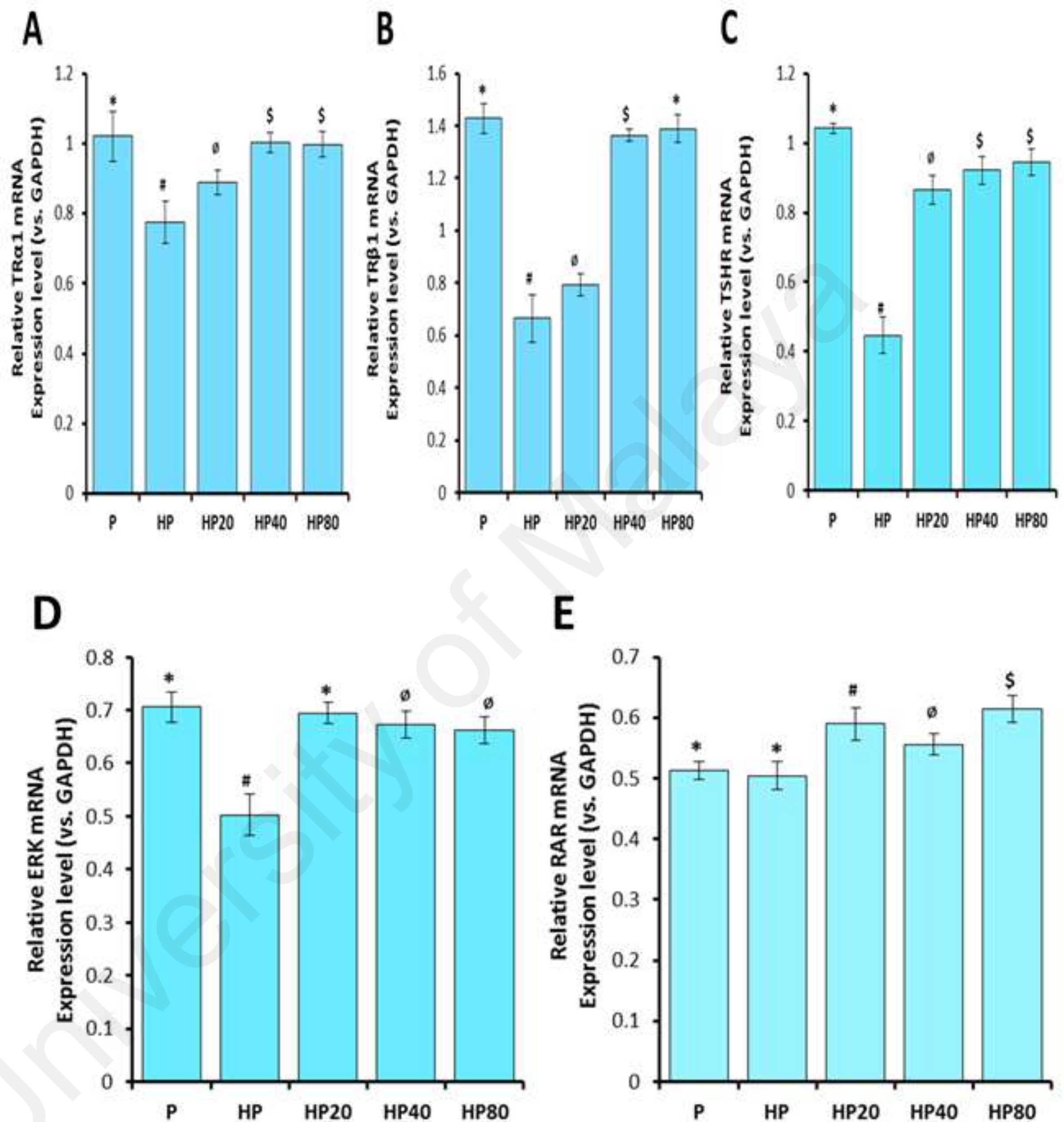

Figure 5.3 Levels of *Trα1*, *Trβ1*, *Tshr, Erk1/2* and *Rar* mRNAs in uterus.
Relative mRNA expression levels for (A) *Trα1*, (B) *Trβ1*, (C) *Tshr*, (D) *Erk1/2* and (E) *Rar* in uterus. Data were presented as mean ± SEM; n = 6 per group. Bars that do not share the same symbols differ significantly at *P*<0.05.

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Levels of *Trα-1* mRNA were significantly decreased in pregnant hypothyroid rats as compared to pregnant euthyroid rats (Fig. 5.3A). In pregnant hypothyroid rats, significant increase in *Tra-1* mRNA levels was observed following treatment with 20

µg/kg/day thyroxine ($p<0.05$). The levels further increased following treatment with 40 and 80 µg/kg/day thy tore approximately 1.5 fold higher as compared to non-thyroxine treated hypothyroid rats.

Levels of *Trβ-1* mRNA also decreased in hypothyroid pregnant rats ($p<0.05$) as compared to euthyroid pregnant rats (Fig. 5.3B). In hypothyroid pregnant rats, treatment with 20 µg/kg/day thyroxine causes a slight but significant increase in *Trβ1* mRNA levels. The levels of *Trβ1* mRNA were increased by more than two folds following treatment with 40 and 80µg/kg/day thyroxine ($p< 0.05$).

In the meantime, hypothyroid pregnant female rats had significantly lower *Tshr* mRNA levels as compared to euthyroid pregnant female rats (Fig. 5.3C). In hypothyroid pregnant female rats, *Tshr* mRNA levels increased following administration of thyroxine ($p<0.05$). Further increase in *Tshr* mRNA levels were observed following treatment with 40 and 80 µg/kg/day thyroxine

Erk1/2 mRNA levels in hypothyroid pregnant rats were significantly lower when compared to euthyroid pregnant rats (Fig. 5.3D). Treatment of hypothyroid rats with 20, 40 and 80 µg/kg/day thyroxine causes significant increase in *Erk1/2* mRNA levels in uterus where the levels were approximately 1.5 fold higher as compared to non-thyroxine treated hypothyroid rats.

In hypothyroid pregnant rats, *Rar* mRNA levels were slightly lower as compared to euthyroid pregnant rats (Fig. 5.3E). In hypothyroid preganat rats, treatment with thyroxine resulted in increased *Rar* mRNA levels. However, no significant changes in *Rar* mRNA levels were noted between different doses of thyroxine.

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5.3 Discussion

To the best of our knowledge, this study is the first to show that expression of TRα1, TRβ1, TSHR, RAR and ERK1/2 in the uterus at the time of embryo implantation were reduced in hypothyroidism and thyroxine treatment could overcome these effects. These findings reaffirmed that thyroxine is indeed essential for the up-regulation of TRα1, TRβ1, TSH, RAR and ERK1/2 in uterus during the implantation period. Previous studies only showed the role of sex-steroids, in particular progesterone in causing expression of these proteins to increase as evidence from administration of mifepristone (RU486), an antiprogestin reduces the expression in uterus (Catalano *et al.*, 2007).

The involvement of thyroid hormone (TH) around the time of implantation in rats (at day 6) has been reported (Cheong *et al.*, 2013; Dey *et al.*, 2004; Norwitz *et al.*, 2001). TH has been reported to participate in increasing endothelial proliferation, which partly requires stimulation by angiogenic factors secreted by the blastocyst (Demir *et al.*, 2010; Staun-Ram & Shalev, 2005). TH has also been reported to be involve in tissue remodeling and angiogenesis, both events occur during implantation (Dey *et al.*, 2004; Plaisier, 2011). TH actions could therefore be enhanced through up-regulation in the expression of TRα1, TRβ1, RAR and ERK1/2 in the uterus during the implantation period.

Our findings that expression of TR increases at the time of implantation were supported by other studies. TRα1 and TRβ1 expression in the glandular and luminal epithelium increases on LH days 6 to 9 in human endometrium, coincide with the time of implantation (Aghajanova Lusine *et al.*, 2011). In view that the tissue we harvested at day 6 also consist of tissue from the implanted blastocyst, therefore increased in TRα and TRβ levels might also be contributed by their expression in the blastocyst. This postulation was supported by a report that TRα was expressed in the blastocyst of

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bovine (Colicchia *et al.*, 2014). Increased in TRα and TRβ levels might also be contributed by the increased in expression of both the isoforms of TR in the uterus, which was well documented in the uterus during implantation (Piccirilli *et al.*, 2018). There is a report that TRα and TRβ are expressed on macrophages and dendritic cells (De Vito *et al.*, 2011). In view of the increased in the number of these cells in uterus during implantation which is associated with the inflammatory processes (Bourdiec *et al.*, 2016), increased levels of both TR isoforms could be attributed to their expression in these cells. Increased TRα1 and TRβ1levels at the day of implantation could also be contributed by their expression in the trophoblast which is form once the embryo implants (Barber *et al.*, 2005).

TH effect on the uterus could be mediated via the genomic pathway (Cheng S. Y. *et al.*, 2010). Upon binding to the nuclear TR, which acts as hormone-dependent transcription factors, this complex than bind to DNA motif located in the promoter regions of TH target genes, known as TRE, the binding which may occur either as homodimers or heterodimers with other members of the nuclear receptor superfamily such as RAR that helps to increase the transcriptional activity of TH (Zhang X. K. & Kahl, 1993). In the absence of TH, the TR/RAR complex binds to TRE where it interacts with co-repressor (CoR), where this inhibits the transcriptional activity of the promoter. Following the binding of TH, structural changes in the TR occur resulting in the release of CoR and the recruitment of coactivator (CoA) which increases the transcriptional activity of the promoter (Cheng S. Y. *et al.*, 2010). Via these mechanisms, TH enhances transcription of DNA, Apart from this, TH can acts via faster nongenomic pathways (Moeller & Broecker-Preuss, 2011), via binding to the cell surface receptor and activates the mitogen-activated protein kinase MAPK-ERK1/2 (Lin *et al.*, 2003). Activation of the ERK1/2 pathway could help to induce angiogenesis and

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promoting cell growth (Davis *et al.*, 2000; Moeller *et al.*, 2006) both events are essential during embryo implantation.

In this study, it was found that expression of TSHR in uterus was also increased during implantation period. It was reported that the G protein-coupled TSHR is widely expressed in the feto-maternal unit during the implantation (Aghajanova L. *et al.*, 2011; Stavreus Evers, 2012) suggesting the importance of TSH in regulating embryo implantation. Furthermore TSHR expression in human endometrial cells increased at the same time as pinopodes i.e. the time of implantation (Aghajanova L. *et al.*, 2011).

There are implications for the increased in expression of TRα1, TRβ1 and RAR in the uterus at the time of implantation. Enhance TH action could help to optimize embryo cleavage, blastocyst formation and it's hatching rates (Ashkar *et al.*, 2010; Costa *et al.*, 2013). This also helps to reduce embryo cell apoptosis as well increases the expansion rate of the blastocoel cavity (Ashkar *et al.*, 2010). TH could interact with leukaemia inhibitory factor (LIF), an important molecule during implantation (Stavreus Evers, 2012). An inverse correlation between grade of hypothyroidism and LIF levels has been reported, implying the important role of LIF for embryo implantation (Ren *et al.*, 1999). TH may enhance angiogenesis (De Vito *et al.*, 2012; Pinto *et al.*, 2011), an event that is crucial for successful implantation, decidualization and placentation (Dey *et al.*, 2004). Upregulated expression of TR which reflects increased TH action in uterus at the time of implantation could also have implications on uterine immunity, which involve the Natural killer (NK) cells (Xiong S. *et al.*, 2013). Uterine NK (uNK) cells are the predominant leukocyte at the time of implantation and provide cytokine support and immunomodulation (King, 2000). Up-regulation of TRα and TRβ could also help TH action in enhancing basal metabolic rate of the endometrium at the time of implantation. This postulation arises based on the findings which showed that TH regulate subcellular

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glucose phosphorylation activity through TRα-1, TRα-2 and TRβ-1 in the skeletal muscle of male mice (Martins Pecanha *et al.*, 2017; Milanesi *et al.*, 2016). In endometrium, increased in basal metabolism is crucial for endometrial receptivity development. Uterine receptivity could not develop if glucose homeostasis was impaired (Shirane *et al.*, 2012). Hence increased TR action helps in TH action on increasing endometrial metabolism.

In the meantime, elevated expression of TSHR in the uterus might indicate enhance action of TSH at the time of implantation. Local TSH is reported to be produced in uterus from sources like T cells, B cells, dendritic cells etc which play a role in uterine immunity (Klein, 2006). TSH helps to enhance the function of NK cells which participates in inflammation (Provinciali *et al.*, 1992). Additionally, TSH also help to increase expression of LIF and its receptor (LIFR) in the endometrial stromal and this is important for endometrial glucose transport, since TSH stimulation causes increased expression of glucose transport protein GLUT1 (Aghajanova L. *et al.*, 2011).

Finally, it was observed the elevated expression of ERK1/2 in the uterus at the time of implantation. ERK1/2 has been reported to participate in the transcription of several angiogenesis-relevant genes, such as fibroblast growth factor (bFGF) and VEGF (Luidens *et al.*, 2010). MAPK-ERK1/2 could activate TRβ1 (MAPK – TRβ1, ERα) signaling' in uterus (Shen *et al.*, 2012). TH-activated ERK1/2 in turn activates members of the signal transduction and activator of transcription (STAT) family, which are involved in vascular growth where these are crucial for implantation.

In conclusion, elevated expression of TRα, TRβ, TSHR, RAR and ERK1/2 in the uterus under the influence of thyroxine could assist in successful implantation where derangement could result in infertility.

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CHAPTER 6: INVESTIGATING THE EFFECT OF THYROID HORMONE ON UTERINE FLUID VOLUME AND AQUAPORIN (AQP) SUBUNITS (AQP-1, 5 AND 7) EXPRESSION IN THE PERI-IMPLANTATION PERIOD

6.1 Introduction

Successful embryo implantation requires precise regulation of the uterine fluid volume mainly by sex-steroids namely estrogen and progesterone (Shahzad H., N. Giribabu, K. Karim, N. Kassim*, et al.*, 2017). A reduction in uterine fluid volume has been reported under progesterone influence, where this helps to establish a close contact between the blastocyst and the receptive uterus (Hoversland & Weitlauf, 1981)). Abnormal amount of fluid in uterus could interefere with embryo implantation, thus could lead to infertility (Altmae *et al.*, 2012; Kaneko *et al.*, 2013). Prior to implantation, precise fluid regulation in uterus is crucial for the reproductive processes such as sperm transport, endometrial lubrication and defense against pathogens (Bhusane *et al.*, 2016).

Changes in uterine fluid volume is due to movement of H_2O between different uterine compartments (Sharkey & Smith, 2003). Fluid accumulation into or fluid loss from the uterine lumen are known to be mediated via AQP, a channel which is reported to be differentially expressed in the uterus under the influence of sex-steroid hormones (Chinigarzadeh *et al.*, 2017). AQP is a small, hydrophobic, integral membrane protein that mediates passive movement of H_2O (Dey *et al.*, 2004). Specific AQP channel subunits have been identified in the uterus which include AQP-1, 5 and 7 (Norwitz *et al.*, 2001). It has been reported that AQP-1 is abundantly expressed in the endometrial stroma blood vessels at the time of implantation in rats (Cheong *et al.*, 2013). The expression of AQP-1 greatly increases the permeability of H_2O into cells (Staun-Ram & Shalev, 2005). In rats, AQP-1 expression in uterus is found to be up-regulated by

testosterone (Demir *et al.*, 2010) and progesterone (Hamilton & Boyd, 1960). AQP-5 was also documented to be expressed at the mesometrial pole of the rat uterus with highest level during peri-implantation period (Huppertz *et al.*, 2014). This subunit was found to be influenced by progesterone (Hamilton & Boyd, 1960). AQP-7, a member of aqua-glyceroporins is involved in the transport of H_2O and glycerol, was reported to be expressed in rat uteri (Gellersen *et al.*, 2007; Palomino *et al.*, 2013). AQP-7 is up-regulated by testosterone and is crucial for mediating uterine fluid reabsorption and decidualization (Demir *et al.*, 2010).

Thyroid hormone (TH) has been identified as an important hormone that control embryo implantation (Ashkar *et al.*, 2010). Low TH levels i.e. hypothyroidism is associated with female infertility (Priya *et al.*, 2015). Several studies indicate TH is involved in embryo implantation. *In-vitro* supplementation of TH has been found to enhance embryo implantation via assisting blastocyst formation and increasing its hatching rate in the uterus in bovine (Costa *et al.*, 2013). TH has also been reported to affect uterine receptivity development (Yoshioka W. *et al.*, 2015). Apart from these documented effects, role of TH in particular in the regulation of fluid in uterus during the peri-implantation period is unknown. There is a possibility that TH could affect uterine fluid transport based on observations that this hormone increases expression of AQP in rat kidney (Taylor J. *et al.*, 2014; Wu *et al.*, 2013). In addition, TH modulates the expression of AQP-8 in liver mitochondria (Marwood *et al.*, 2009) and AQP-5 in lung of rats (Goulart *et al.*, 2004).

We hypothesized that TH could also interfere with the fluid volume in uterus during peri-implantation period as well as expression of AQP subunits in uterus that are involved in the fluid volume regulation. Therefore, this study aims to investigate changes in the amount of fluid in uterus and the levels of AQP-1, 5 and 7 during peri-

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implantation period in a state of low thyroid hormone levels and following thyroxine treatment. This study is important as it could provide additional mechanisms underlying hypothyroid-related infertility in female which could be related to derangement in fluid transport during the early pregnancy period.

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6.2 Results

6.2.1 Effects of hypothyroidism and thyroxine treatment on uterine inner/outer circumference during peri-implantation period

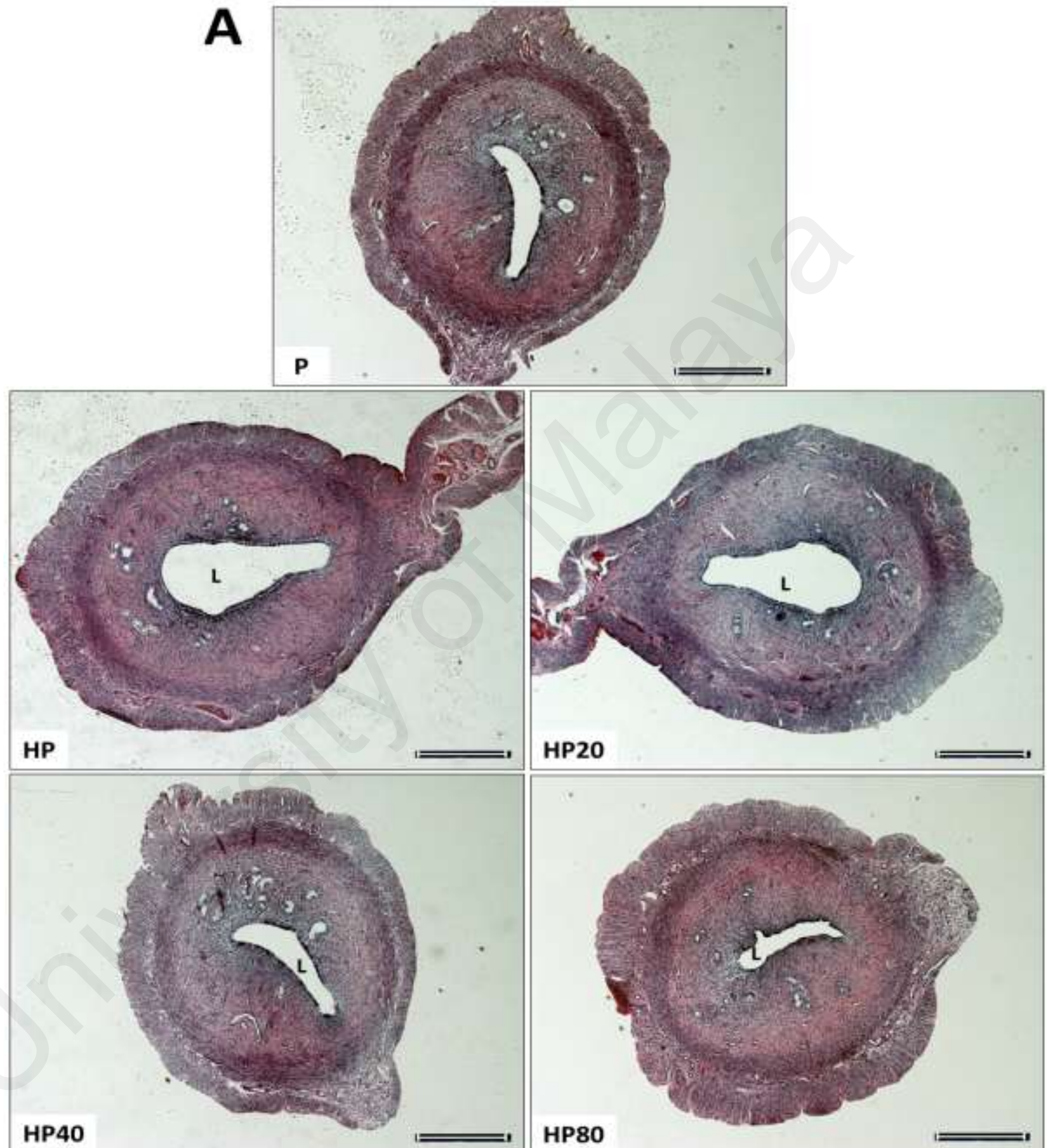

Figure 6.1 (A) Representative cross-sectional image of uterus (In next page) (B) Analyses of the ratio of inner/outer uterine circumference.

Data were expressed as mean ± SEM; n= 6 per group; *p<0.05 compared to P; #, $, Ø p<0.05 compared to HP. Scale bars represent 50 μm, L=lumen.

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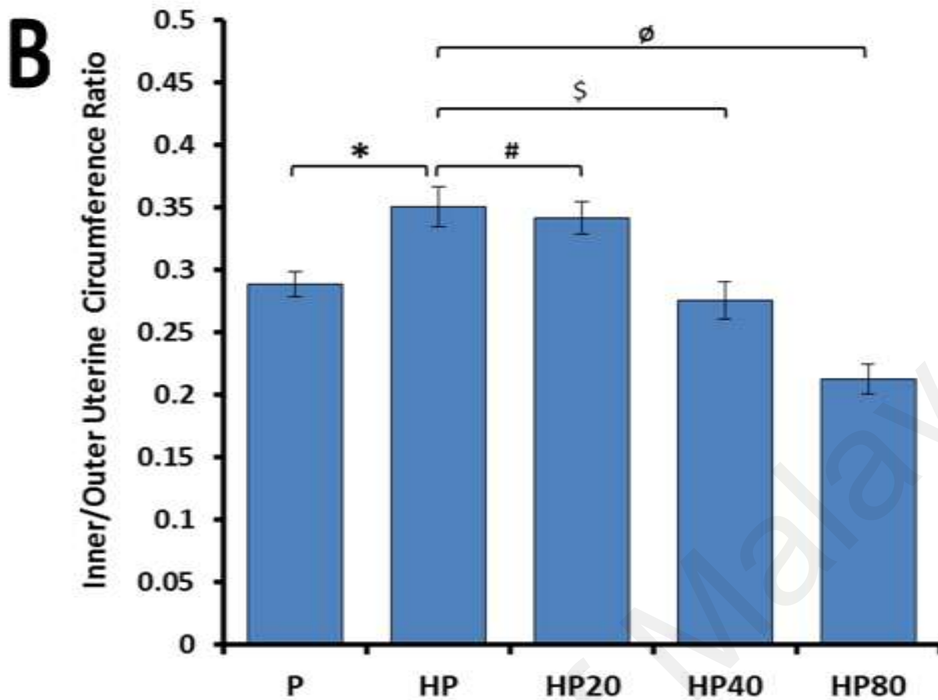

Figure 6.1 (A) Representative cross-sectional image of uterus (B) Analyses of the ratio of inner/outer uterine circumference.

Data were expressed as mean ± SEM; n= 6 per group; $^{*}p<0.05$ compared to P; $^{\#, \$, Ø}\ p<0.05$ compared to HP. Scale bars represent 50 µm, L=lumen.

Representative cross sectional images of the uterus showed uterine lumen was largest in hypothyroid pregnant rats relative to euthyroid pregnant rats (Fig. 6.1A). In hypothyroid pregnant rats which received 20 µg/kg/day thyroxine, the amount of fluid in uterus was not significantly different when compared to non-thyroxine treated hypothyroid rats. However, in pregnant hypothyroid rats receiving 40 and 80 µg/kg/day thyroxine, the amount of fluid in uterus was markedly lower as compared to non-thyroxine treated and 20 µg/kg/day thyroxine-treated hypothyroid rats, where the effect of 40 µg/kg/day thyroxine resemble that of the euthyroid pregnant rats. Analyses of inner/outer uterine circumference ratio confirm the morphological observation (Fig. 6.1B).

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6.2.2 Effects of hypothyroidism and thyroxine treatment on the levels of AQP-1, 5 and 7 proteins in uterus during peri-implantation period

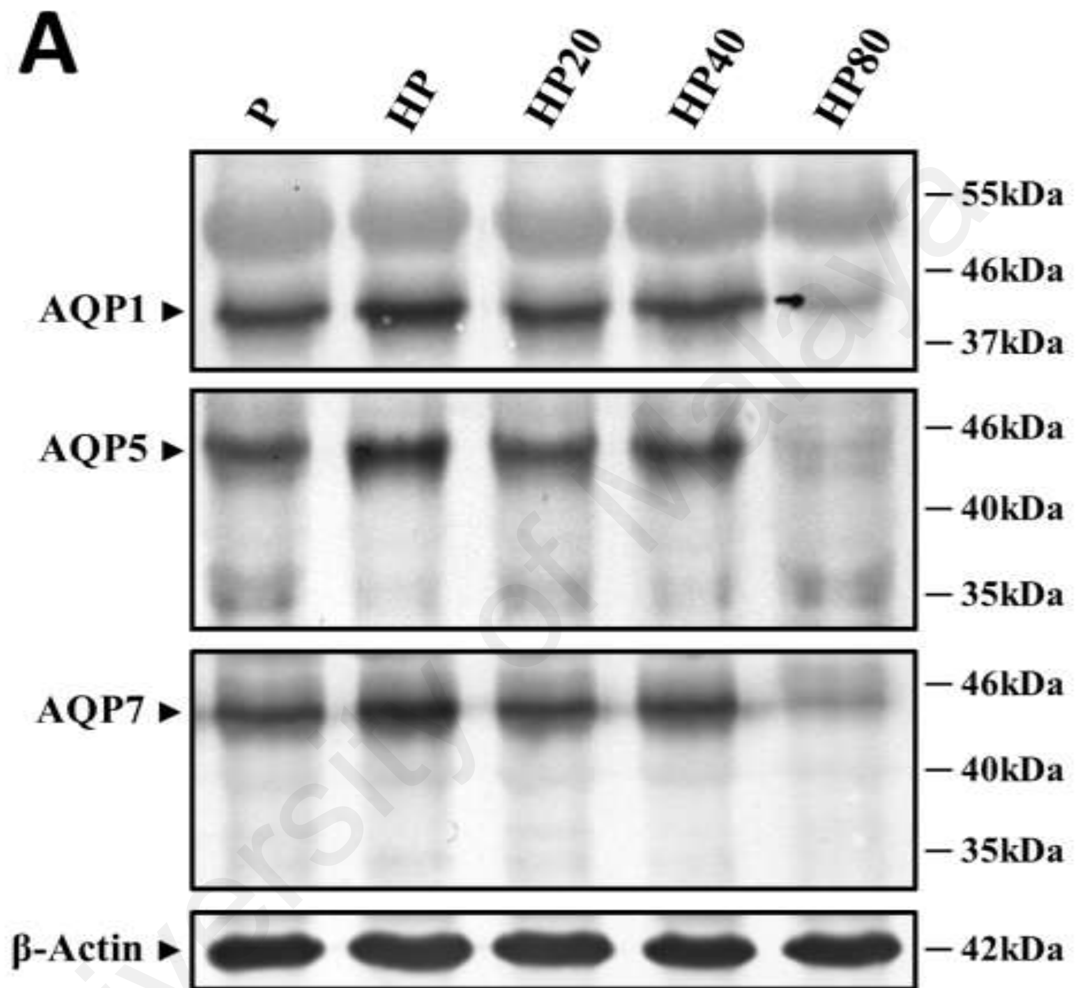

Figure 6.2 Expression level of AQP-1, AQP-5 and AQP-7 proteins in uterus

(A) Representative immunoblot images of AQP-1, AQP-5 and AQP-7 in uterus. (In next page) (B) Ratio of band intensity of AQP-1, AQP-5 and AQP-7 proteins versus β-actin protein. Data were presented as mean ± SEM; n = 6 per group; $^{*}p<0.05$ compared to P; $^{\#, \$, Ø}p<0.05$ compared to HP.

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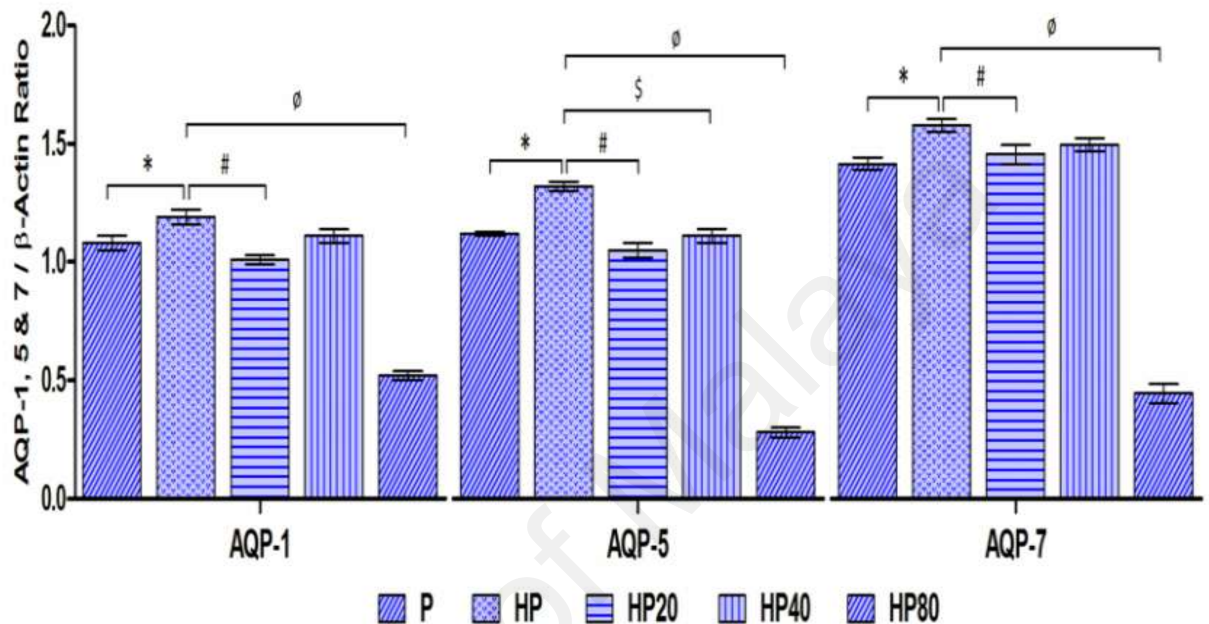

Figure 6.2 Expression level of AQP-1, AQP-5 and AQP-7 proteins in uterus

(A) Representative immunoblot images of AQP-1, AQP-5 and AQP-7 in uterus. (B) Ratio of band intensity of AQP-1, AQP-5 and AQP-7 proteins versus β-actin protein. Data were presented as mean ± SEM; n = 6 per group; $^{*}p<0.05$ compared to P; $^{\#, \$, Ø}$ $p<0.05$ compared to HP.

Higher expression of AQP-1 protein was observed in hypothyroid pregnant rat uterus as compared to euthyroid pregnant rat uterus (Fig. 6.2A and 6.2B). In 20 and 40 µg/kg/day thyroxine-treated hypothyroid pregnant rats, levels of AQP-1 protein in uterus were slight but significantly lower when compared to untreated pregnant hypothyroid rats. The levels were markedly decreased in pregnant, hypothyroid rats following treatment with 80µg/kg/day thyroxine.

Expression levels of AQP-5 protein were higher in pregnant, hypothyroid rats when compared to pregnant, euthyroid rats ($p<0.05$). In the former, AQP-5 levels

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decreased significantly following treatment with 20 and 40µg/kg/day thyroxine. Marked decreases in AQP-5 levels were observed in pregnant hypothyroid rats following treatment with 80µg/kg/day thyroxine (Fig. 6.2A and 6.2B).

In the meantime, levels of AQP-7 in uterus were found to slightly increase in hypothyroid condition as compared to euthyroid condition in early pregnancy (Fig. 6.2A and 6.2B). In hypothyroid, pregnant rats, slight but significant decreases in AQP-7 levels were observed following treatment with 20 and 40 µg/kg/day thyroxine. A marked decrease in AQP-7 levels was observed following treatment with 80 µg/kg/day thyroxine.

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6.2.3 Effects of hypothyroidism and thyroxine treatment on distribution of AQP-1 proteins in uterus

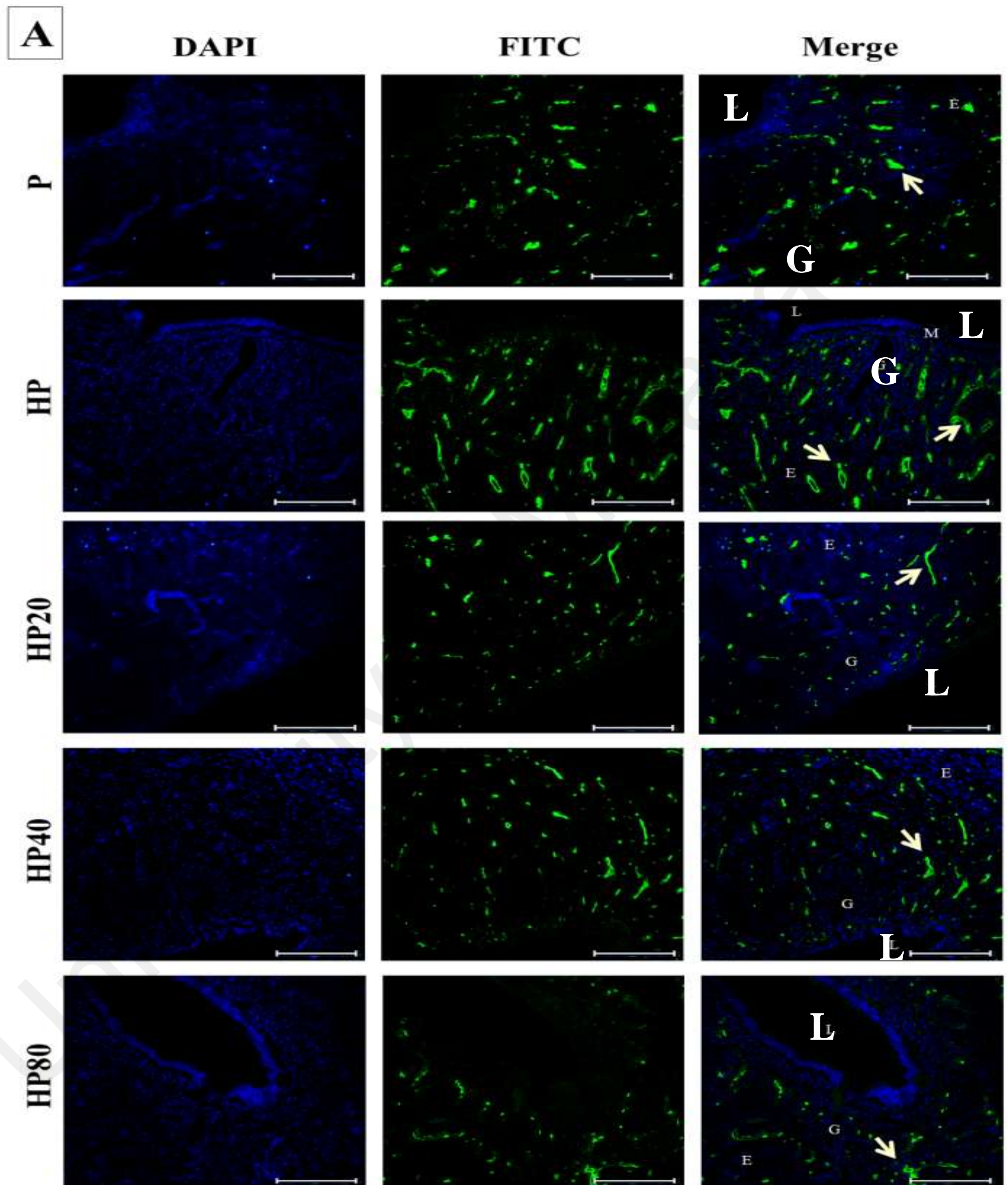

Figure 6.3 Representing fluorescence images showing expression of (A) AQP-1 and (In next page) (B) AQP-5 in uterus.

Green fluorescence signals indicate sites where AQP-1 was distributed and red fluorescence signals indicate sites where AQP-5 was distributed. Analysis was done qualitatively. Scale bars represent 50 µm. Arrows show protein distribution sites. *DAPI*, 4′,6-diamidino-2-phenylindole dihydrochloride; *FITC*, fluorescein isothiocyanate; *PE*, phycoerythrin; E, endometrium; L, lumen; G, gland; M, endometrial muscle.

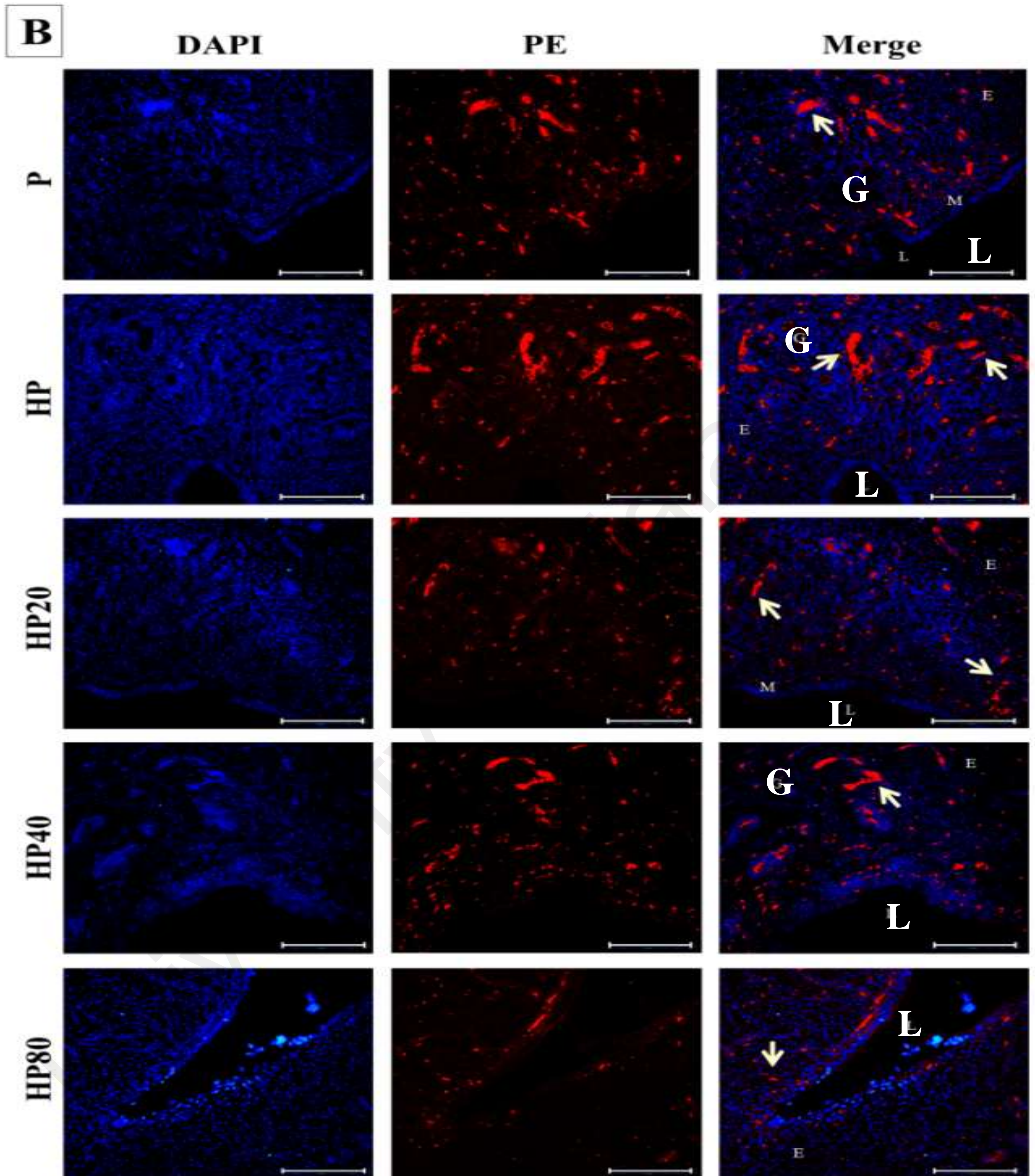

Figure 6.3 Representing fluorescence images showing expression of (A) AQP-1 and (B) AQP-5 in uterus.

Green fluorescence signals indicate sites where AQP-1 was distributed and red fluorescence signals indicate sites where AQP-5 was distributed. Analysis was done qualitatively. Scale bars represent 50 µm. Arrows show protein distribution sites. *DAPI*, 4′,6-diamidino-2-phenylindole dihydrochloride; *FITC*, fluorescein isothiocyanate; *PE*, phycoerythrin; E, endometrium; L, lumen; G, gland; M, endometrial muscle.

Immunofluorescence images showed that AQP-1 protein is distributed in the stromal blood vessels (Fig. 6.3A). A relatively higher distribution was observed in hypothyroid pregnant rats as compared to euthyroid pregnant rats. In hypothyroid pregnant rats, a relatively lower distribution was observed following treatment with 20 and 40µg/kg/day thyroxine. The distribution was markedly reduced following treatment with 80µg/kg/day thyroxine

6.2.4 Effects of hypothyroidism and thyroxine treatment on distribution of AQP-5 protein in uterus

In Fig. 6.3B, immunofluorescence images showed AQP-5 protein was distributed in the glands. A relatively higher distribution was observed in hypothyroid pregnant rats as compared to euthyroid pregnant rats. In hypothyroid pregnant rats, distribution was relatively lower following treatment with 20 and 40µg/kg/day thyroxine. The distribution was markedly reduced following treatment with 80µg/kg/day thyroxine.

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6.2.5 Effects of hypothyroidism and thyroxine treatment on distribution of AQP-7 protein in uterus

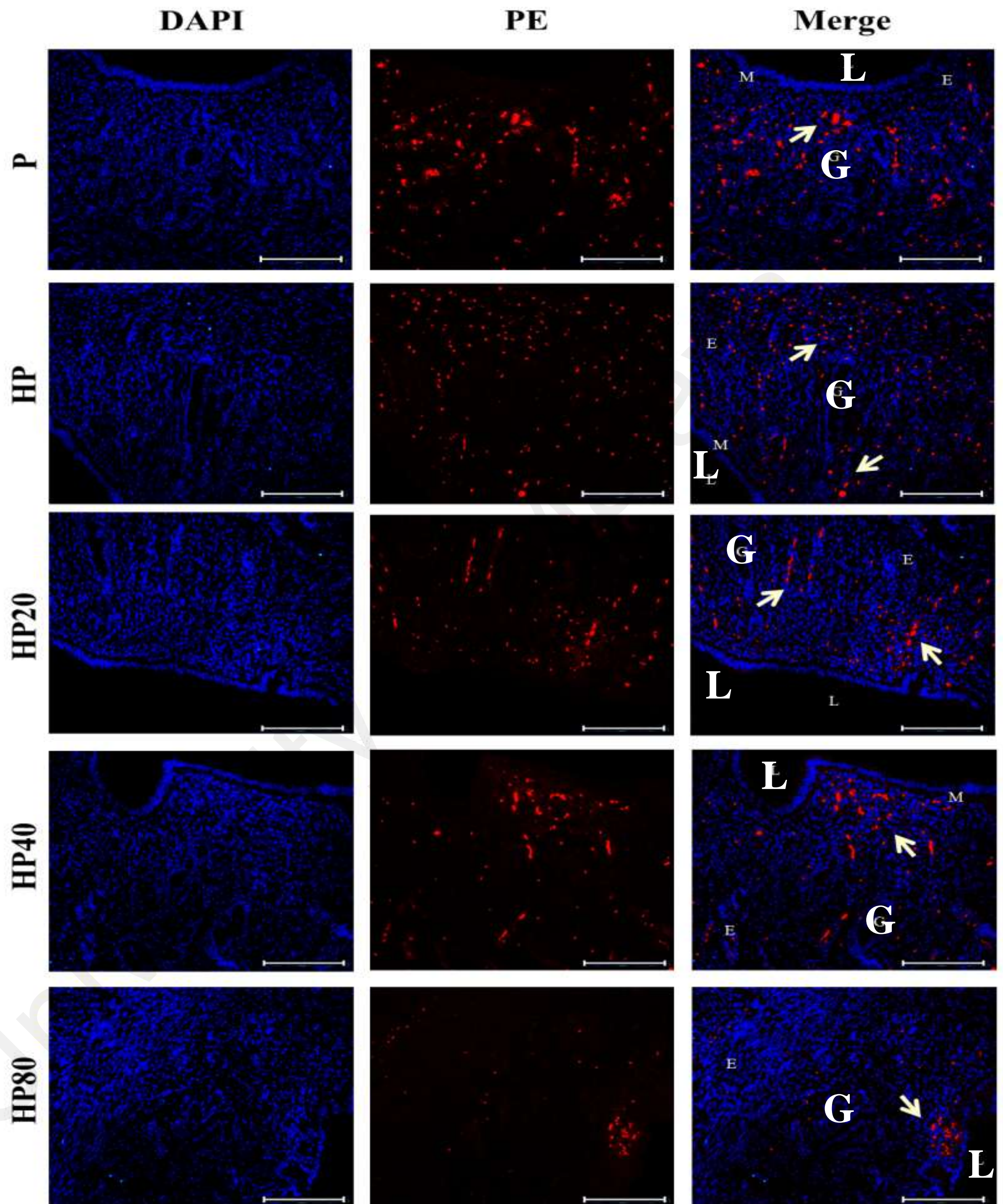

Figure 6.4 Representing fluorescence images showing expression of AQP-7 in uterus.

Red fluorescence signals indicate sites where AQP-7 was distributed. Analysis was done qualitatively. Scale bars represent 50 µm. Arrows show protein distribution sites. *DAPI*, 4′,6-diamidino-2-phenylindole dihydrochloride; *FITC*, fluorescein isothiocyanate; *PE*, phycoerythrin; E, endometrium; L, lumen; G, gland; M, endometrial muscle.

In Fig. 6.4, immunofluorescence images showed AQP-7 protein was distributed in the glands. A relatively higher distribution was observed in hypothyroid pregnant rats as compared to euthyroid pregnant rats. In hypothyroid pregnant rats, distribution was relatively lower following treatment with 20 and 40µg/kg/day thyroxine. The distribution was markedly reduced following treatment with 80µg/kg/day thyroxine.

6.3 Discussion

In this study, it was found that uterine fluid volume as reflected by inner/outer uterine circumference in early pregnancy increased when euthyroid rats were rendered hypothyroid and that thyroxine treatment in hypothyroid rats caused the fluid volume to decrease, depending on thyroxine doses where higher dose causes greater decrease as compared to lower doses. In addition, in this study, we found that expression of AQP-1, 5 and 7 proteins in the uterus markedly increased in hypothyroid condition as compared to euthyroid condition. Thyroxine treatment causes expression of AQP-1, 5 and 7 proteins in the uterus in hypothyroid state to decrease.

Changes in AQP subunits expression in the uterus could provide explanation for the changes observed in uterine fluid volume in different thyroid hormone conditions. Increased expression of AQP-1, which is mainly distributed in the stromal blood vessels, could contribute towards higher fluid volume in uterus. Distribution of AQP-1 in stromal blood vessels as observed in this study was consistent with the report of its similar distribution in the uterus in humans (Hildenbrand *et al.*, 2008; Mints *et al.*, 2007). Higher AQP-1 expression could result in greater H_2O imbibition into the lumen, with subsequent increase in volume of uterine fluid. As a consequence of the down-regulation of AQP-1 expression, lesser H_2O imbibition from blood vessels into the lumen would occur in hypothyroid pregnant rats receiving thyroxine treatment,

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particularly at higher dose. This would result in a decrease in uterine fluid volume and a smaller size lumen.

Additionally, this study showed that expression of AQP-5 in uterus was significantly in hypothyroidism which decreased following thyroxine treatment, particularly at high dose (80mg/kg/day). AQP-5 was also found to be distributed in endometrial glands, which is congruent with other reports (Chinigarzadeh *et al.*, 2016; Granfors *et al.*, 2013; Stavreus Evers, 2012). This localization would help to facilitate H_2O movement from the stromal compartments into the lumen of the uterus. Therefore, its increased expression under hypothyroid condition could help to explain the increased in uterine fluid while its reduced expression following thyroxine treatment would result in the *vice versa* effects.

Finally, we found that AQP-7 expression was also increased in hypothyroid pregnant rats and was reduced following thyroxine treatment, particularly at highest dose. Localization of AQP-7 in the glands which was highest in hypothyroid condition would also help in the H_2O imbibition process from stroma into the lumen. Our findings were similar with the findings by (Chinigarzadeh *et al.*, 2016) which also indicated that AQP-7 was expressed in the glands under the influence of sex-steroids. Expression of AQP-7 in the endometrium at the time of implantation was consistent with other finding (Degrelle *et al.*, 2013). Reduced expression of this AQP subunit following thyroxine treatment in early pregnant hypothyroid rats would result in lesser movement of H_2O into the lumen, consequently a smaller size lumen.

In general, increased expression levels of AQP 1, 5 and 7 subunits and their considerably higher distributions in the uterus in hypothyroid condition in early pregnancy period was consistent with the findings of an increased in expression of the AQP-5 protein in the lungs in rats (Goulart *et al.*, 2004). The observed decreased in

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expressions of AQP subunits and their distribution following thyroxine treatment suggested that down-regulation of AQP subunits by thyroxine could be due to interaction of TH-dependent TR its with putative negative thyroid hormone response elements (nTREs) (Cross *et al.*, 1994) which could be present in the promoter of AQP subunits gene. TH implements this regulatory mechanism when acting on its negatively regulated target genes (Kim M. *et al.*, 2013; Sun *et al.*, 2010). The postulation of TH down-regulation mediated by nTREs is consistent with a study whereby tri-iodothyronine (T3) down-regulated the transcription of AQP-8 within a short span of time (Kilic *et al.*, 2008). Further investigations are needed to elucidate the precise mechanisms underlying TH regulation of AQP expression in uterus.

In conclusions, higher than normal AQP- 1, 5 and 7 expression in the peri-implantation uterus in hypothyroid condition could result in excessive accumulation of fluid in the uterine lumen which could interfere with blastocyst attachment to the uterine wall. Failure of attachment would compromise the establishment of pregnancy that might explain the high infertility rate associated with hypothyroid state. Thyroxine treatment could reverse the hypothyroid effects, albeit high dose thyroxine treatment might also result in adverse effect on implantation by causing excessive reduction in the amount of fluid in uterus.

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CHAPTER 7: INVESTIGATE THE EFFECTS OF THYROID HORMONE ON THE EXPRESSION OF UTERINE RECEPTIVITY PROTEINS (INTEGRIN AVB3, E-CADHERIN, MSX-1 AND IHH) IN RAT DURING PERI-IMPLANTATION PERIOD

7.1 Introduction

Development of uterine receptivity is crucial for successful embryo implantation (Costa *et al.*, 2013). Transformation of uterus into a receptive state involves functional and morphological changes, particularly to the endometrium (Shahzad H., N. Giribabu, K. Karim, S. Muniandy*, et al.*, 2017). During the receptivity period, complex interactions occur between hormones, adhesion molecules, extracellular matrix, and paracrine factors (Lindsay L. A. & C. R. Murphy, 2007). Several endometrial proteins have been identified to be expressed exclusively during the uterine receptivity period, thus serve as markers for uterine receptivity. These proteins molecules include Integrin αvβ3, e-cadherin, Msx-1 and Ihh (Inuwa & Williams, 1996b).

Integrin is a member of proteins belongs to cell adhesion family which is heterodimeric glycoprotein formed by both α and β subunits structurally (Achache & Revel, 2006). Integrin αvβ3 is essential regulator of cell to cell interaction, cell adhesions and cell survival (Trolice & Amyradakis, 2012). Defect in expression of integrin αvβ3 is associated with unexplained infertility (Gupta *et al.*, 2008) Integrin αvβ3 is reported to be expressed in the luminal and glandular epithelia in normal fertile uterus in women during implantation window period (Zhang D. *et al.*, 2012). It is reported to mediate cell proliferation, apoptosis and modulation of inflammatory changes in endometrial decidualization (Mangale *et al.*, 2008) and its expression can be influenced by sex-steroids (Kirsten, 2000).

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Cadherins are the family of Ca^{2+} dependent surface cell adhesion (Achache & Revel, 2006). Among all cadherin's molecules, E-cadherin is the most investigated and have been categorized as crucial receptivity molecule for human trophoblastic invasion (Rahnama *et al.*, 2006). E-cadherin plays a vital role in the maintenance and function of endometrial epithelium (Li Q. *et al.*, 2002). It has been showed that alterations in E-cadherin gene could result in impaired development of uterine receptivity in mouse during the peri-implantation period (Riethmacher *et al.*, 1995). Expression of E-cadherin was reported to increase in canine endometrium during uterine receptivity period (Stathatos, 2012). E-cadherin expression in endometrium could be influence by progesterone (Brent, 2012).

Muscle segment homebox (Msx) genes are one of the most conserved families of homeobox genes (Finnerty *et al.*, 2009). It acts as downstream targets of bone morphorgenic protein (BMPs) during development (Bei & Maas, 1998; Timmer *et al.*, 2002). Msx family of three different genes, such as, Msx-1, Msx-2 and Msx-3 have been found in mice (Davidson, 1995). Msx plays crucial role in determining endometrial cell polarity that is required for blastocyst attachment to the endometrium. Msx-1 expression in uterus was found during implantation window period and influenced by sex-steroids (Davis *et al.*, 2010). It has been reported that Msx genes critically regulate embryo implantation via controlling Ihh paracrine signaling between uterine stroma and epithelium (Davis P. J. *et al.*, 2016).

Mammalian Hedgehog proteins synthesized by the transcriptions of three hedgehog genes, i.e. Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh) (Pathi *et al.*, 2001). Hedgehog family plays several roles in mediating cellular processes from embryonic to adult many species (Ingham & McMahon, 2001; Walterhouse *et al.*, 2003; Walterhouse *et al.*, 1999). Ihh was reported to be expressed at

high levels in uterine luminal and glandular epithelia prior to receptivity in rats and mice (Paria *et al.*, 2001). The expression of Ihh in uterus was found to be regulated by progesterone (Bergh *et al.*, 2005).

Besides sex-steroids, thyroid hormone (TH) has been proposed to play a role in endometrial receptivity development prior to implantation (Ashkar *et al.*, 2010). This was based on the observation that the levels of TH receptor proteins (TRα-1, TRα-2 and TRβ1) in the endometrium increased concomitantly with expression of pinopodes and other endometrial receptivity markers (Aghajanova L. *et al.*, 2011). Therefore, in this study, effects of thyroxine on uterine receptivity marker (integrin αV/β3, E-cadherin, Msx-1 and Ihh) expression were investigated in hypothyroid condition.

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7.2 Results

7.2.1 Levels of integrin αvβ3, E-cadherin, Msx-1 and Ihh proteins in uterus during peri-implantation period

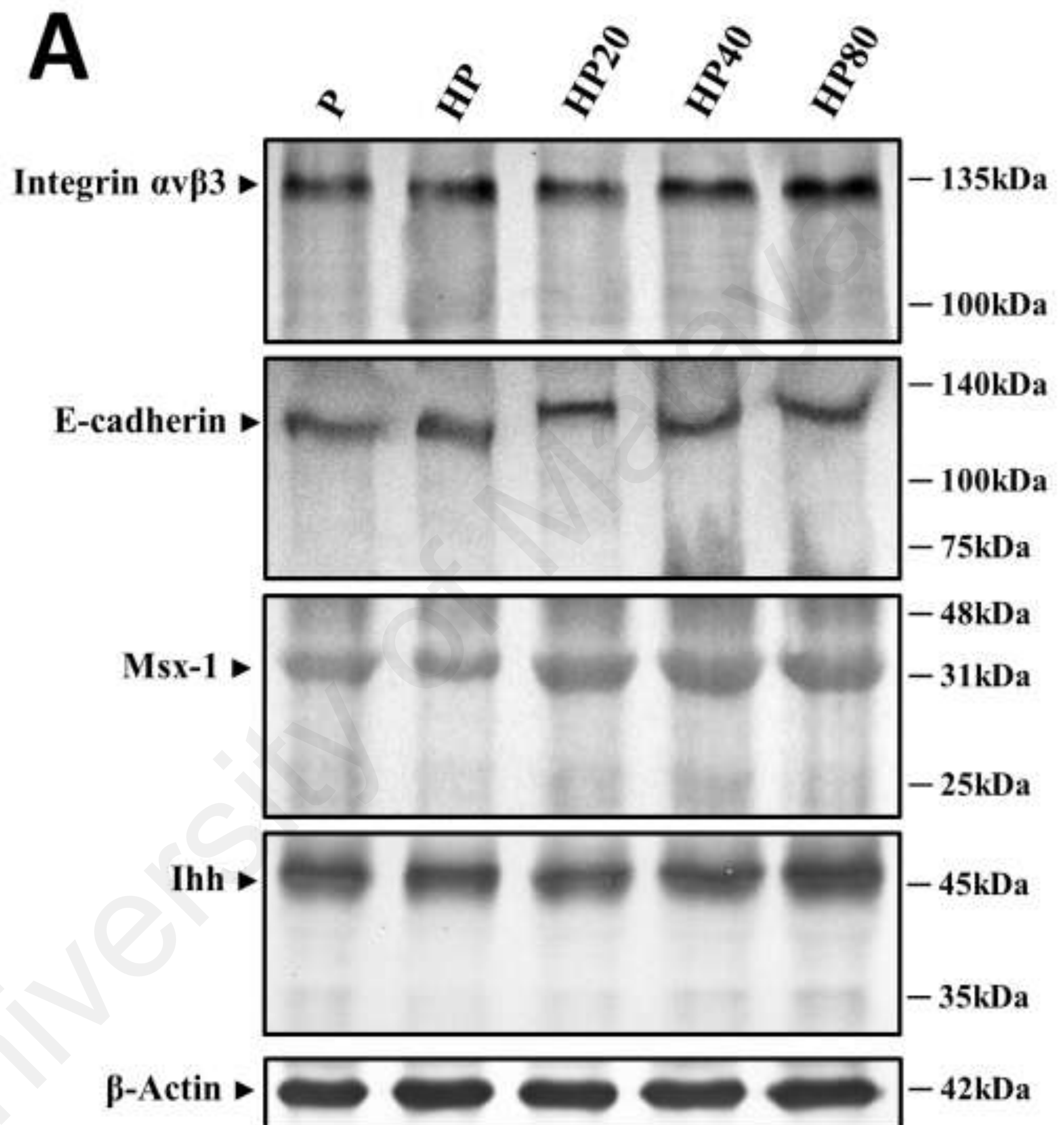

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Figure 7.1 Expression level of Integrin αvβ3, E-cadherin, Msx-1 and Ihh proteins in uterus.

(A) Representative immunoblot images of Integrin αvβ3, E-cadherin, Msx-1 and Ihh in uterus. (In next page) (B) Ratio of band intensity of Integrin αvβ3 and E-cadherin proteins versus β-actin protein. (C) Ratio of band intensity of Msx-1 and Ihh proteins versus β-actin protein. Data were presented as mean ± SEM; n = 6 per group. Bars that do not share the same symbols differ significantly at $P<0.05$.

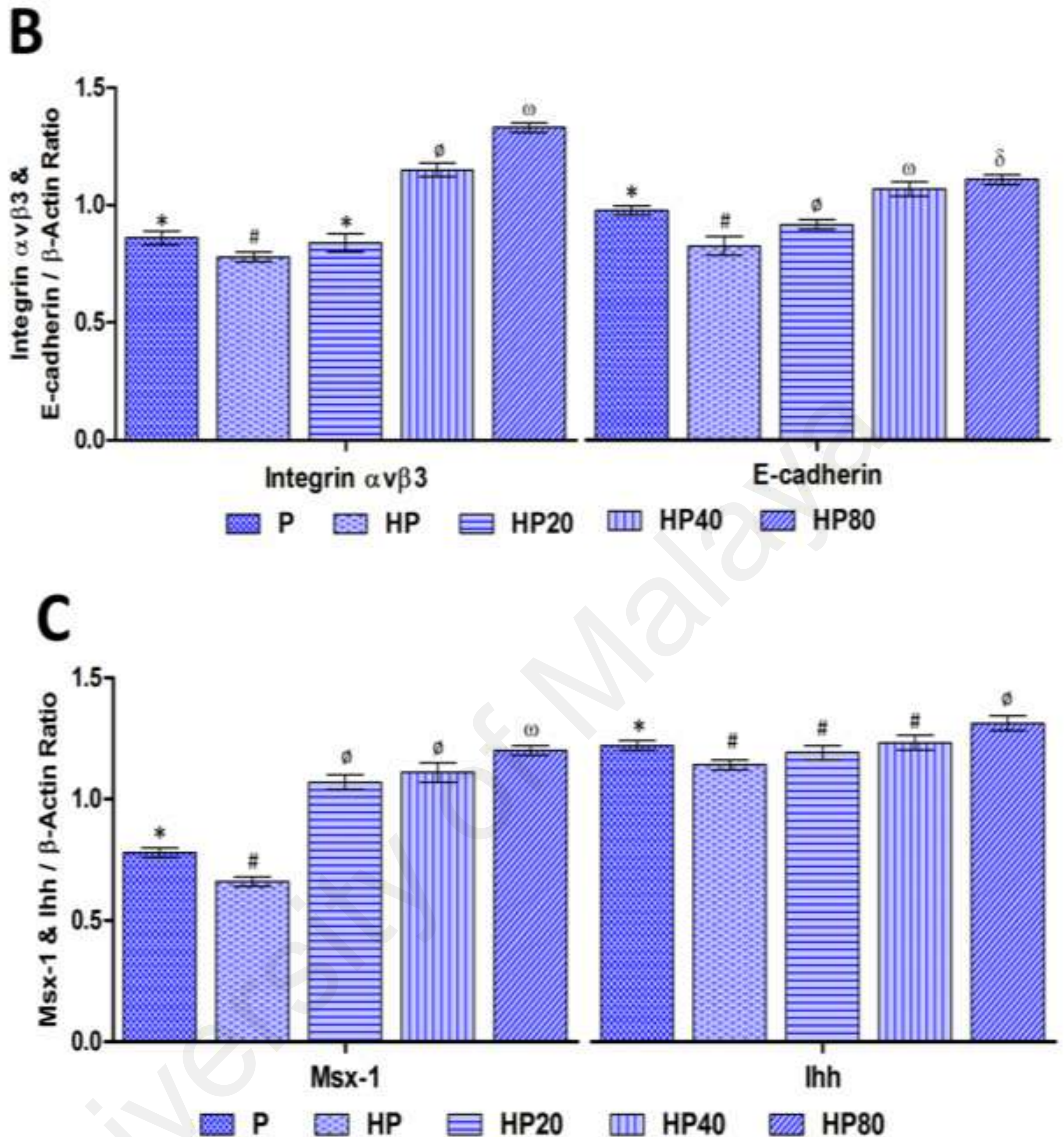

Figure 7.1 Expression level of Integrin αvβ3, E-cadherin, Msx-1 and Ihh proteins in uterus.

(A) Representative immunoblot images of Integrin αvβ3, E-cadherin, Msx-1 and Ihh in uterus. (B) Ratio of band intensity of Integrin αvβ3 and E-cadherin proteins versus β-actin protein. (C) Ratio of band intensity of Msx-1 and Ihh proteins versus β-actin protein. Data were presented as mean ± SEM; n = 6 per group. Bars that do not share the same symbols differ significantly at *P*<0.05.

Levels of integrin αvβ3 protein expression in hypothyroid pregnant rat uterus were lower as compared to euthyroid pregnant rats (Fig. 7.1A and 7.1B). In 20 μg/kg/day thyroxine-treated hypothyroid pregnant rats, levels of integrin αvβ3 protein

in uterus were slightly increased. But the levels were significantly increased in pregnant, hypothyroid rats following treatment with 80µg/kg/day thyroxine.

Expression levels of E-cadherin protein in uterus were lower in pregnant, hypothyroid rats when compared to pregnant, euthyroid rats ($p<0.05$). E-cadherin levels increased significantly following treatment with 20, 40 and 80 µg/kg/day thyroxine in a dose dependent manner (Fig. 7.1A and 7.1B).

Levels of Msx-1 in hypothyroid pregnant rats uteri were significantly lower as compared to pregnant, euthyroid rats ($p<0.05$). In hypothyroid pregnant rats, significant increases in Msx-1 levels were observed following treatment with 20 and 40 µg/kg/day thyroxine (Fig. 7.1A and 7.1C). A marked increase in Msx-1 levels was observed following treatment with 80 µg/kg/day thyroxine.

Expression levels of Ihh protein were slightly decreased in the uterus of hypothyroid pregnant rats as compared to euthyroid pregnant rats. Following treatment with 20 µg/kg/day thyroxine levels of Ihh expression in hypothyroid pregnant rats were increased slightly (Fig. 7.1A and 7.1C). Significant increases in the level of Ihh expression were observed following treatment with 40 and 80µg/kg/day thyroxine.

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7.2.2 Distribution of integrin αv/β3 and E-cadherin proteins expression in the endometrium

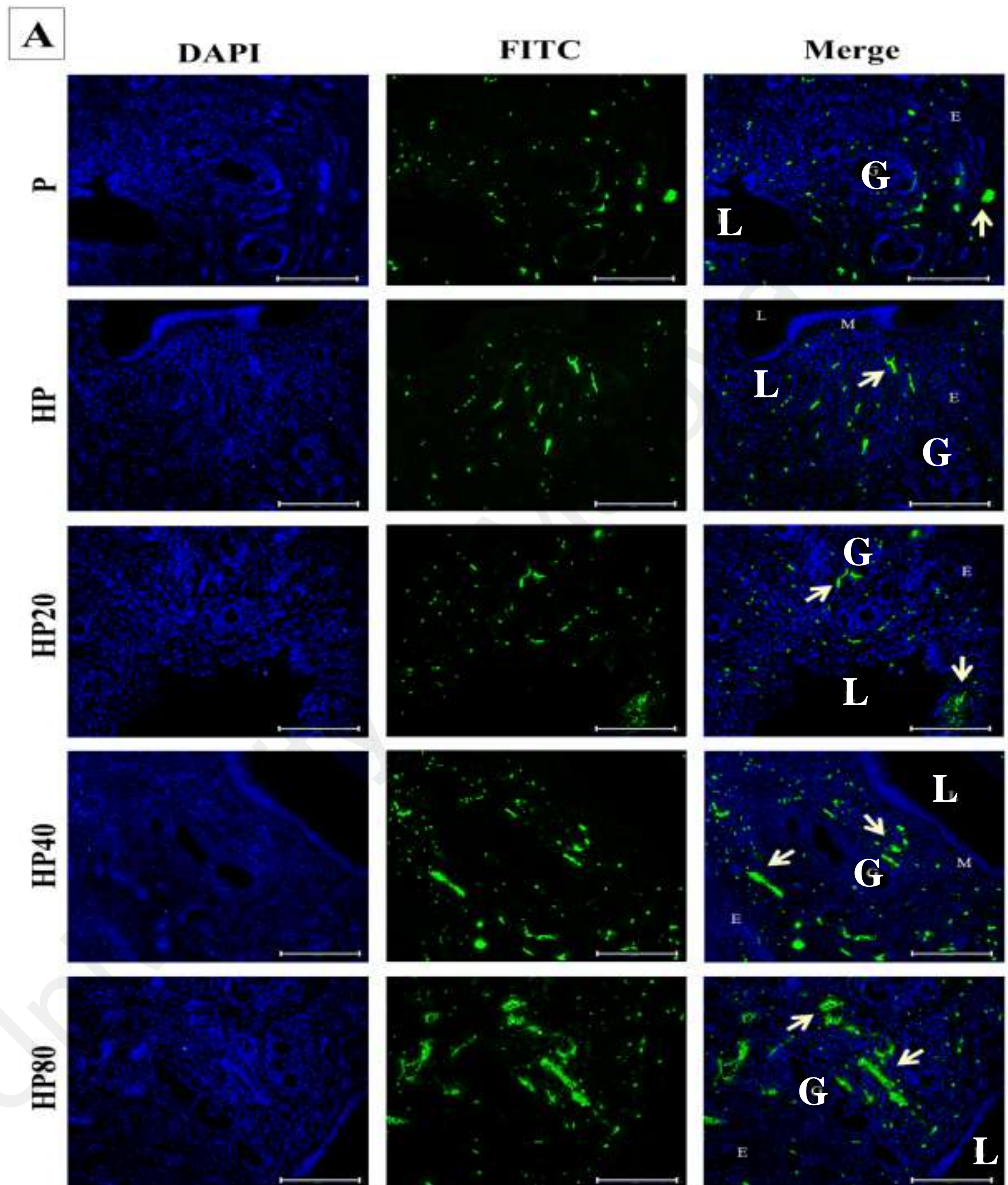

Figure 7.2 Representing fluorescence images showing expression of (A) Integrin αvβ3 and (In next page) (B) E-cadherin in uterus.

Green fluorescence signals indicate sites where Integrin αvβ3 and E-cadherin were distributed. Analysis was done qualitatively. Scale bars represent 50 µm. Arrows show protein distribution sites. *DAPI*, 4′,6-diamidino-2-phenylindole dihydrochloride; *FITC*, fluorescein isothiocyanate; E, endometrium; L, lumen; G, gland; M, endometrial muscle.

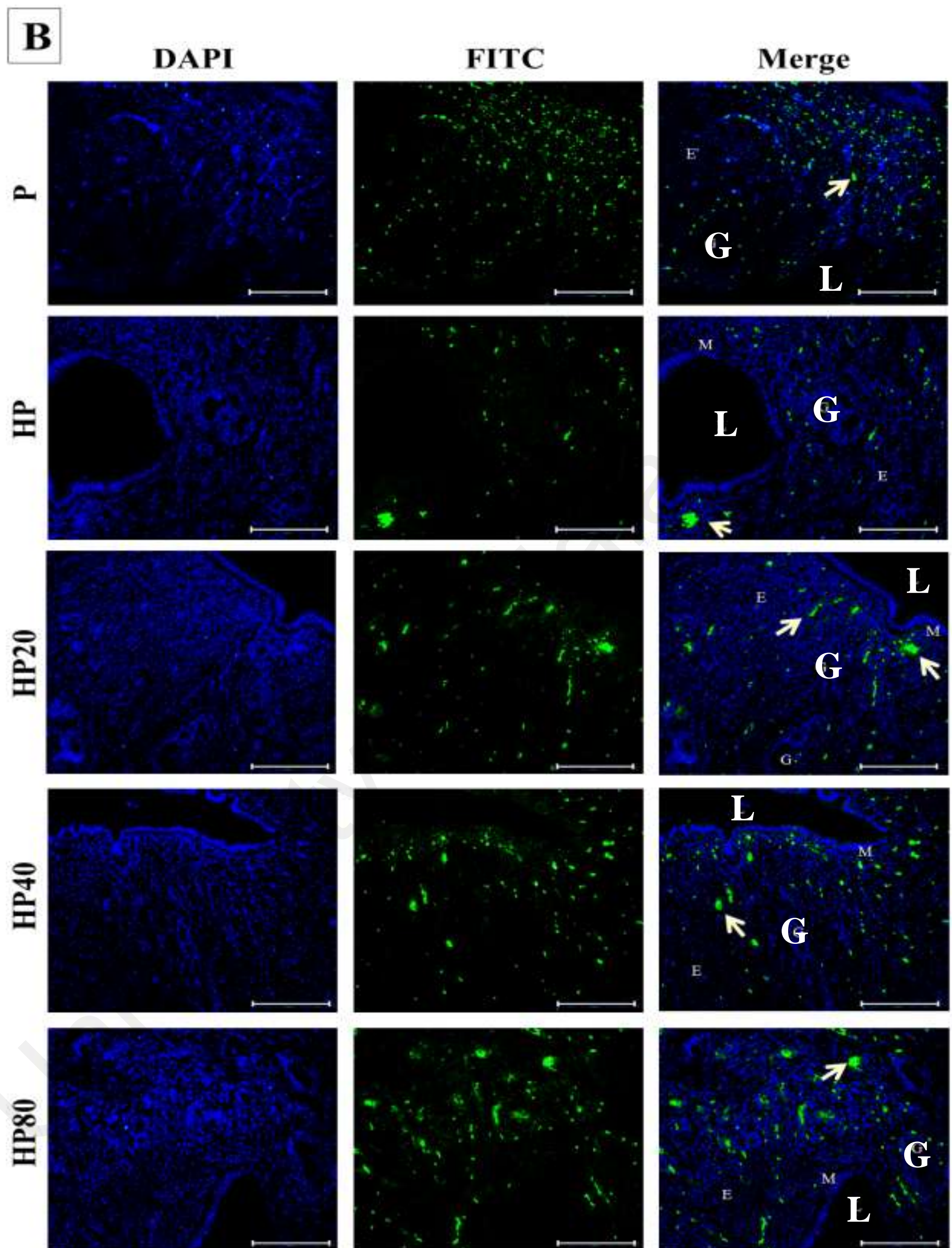

Figure 7.2 Representing fluorescence images showing expression of (A) Integrin αvβ3 and (B) E-cadherin in uterus.

Green fluorescence signals indicate sites where Integrin αvβ3 and E-cadherin were distributed. Analysis was done qualitatively. Scale bars represent 50 µm. Arrows show protein distribution sites. *DAPI*, 4′,6-diamidino-2-phenylindole dihydrochloride; *FITC*, fluorescein isothiocyanate; E, endometrium; L, lumen; G, gland; M, endometrial muscle.

In hypothyroid pregnant rats, distribution of integrin αvβ3 in uterus was relatively lower as compared to euthyroid pregnant rats (Fig. 7.2A). In the former, distribution of integrin αvβ3 relatively increases following treatment with thyroxine, with higher dose of thyroxine causes higher integrin αvβ3 distribution. This protein could be seen to be distributed in the stroma.

E-cadherin protein was found to be distributed in the stroma, particularly in the blood vessels (Fig. 7.2B). The distribution was relatively decreased in hypothyroid pregnant rats. In these rats, a relatively higher E-cadherin distribution was observed in the uterus following thyroxine treatment. The distribution increases with increasing dose of thyroxine.

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7.2.3 Distribution of Msx-1 and Ihh proteins expression in the endometrium

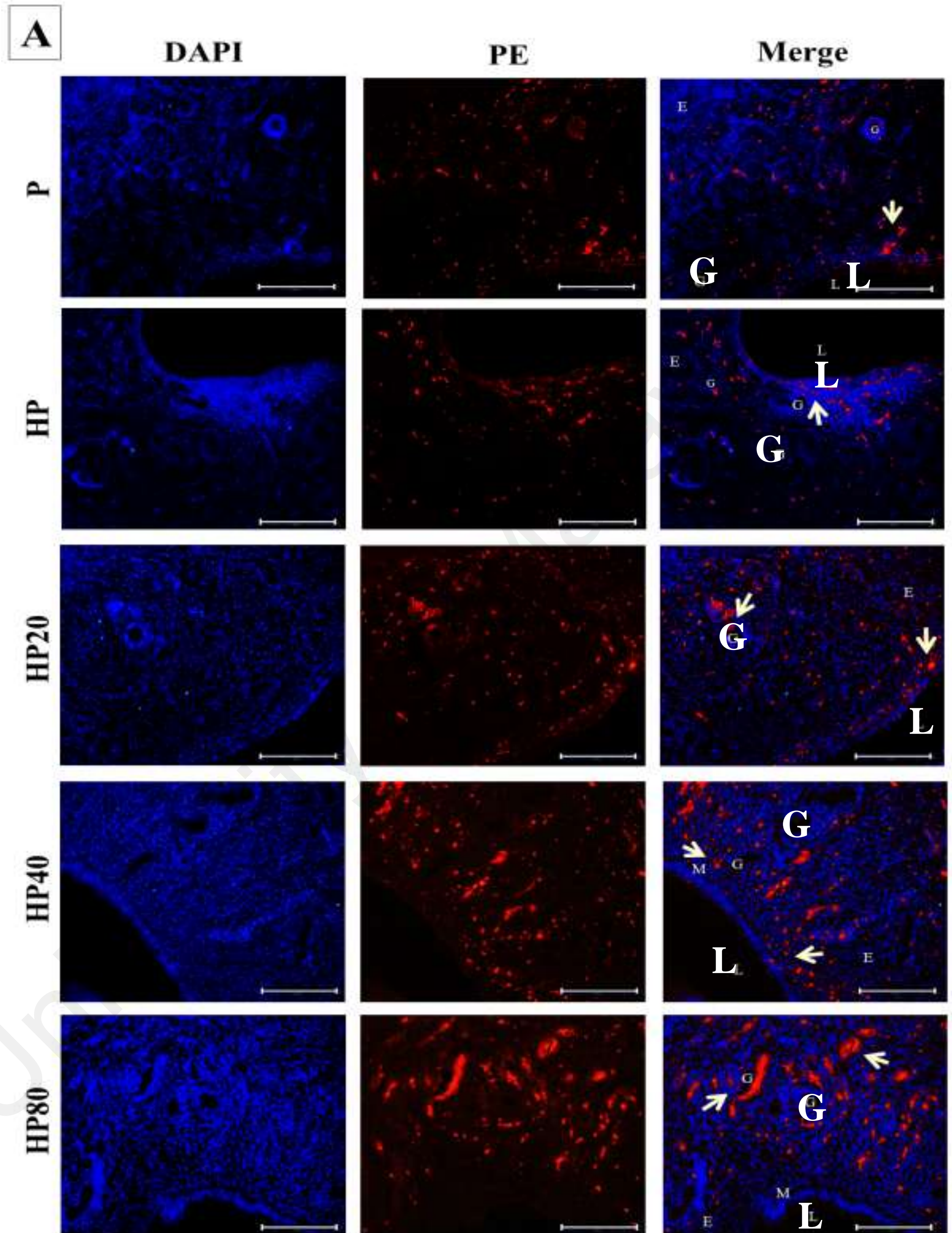

Figure 7.3 Representing fluorescence images showing expression of (A) Msx-1 and (In next page (B) Ihh in uterus.

Red fluorescence signals indicate sites where Msx-1 and Ihh were distributed. Analysis was done qualitatively. Scale bars represent 50 µm. Arrows show protein distribution sites. *DAPI*, 4′,6-diamidino-2-phenylindole dihydrochloride; *PE*, phycoerythrin; E, endometrium; L, lumen; G, gland; M, endometrial muscle.

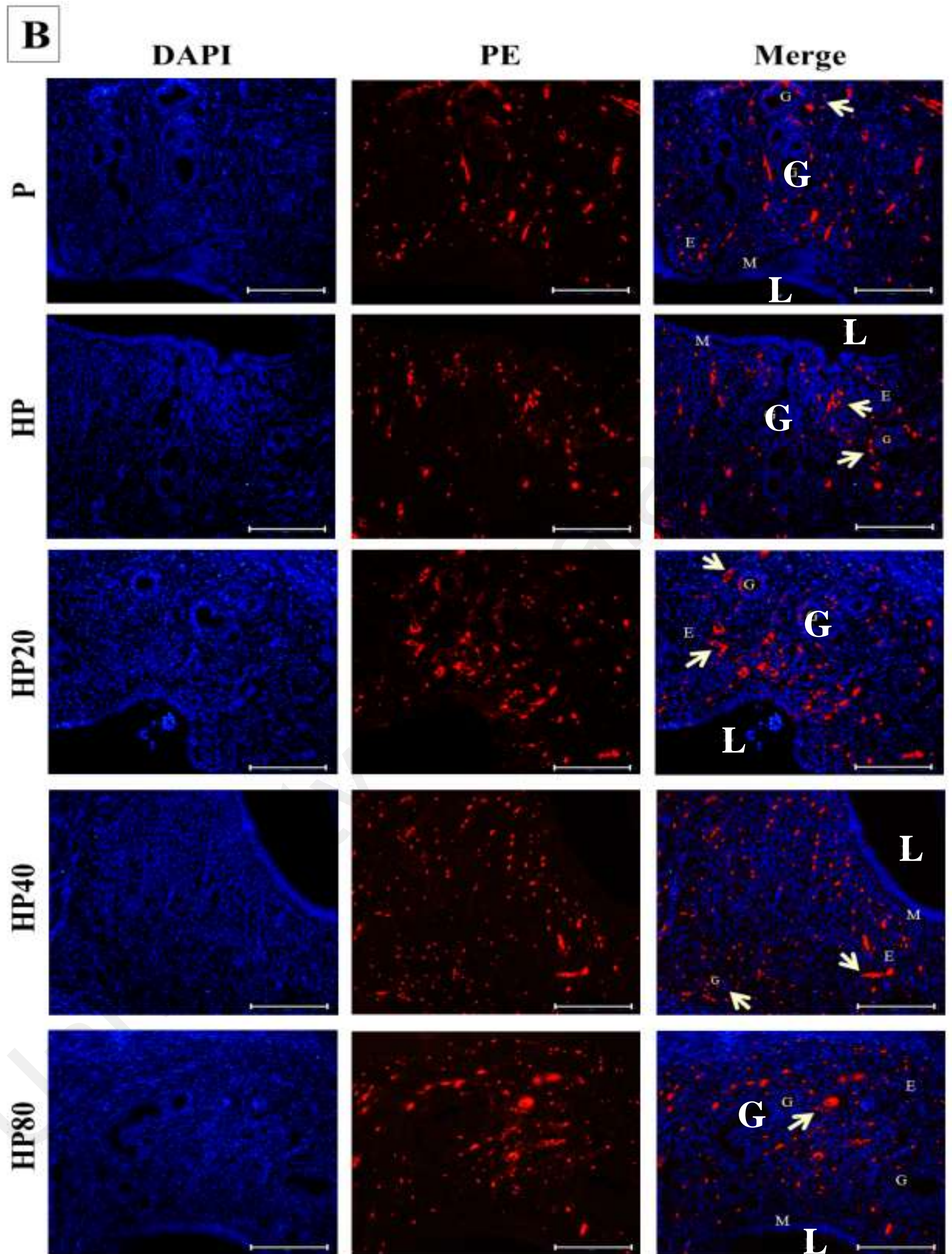

Figure 7.3 Representing fluorescence images showing expression of (A) Msx-1 and (B) Ihh in uterus.

Red fluorescence signals indicate sites where Msx-1 and Ihh were distributed. Analysis was done qualitatively. Scale bars represent 50 µm. Arrows show protein distribution sites. *DAPI*, 4′,6-diamidino-2-phenylindole dihydrochloride; *PE*, phycoerythrin; E, endometrium; L, lumen; G, gland; M, endometrial muscle.

In Fig. 7.3A, distribution of Msx-1 was relatively decreased in hypothyroid pregnant rat uterus when compared to euthyroid pregnant rats. Msx-1 could be seen to be distributed in the luminal and glandular epithelium and stroma. Msx-1 distribution was relatively increased following treatment with thyroxine, in a dose-dependent manner.

Ihh protein could be seen to be distributed in the stroma (Fig. 7.3B). The distribution was relatively similar in all groups i.e. euthyroid pregnant rats, hypothyroid pregnant rats and hypothyroid pregnant rats treated with 20, 40 and 80 µg/kg/day thyroxine.

7.3 Discussion

In this study, thyroxine treatment increased expression of uterine receptivity molecules including integrin αvβ3, E-cadherin and Msx-1, however only a slight increase in Ihh protein expression was observed following treatment with highest dose of thyroxine. The expression of all proteins except Ihh was decreased in hypothyroid condition.

We have shown that expression of E-cadherin in uterus during the peri-implantation period increased under the influence of thyroxine. This protein was found to be distributed in the stroma, mainly in the blood vessels and deep glands. Our findings were consistent with the finding by Jha et al, who reported expression of this protein in uterus increased in the peri-implantation period (Medici *et al.*, 2017). Our findings were also supported by a report that E-cadherin expression is the lowest in the surface epithelium which progressively increases toward the deepness of the endometrial glands (Payan-Carreira *et al.*, 2016). In addition, the presence of E-cadherin expression could also be due to the remodeling of the endometrial epithelia and stroma during peri-implantation period (Pawar *et al.*, 2013). It was previously reported that E-

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cadherin expression can be influence by sex-steroids as observed in the uterus of rats (Shahzad Huma *et al.*, 2017) and in human prostatic cell lines (Carruba *et al.*, 1995). Estrogen was found to decrease the levels of E-cadherin and the levels of this protein was also found to decreased in endometrium during the proliferative phase of the menstrual cycle, under the influence of E2 (Fujimoto *et al.*, 1998b). On the other hand, progesterone was found to increase E-cadherin expression (Fujimoto *et al.*, 1996). In addition to sex-steroids, plant-derived estrogen-like compound like quercetin was also reported to affect E-cadherin expression in rat uterus (Shahzad Huma *et al.*, 2017). Since E-cadherin plays a role in cell to cell adhesion, its increased expression in the stromal blood vessels indicates the possible decreased in vascular permeability (Fujimoto *et al.*, 1998a). Thus, there is a possibility that under the influence of thyroxine, decreased in stromal vascularity would result in lesser imbibition of water into the uterine lumen, consequently decreasing the uterine fluid volume. A reduction in uterine fluid volume during the peri-implantation period which is documented to assist in blastocyst attachment (Naftalin *et al.*, 2002) could affect the implantation process. Additionally, increased E-cadherin expression during the peri-implantation period could be due to the remodeling of the endometrial epithelia and stroma in preparation of blastocyst attachment and invasion (Pawar *et al.*, 2013). Collectively, low expression of E-cadherin in hypothyroid state might interfere with the amount of fluid in the uterus, as well as could interfere with endometrial epithelia remodeling, whereby dysregulation of both parameters could adversely affect blastocyst attachment. The outcomes of low expression of E-cadherin in uterus has been reported in women where this increases the in risk of recurrent failure in implantation (Mullur *et al.*, 2014)

Meanwhile, expression of integrin αvβ3 in uterus during peri-implantation period was also found to increase following thyroxine treatment. This protein was observed in the stroma. Our findings were consistent with the other findings which

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reported the expression of integrin αvβ3 in endometrium during the peri-implantation period (Davis *et al.*, 2011). . It was demonstrated that expression of integrin αvβ3 in swine uterus was influenced by sex-steroids (Poppe K. & B. Velkeniers, 2004). Besides, the influence of sex-steroids on integrin αVβ3 expression in uterus of women was proposed based on the observation that this protein was differentially expressed in endometrium throughout the menstrual cycle (Abdalla & Bianco, 2014). Integrin αVβ3 was reported to be highly expressed in the endometrium of fertile women as compared to infertile women which suggest that this protein might play a role in uterine receptivity development (Visser *et al.*, 2011).

Recently, Wang et al, reported the important role integrin αvβ3 played in decidua where suppression of this protein compromised stromal cellular proliferation, an event deemed essential during the implantation process (Fedail *et al.*, 2014). Hence, it is likely that increased expression of integrin αvβ3 in stroma under thyroxine influence would assist stromal cell proliferation, which increases endometrial thickness in preparation for blastocyst attachment. Lower expression of integrin αvβ3 in uterus has been found to impair endometrial receptivity development (Andreeva, 2014) which could be due to a decrease in endometrial thickness (Du & Li, 2013).

In this study, it was found that expression and distribution of Msx-1 in the endometrial luminal and glandular epithelia as well as stroma were increased following thyroxine treatment which could also help in the uterine receptivity development. Msx-1 is a signaling molecule which has been reported to play a role in the implantation process (Rosen *et al.*, 2011). The level of Msx-1 was found to increase in the uterus in mice during the period of receptivity (Zhang S. S. *et al.*, 1997). Consistently, infertile mice were found to have abnormal uterine expression of Msx-1 (Cheng S. Y. *et al.*,

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2010). In addition, it has also been reported that reduced expression of Msx-1 in human endometrial tissue is linked to infertility (Hulchiy M. *et al.*, 2012).

Deletion of Msx genes in the uterus causes failure of blastocyst attachment and subsequently implantation (Zarnani A. H. *et al.*, 2010). In a conditional ablation of Msx1/Msx2 gene in the uterus in mice, it was observed that uterine epithelium exhibited persistent proliferative activity due to stromal cells' activated fibroblast growth factors (FGFs), which ultimately resulted in failure of blastocyst attachment (Jeddi-Tehrani *et al.*, 2009). Since Msx genes critically regulate blastocyst implantation by controlling signaling between uterine stroma and epithelia, its increased expression under thyroxine influence could enhance the endometrial receptivity development.

In addition, our study has also showed that expression of Ihh in the stroma was increased following thyroxine treatment. Ihh signaling pathway has been reported to be essential for implantation and decidualization (Rosen *et al.*, 2011). Ihh expression in uterus was up-regulated by progesterone and its level was maintained high until the initiation of implantation in mice (Shahbazi *et al.*, 2011). It has also been documented that Ihh is a major mediator for progesterone effects on uterine function through epithelial-stromal cross-talk and mediates communication between the uterine epithelium and stroma during blastocyst implantation (Ortiga-Carvalho *et al.*, 2014). Therefore, its unregulated levels by thyroxine could enhance the signaling between uterine epithelia and stroma thus could improve implantation process.

In conclusions, thyroxine is essential for up-regulation of the important molecules during the peri-implantation period that are involved in uterine receptivity development. Low level of thyroxine could impair up-regulation of these proteins which could result in failure of receptivity development, hence infertility.

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CHAPTER 8: INVESTIGATING DIFFERENTIAL EXPRESSION OF THE RECEPTORS FOR THYROID HORMONE, THYROID STIMULATING HORMONE, VITAMIN D AND RETINOIC ACID AND EXTRACELLULAR SIGNAL-REGULATED KINASE IN UTERUS OF RATS UNDER INFLUENCE OF SEX-STEROIDS

8.1 Introduction

Sex-steroids play important role in regulating the uterine functions. Under the influence of E2, uterus undergoes a remarkable growth and proliferation, while under the influence of P, extensive secretion and stromal decidualization occurs in preparation for pregnancy (Winuthayanon *et al.*, 2017; Xin *et al.*, 2018; Xiong Y. *et al.*, 2017). E2 and P exert their action via binding to sex-steroid receptor, following which the intracellular signalling is activated, leading to transcription of genes that encode proteins involved in many uterine functions (Gentilini *et al.*, 2007; Tapia-Pizarro *et al.*, 2017). Among the proteins which expression have been reported to be influence by sex-steroids are the receptors for thyroid hormone, TRα and TRβ (Aghajanova L. *et al.*, 2011; Oner & Oner, 2007), receptor for thyroid stimulating hormone, TSHR (Hulchiy Mariana *et al.*, 2012), receptor for vitamin D, VDR (Emam M. A. *et al.*, 2016), receptor for retinoic acid, RAR (Boehm *et al.*, 1997) and ERK1/2 signaling protein (Xiao *et al.*, 2009). These proteins participate in thyroid hormone actions.

Besides sex-steroids, thyroid hormone (TH) is also important for many uterine functions (Choksi *et al.*, 2003; Poppe Kris & Brigitte Velkeniers, 2004). This was evidence from deficiency of thyroid hormone which can cause menstrual irregularities and failure of the embryo to implant, that would eventually impair fertility (Poppe & Velkeniers, 2002). There are two forms of thyroid hormones i.e. thyroxine (T4) and tri-

iodothyronine (T3) which mediate their action via binding to specific thyroid hormone receptors (TR) (Aghajanova *et al.*, 2009; Aghajanova L. *et al.*, 2011) that are located intracellularly (Hones *et al.*, 2017) or in the membrane (Taylor E. & Heyland, 2017). Currently, two isoforms of TR i.e. TRα and TRβ have been identified (Cheng S. Y. *et al.*, 2010; Yen, 2001). Expression of TR has been detected in the endometrium in humans and rodents (Aghajanova L. *et al.*, 2011; Oner & Oner, 2007).

Additionally, thyroid stimulating hormone (TSH) also plays important role in the uterus (Mizukami *et al.*, 1994). Among its uterine actions include increases the expression of leukemia inhibitory factor (LIF) and its receptor (LIFR) in the endometrial stroma of humans (Aghajanova L. *et al.*, 2011) and primates (Ren *et al.*, 1999). TSH is also involved in endometrial glucose transport, in view that expression of *Glut-1* mRNA was up-regulated by TSH in human endometrial stromal cells and Ishikawa (uterine adenocarcinoma) cells (Aghajanova L. *et al.*, 2011). TSH might have direct actions on the uterine functions, since receptors for thyrotropin-releasing hormone (TRH) and TSH were detected in the monkey uterus following long term treatment with sex-steroids (Hulchiy M. *et al.*, 2012). TSH acts via binding to TSHR which has been found to be expressed in the uterus in humans and rabbits (Catalano *et al.*, 2007; Rodríguez-Castelán *et al.*, 2017). Expression of TSHR and TRs in the uterus was reported to be influence by sex-steroids in which chronic administration of conjugated equine estrogen and medroxyprogesterone acetate to ovariectomized cynomolgus macaques caused up-regulation of TSHR and TR expression in uterine compartments (Hulchiy Mariana *et al.*, 2012). However, direct effects of individual sex-steroids i.e E2 and P on TSHR and TR expression in the uterus have never been identified.

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In the meantime, Vitamin D has been reported to play important role in uterine functions including regulating uterine smooth muscle contraction (Thota *et al.*, 2014) and uterine cell proliferation (Yoshizawa *et al.*, 1997). Expression of VDR and enzyme that convert vitamin D to its active form (1α-hydroxylase) has been reported in the uterine tissue (Muscogiuri *et al.*, 2017). In uterus, VDR is found to be expressed in the follicular and luteal phases of the menstrual cycle (Emam M. A. *et al.*, 2016). VDR can be localized in both endometrium and myometrium in humans (Vienonen *et al.*, 2004). VDR is a transcription factor located in the nuclei, and this protein mediates the genomic effect of vitamin D (1,25(OH)2D3) (Shahrokhi *et al.*, 2016). Lack of VDR expression in thyroid disorders has been linked to infertility and pregnancy loss (Twig *et al.*, 2012). It was also reported that mice lack VDR had impaired fertility (Yoshizawa *et al.*, 1997).

Retinoic acid (RA) is a low molecular weight acid that is a lipophilic metabolite in terms of Vitamin A. RA is crucially involved in maintenance of the female reproductive system functions, including cell proliferation and differentiation (Grenier *et al.*, 2007). RA has important role in regulating expression of matrix metalloproteinases (MMP) produced by endometrial stromal cells during decidualization (Osteen *et al.*, 2003; Zheng *et al.*, 2000). In addition, RA has protective role on the uterus, since growth of cancer cells in the endometrium was reduced following treatment with RA (Saidi *et al.*, 2006). RA binds to RAR, which is a nuclear receptor (Chambon, 1996). Expression of RAR has been reported in the uterine stroma of mice (Nakajima *et al.*, 2016) and uterine epithelium of rats (Boehm *et al.*, 1997) and humans (Kumarendran *et al.*, 1996). Expression of RAR in uterus was found to be influence by ovarian steroids, as documented in rats (Boehm *et al.*, 1997) and humans (Kumarendran *et al.*, 1996). The functional heterodimer complex of TR and retinoid

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acid receptor (RAR) interact with specific thyroid hormone responsive element (TRE) on DNA, initiating protein synthesis (Liu Y. Y. & Brent, 2010).

ERK1/2, a member of well-known mitogen-activated protein kinase (MAPK), is reported to also be involve in many uterine functions, including proliferation and decidualization (Paul *et al.*, 2011; Shan *et al.*, 2013). ERK1/2 plays critical role in embryo implantation, in mice and humans (Lee C. H. *et al.*, 2013). ERK1/2 is also involved in mediating the non-genomic effects of thyroid hormone (Chen *et al.*, 2014; Deb & Das, 2011; Iordanidou *et al.*, 2010). Both E_2 and P were reported to regulate ERK1/2 expression in the smooth muscle of uterine artery (Xiao *et al.*, 2009).

In view of the important role of E2 and P for the uterus, it was hypothesized that both hormones could affect uterine expression of TR isoforms, TSHR, VDR, RAR and ERK1/2. As there was currently inadequate information pertaining to the effect of individual sex-steroids on expression of these proteins in the uterus, our study aims to identify changes in expression and distribution of these proteins under different sex-steroid influence.

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8.2 Results

8.2.1 Levels of TRα-1 protein expression and its distribution in uterus

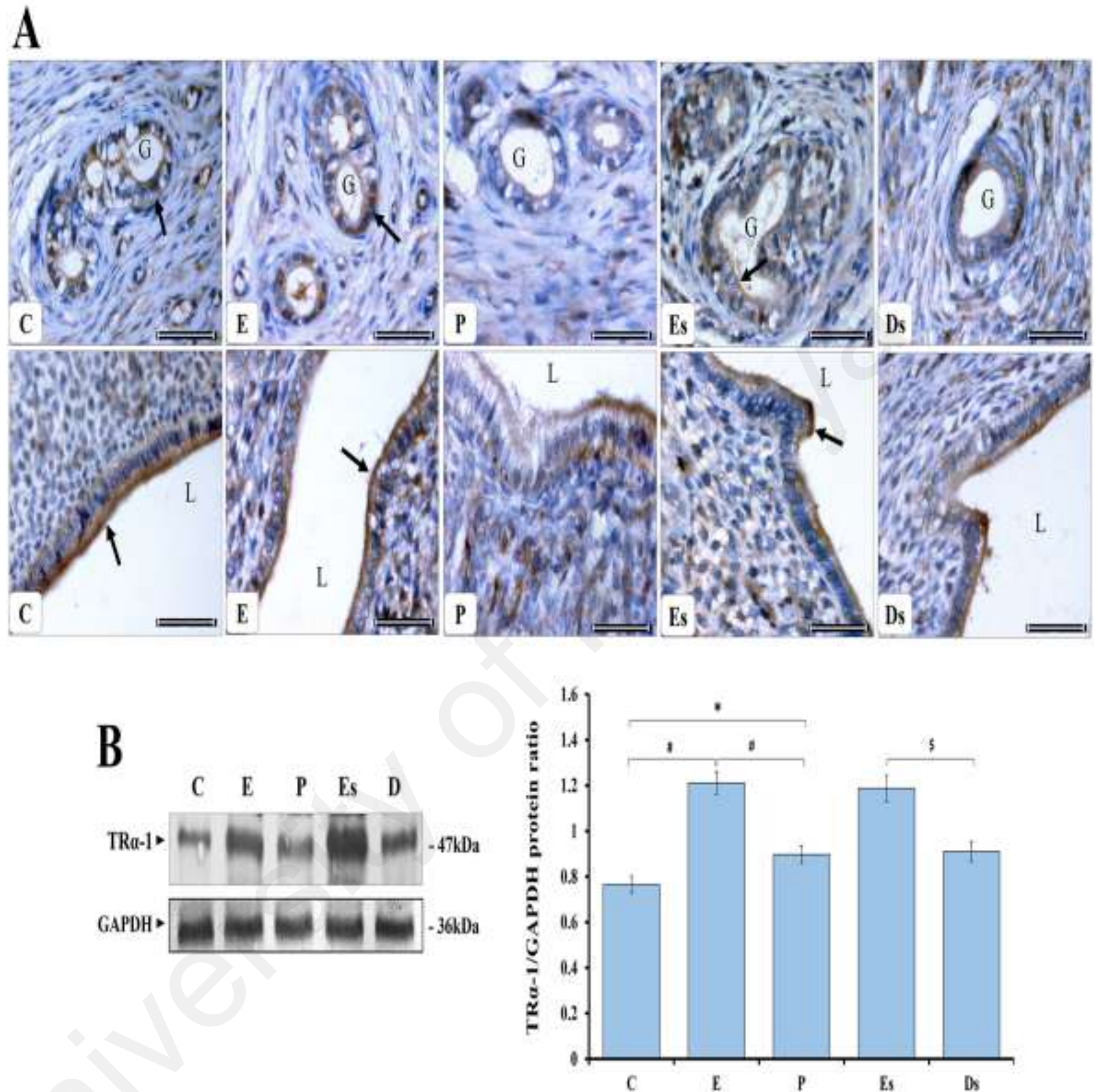

Figure 8.1 Representative immunoperoxidase images showing distribution of (A) TRα-1 in the glandular and luminal epithelium of endometrium and representative western blot band and analysis of expression level of (B) TRα-1 protein in uterus.

In (A) relatively higher expression of proteins could be seen under E influence as compared to P influence. Images were taken at magnification of 40X. Scale bar= 50µm. In (B) $^{*,\#}p < 0.05$ compared to C, $^{Ø}p < 0.05$ compared to P, $^{\$}p < 0.05$ compared to Ds. Data were expressed as mean ± standard error of mean with n= 6 per group. Molecular weight of TRα-1= 47 kDa and GAPDH= 36 kDa.

A

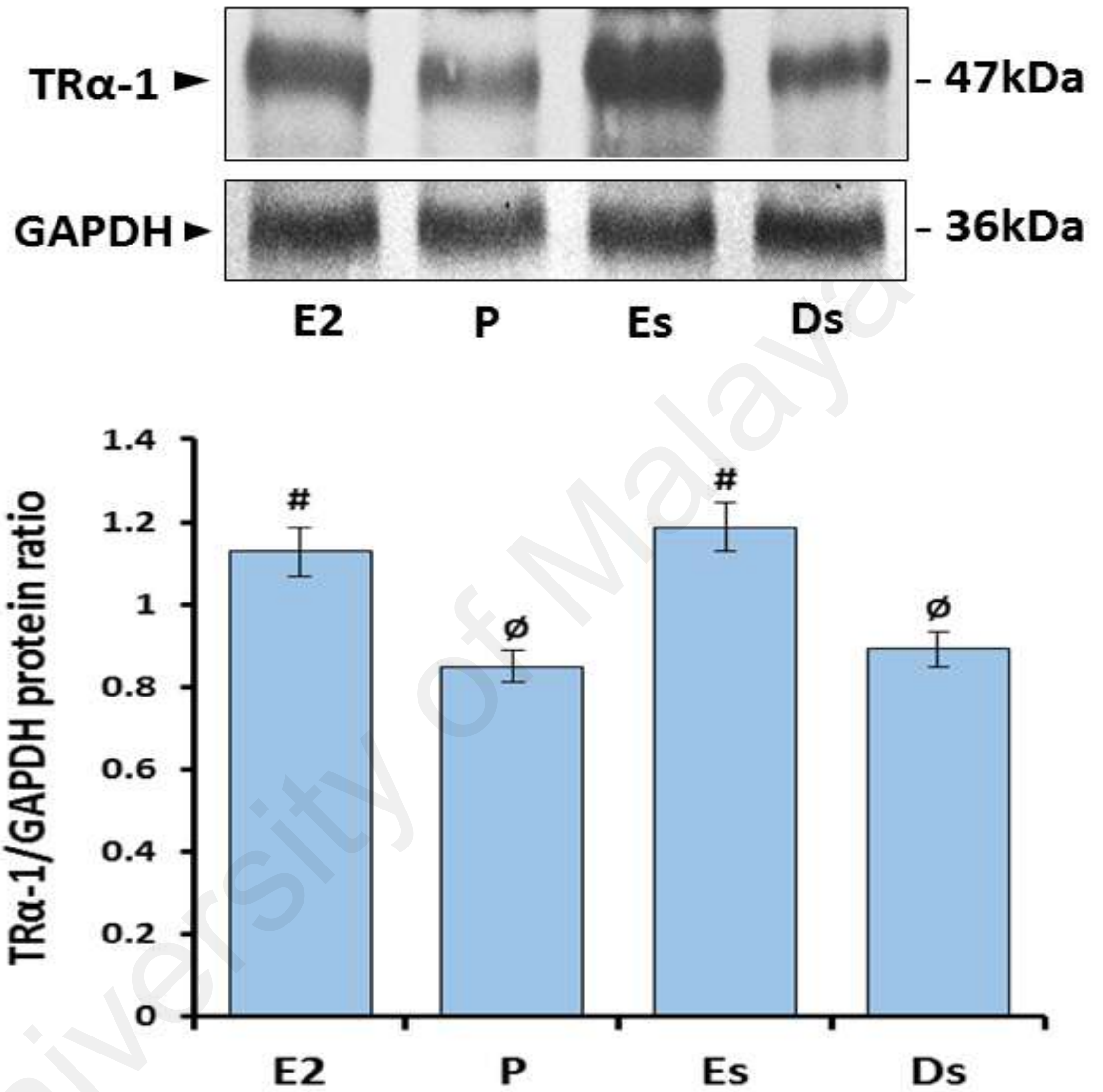

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Figure 8.2 (A) Representative western blot band and analysis of expression level of TRα-1 protein in the uterus and (In next page) (B) immunoperoxidase images showing distribution of TRα-1 in the uterus.

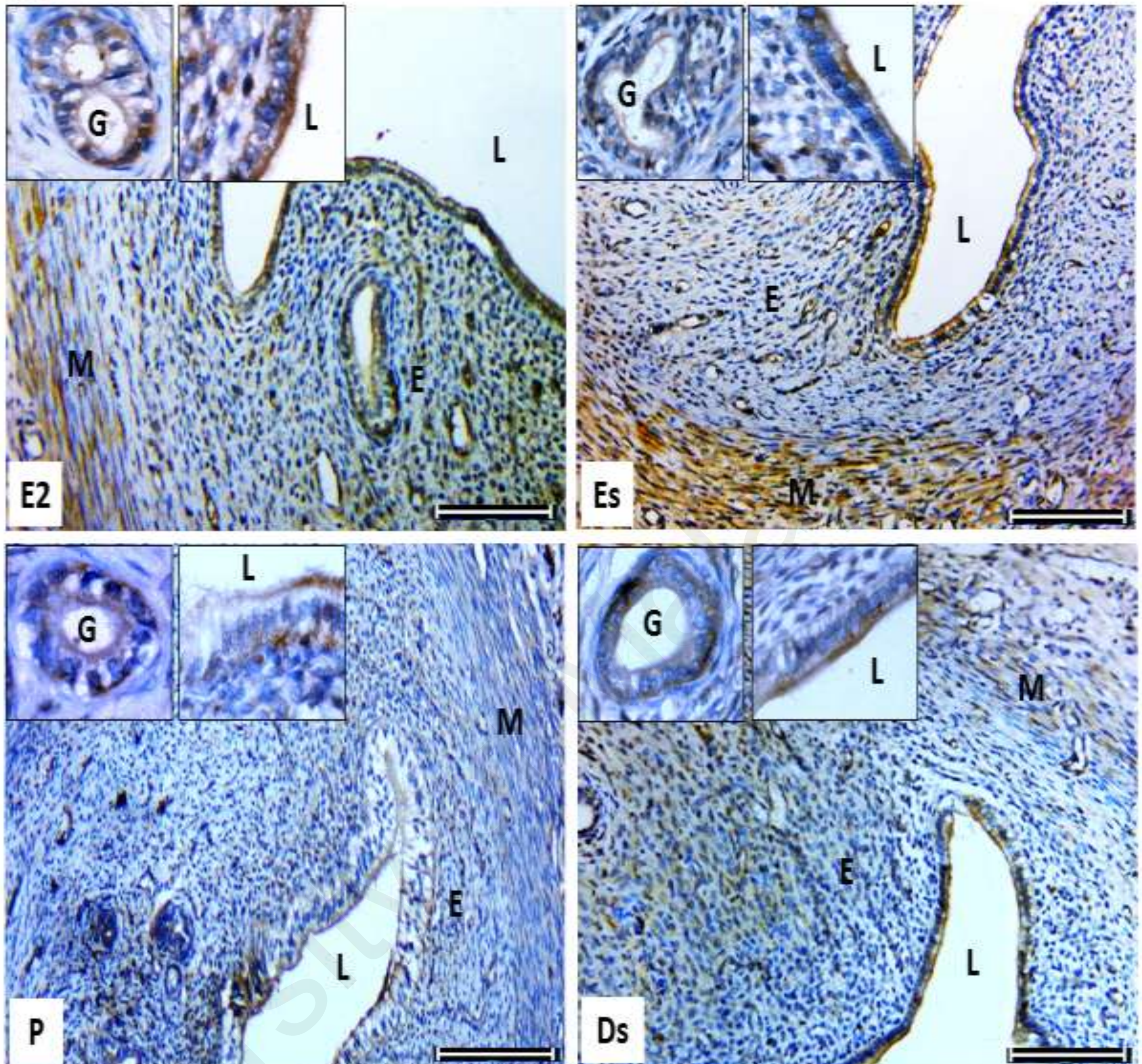

Figure 8.2(A) Representative western blot band and analysis of expression level of TRα-1 protein in the uterus and (B) immunoperoxidase images showing distribution of TRα-1 in the uterus.

TRα-1 could be seen to be distributed in the endometrial luminal and glandular epithelia and myometrium. Larger images and smaller images were taken at magnification of 10X and 40X respectively. Scale bar = 50μm. Bars that do not share the same symbols differ significantly at *P*<0.05. Data were expressed as mean ± standard error of mean with n= 6 per group. Es: estrus, Ds= diestrus, E_2= estradiol, P= progesterone. M= myometrium, E= endometrium, G= Gland, L= lumen. Molecular weight of TRα-1= 47 kDa and GAPDH= 36 kDa.

Levels of expression of TRα-1 protein in uterus in rats receiving E2-treatment were higher than P-treatment (Fig. 8.2A). Higher TRα-1 expression was observed in uterus at Es phase as compared to Ds phase. Expression levels of TRα-1 protein in E2-treated rats were not significantly different as compared to Es phase, but the levels were higher than Ds phase ($p < 0.05$). No significant different in TRα-1 levels was observed between P-treated rats and rats in Ds phase. In P-treated rats, expression level of TRα-1 was lower than E2-treated rats and rats at Es phase. However, in these rats, TRα-1levels were not significantly different when compared to rats at Ds phase.

A relatively higher TRα-1 distribution was obserdved in the luminal and glandular epithelia, stroma and myometrium in E2-treated rats as compared to P-treated rats (Fig 8.2B). Similarly, TRα-1 distribution in luminal and glandular epithelia, stroma and myometrium at Es phase was relatively higher than Ds phase. TRα-1 could be seen to be distributed both intracellularly and at the plasma membrane of the endometrial epithelium.

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8.2.2 Levels of TRβ-1 protein expression and its distribution in uterus

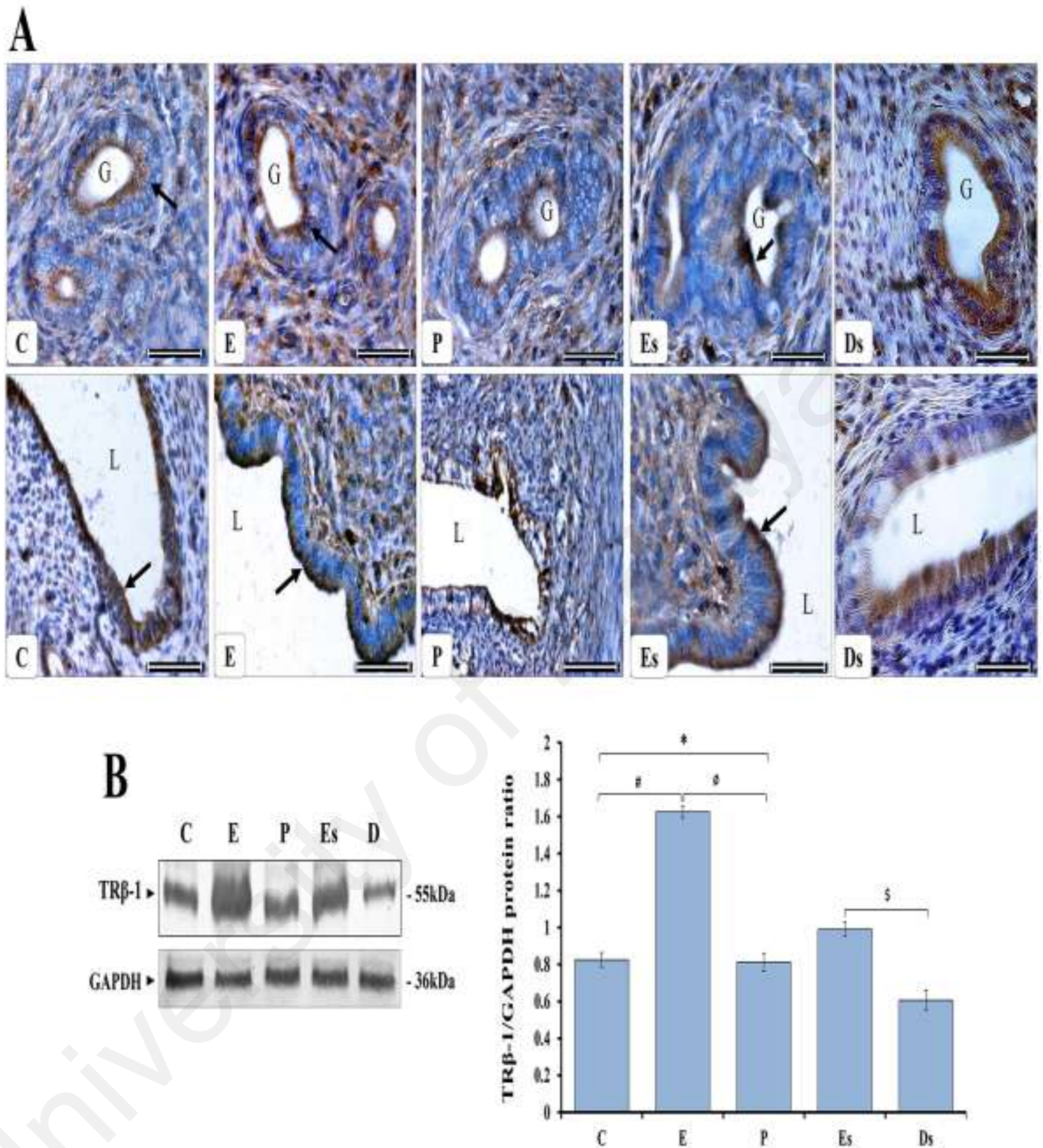

Figure 8.3 Representative immunoperoxidase images showing distribution of (A) TRβ-1 in glandular and luminal epithelium of endometrium and representative western blot band and analysis of expression level of (B) TRβ-1 protein in uterus.

In (A) relatively higher expression of proteins could be seen under E influence as compared to P influence. Images were taken at magnification of 40X. Scale bar= 50μm. In (B) $^{*,\#}p < 0.05$ compared to C, $^{\varnothing}p < 0.05$ compared to P, $^{\$}p < 0.05$ compared to Ds. Data were expressed as mean ± standard error of mean with n= 6 per group. Molecular weight of TRβ-1= 55 kDa.

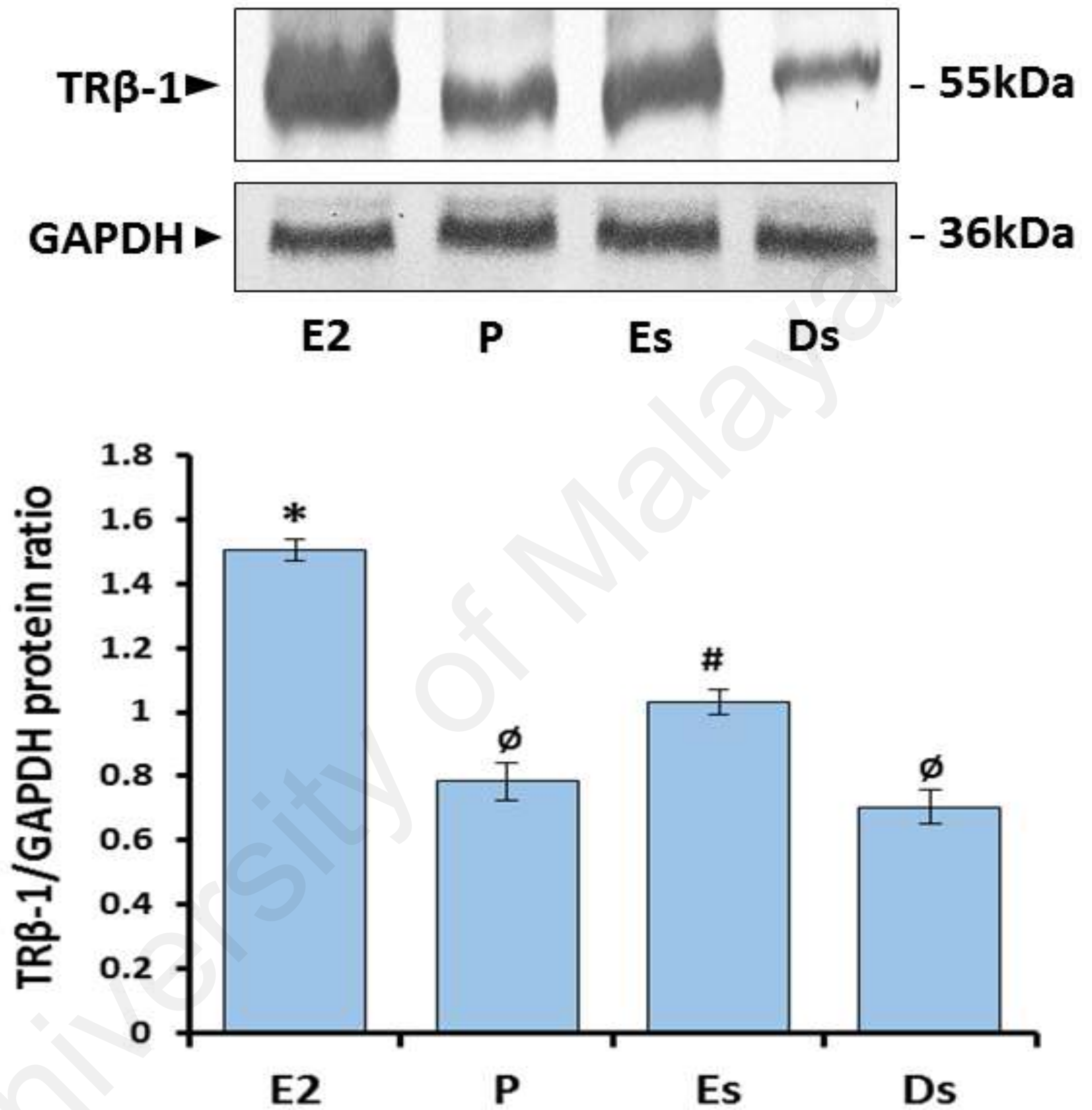

Figure 8.4 (A) Representative western blot band and analysis of expression level TRβ-1 protein in the uterus. (In next page) (B) Representative immunoperoxidase images showing distribution of TRβ-1.

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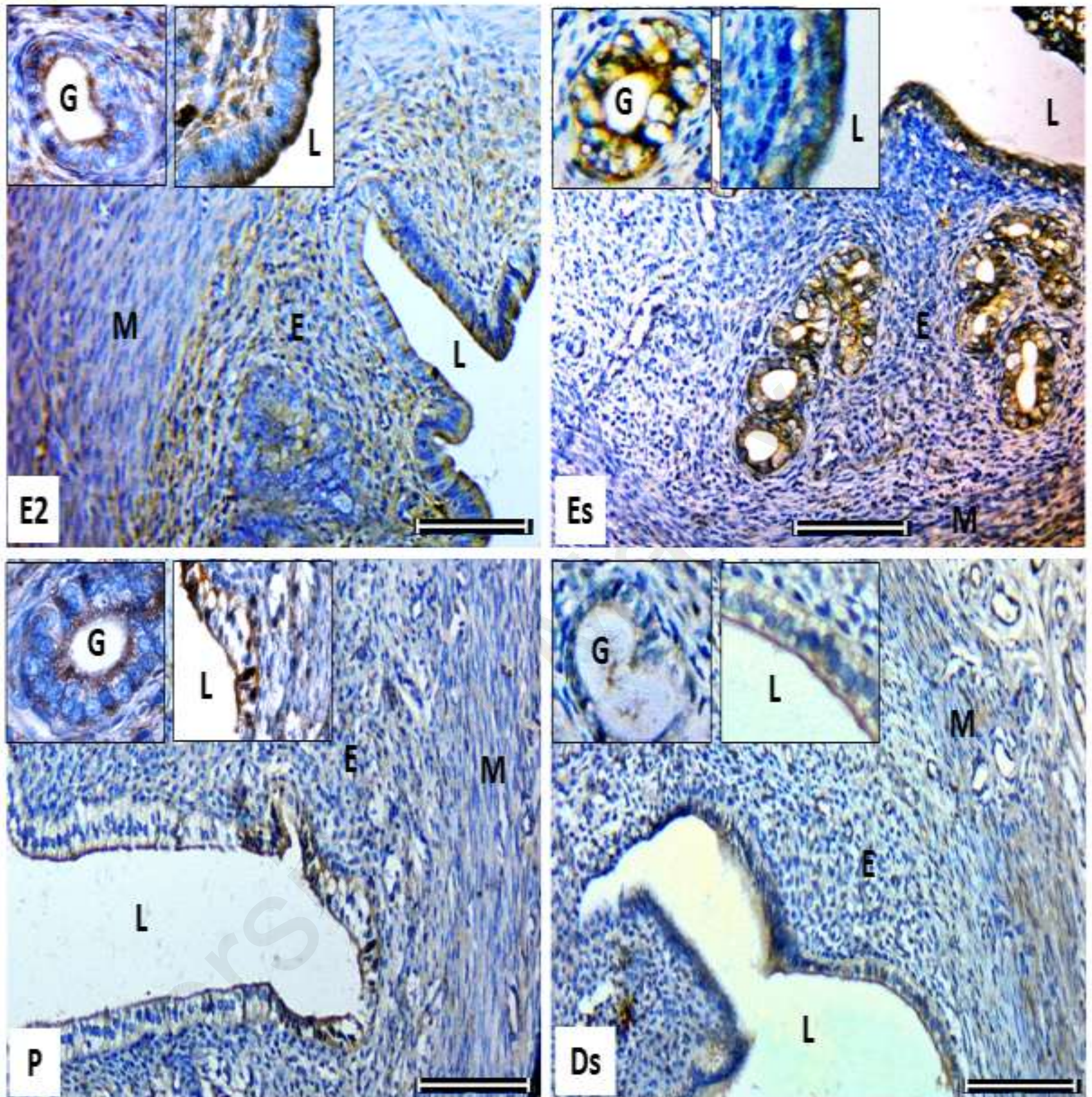

Figure 8.4 (A) Representative western blot band and analysis of expression level TRβ-1 protein in the uterus. (B) Representative immunoperoxidase images showing distribution of TRβ-1.

TRβ-1 could be seen to be distributed in the stroma and luminal and glandular epithelia. No myometrial distribution could be seen. Larger images and smaller images were taken at magnification of 10X and 40X respectively. Scale bar = 50µm. Bars that do not share the same symbols differ significantly at $P<0.05$. Data were expressed as mean ± standard error of mean with n= 6 per group. Es: estrus, Ds= diestrus, E_2= estradiol, P= progesterone, M= myometrium, E= endometrium, G= Gland, L= lumen. Molecular weight of TRβ-1= 55 kDa.

The level of TRβ-1 in the uterus of E2-treated rats was significantly higher when compared to P-treated rats and rats at Es phase. In intact rats, TRβ-1 level was higher at Es phase than Ds phase ($p < 0.05$) (Fig. 8.4A). In P-treated rats, levels of TRβ-1 expression were lower than rats at Es phase however was not significantly different when compared to rats at Ds phase (Fig. 8.4A).

A relatively higher TRβ-1 distribution was observed in luminal and glandular epithelia and in the stroma of E2-treated rats than P-treated rats. TRβ-1 distribution was also higher in luminal and glandular epithelia and in the stroma of rats at Es phase as compared to Ds phase (Fig 8.4B). TRβ-1 could be seen to be distributed both intracellularly and at the plasma membrane of the endometrial epithelium.

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8.2.3 Levels of TSHR protein expression and its distribution in uterus

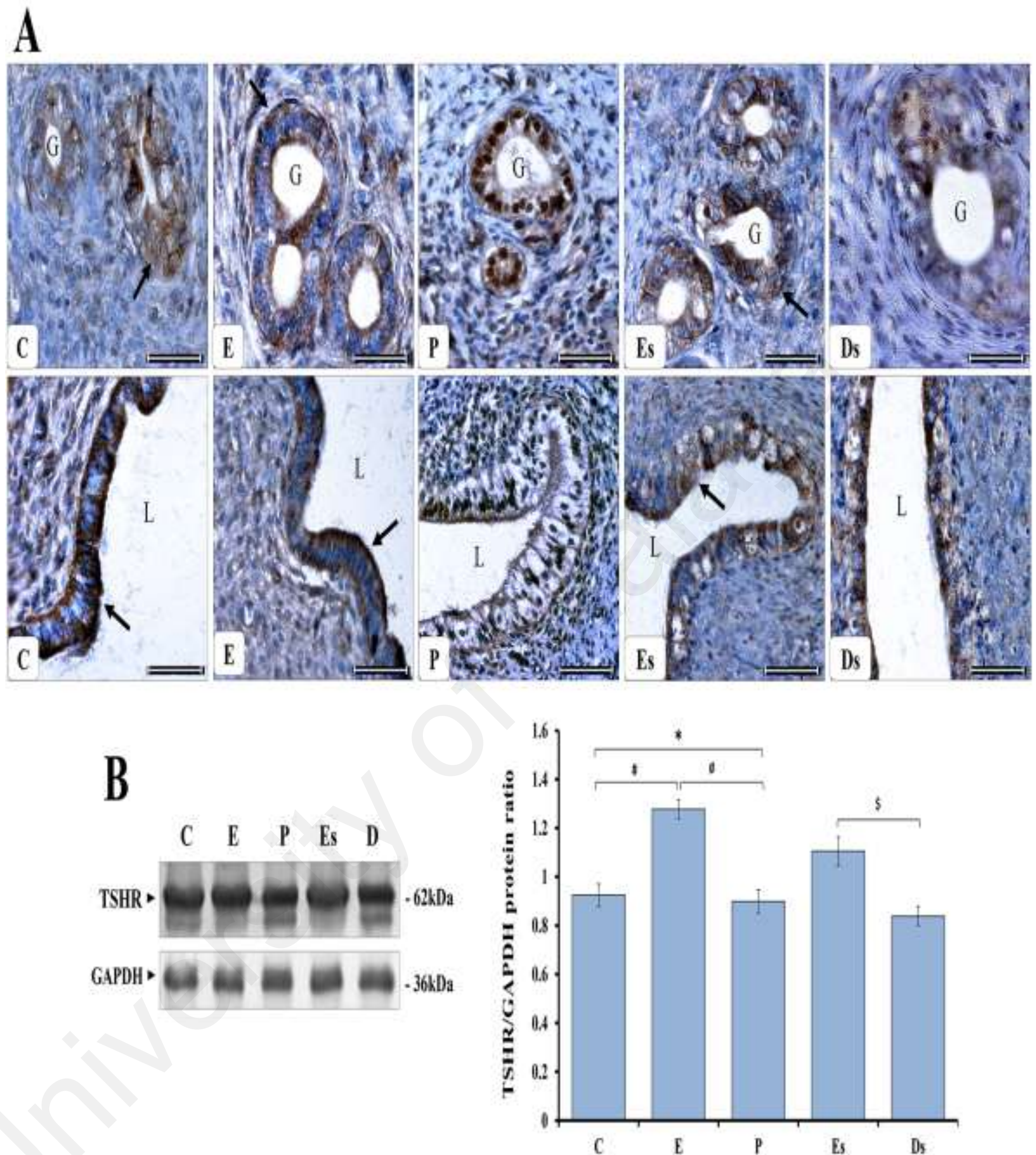
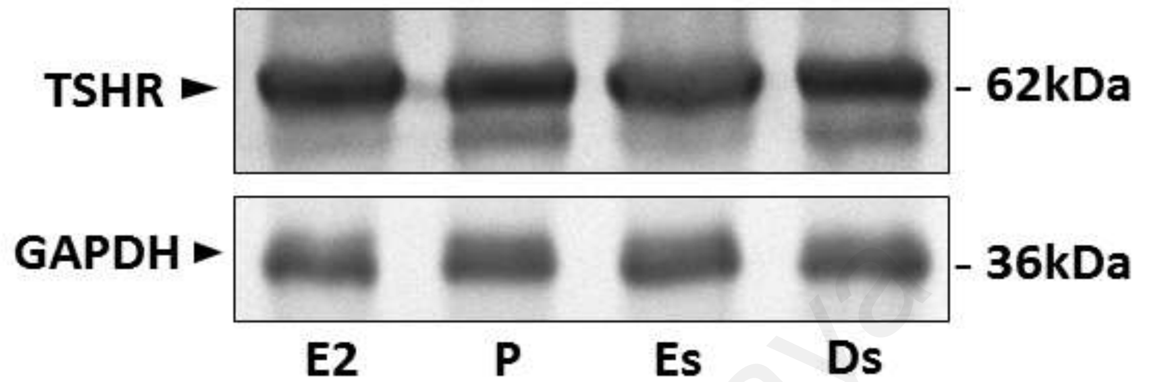


Figure 8.5 Representative immunoperoxidase images showing distribution of (A) thyroid-stimulating hormone receptor (TSHR) in glandular and luminal epithelium of endometrium and representative western blot band and analysis of expression level of (B) TSHR protein in uterus.

In (A) relatively higher expression of proteins could be seen under E influence as compared to P influence. Images were taken at magnification of 40X. Scale bar= 50µm. In (B) $^{*}p<0.05$ compared to C, $^{Ø}p<0.05$ compared to P, $^{\$}p<0.05$ compared to Ds. Data were expressed as mean ± standard error of mean with n= 6 per group. Molecular weight of TSHR= 62 kDa.

A



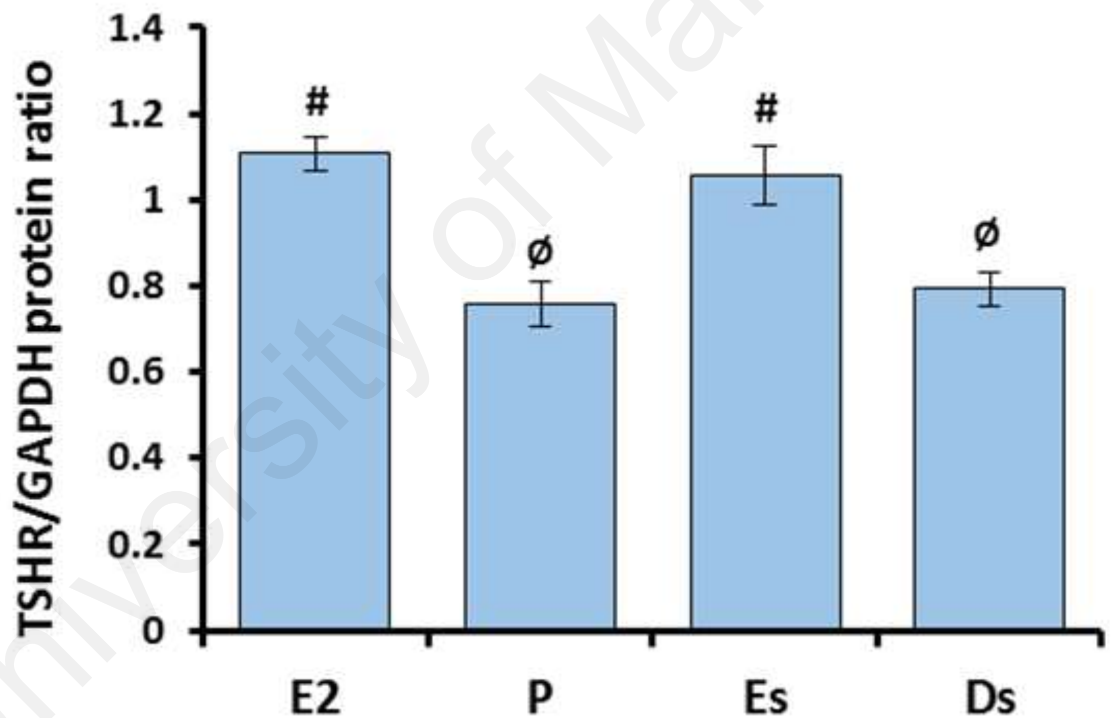


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Figure 8.6 (A) Representative western blot band and analysis of expression level of TSHR protein in the uterus. (In next page) (B) Representative immunoperoxidase images showing distribution of TSHR in the uterus.

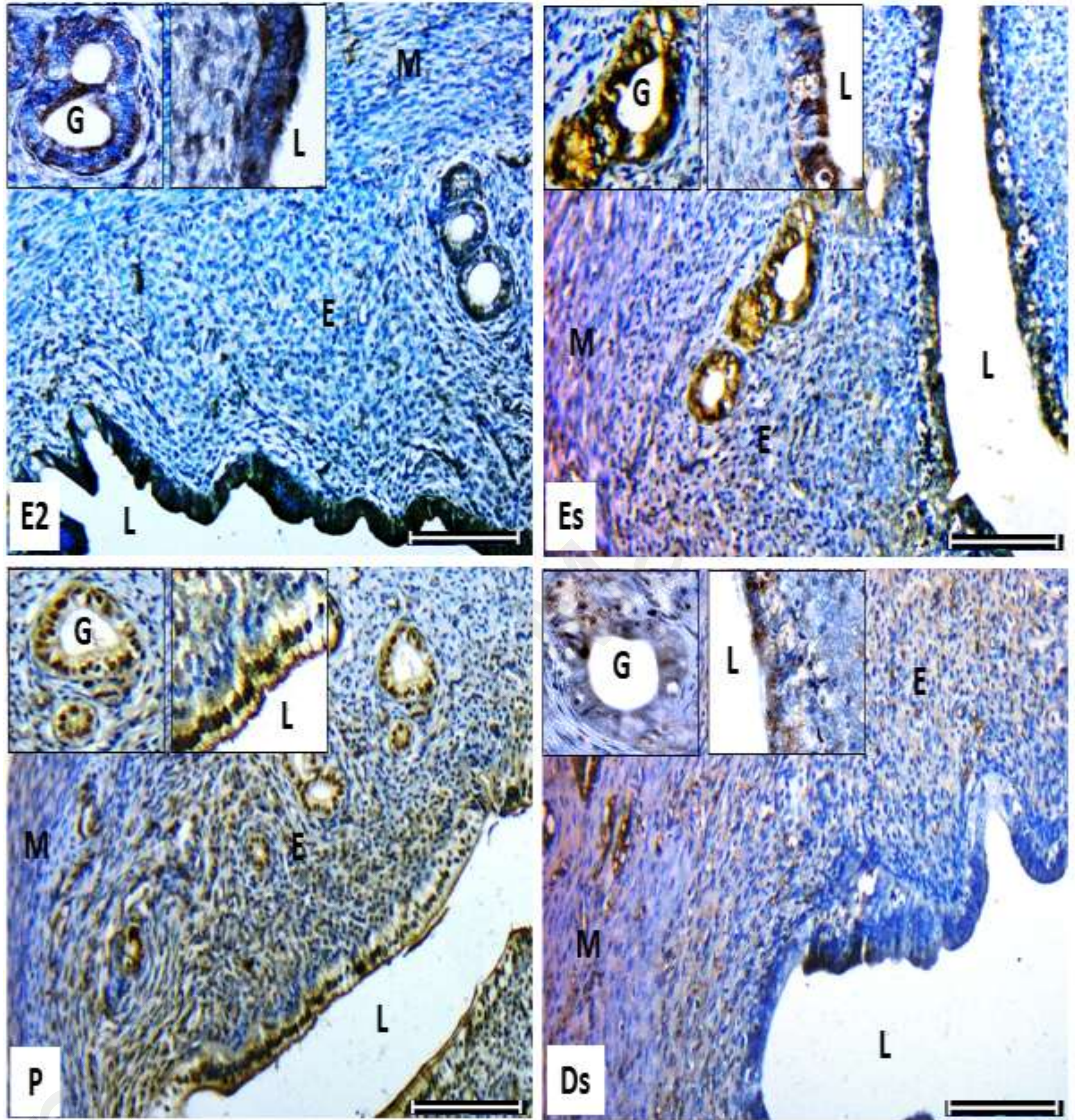

Figure 8.6(A) Representative western blot band and analysis of expression level of TSHR protein in the uterus. (B) Representative immunoperoxidase images showing distribution of TSHR in the uterus.

TSHR could be seen to be distributed in the endometrium, both in the stroma and luminal and glandular epithelia. No myometrial distribution could be seen. Larger images and smaller images were taken at magnification of 10X and 40X respectively. Scale bar = 50µm. Bars that do not share the same symbols differ significantly at *P*<0.05. Data were expressed as mean ± standard error of mean with n= 6 per group. Es: estrus, Ds= diestrus, E_2= estradiol, P= progesterone, M= myometrium, E= endometrium, G= Gland, L= lumen. Molecular weight of TSHR= 62 kDa.

Expression level of TSHR protein in uterus of ovariectomized rats treated with E2 was higher than ovariectomized rats treated with P (Fig. 8.6A). In the meantime, expression level of TSHR in rats at Es phase was higher than Ds phase ($p<0.05$). Although the level of THSR in E2-treated rats were not significantly different as compared to its level at Es phase, the former had significantly higher TSHR expression level as compared to P-treated rats and rats at Ds phase ($p< 0.05$). However, in P-treated rats, levels of TSHR expression were not significantly different as compared to rats at Ds phase (Fig. 8.6A).

A relatively higher TSHR distribution was observed in the luminal and glandular epithelia in E2-treated rats than P-treated rats (Fig 8.6B). However, higher TSHR distribution was observed in the stroma of P-treated rats than E2-treated rats. TSHR distribution in the luminal and glandular epithelia in rats at Es phase was relatively higher than rats at Ds phase, however stromal distribution was relatively higher in the latter as compared to the former. TSHR could be seen to be distributed mainly at the plasma membrane.

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8.2.4 Levels of VDR protein expression and its distribution in uterus

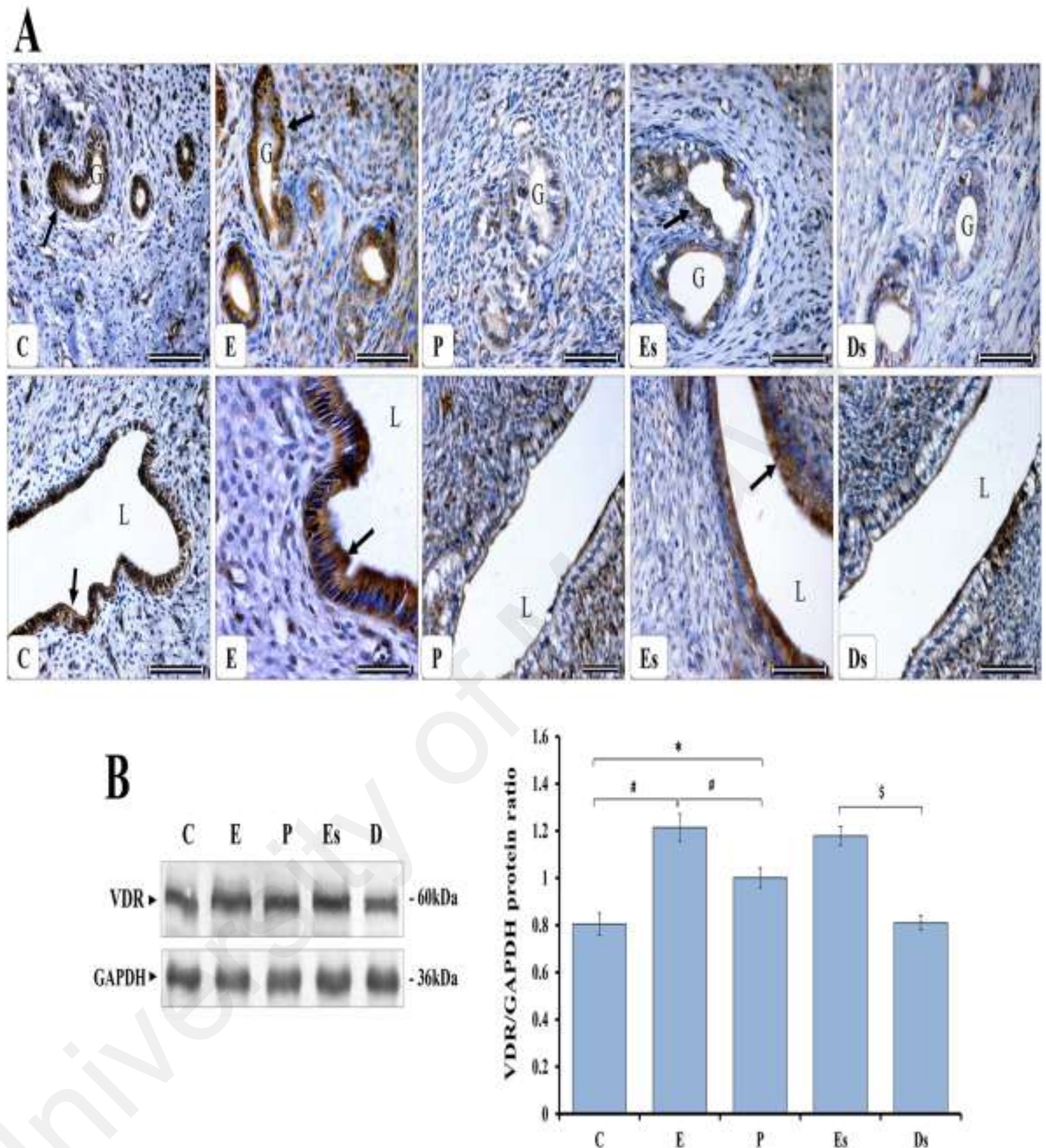

Figure 8.7 Representative immunoperoxidase images showing distribution of (A) VDR in glandular and luminal epithelium of endometrium and representative western blot band and analysis of expression level of (B) VDR protein in uterus.

In (A) relatively higher expression of proteins could be seen under E influence as compared to P influence. Images were taken at magnification of 40X. Scale bar= 50µm. In (B) $^{*,\#}p< 0.05$ compared to C, $^{Ø}p< 0.05$ compared to P, $^{\$}p< 0.05$ compared to Ds. Data were expressed as mean ± standard error of mean with n= 6 per group. Molecular weight of VDR= 60 kDa.

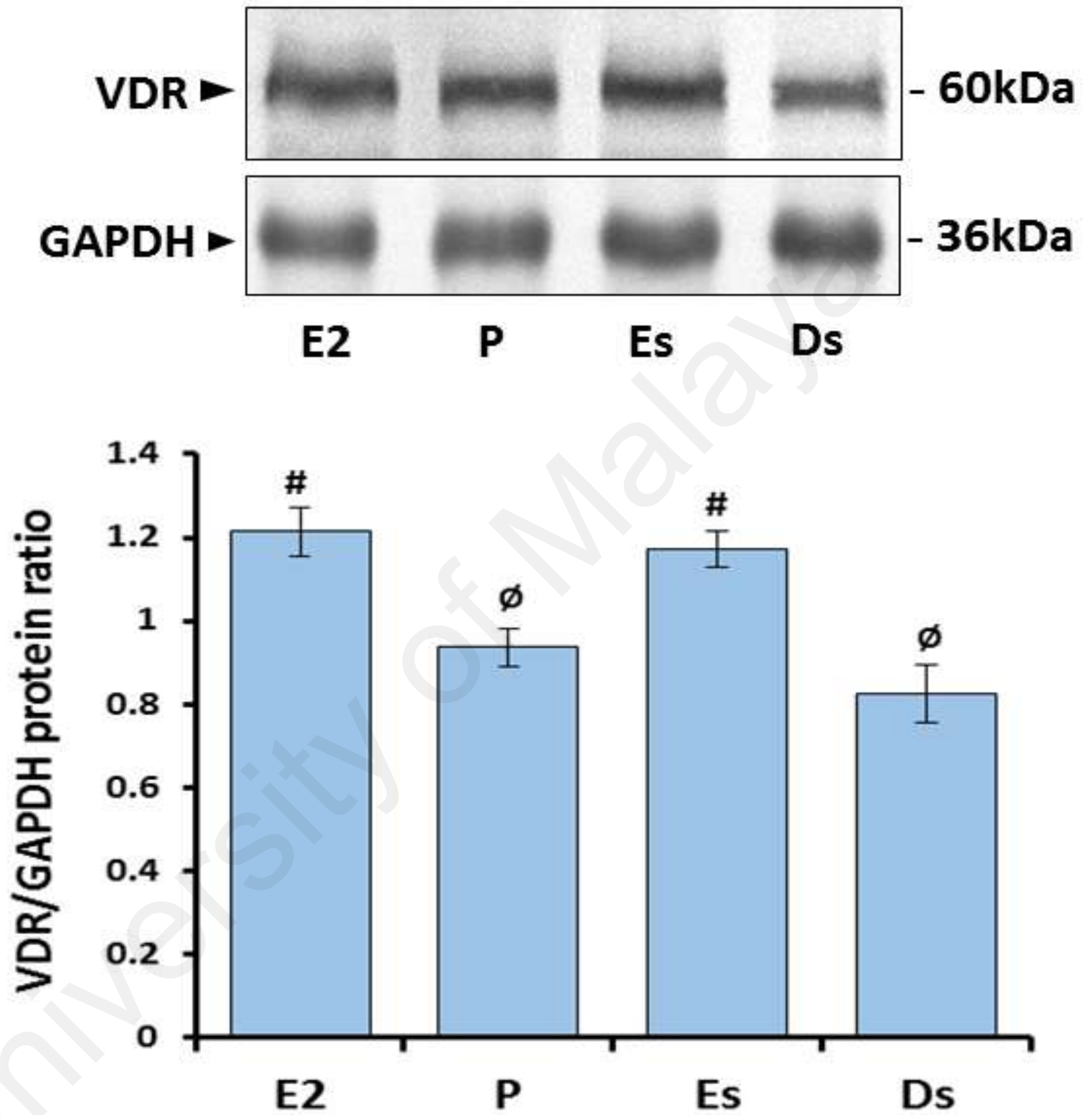

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Figure 8.8 (A) Representative western blot band and analysis of expression level of VDR protein in the uterus and (in next page) (B) Representative immunoperoxidase images showing distribution of VDR.

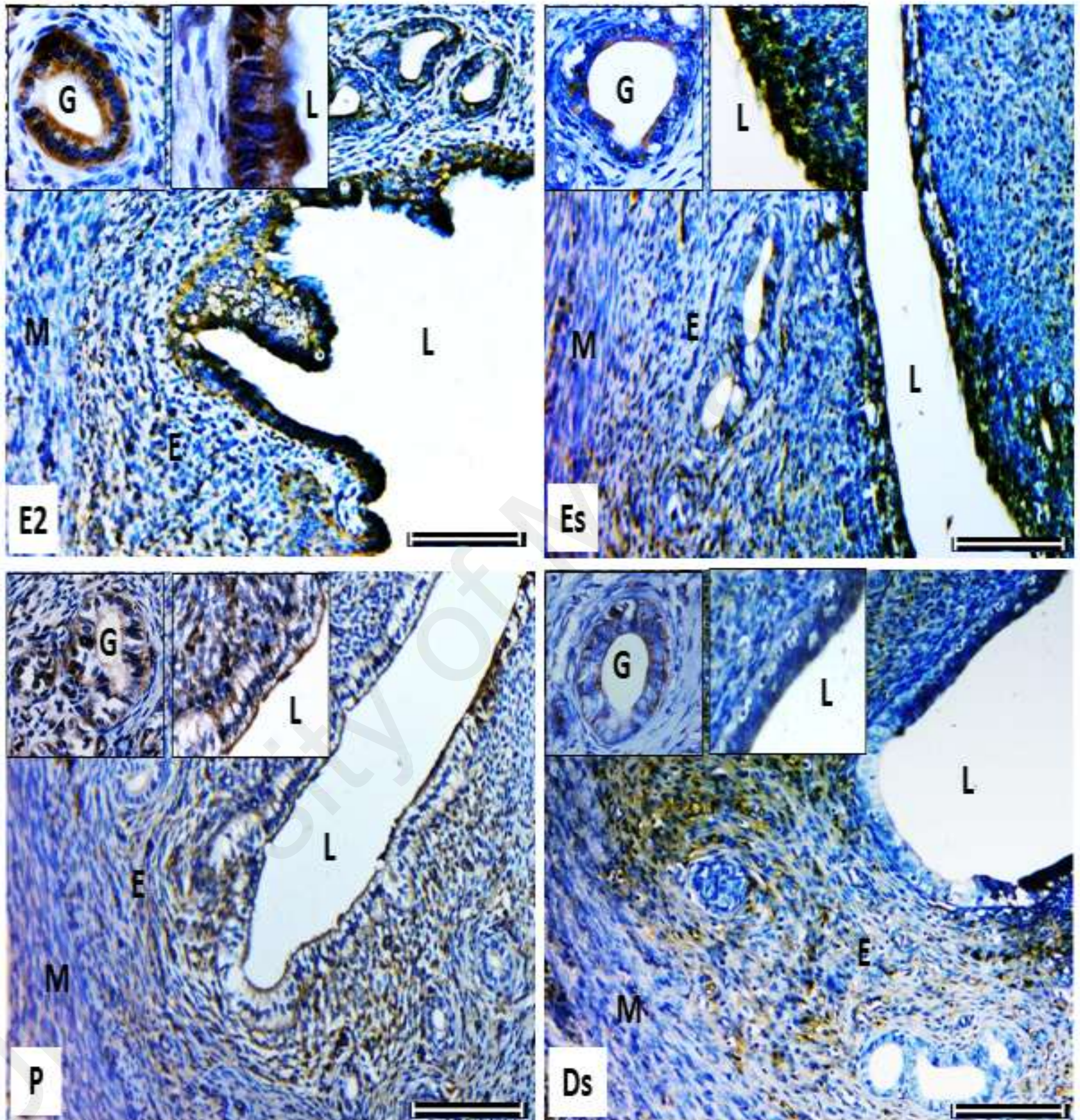

Figure 8.8(A) Representative western blot band and analysis of expression level of VDR protein in the uterus (B) Representative immunoperoxidase images showing distribution of VDR.

VDR could be seen to be distributed in the endometrium, both in the stroma and luminal and glandular epithelia, No myometrial distribution could be seen. Larger images and smaller images were taken at magnification of 10X and 40X respectively. Scale bar = 50µm. Bars that do not share the same symbols differ significantly at $P<0.05$. Data were expressed as mean ± standard error of mean with n= 6 per group. Es: estrus, Ds= diestrus, E_2= estradiol, P= progesterone. M= myometrium, E= endometrium, G= Gland, L= lumen. Molecular weight of VDR= 60 kDa.

Expression level of VDR protein in the uterus of E2-treated rats was higher than P-treated rats (Fig. 8.8A). Similarly, expression levels of VDR protein in the uterus at Es phase were higher than Ds phase ($p<0.05$). However, levels of VDR protein in E2-treated rats were not significantly different as compared to rats at Es phase but were higher than rats at Ds phase (Fig. 8.8A). In P-treated rats, VDR expression level was not significantly different as compared to rats at Ds phase.

A relatively higher VDR distribution was observed in the luminal and glandular epithelia of E2-treated rats as compared to P-treated rats (Fig 8.8B). Similarly, VDR distribution in the luminal and glandular epithelia at Es phase was relatively higher than Ds phase. However, VDR distribution in the stroma was relatively higher in P-treated rats when compared to E2-treated rats. Similarly, stromal distribution of VDR was higher in rats at Ds phase as compared to rats at Es phase. VDR could be seen intracellularly and at the plasma membrane.

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8.2.5 Levels of RAR protein expression and its distribution in uterus

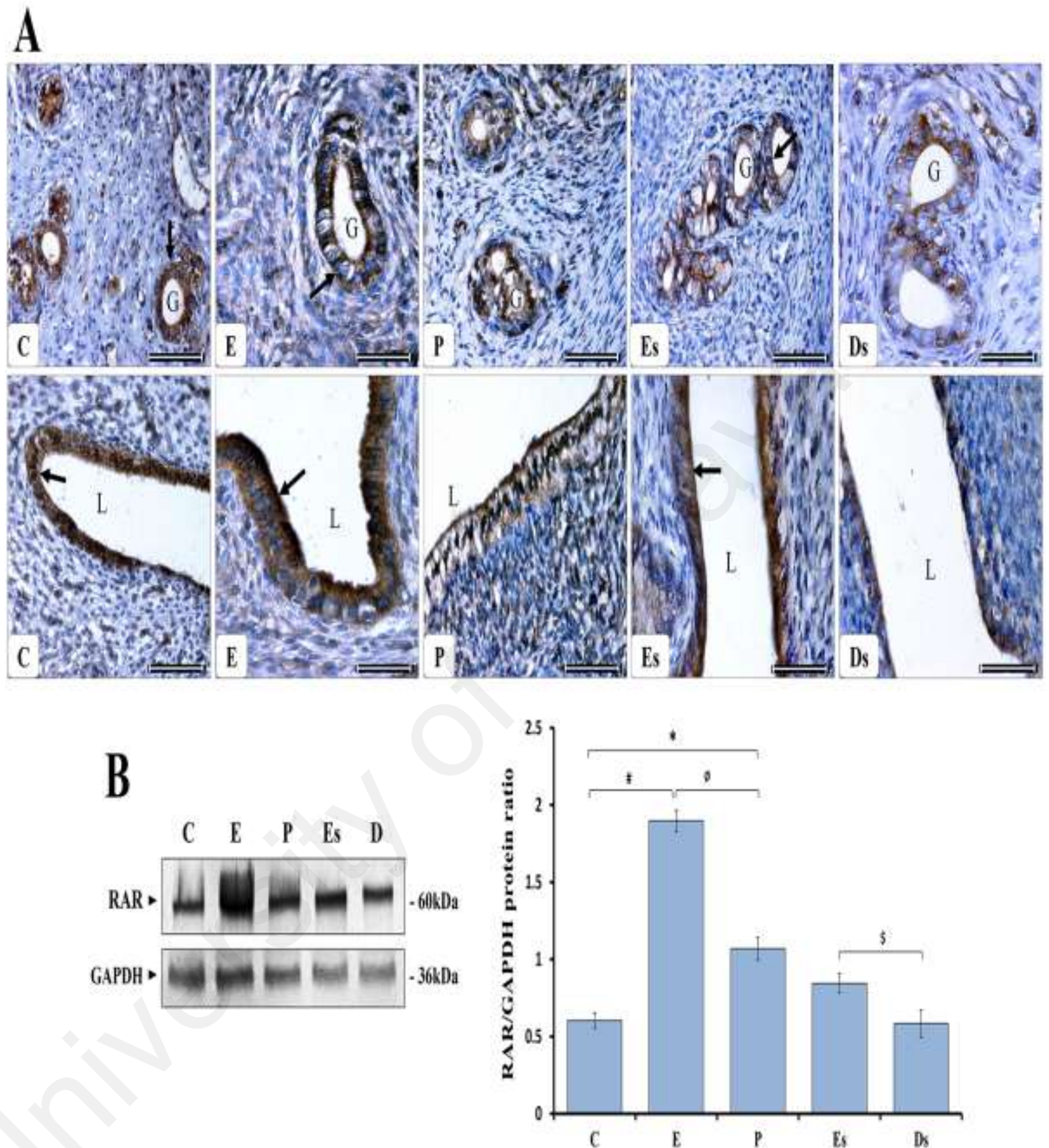

Figure 8.9 Representative immunoperoxidase images showing distribution of (A) RAR in glandular and luminal epithelium of endometrium and representative western blot band and analysis of expression level of (B) RAR protein in uterus.

In (A) relatively higher expression of proteins could be seen under E influence as compared to P influence. Images were taken at magnification of 40X. Scale bar= 50µm. In (B) $^{*,\#}p<0.05$ compared to C, $^{Ø}p<0.05$ compared to P, $^{\$}p<0.05$ compared to Ds. Data were expressed as mean ± standard error of mean with n= 6 per group. Molecular weight of RAR= 60 kDa.

A

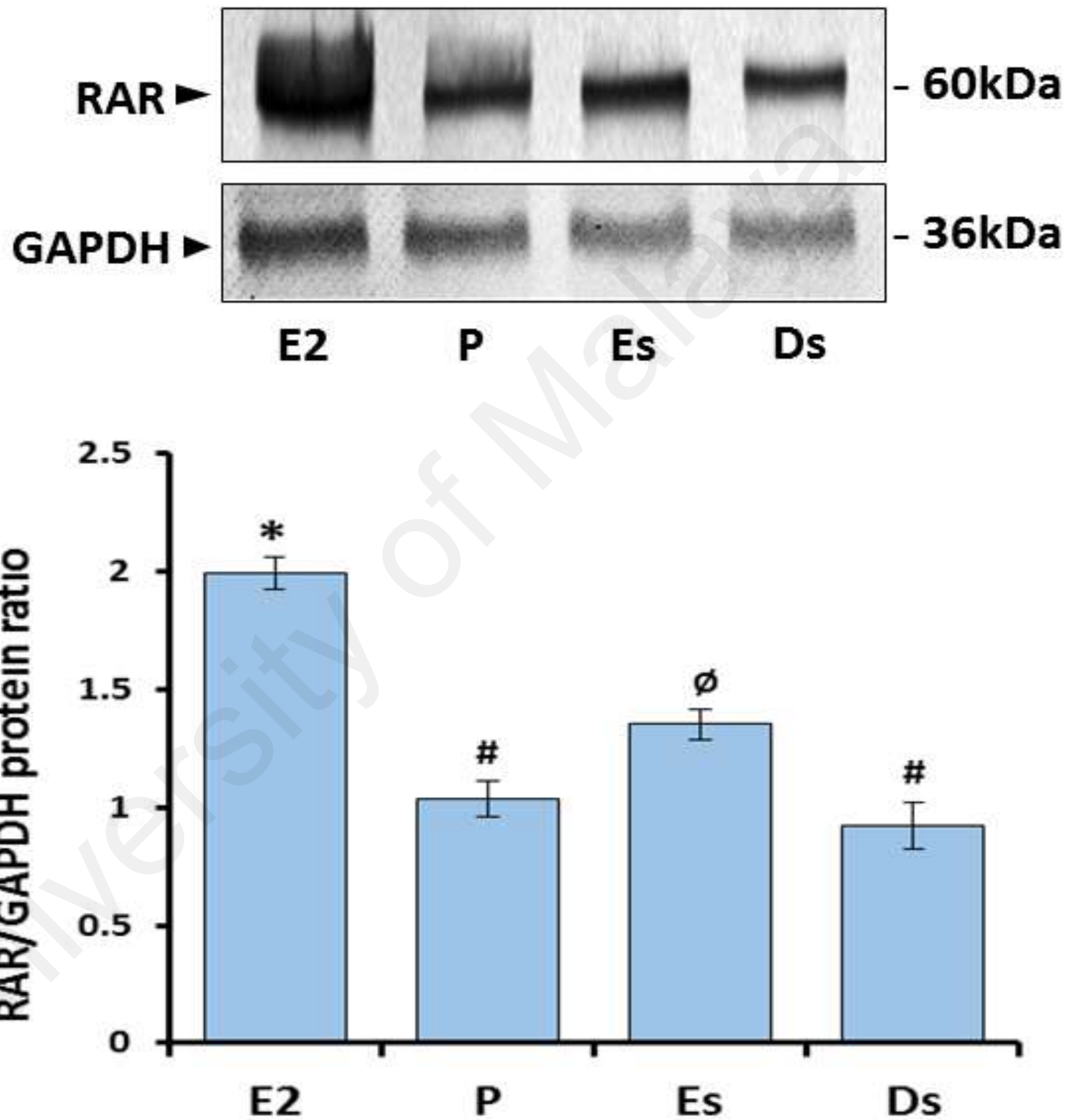

Figure 8.10 (A) Representative western blot band and analysis of expression level RAR protein in the uterus. (In next page) (B) Representative immunoperoxidase images showing distribution of RAR.

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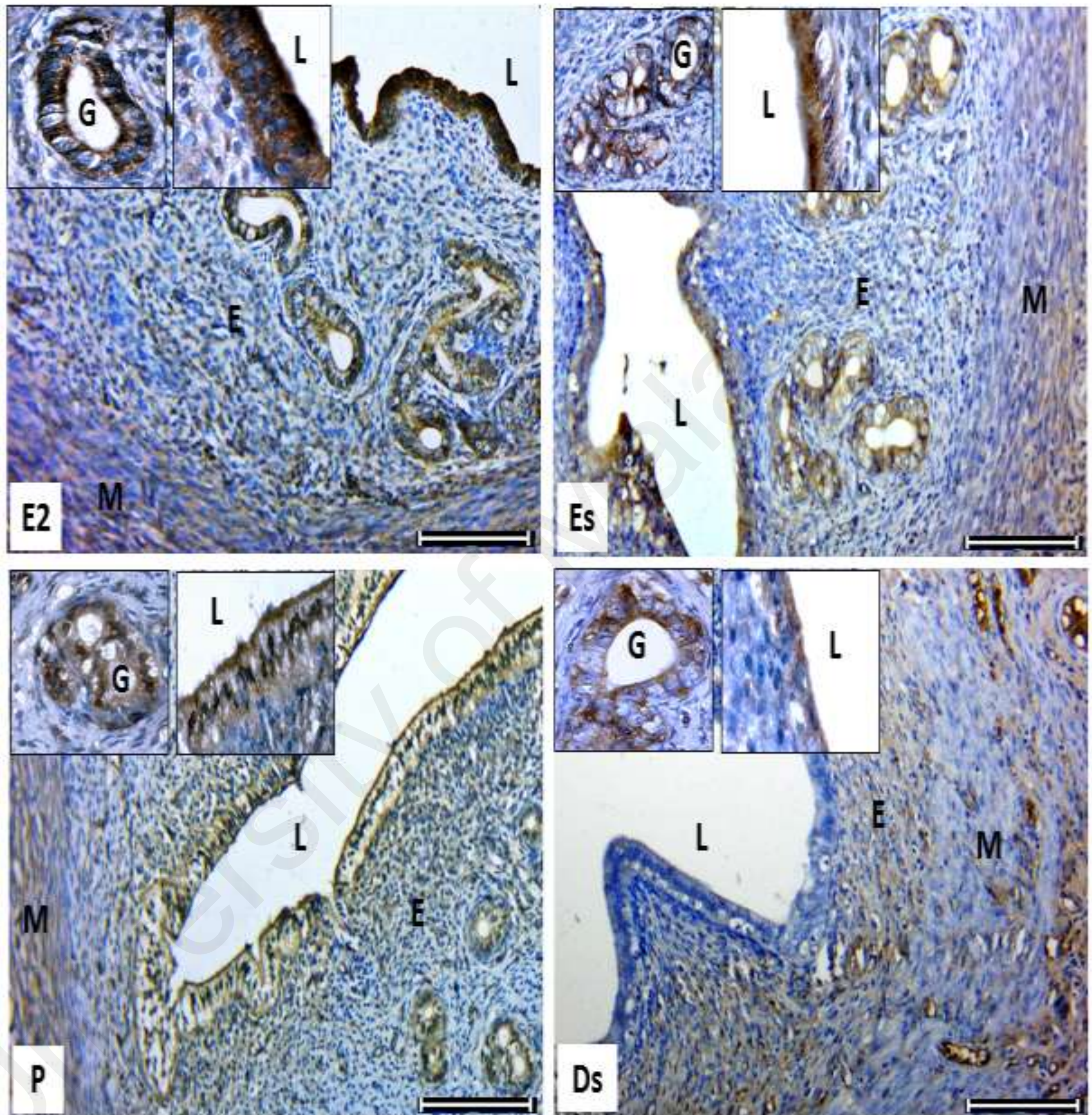

Figure 8.10 (A) Representative western blot band and analysis of expression level RAR protein in the uterus. (B) Representative immunoperoxidase images showing distribution of RAR.

RAR could be seen to be distributed in the endometrium, both in stroma and luminal and glandular epithelia, myometrium. Larger images and smaller images were taken at magnification of 10X and 40X respectively. Scale bar = 50µm. Bars that do not share the same symbols differ significantly at *P*<0.05. Data were expressed as mean ± standard error of mean with n= 6 per group. Es: estrus, Ds= diestrus, E_2= estradiol, P= progesterone. M= myometrium, E= endometrium, G= Gland, L= lumen. Molecular weight of RAR= 60 kDa.

Expression levels of RAR protein in uterus were significantly higher in E2-treated rats as compared to P-treated rats ($p < 0.05$) (Fig. 8.10A). Rats at Es phase have higher uterine RAR expression level as compared to Ds phase. Expression levels of RAR in E2-treated rats were significantly higher than rats at Es and Ds phases ($p < 0.05$). However, in P-treated rats, RAR expression level was not significantly different as compared rats at Ds phase.

A relatively higher RAR distribution was observed in the luminal and glandular epithelia in E2-treated rats than P-treated rats (Fig 8.10B). Distribution of RAR protein in the luminal and glandular epithelia was also higher at Es phase as compared to Ds phase. However, distribution of this protein in the stroma was relatively higher in P-treated rats as compared to E2-treated rats and was also higher in rats at Ds phase as compared to Es phase. RAR could be seen to be distributed intracellularly.

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8.2.6 Levels of ERK1/2 protein expression and its distribution in uterus

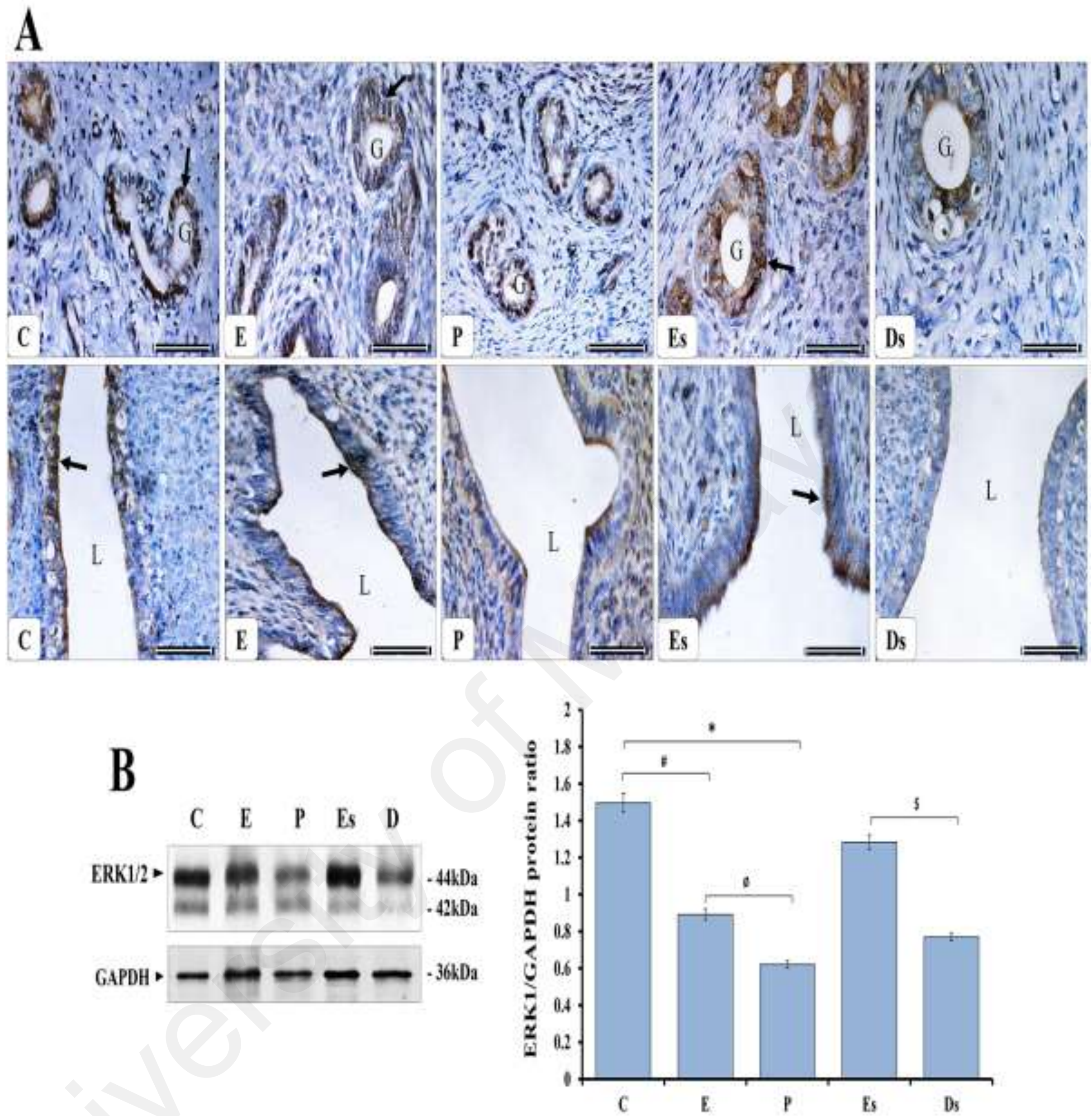

Figure 8.11 Representative immunoperoxidase images showing distribution of (A) ERK1/2 in glandular and luminal epithelium of endometrium and representative western blot band and analysis of expression level of (B) ERK1/2 protein in uterus.

In (A) relatively higher expression of proteins could be seen under E influence as compared to P influence. Images were taken at magnification of 40X. Scale bar= 50µm. In (B) $^{*,\#}p<0.05$ compared to C, $^{Ø}p<0.05$ compared to P, $^{\$}p<0.05$ compared to Ds. Data were expressed as mean ± standard error of mean with n= 6 per group. Molecular weight of ERK1/2= 44/42 kDa.

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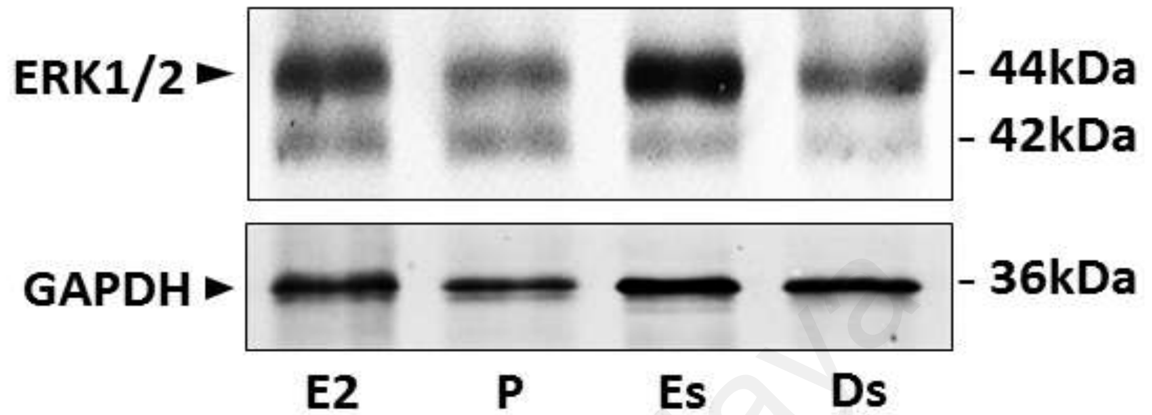
A



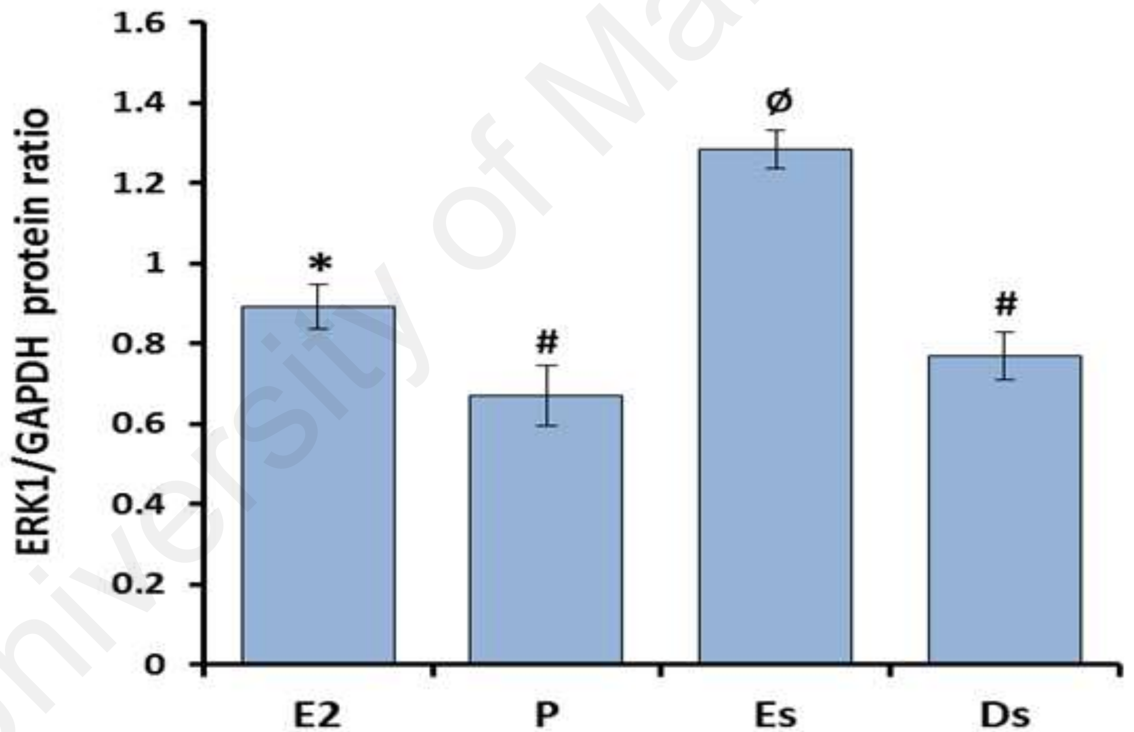


Figure 8.12 (A) Representative western blot band and analysis of expression level of ERK1/2 protein in the uterus. (In next page) (B) Representative immunoperoxidase images showing distribution of ERK1/2.

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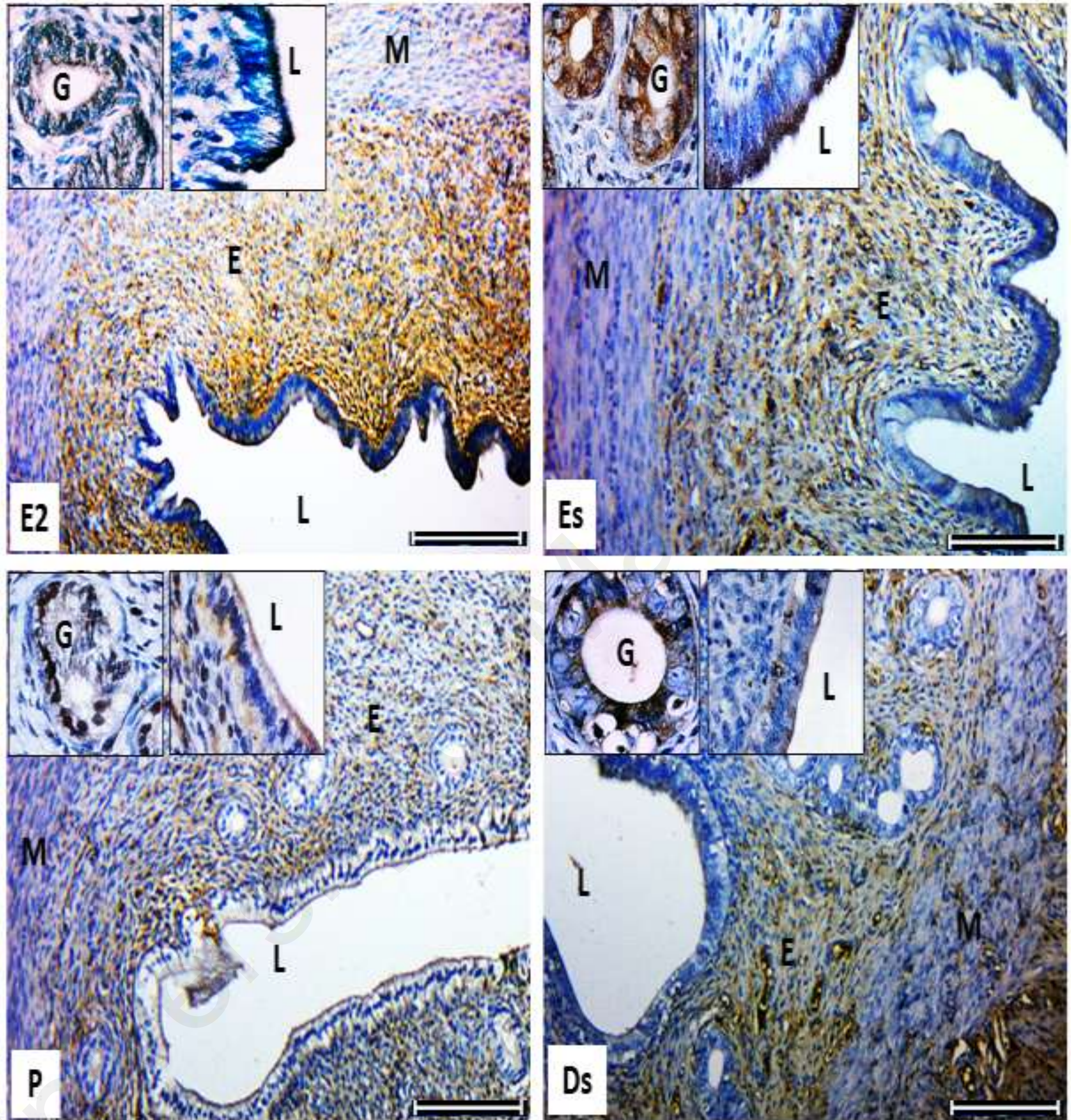

Figure 8.12(A) Representative western blot band and analysis of expression level of ERK1/2 protein in the uterus. (B) Representative immunoperoxidase images showing distribution of ERK1/2.

ERK1/2 could be seen to be distributed in the endometrium, both in the stroma and luminal and glandular epithelia. No myometrial distribution could be seen. Larger images and smaller images were taken at magnification of 10X and 40X respectively. Scale bar = 50µm. Bars that do not share the same symbols differ significantly at $P<0.05$. Data were expressed as mean ± standard error of mean with n= 6 per group. Es: estrus, Ds= diestrus, E_2= estradiol, P= progesterone. M=myometrium, E= endometrium, G= Gland, L= lumen. Molecular weight of ERK1/2= 44/42 kDa, where 44 kDa has been used for analysis.

Expression level of ERK1/2 protein in uterus of E2-treated rats was higher than P-treated rats ($p<0.05$) (Fig. 8.12A). A significantly higher ERK1/2 protein expression level was observed in rats at Es phase as compared to Ds phase ($p< 0.05$). In E2-treated rats, levels of ERK1/2 protein were significantly lower when compared to rats at Es phase but higher when compared to rats at Ds phase. Lower ERK1/2 protein expression levels were observed in P-treated rats as compared to rats at Es phase and in the latter was not significantly different when compared to rats at Ds phase.

A relatively higher distribution of ERK1/2 was observed in the luminal and glandular epithelia and stroma of E2-treated rats as compared to P-treated rats (Fig 8.12B). Similarly, distribution of ERK1/2 was relatively higher in the luminal and glandular epithelia and in the stroma at Es phase as compared to Ds phase. ERK1/2 protein could be seen to be distributed intracellularly and at the plasma membrane.

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8.3 Discussion

To the best of our knowledge, this study is the first to show the individual effect of female sex hormones i.e E2 and P which were found able to differentially affect expression and distribution of TRα-1, TRβ-1, TSHR, VDR, RAR and ERK1/2 proteins in rat uterus. This study also showed that in intact rats, differential distributions of these proteins were observed at different phases of oestrous cycle. Under E2 influence, expression level of TRα-1, TRβ-1, TSHR, VDR, RAR and ERK1/2 were higher than under P influence which indicated that uterine effect of thyroid hormone could be greater under E2 influence as compared to P influence. It was also found that distribution of these proteins in uterine compartments were markedly different under the different hormonal condition. A relatively higher TRα-1, TRβ-1, TSHR, VDR, RAR and ERK1/2 proteins were distributed in endometrial luminal and glandular epithelia under E2 influence; however a relatively higher TSHR, VDR and RAR were distributed in the stroma under P influence. Meanwhile, only TRα-1 and TR-β were found to be distributed in the myometrium, particularly under E_2 influence.

Increased in expression of these proteins in the endometrial luminal and glandular epithelia in particular under E2 influence could have implications on growth of the endometrium under this hormonal condition. Under E2 influence, growth and proliferation have been reported in both endometrium and myometrium (Dong *et al.*, 2017; Greening *et al.*, 2016; Korzekwa *et al.*, 2016; Vercellini *et al.*, 2016). Besides, E2 was reported to regulate stromal growth through the epithelium (Carpenter *et al.*, 2003) via this mechanism, thyroid hormone could exert its effect on the uterine stroma. Under E2 influence, increased in growth and proliferation of the uterus is associated with increased in the basal metabolic rate that can be achieved via the action of thyroid hormone. Therefore, an increased in the number of thyroid hormone receptors reflect increased uterine metabolism. The important role of thyroid hormone on uterine growth

could be seen in a condition related to thyroid hormone deficiency i.e hypothyroidism where an absolute decreased in the volume of epithelial cells and nuclei were observed (Inuwa I. & M. Williams, 2006; Sayem A. S. M. *et al.*, 2017). A study has shown that E2 stimulates mitogenic response in endometrial cells via up-regulating pyruvate kinase expression that co-activate estrogen receptor-α. This integrates the metabolic reprogramming leading to a shift in the glucose metabolism toward aerobic glycolysis (Salama *et al.*, 2014). These E2 effect could be accomplished via the action of thyroid hormone.

Our findings which indicated differential expression of thyroid hormone receptors; TRα1, TRβ1 and TSHR in different endometrial compartments were supported by the reported differential expression of these receptors in the glandular and luminal epithelial, and the stroma throughout the menstrual cycle in humans (Aghajanova L. *et al.*, 2011). Furthermore, similar pattern of uterine distribution was observed following sex hormone treatment in macaque (Hulchiy Mariana *et al.*, 2012). A study has also shown that TSHR distribution in the endometrial luminal epithelia and TRα1 and TRβ1 distribution in the endometrial luminal and glandular epithelia increased significantly on luteinizing hormone (LH) days 6 to 9 in humans, coincide with high E2 levels (Aghajanova Lusine *et al.*, 2011).

In this study, VDR expression was observed to be high in the epithelium under the influence of E2. Our findings were consistent with a report by Emam et al, (Emam Mahmoud Abdelghaffar *et al.*, 2016) which shown that in the uteri of cows, expression of VDR was restricted to the luminal and glandular epithelia of endometrium under E2 influence which indicated that this receptor plays important role in endometrial epithelial functions. Further, they found that *Vdr* mRNA was expressed in the

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endometrium throughout the estrous cycle with a relatively high expression at Es phase, under the influence of E2, the findings that were similar to ours.

Immunohistochemical analysis revealed luminal and glandular epithelia of endometrium strongly express VDR, particularly under E2 influence. Our findings were supported by others who showed similar pattern of VDR expression in the endometrium (Zarnani Amir Hassan *et al.*, 2010). VDR mediates the action of Vitamin D which participates in many uterine events. For example, transport of calcium across the uterine epithelium is vitamin D dependent. The role of vitamin D in uterine calcium transport is further supported by a study which showed that expression of vitamin D calcium binding protein (CaBP or calbindin-D9k) in the uteri of 21-day-old non-pregnant rats increased following administration of tamoxifen or physiological doses of estrogens (Bruns *et al.*, 1988). Activation of vitamin D by cytochrome (CYP27A1 and CYP2R1) enzymes were also reported to be involved in controlling endometrial epithelial growth (Bergadà *et al.*, 2014). Therefore, up-regulated VDR expression as observed under E2 influence could play a role in endometrial epithelial growth that occurs under this hormonal influence.

In this study, we have shown that RAR expression in the uterus was enhanced under the influence of E2, particularly in the luminal and glandular epithelia. Increased RAR expression suggests its involvement in growth and proliferation of endometrial epithelia under the influence of E2. In postmenopausal women receiving estrogen replacement therapy, increased expression of RAR in the uterus was observed while in premenopausal women, levels of RAR in the endometrium increased during the proliferative phase, which is associated with high E2 level (Deng *et al.*, 2003). Similar study by Fukunaka et al, (Fukunaka *et al.*, 2001) showed that RAR was strongly

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expressed in the nuclei of endometrial epithelium in the proliferative phase of the menstrual cycle.

We have shown that the level of ERK1/2 in uterus increased under E_2 influence and this protein was found mainly in the luminal and glandular epithelia. ERK1/2 protein could mediate growth and proliferative effects of E2. Our findings were consistent with a report by Wang et al, (2015) (Wang *et al.*, 2015) who showed that E2 regulates protein synthesis in the uterine epithelial cells through activation of protein kinase C (PKC) that in turn stimulates ERK1/2 to phosphorylate and activate the central regulator of protein synthesis, mTOR. Besides mediating growth and proliferative effects, ERK1/2 also mediates several E_2 effects in the endometrial epithelia such as MMP-2/9 expression (Shan *et al.*, 2013) and expression of early growth response 1 (Egr1), a zinc finger transcription factor that regulates cell growth, differentiation and apoptosis in the uterus (Kim H.-R. *et al.*, 2014).

Meanwhile, under P influence, expression of TRα1, TRβ1, TSHR, VDR, RAR and ERK1/2 in the epithelia was lower than under E2 influence, with exception of the stroma where expression of TSHR, VDR and RAR were higher under P influence than under E2 influence. Aghajanova et al, (Aghajanova Lusine *et al.*, 2011) showed that human endometrial stroma expressed mRNA for *Tshr*. Our findings indicated that under P influence, thyroid hormone exerts lesser action on uterine epithelium. However, high expression of TSHR, VDR and RAR in the stroma under P influence indicated that thyroid stimulating hormone (TSH), vitamin D and retinoid acid plays important role in stromal functions under this hormonal influence.

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We have shown that P downregulates epithelial expression of TR and TSHR but up-regulates expression of TSHR in the stroma which were consistent with the findings by Hulchiy et al, (Hulchiy Mariana *et al.*, 2012) who reported that in uterine stroma of macaque, TSHR expression was increased following administration of conjugated quine estrogen and medroxyprogesterone acetate (CEE + MPA). The role of TSH in endometrial physiology has been proposed. Aghajanova (Aghajanova Lusine *et al.*, 2011) reported that administration of TSH to cultured human endometrial stroma cells significantly increase leukemia inhibitory factor (LIF) and LIF receptor (*Lifr*) mNA levels. Furthermore, glucose transporter 1 (*Glut-1*) mRNA levels were also enhanced by TSH in Ishikawa (uterine adenocarcinoma) cells. The importance of TSH has been documented in women where its level correlates with the clinical pregnancy outcomes (Karmon *et al.*, 2016).

In the meantime, expression on VDR in the stroma was higher under P than under E2 influence. It was shown previously that in cow uteri, VDR expression was high at luteal (P dominant) phase (Emam Mahmoud Abdelghaffar *et al.*, 2016), supporting our findings that this could be due to high expression in the stromal compartment. It was recently reported that the concentrations of calcitriol, an active form of vitamin D in endometrial tissues was higher during early pregnancy which could affect expression of several genes related to implantation, vitamin D metabolism, calcium ion regulation, PG metabolism, and calcium-binding proteins in the endometrium (Jang *et al.*, 2017). Thus, high VDR expression, in particular in the stroma could play a role in mediating vitamin D effect in preparation for pregnancy. Vitamin D also play a pivotal role in normal decidual immune function via promoting the innate responses to infection (Tamblyn *et al.*, 2015) which could explain the reason for the increased VDR expression in the stroma under P influence. Vitamin D treatment was

found to decrease inflammation-induced cytokines and contractile-associated factors in uterine myometrial smooth muscles (Thota *et al.*, 2014).

Besides the increased in stromal TSHR and VDR, expression of RAR in stroma also increased under P influence. Our findings were consistent with the report that RAR plays important role in uterine stromal development from Mullerian duct in mouse embryo (Nakajima *et al.*, 2016). Ozaki et al, (Ozaki *et al.*, 2017) also shows that RAR and the retinoic acid pathway are involve in decuadualization in human endometrial stromal cells, an event which occur under P influence. In the meantime, our findings that RAR expression in uterine epithelium was low under P influence was consistent with a report by Fukunaka et al, (2001) (Fukunaka *et al.*, 2001) who observed that RAR expression was drastically reduced in epithelial nuclei during the secretory phase in association with reduced in serum E2 level.

In this study, we observed that TRα1, TRβ1, VDR and RAR were distributed intracellularly as well as at or near the plasma membrane. Thyroid hormone can mediate both genomic and non-genomic effects. Among the non-genomic effects are a set of actions initiated at the cell surface receptor that are relevant to intracellular trafficking of proteins, serine phosphorylation and acetylation, assembly within the nucleus and transcription of specific genes (Davis Paul J. *et al.*, 2016). In the meantime, genomic effects of thyroid hormone involve thyroid hormone response elements (TRE) on specific genes, complexes of nuclear thyroid hormone receptors (TRs) and 3,5,3'-triiodo-L-thyronine (T(3)), coactivator or corepressor nucleoproteins, and histone acetylases or deacetylases (Davis *et al.*, 2013). Additionally, it was reported that TR especially TRβ1 isoform may be found in the cytoplasm complexed with other proteins, such as mitogen-activated protein kinase (MAPK). Formation of such complexes may facilitate nuclear import of TR (Davis *et al.*, 2008). Furthermore, nuclear retention of

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TR occur only following binding to thyroid hormone (Davis *et al.*, 2008). It is likely that the receptors found in the cytoplasm or at the plasma membrane are TRs that are not complex to thyroid hormone.

In the meantime, our findings that VDR is located at the plasma membrane is also consistent with a report in the neuron where VDR was localized to neuronal plasma membrane which involve in the non-genomic effect of vitamin D (Dursun & Gezen-Ak, 2017). Similarly, RAR has been reported to be retained in the cytoplasm and moves towards the nucleus only when retinoic acid is present (Park *et al.*, 2010) and this could support our observation for the cytoplasmic distribution of RAR, rather than its distribution in the nucleus of the uterine epithelial cells.

In this study, it was found that the level of E2 in ovariectomized rats was higher than intact rats either at estrus or diestrus phases of the cycle. The likely reason was that following ovariectomy, there could be a compensatory production of E2 from the adrenal gland (Alagwu & Nneli, 2005) and this would contribute towards the high E2 levels. Progesterone treatment did not have any effect on serum E2 levels. In this study, it was also found that the levels of expression of TRβ1 and RAR in uterus at Es phase was lower than following E2 treatment, while the opposite was observed in the ERK1/2 level where its level was higher at Es phase as compared to following E2 treatment. However, no difference in TRα1 and VDR levels were observed between Es phase and E2 treatment. The likely explanation was that higher serum E2 levels could induce transcription of TRβ1 and RAR while higher ERK1/2 level in intact as compared to ovariectomised, E2-treated rats suggests that in addition to E2, other factors/hormones from the ovary could induce ERK1/2 expression in the uterus.

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In conclusions, our study has shown the differential effects of E2 and P on TRα1 and TRβ1, TSHR, VDR, RAR and ERK1/2 expression and distribution in the uterus which could facilitate the changing functions of this organ under these different sex-steroid conditions. The changes in expression of these proteins could also mediate uterine effects of thyroid hormone that could be different under the influence of E2 and P.

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CHAPTER 9: CONCLUSION

Hypothyroidism can cause female infertility. In this study, the possible mechanisms could be due to down-regulation of the receptors related to TH, TSH, Vitamin D and retinoic acid functions in the uterus as well as down-regulation of the expression of ERK1/2 signaling molecule which is involve in many uterine functions. Additionally, hypothyroidism also causes down-regulation of expression of uterine receptivity molecules but increases the expression of water channel in the uterus during the peri-implantation period.

This study also shown that in hypothyroidism, the expressions of TR, TSHR, RXR, VDR and ERK1/2 in uterus were down-regulated at the time on embryo implantation. Collectively, changes in expression of these proteins in the uterus during both the peri-implantation and implantation period could lead to decrease in embryo implantation as reflected by decreased in implantation rate. These could contribute to infertility as reported in hypothyroidism (Priya *et al.*, 2015).

To the best of my knowledge, this is the first study to report the expressions of the proteins that are involved in mediating thyroid hormone function in uterus are under the influence of thyroxine. Besides this study could be the first to elucidate the role of thyroxine in uterine fluid regulation during the peri-implantation period, although other studies reported the involvement of thyroxine in the development of a competent blastocyst during peri-implantation period.

Below, the findings from this study are summarized in diagrams:

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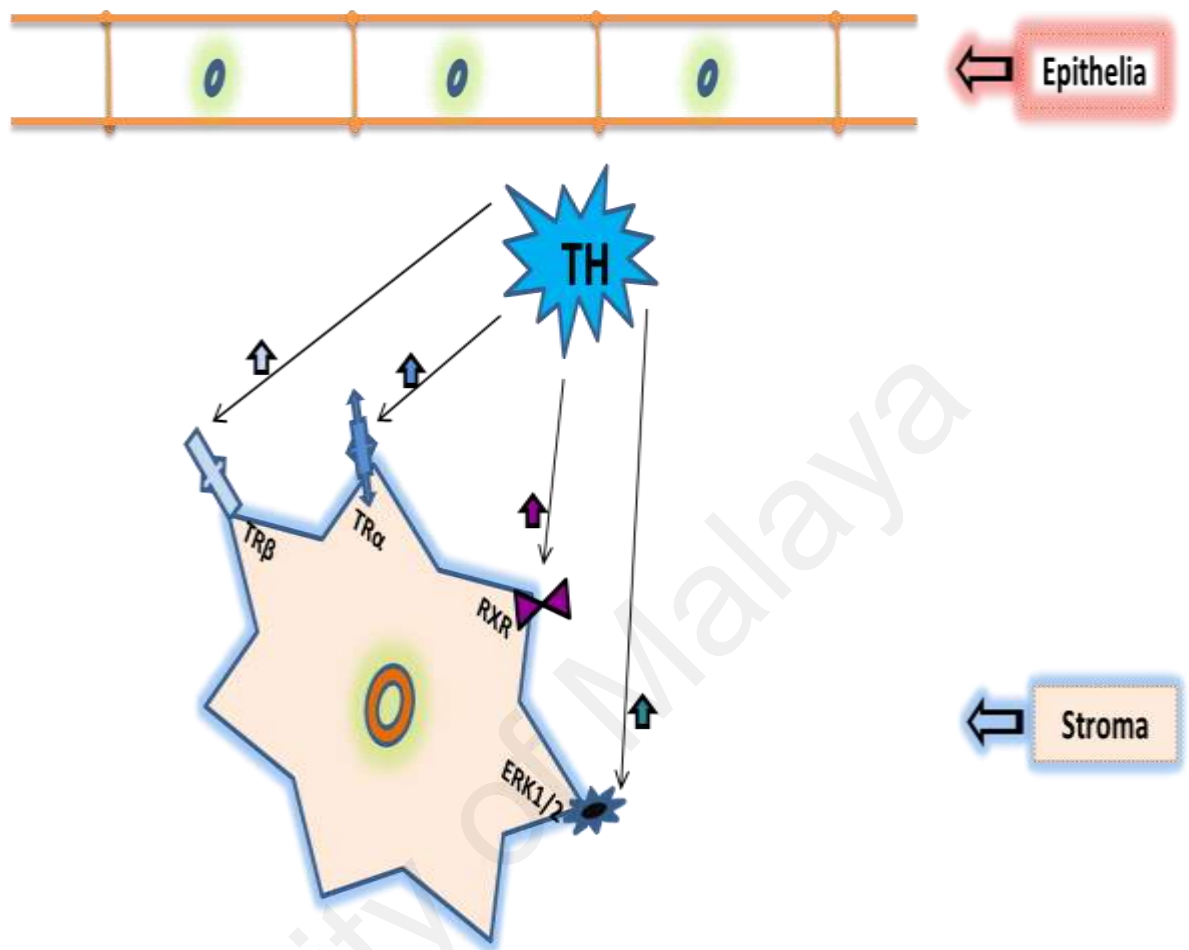

Figure 9.1 Effect of TH on receptors related to TH function during peri-implantation period.

Thyroxine up-regulates the expression of TRα1, TRβ1, RXR and ERK1/2 in the uterine stroma during peri-implantation period (Fig. 9.1).

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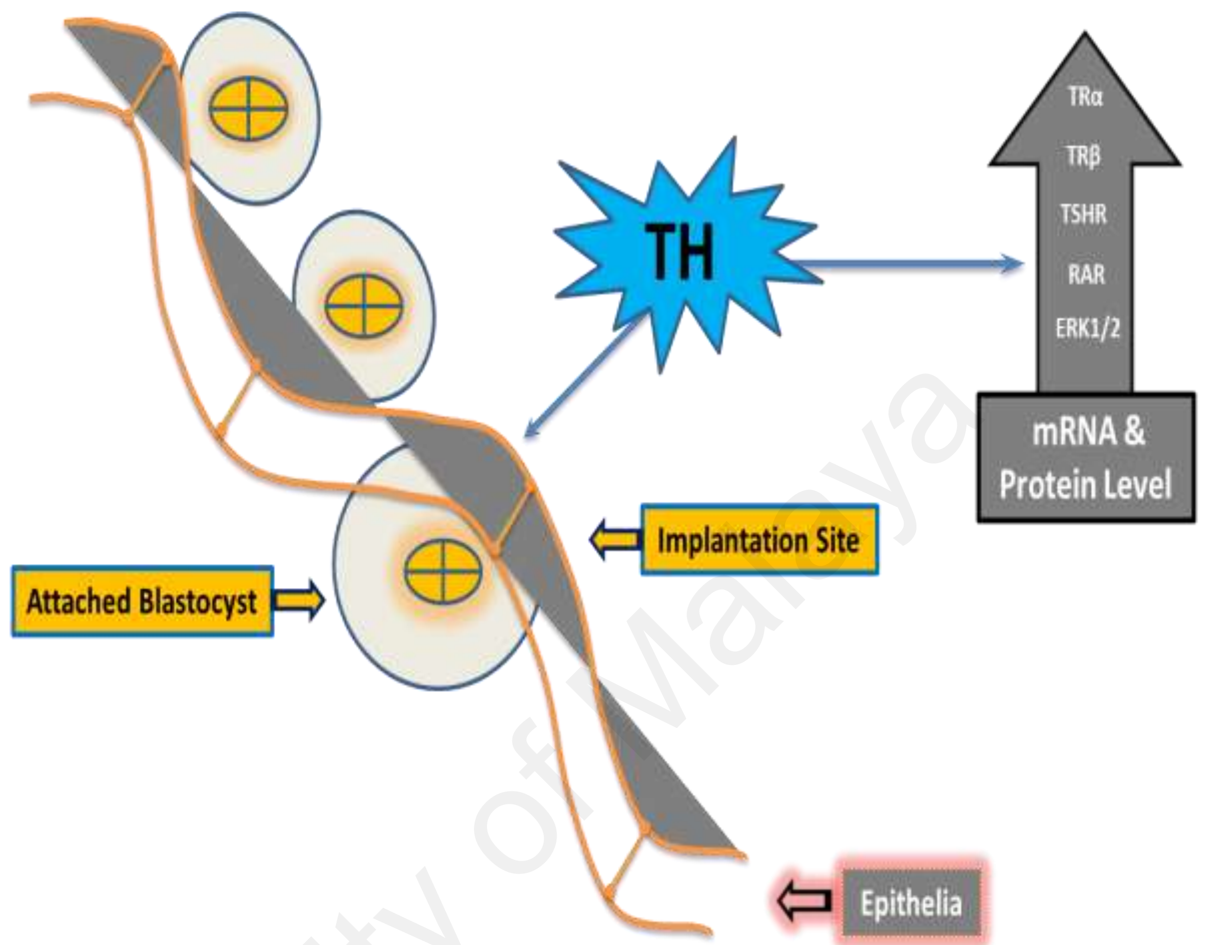

Figure 9.2 Effect of TH on receptors related to TH function & implantation site during implantation period

During implantation period, mRNA levels for TRα1, TRβ1, TSHR, RAR and ERK1/2 in uterus were up-regulated following thyroxine treatment (Fig. 9.2).

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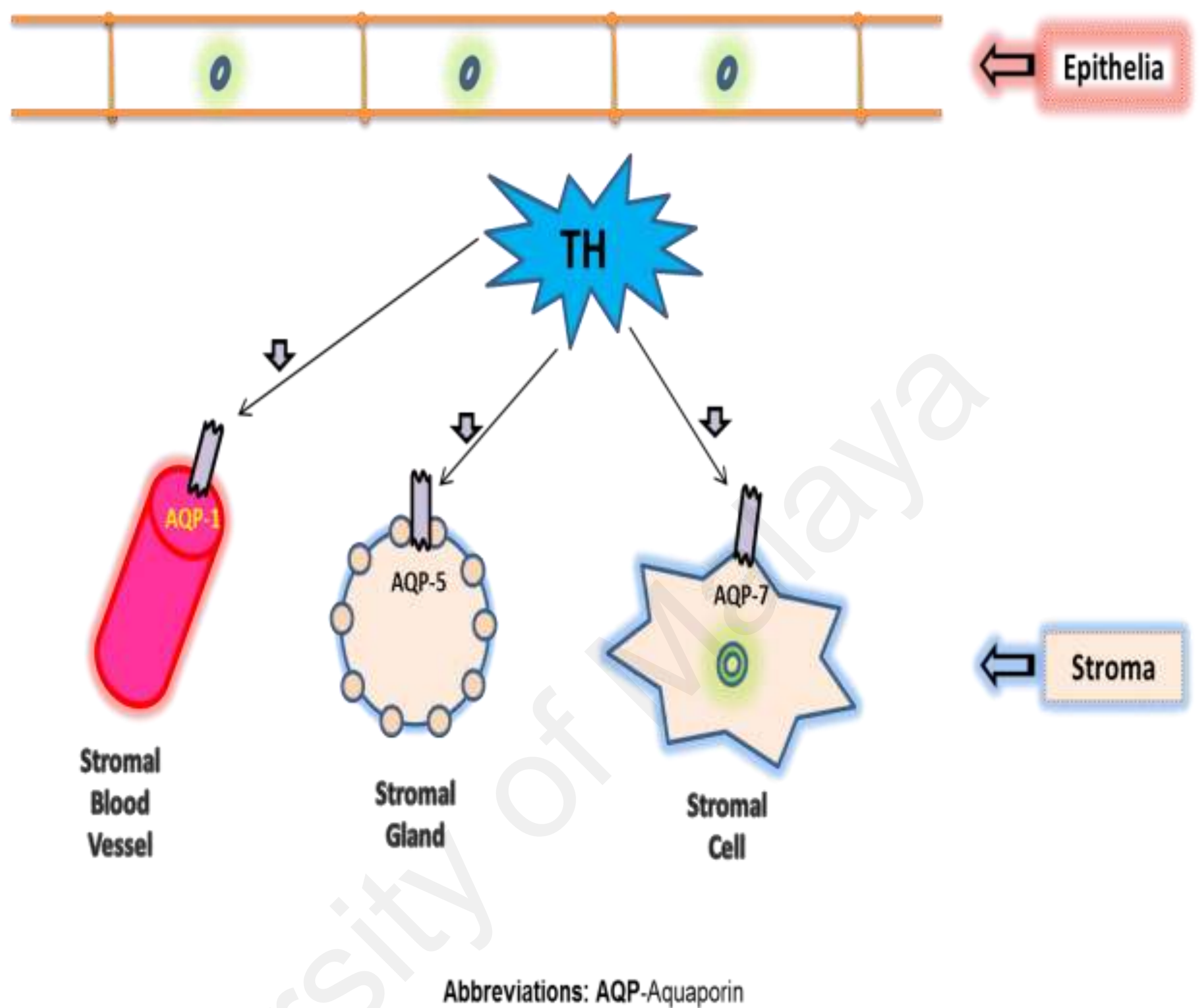

Figure 9.3 Effect of TH on uterine water channel expression during peri-implantation period.

Thyroxine treatment down-regulates AQP-1, 5 & 7 in the stromal blood vessels, glands and cells respectively (Fig. 9.3).

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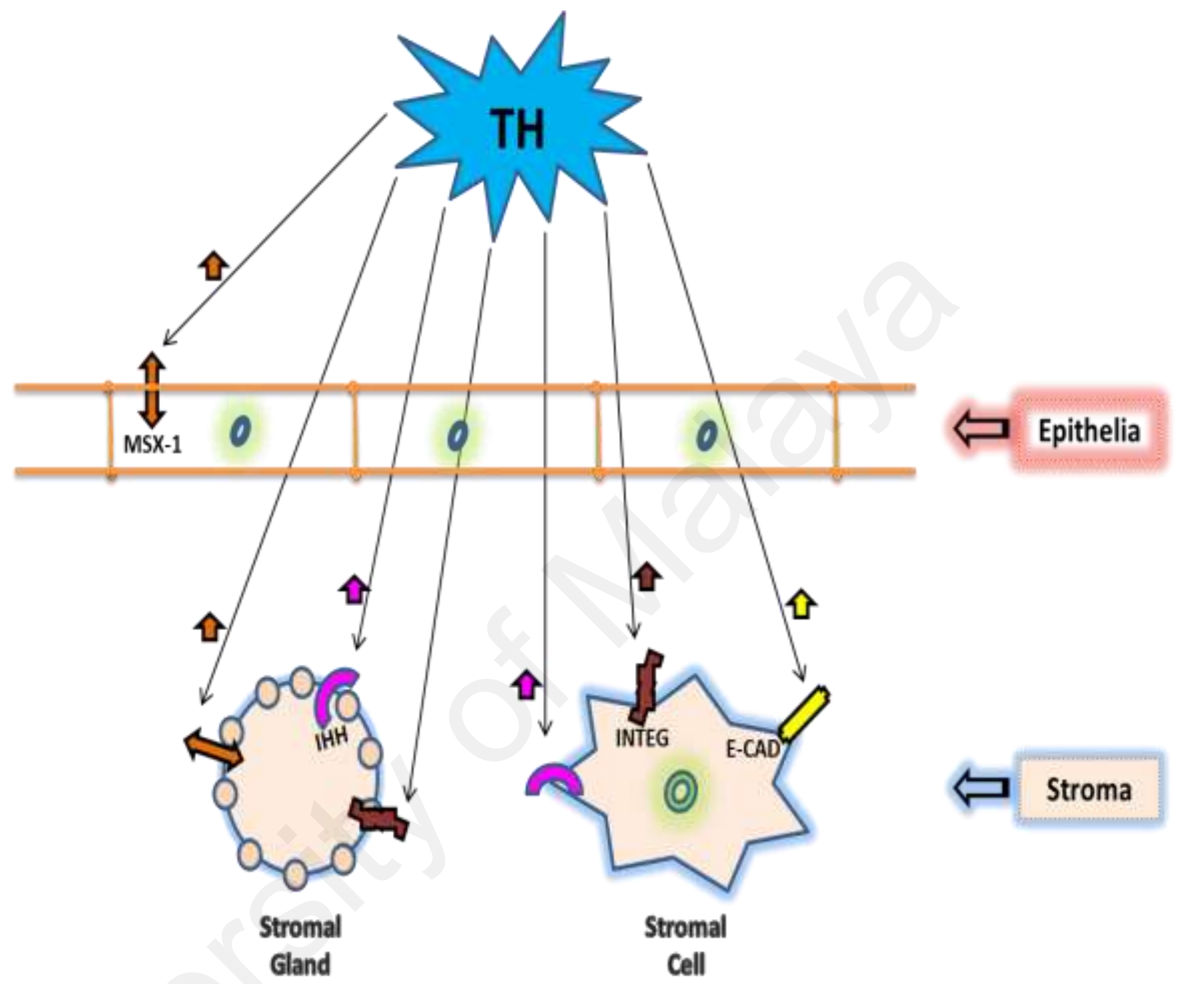

Figure 9.4 Effect of TH on uterine receptivity molecules during peri-implantation period

Thyroxine up-regulates the expression of Msx-1 in epithelia and stromal glands, Ihh, Integrin $\alpha v\beta 3$, and E-cadherin in stroma during peri-implantation period (Fig. 9.4).

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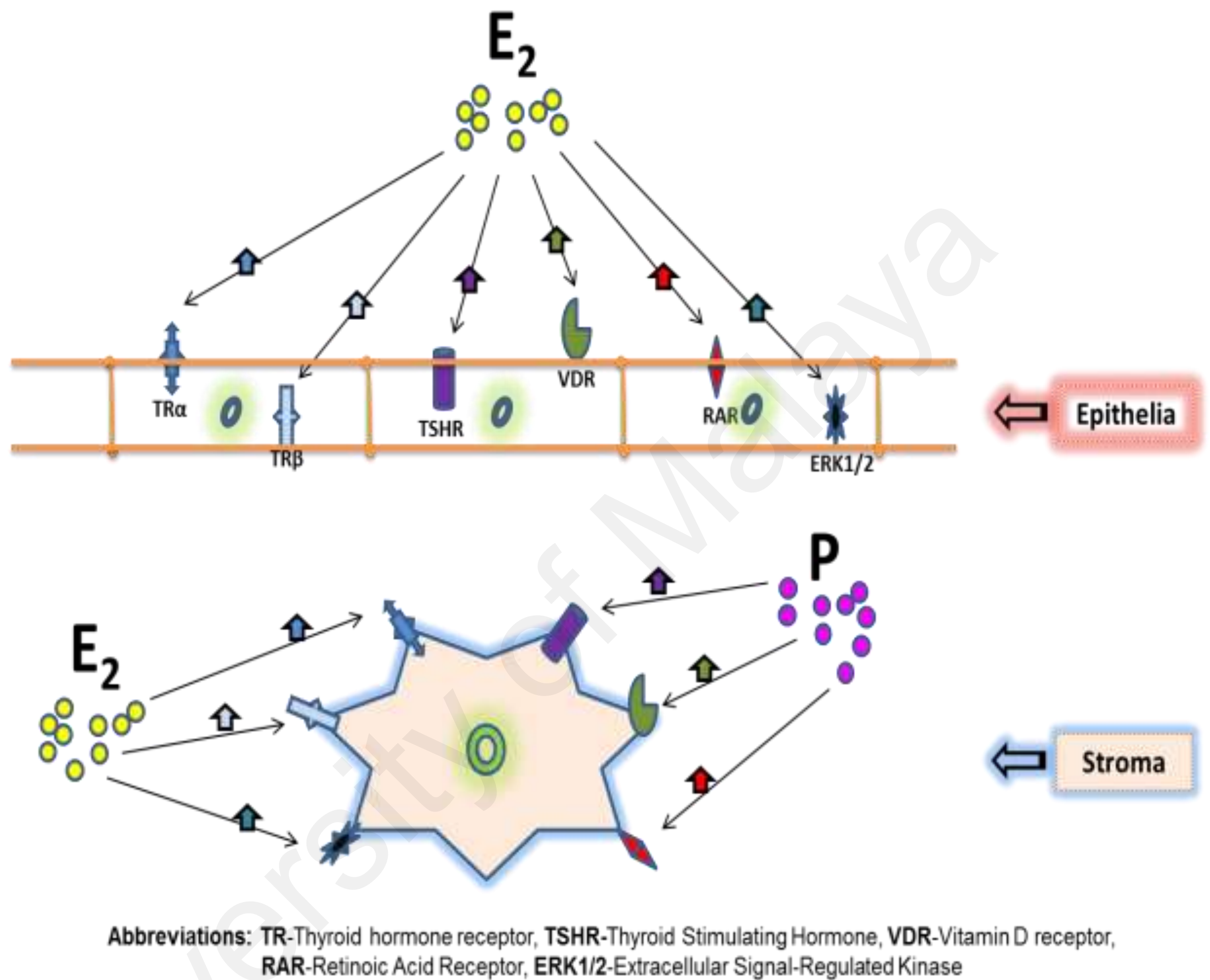

Figure 9.5 Effects of sex-steroids on expression of receptors related to TH function

E2 up-regulates the expression of TR-α, TR-β, TSHR, VDR, RAR and ERK1/2 in the epithelia, and TR-α, TR-β, ERK1/2 in the stromal cell, whereas P only up-regulates TSHR, VDR, RAR in the stromal cell (Fig. 9.5).

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9.1 Limitations of the study

Below are some limitations of the study:

(i) The volume of fluid in the uterus as identified by measuring the inner/outer uterine ratio was only estimation. Measurement of the ratio allows different animals with different uterine size to be compared. Perfusion experiment which will give an accurate amount of fluid volume was not done, however will be performed in future studies.

(ii) Like distributions of proteins in uterus during peri-implantation period were studied, protein distributions in uterus during implantation period could also be studied by immunohistochemistry or immunofluorescence. In our study, levels of protein expressions and mRNA levels in uterus during implantation period were measured by western blot and Real time PCR respectively.

(iii) Changes of protein expressions were analyzed in the uterus of pregnant rats and were not compared with pseudopregnant animals or non-pregnant animals which could be performed in future study.

(iv) Immunofluorescence images would be better visualize under high magnification. However these could not be performed as suggested by the thesis examiner due to lack of funding for further studies as well as the signals in the existing slides faded away as a result of long-term storage.

(v) Estradiol levels were high in ovariectomized models. Because this could probably be due to adrenal gland hyperplasia that has been reported in sex-steroid deficient state in females.

(vi) Sex-steroid results were described in general which applied to few result chapters.

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(vi) In this study, qualitative analysis was performed despite of quantitative analyses. In the future, quantitative analysis would give a more acurate interpretation of the result of immunofluorescence.

(vii) In this study, the effcts of TH on uterus during implantation process have been studied. However thease TH effects could be linked with other reproductive events, like oocyte/embryo quality and quantity. Therefore further study will investigate the effect of TH on the ovarian developments.

9.2 Future Directions

In this study, we have investigated the effects of thyroid hormone on thyroid hormone receptors and the signaling proteins which are involved in mediating thyroid hormone functions during peri-implantation and implantation period, and expression of these proteins in uterus under the influence of sex-steroid. In addition, we have also investigated the effects of thyroid hormone on uterine fluid volume and receptivity proteins during peri-implantation period. We further intend to investigate parameters related to thyroid hormone functions during implantation period.

During embryo implantation, there is inflammation in the uterus (Dekel *et al.*, 2014). This is a normal physiological event. It is known that inflammatory proteins can be affected by sex-steroid. However, thyroid hormone could also be involved in inflammation during embryo invasion into the uterine wall (Dekel *et al.*, 2014). Therefore in this study, we will further investigate changes in several inflammatory markers such as IL-1β, TNF-α, NFK-β during this period. In addition, thyroid hormone response elements (TRE) may play important role in the TH signaling. Therefore, presence of TRE will be further studied by bioinformatics analyses.

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In future, many study can be carried out to look that the influence of other importance of hormone or neuropeptides certain medicinal herbs, nutritional value or diet and age related that can further enhance the effect of oestrogen and progesterone to improve the infertility in women.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

❖ **Publications:**

Article 1:

Sayem, A. S. M., Giribabu, N., Muniandy, S., & Salleh, N. (2017). Effects of thyroxine on expression of proteins related to thyroid hormone functions (TR-alpha, TR-beta, RXR and ERK1/2) in uterus during peri-implantation period. *Biomed Pharmacother, 96*, 1016-1021. doi: 10.1016/j.biopha.2017.11.128

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Effects of thyroxine on expression of proteins related to thyroid hormone functions (TR-α, TR-β, RXR and ERK1/2) in uterus during peri-implantation period

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Keywords:
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ABSTRACT

Introduction: Thyroid hormone is known to play important role during embryo implantation, however mechanisms underlying its actions in uterus during peri-implantation period has not been fully identified. In this study, we hypothesized that thyroid hormone could affect expression of proteins related to its function, where these could explain mechanisms for its action in uterus during this period.
Methods: Female rats, once rendered hypothyroid *via* oral administration of methimazole (0.03% in drinking water) for twenty-one days were mated with fertile euthyroid male rats at 1:1 ratio. Pregnancy was confirmed by the presence of vaginal plug and this was designated as day-1. Thyroxine (20, 40 and 80 μg/kg/day) was then subcutaneously administered to pregnant, hypothyroid female rats for three days. A day after last injection (day four pregnancy), female rats were sacrificed and expression of thyroid hormone receptors (TR-α and β), retinoid X receptor (RXR) and extracellular signal-regulated kinase (ERK1/2) in uterus were quantified by Western blotting while their distribution in endometrium was visualized by immunofluorescence.
Results: Expression of TRα-1, TRβ-1 and ERK1/2 proteins in uterus increased with increasing doses of thyroxine however no changes in RXR expression was observed. These proteins were found in the stroma with their distribution levels were relatively higher following thyroxine treatment.
Conclusions: Increased expression of TRα-1, TRβ-1 and ERK1/2 at day 4 pregnancy in thyroxine-treated hypothyroid pregnant rats indicate the importance of thyroxine in up-regulating expression of these proteins that could help mediate the uterine changes prior to embryo implantation.

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Article 2:

Sayem, A. S. M., Giribabu, N., Karim, K., Si, L. K., Muniandy, S., & Salleh, N. (2018). Differential expression of the receptors for thyroid hormone, thyroid stimulating hormone, vitamin D and retinoic acid and extracellular signal-regulated kinase in uterus of rats under influence of sex-steroids. Biomed Pharmacother, 100, 132-141. doi: 10.1016/j.biopha.2018.02.008

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Differential expression of the receptors for thyroid hormone, thyroid stimulating hormone, vitamin D and retinoic acid and extracellular signal-regulated kinase in uterus of rats under influence of sex-steroids

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ABSTRACT

Sex-steroids play important role in modulating uterine functions. We hypothesized that these hormones affect expression of proteins in the uterus related to thyroid hormone action. Therefore, changes in expression levels of receptors for thyroid hormone (TRα-1 and TRβ-1), thyroid stimulating hormone (TSHR), vitamin D (VDR) and retinoid acid (RAR) as well as extracellular signal-regulated kinase (ERK1/2) in uterus were investigated under sex-steroid influence.

Methods: Two rat models were used: (i) ovariectomised, sex-steroid replaced and (ii) intact, at different phases of oestrous cycle. A day after completion of sex-steroid treatment or following identification of oestrous cycle phases, rats were sacrificed and expression and distribution of these proteins in uterus were identified by Western blotting and immunohistochemistry, respectively.

Results: Expression of TRα-1, TRβ-1, TSHR, VDR, RAR and ERK1/2 in uterus was higher following estradiol (E_2) treatment and at estrus phase of oestrous cycle when E_2 levels were high. A relatively lower expression was observed following progesterone (P) treatment and at diestrus phases of oestrous cycle when P levels were high. Under E_2 influence, TRα, TRβ, TSHR, VDR, RAR and ERK1/2 were distributed in luminal and glandular epithelia while under P influence, TSHR, VDR abo RAR were distributed in the stroma.

Conclusions: Differential expression and distribution of TRα-1, TRβ-1, TSHR, VDR, RAR and ERK1/2 in different uterine compartments could explain differential action of thyroid hormone, TSH, vitamin D, and retinoic acid in uterus under different sex-steroid conditions.

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❖ Presentations:

1. **Abu Sadat Md Sayem,** Nelli Gribabu, Sekaran Muniandy and Naguib Salleh 2017. "Differential Expression of Thyroid Hormone, Thyroid Stimulating Hormone, Vitamin D and Retinoic Acid Receptors and Extracellular Signal-Regulated Kinase in the Uterus under Sex-Steroid Influence and Oestrous Cycle Phases in Rodents".

The 4th Asian Regional Conference on Systems Biology 2017 (International conference-Oral presentation).

2. **Abu Sadat Md Sayem,** Nelli Gribabu, Sekaran Muniandy and Naguib Salleh 2016. "Role of Thyroid Hormone in the Regulation of Uterine Fluid Environment and Development of Uterine Receptivity in Rats".

30th Scientific Meeting of Malaysian Society of Pharmacology and Physiology 2016 (International Conference).

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APPENDIX

Buffer Preparation

30%Acryamide solution

30g acrylamide

.8g N'N'-BIS-methylee-acrylaide

Dluteto 100mL with distilled water. Sore n the dark room at 4 degree C

Tris buffer 4x Resolving

18.17g Tris base

40 L 10% SDS (optional)

Tris buffer 4x Stacking

6.06 g Tris base

40 mL1 % SDS (optional).

Adjust pH to 6.8with concentrated HCl. Dilute to 100mL and store 4degree C.

Sample buffer (Loading dye)

3.9 mL distilled water

1.0mL 0.5Tis pH 6.8

0.8 ml Glycerol

1.6ml 10 % SDS

0. ml – mercapthanol

04ml 1% bromphenol blue. Store at room temperature.

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10% SDS

Dissolve 50 SDS in 450 mLdistilled water with gentle stoirring and bring up to 500mL. Store at room temperature.

Ammonium persulfate solution (APS) make fresh daily

Dissole 0.1 g APS (lectrophoreosis grade) in mL distilled water.

Running buffer 1L

14.4 g glycine

3.03 g Tris base

2.0 g SDS

1000mL istilled water

Dissole using magnetic stirrer.

Towbin buffer 1x

3.03 gTris base

14.41g Glycine

200mL 20% methanol

800 mL ditilled water.

Posphate buffer saline (PBS)

8.0 NACL

0.2 G KCl

1.4 G Na2HP4

0.24 g KH2PO4

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PBST (Phosphate buffer saline Tween20)

20Ml PBS

2.5m BSA 1%

250 µL Tween 20.

BSA 1 %

1g BSA

100 mL PBST

BSA 5%

5 g BSA

100 mL PBST.

Perfusion buffer

Stock concentration (mmol/l):

110 NaCl (6.43g), 143 $NaHCO_3$ (1.2g), 1 $NaHPO_4$ (0.15g), 20 KCl (1.5g), 0.8 $MgSO_4$ (0.096g), 1.8 $CaCl_2$ (0.2g), 5.5 glucose (0.99g), 10 HEPES.

All mixed and dissolved in dH20 and pH was then adjusted to 7.34.

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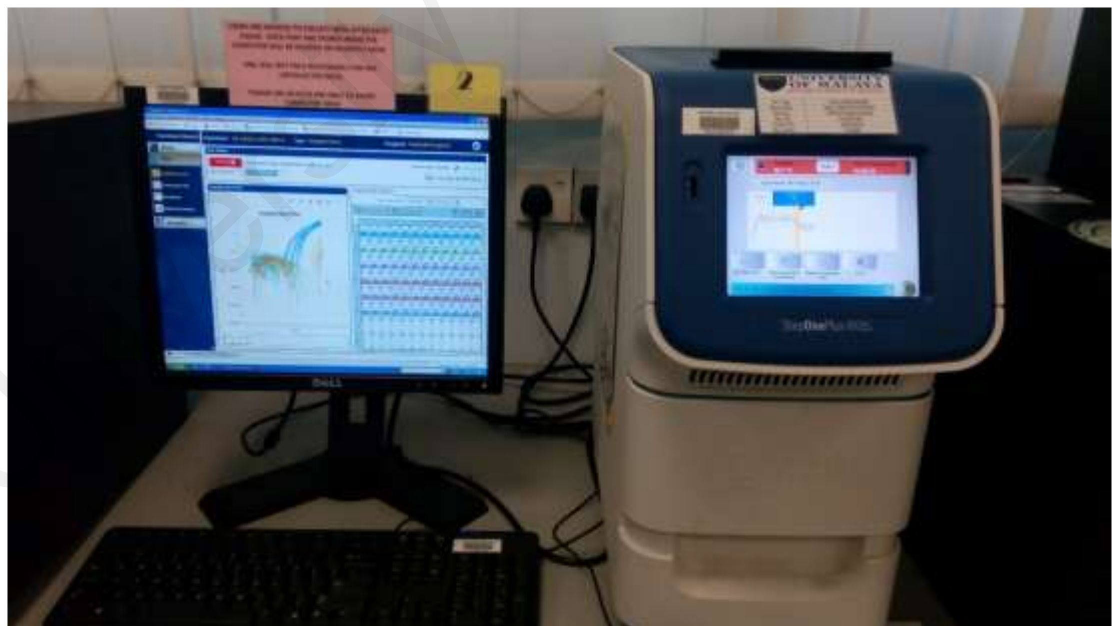
Agarose gel running at Protein lab, departemt of Molecular medicines

Real time PCR running



Western Blot running

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