TO STUDY THE ROLE OF TIGHT, ADHERENCE AND GAP JUNCTIONS IN FEMALE PRIMARY INFERTILITY

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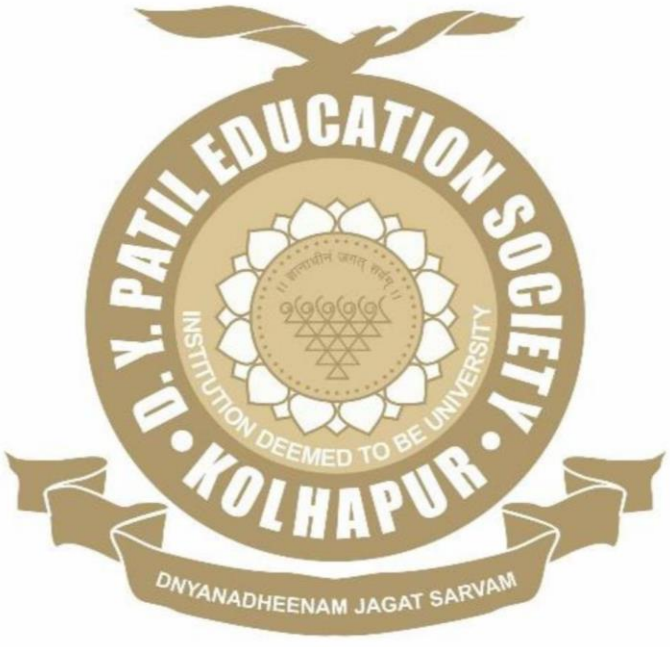

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UNDER THE GUIDENCE

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DECLARATION

I hereby declare that the work presented in this thesis entitled **"To study the role of tight, adherence and gap junctions in female primary infertility"** is entirely original and was carried out by me independently in the D. Y. Patil Education Society (Deemed to be University), Kolhapur under the guidance of Dr. Meghnad G Joshi, Associate Professor, Department of stem cells and regenerative medicine, D.Y. Patil University, Kolhapur (M.S.) India and Co-guide Dr. Rakesh Sharma, Prof. & Dean, D. Y. Patil Medical College, Kolhapur (M.S.) India. I further declare that present work has not form the basis for the award of any degree, diploma, fellowship or similar title of any University or institutions. The extend information derived from the existing literature has been indicated in the body of the thesis at appropriate places giving the references.

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4) Kshersagar J and Joshi M. Genital Hygiene and Strategies for HPV Prevention. Novel approches in cancer study. 1,1-3 (2018) (I. F. – 5.79).
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15) Bhamare N, Tardalkar K , Kshersagar J, Desai S, Marsale T, Nimbalkar M, Sharma S, Joshi M. Tissue engineered human ear pinna derived from decellularized goat ear cartilage: clinically useful and biocompatible auricle construct. Cell Tissue Bank (2021)10.1007/s10561-021-09911-1
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3) 5th International conference: Angiogenesis research targeted anti-angiogenic therapy. Center of Interdisciplinary Research, D. Y. Patil Education Society (Deemed to be University), Kolhapur held on October-26-27, 2018.
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7) Two Days Training Program on Flow Cytometry organized by D Y Patil Medical College and Center of Interdisciplinary Research, D. Y. Patil Education Society (Institution Deemed to be university), Kolhapur held on September 18-19, 2019.

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Abbreviations

% - Percentage

°C – degree Celsius

µm – micrometer

13- plex - LEGENDplex Rat Th Cytokine Panel

AJs - Adherent junctions

aPRP- activated PRP

ART - Assisted Reproductive Techniques

ASA - Anti-sperm antibodies

ASCs – Adult stem cells

BM- bone marrow

BMDCs- Bone marrow-derived cells

BM-MNCs - bone marrow derived mononuclear cells

BM-MSCs -Bone marrow derived mesenchymal stem cells

BSA – bovine serum albumin

CADs – Cadherins

CaM – Calmodulin

CAM – Cell adhesion molecules

cAMP - cyclic AMP

CCL2 - C-C motif ligand 2

CD – Cluster of differentiation

CK- cytokeratin

CK- Cytokeratin

Cla / CLDN – Claudin

Cla-1- Claudin -1

HB-EGF - heparin binding-epidermal

Cm – Centimeter

CRH - Corticotropin releasing hormone

Cx – Connexin

CXCL - C-X-C motif ligand

Cyt P450 - Cytochrome P450

D/W – distilled water

Da – Dalton

DAPI - 4',6-diamidino-2-phenylindole

DE – Disturbed endometrium

DLK - Delta like Homolog

DMSO – Dimethyl sulfoxide

DUB - Dysfunctional uterine bleeding

E and P – Estrogen and progesterone

E-Cad- E cadherin

ECM – Extracellular matrix

EGF - epidermal growth factor

EGFR - epidermal growth factor receptor

EMMPRIN - extracellular matrix metalloproteinase inducer

EMT - epithelial to mesenchymal transition

EPCs- endothelial progenitor cells

ER – Estrogen receptor

ERA – Endometrial receptive array

ERK - extracellular-signal-regulated kinase

FACS – Fluorescence assisted cell sorting

FSH - follicular stimulating hormone

g/gm – grams

GCs- precursors of germ cells

growth factor

hCG - Human chorionic gonadotropin

HCV – Hepatitis C virus

HE- hematoxylin-eosin staining

hMTtx- human placenta derived mitochondria transplantation

HOXA – homeobox A cluster

HPA – Hypothalamo-Pituitary Axis

HSCs- hematopoietic stem cells

HSG – hysterosalpingography

ICM – Inner cell mass

ICMART - International Committee for Monitoring Assisted Reproductive Technology

ICSI - Intracytoplasmic Sperm Injection

IFN-γ - interferon-GAMMA

IGF-1 - insulin like growth factor-1

IGFBP1 - insulin like growth factor binding protein 1

IHC – Immunohistochemistry

IL- Interleukin

IVF - In Vitro Fertilization

KD – Kilodalton

L.S.C.S.- lower segment caesarian section

LH - Luteinizing hormone

LIF – Leukemia inhibiting factor

LN2 – liquid nitrogen

Raf - Rapidly Accelerated Fibrosarcoma

Ras - Rat sarcoma virus

RBCs- red blood cell

RIF - recurrent implantation failure

rpm – revolutions per minute

G-CSF – granulocyte-colony stimulating factorGF – Growth factors

GFs- Growth factor

GnRH - gonadotropin releasing hormone

GPCR - G-protein-coupled receptor

LRCs - label-retaining cells

mAbs - monoclonal antibodies

MAGUKs - membrane-associated guanylate kinases

MAPCs- multipotent adult progenitor cells

MAPK - mitogen-activated protein kinase

MASCs- multipotent adult stem cells

MDCK - Madin-Darby canine kidney

MEK - Mitogen-activated protein kinase

MenSCs - menstrual blood-derived stem cells

MIAMI- marrow isolated adult multilineage inducible

Min – minute

miRNA – micro ribonucleic acid

ml – milliliter

Mm - Millimeter

Mmol – millimole

MMPs - matrix metalloproteinases

MSCs- mesenchymal stem cells

mtDNA- mitochondria DNA

MUPP1 – Multi PDZ domain protein 1

NADH cyt C red - NADH-cytochrome c reductase

NEC- non-enzyme control

nM – nano molar

NTC- non-template control

OCLN - Occludin

PATJ – PDZ associated tight junction

SD- Standard Deviation

SH3 - Src homology-3

SMA – Smooth muscle actin

SP - side population cells

SSEA1+ - Stage-specific embryonic antigen 1 marker

TB – tuberculosis

TGF-β - Transforming growth factor beta

Thy - T cell marker

Tie-2 - TEK tyrosine kinase

TIMPs - tissue inhibitors of metalloproteinases

TJ – Tight Junction

TNF- α - tumor necrosis factor-alpha

TNF- α- tumor necrosis factor-alpha

Tx – transplant

uNK cells - Uterine natural killer cells

WHO - World Health Organization

wk – weeks

WOI - window of implantation

wt – weight

Yrs. - Years

ZO – Zonal Occludens

ZO-1- Zona Occludin 1

PBS – Phosphate buffer saline

PCOS - polycystic ovarian syndrome

PDGF - platelet-derived growth factor

PGE2 - Prostaglandin E2

PGF - platelet growth factor

PKA - protein kinase A

PP - Protein phosphatase

PR - progesterone receptors

PRL – prolactin

PRP - Platelet rich Plasma

USG – ultrasonography

v/v – volume by volume

VAP- Vascular Angiogenic Precursor

VEGF - vascular-endothelial growth factor

IGF - insulin-like growth factor

VSELs / VSELSCs - very small embryonic like stem cells

α and β – Alpha and Beta

Chapter – 1

Introduction

1.1. Introduction

Infertility is a serious issue worldwide that has intense socioeconomic and health implications on both the individual and society. Despite the significant consequences of infertility, estimation of its prevalence is limited. It affects approximately 10%–15% of couples worldwide. Out of 60–80 million infertile couples suffering from every year worldwide, approximately 25% are in India alone(1).

According to information by the World Health Organization (WHO), one in every four couples in developing countries is concerned with infertility. Infertility is a distressing condition which prevents reproduction in a couple in the reproductive age. It is not only a medical problem, but has also social-psychological implications. Although several underlying causes of infertility have been overcome by assisted reproductive techniques (ART), the implantation remains rate-limiting step with regards to the accomplishment of treatment (2). A prerequisite for implantation further relies upon a two-way communication between the embryo and the uterus.

In order to increase success rates, there is a continuing need to understand the molecular changes in implantation. Studies on endometrium endow with useful information on endometrium for implantation. Still, there is a lack of diagnostic and therapeutic tools for implantation dysfunction, and an optimal marker for defining a state of endometrial receptivity is needed.

Here, we assess the complex network of junction protein interactions in endometrial luminal epithelium; its potential roles in implantation process and emphasize how the molecular interactions may regulate endometrium.

This study summarizes the knowledge of impaired protein expression in unreceptive endometrium and assessment of endometrial receptivity

during implantation window prior to treatment. It would facilitate further development and a better understanding of uterine of reproductive technology.

1.1.1. Fertility and Infertility

Fertility is the natural ability to give birth. It can be measured by the time taken to achieve pregnancy. It is regulated by an intricate coordination and synchronization in the hypothalamic-pituitary-ovarian axis. Fertility can be influenced by dysfunctions of reproductive tract, endocrine system, and immune system or by disorders in ovulation (most commonly polycystic ovarian syndrome, PCOS), tubal factor infertility, endometriosis and unexplained infertility(3).

Infertility is the inability to conceive a clinical pregnancy naturally after regular and unprotected intercourse for 12 months or more of regular unprotected sexual intercourse, based on criteria of International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO). It affects approximately 10–15% of couples worldwide (4,5).

It affects 10–15% of couples attempting to conceive. More than half of all cases of infertility are a attributed to female factors, while the remaining cases can be related to male conditions, and to a both male and female factors or is unexplained in approximately 20% cases. It has been estimated that 79% of the population is fertile, 18% is infertile and 3% is superfertile. Over 72 million women worldwide between age group 20 to 44 yrs. are infertile (6,7). Infertility is again categorized as primary and secondary infertility. The primary infertility applies to a woman who has never been diagnosed with a clinical pregnancy and assembles the criteria of infertility. Secondary female infertility applies to a woman unable to ascertain

a clinical pregnancy but has been diagnosed with a clinical pregnancy previously(8).

For the further development and a better understanding of uterine reproductive potential, the search for knowledge of impaired junction protein expression in unreceptive endometrium and assessment of endometrial receptivity during implantation window prior to treatment is a challenging issue.

It outlines and discuss the way forward in using junction protein expression as novel biomarkers of receptivity, which will predictive and diagnostic tools for improving pregnancy outcomes and ameliorating the infertility.

1.1.2. Female primary infertility

The World Health Organization describes infertility as a disease of the reproductive system defined by biological inability of a female to achieve a clinical pregnancy after 12 months or more of regular intercourse without the use of contraception. Female primary infertility is infertility in a female who have never had a child but often there is no obvious underlying cause. Secondary infertility is failure to conceive following a previous pregnancy (9).

The consequences of infertility are multiple and can include societal impacts and personal suffering. Advances in assisted reproductive technologies can offer hope to many couples.

The most frequent causes of female primary infertility are summarized in Table 1.1. Around 10–30% of infertile couples suffer from Anti-sperm antibodies (ASA) Immune infertility. Autoimmunity associated with infertility influence the fertility complications. ASA production in male are directed against surface antigens on sperm that interfere with sperm motility and transport through the genital tract, hindering capacitation and acrosome reaction,

impaired implantation and impaired development of the foetus. ASA production in women causes disturbed normal immunoregulatory mechanisms, infections (10). Sexually transmitted pathogens, Chlamydia trachomatis and Neisseria gonorrhoeae have a negative effect on fertility. These pathogen infections in female reproductive tract are associated with increased risk of infertility(11,12).

Smoking, radiation or chemotherapy is responsible for the DNA damage thus reduces the chances of fertility in female(13,14). Many other general factors such as diabetes mellitus, thyroid disorders, undiagnosed and untreated coeliac disease, adrenal disease increase the risk of female primary infertility(15–18).

Abnormally high levels of prolactin in the blood and decreased secretion of one or more hormones produced by pituitary gland is associated with an increased risk of primary infertility. Many other prevalent factors related to female primary infertility are premature ovarian failure, Polycystic ovary syndrome (PCOS) and Endometriosis(19).

Table 1.1 Etiology of female infertility

Etiology	Causes
Immune infertility	Anti-sperm antibodies Autoimmunity associated with infertility
Sexually transmitted infections	Chlamydia trachomatis Neisseria gonorrhoeae
DNA damage	Smoking radiation chemotherapy
General factors	Diabetes mellitus Thyroid disorders Untreated coeliac disease Adrenal disease
Hypothalamic-pituitary factors	Hyperprolactinemia Hypopituitarism

Tubo peritoneal infertility	Tubal factor infertility Premature ovarian failure PCOS, Endometriosis
Uterine abnormalities	Malformations Myomas

1.1.3. Uterus

Uterus is hollow pyriform muscular organ situated in the pelvis between the bladder and rectum. It is divided into an upper part- the body and a lower part –the cervix that is continuous with upper vagina. Body wall consists of three layers from outside inwards- Perimetrium, Myometrium and Endometrium. Perimetrium is a serous coat which invests the entire organ except on the lateral borders. Myometrium consists of bundle of smooth muscle fibers held by connective tissues and are arranged in various directions.

During pregnancy three distinct layers can be identified-outer longitudinal, middle interlacing and inner circular. Endometrium is mucus lining of the cavity. As there is no sub mucous coat, endometrium is directly opposed to the muscle coat. Endometrium is a complex and dynamic tissue that undergoes regeneration, differentiation, and shedding in each menstrual cycle during the reproductive life of a woman.

1.1.4. Human endometrium

The endometrium is an endocrine organ which produces several hormones, growth factors and cytokines. It is a mucosal lining of the uterine cavity. Endometrium is directly opposed to the muscle layer. The endometrium permits an implantation of a viable embryo, and endow with a nourishment to supports foetal development. In absence of pregnancy, Endometrium sheds off and protect itself against invading pathogens (20).

Histologically endometrium is divided into the lower one third basal layer-stratum basalis and upper two third functional layer-stratum functionalis (Figure 1.1). The basalis layer is adjacent to the myometrium. It undergoes only limited changes during the menstrual cycle and remains after menstruation (21). After menstruation, endometrium regenerates from this stratum basalis layer.

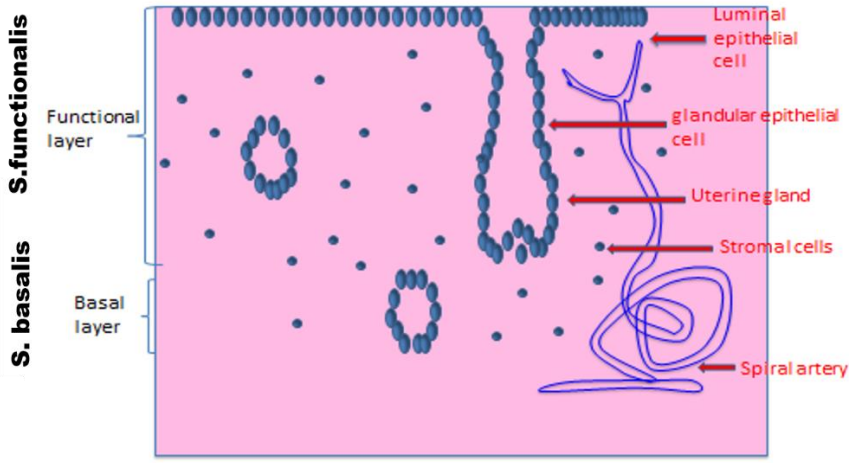

Figure 1.1: Schematic representation of endometrium.

The functionalis layer is highly sensitive to the hormones and is responsive to oestrogen, progesterone and androgens. It is subsequently shed during each menstrual cycle. This layer undergoes series of proliferation, secretion and degeneration. Post menstruation, functionalis layer is regenerated from basalis layer.

The purpose is to prepare the endometrium for embryo implantation. Endometrium is composed of lamina propria and surface epithelium. The lamina propria contains stromal cells, endometrial glands, vessels, nerves and to a lesser extent, stem cells, endothelial cells and immune cells - macrophages and uterine

natural killer cells (uNK cells). The glands are simple, tubular and lined by mucus secreting nonciliated secretory columnar cells (22).

The surface epithelium is luminal epithelium that covers the endometrial surface. Surface epithelium is classified into the luminal epithelial layer and glandular epithelium within the stromal compartment.

Luminal epithelium provides the sites of implantation, as it is the first maternal surface for the trophoblast cells of the implanting embryo to encounter during window of implantation (WOI) (23).

Outside WOI these cells are refractory to implantation due to hormonally controlled protective glycocalyx molecules. Glandular epithelial cells serve as a barrier against infections and secrete several autocrine and paracrine factors required for endometrial maturation and embryo implantation.

Glandular epithelium is composed of phenotypically different single layer of ciliated columnar epithelial cells. The morphology of glandular epithelium changes continuously depending upon the ovarian hormone secretions throughout different phases of menstrual cycle. During estrogen dominant proliferative phase, the glands appear straight and long and during progesterone dominant secretory phase, the glands become coiled and produce secretions(24,25).

Stromal cells are fibroblastic in nature, are located in the connective tissue of the endometrium made up of proteoglycans and collagen. Stromal cells are loosely packed in the functionalis layer to facilitate blastocyst implantation whereas densely packed in the basalis layer of the endometrium. Fibroblasts are the dominant cell type of the stroma, and produce extracellular matrix, metalloproteinases (MMPs), and other proteins (26). It secretes matrix

metalloproteinases (MMP-2 and MMP-4), tissue inhibitors of metalloproteinases (TIMPs), growth factors and cytokines.

MMPs expression in the stroma is mainly regulated by a cell surface glycoprotein present in the luminal and glandular epithelium known as extracellular matrix metalloproteinase inducer (EMMPRIN). Thickness of stromal compartment is influenced by ovarian hormones. During the proliferative phase, estrogen increases the proliferation of stromal cells and during secretory phase, progesterone reduces stromal proliferation. Stromal endothelial cells are essential for the formation of new vessels from the existing ones. Upon successful blastocyst implantation, stromal endometrium is changed to decidua and terminally differentiate during pregnancy(27).

1.1.5. Menstrual cycle

A menstrual cycle consists of recurrent changes which repeatedly take place over a certain period of time in the female reproductive system once every 28 days during the reproducing years of each woman. The duration of the cycle might vary from person to person. During every coordinated menstrual cycle, changes occur in the ovum producing ovaries and the uterus, which is necessary for uterine preparation for pregnancy.

This cycle is governed by changes in the levels of ovarian E and P. In human females, a normal cycle of menstruation lasts about 28 days on an average with a variation in length from 21-35 days (mean ± standard deviation of 28 ± 7 days) (28).

The onset of menstrual bleeding is denoted as day 1 of the cycle while ovulation occurs on day 14. Based on the occurrence of changes in the endometrium that correlate with the functional changes in the ovaries, a menstrual cycle can further be

apportioned into three main phases, viz the proliferative phase, secretory phase and menstrual phase (29).

Periodically the endometrium regenerates itself in the proliferative phase, while proliferation occurs in the stroma and epithelia under the influence of E secreted by the developing follicles. During this phase, the thickness of the endometrium increases with glands lined with stratified columnar cells. Additionally, estrogen receptors (ER) a and β and progesterone receptors (PR) A and B are expressed in the endometrium. This phase, while it lasts for 11 days and ends at ovulation (30). Whereas, the secretory phase occurs after ovulation and lasts for 12 days. This is a phase that is related to an increase in P production by the corpus luteum in addition to E. At this stage, the endometrium is fully matured and undergoes secretory transformation under the combined effect of E and P. In the lumen of the uterine glands, P induces development of secretory glands and enhances 22 secretions of fluid, electrolytes and other molecules. During this phase, changes in endometrial lining provide an appropriate environment for the implantation of an embryo. Due to the high level of P in the mid-secretory phase, expression of ERa in glandular and luminal epithelia is down-regulated (31).

If no embryo implantation takes place in the endometrium, the corpus luteum will start degenerating followed by an abrupt decline in the circulating levels of E and P. With the breakdown and degeneration of the endometrium, the menses phase commences and blood starts leaking in the lumen. This duration roughly lasts for 4-5 days and later the endometrium will again start to proliferate under the action of E (32).

1.1.6. Ovarian cycle

The simultaneous sequential changes that occur in the ovary during the menstrual cycle is denoted as the ovarian cycle. And is further classified into the follicular phase and luteal phase with the 14th day being the day of ovulation in a 28 days menstrual cycle (33).

At the beginning of every cycle, 6-12 primary follicles start developing under the influence of follicular stimulating hormone (FSH). During the early follicular phase, the primary follicles start secreting follicular fluid rich in estrogens and the fluid cavity, known as appearance of antrum(34).

High estrogen concentration along with pituitary FSH from the antral follicle promotes the appearance of LH receptors and stimulates the secretion of Luteinizing hormone (LH) in the granulosa cells of an ovum. The growth of the antral follicle is subsequently promoted by estrogens, in combination with LH and show an increase in size by 3-4 times (35). By the second week, one of the follicles becomes dominant and all other follicles undergo atresia. More estrogen is secreted by the dominant follicle so that the plasma concentration of estrogen starts increasing, and the dominant follicle by the time of ovulation reaches a diameter of 1.5-2 cm and is termed as a mature follicle. Increased concentration of estrogen during the late follicular phase stimulates gonadotropin releasing hormone (GnRH) secretion and enhances the LH hormone secretion from the pituitary which leads to a surge in the production of LH known as LH peak (36).

Later in the midcycle, LH surge induces ovulation, that approximately occurs on day 14, 10-12 hours post LH peak, and 24-36 hours after peak estrogen level. The LH surge further stimulates the remaining granulosa cells and theca cells of the mature follicle in

the luteal phase to transform into lutein cells that are filled with lipid inclusions and appear yellow in color (37).

This process is called 'luteinization' and a complete follicle is termed as corpus luteum. And when the fertilization fails to occur after 7-8 days of ovulation, the corpus luteum starts involuting and by losing its secretory activity it becomes corpus albicans. As a result, plasma concentrations of progesterone and estrogen start declining and an inhibitory effect of these ovarian hormones on GnRH secretion is released, starting a new cycle with rising FSH and LH hormones. Whereas, on successful fertilization and implantation, the corpus luteum is maintained as corpus luteum of pregnancy that secretes high levels of progesterone and estrogen to maintain pregnancy(38).

1.1.7. Endometrial cycle

An endometrial cycle is a sequential morphological and molecular change that takes place in the endometrium during the menstrual cycle. It is divided into proliferative and secretory phase separated by the day of ovulation(39).

During the first five days of the cycle, endometrium starts to shed off in the form of menstrual blood. After the menstrual phase, the thickness of the endometrium remains less than 2 mm, while under the influence of estrogens, the proliferation of glands, blood vessels, stroma and luminal epithelium takes place, termed as proliferative phase, and by the day of ovulation, the endometrium increases in thickness of 4-5 mm. During the secretory phase or progestational phase, corpus luteum secretes much higher quantities of progesterone than estrogen. While the estrogen during this phase causes a minimal additional cellular proliferation in endometrium,

high progesterone concentrations cause a marked swelling and an increase in the secretory activity of the cells (40,41).

Under the influence of progesterone, the tortuosity in the glands increase and large quantity of secretory substances are deposited in the glands. The cytoplasm of stromal cells is packed with glycogen and lipids 7 and transform the structure, a process termed as decidualization. Increased blood supply ensues to endometrium with developing secretory activity. The purpose of all these observed changes of the secretory phase is to produce a secretory endometrium containing sizeable amounts of accumulated nutrients to provide optimal conditions for implantation of the embryo and for accommodating subsequent embryo development(42).

1.1.8. Decidualization

Decidualization is the morphological change that is observed in stromal cells at the end of the luteal phase. This process starts around day 23 of the monthly cycle and is independent of the presence or absence of blastocyst. If incase there is an implantation, then, decidualization plays an important role in the formation of placenta by mediating the invasion of trophoblasts, while lack of decidualization leads to a failed placentation (43).

Decidualization is characterized by transformation of elongated stromal cells or fibroblasts into larger and circular phenotype by the accumulation of glycogen and lipids, secreting numerous cellular products. Further changes that are seen during decidualization include presence of leukocytes and vascular changes in the maternal arteries. If no implantation occurs, the decidualized endometrial lining sheds off during menstruation. In in vitro, the stromal cells can be decidualized by progesterone treatment of

estrogen primed cells, ligands of cyclic AMP (cAMP) pathway like Prostaglandin E2 (PGE2), LH, FSH and Relaxin hormone. cAMP alone can induce decidualization if the stromal cells are obtained from the late luteal phase biopsy. In general prolactin (PRL), insulin like growth factor binding protein 1 (IGFBP1) and notch1 are considered as markers (44,45).

1.1.9. Hormonal regulation of endometrial cycle

1.1.9.1. Estrogen

The endometrium is governed by estrogen and has domino effect in endometrial proliferation and induction of progesterone receptors that allow progesterone consequently to provoke endometrial receptivity. Although not necessarily in a large quantity, but existence of estrogen is important for normal development of the endometrium (46). A high concentration of estrogen increases the risk of abnormal placentation and other pregnancy complications. Estrogen receptors (ER) exist in two ERa and ERβ isoforms. It acts on uterus through the ERs. ERa is considered as a dominant receptor. ERa deficiency leads to hypoplastic uterus and shows no response to estrogen treatment. ERβ receptor deficiency does not show such effects (47,48).

Estrogen endorses the release of pro-heparin binding-epidermal growth factor (HB-EGF) by binding to membrane-associated G-protein-coupled receptor (GPCR). HB-EGF binds to epidermal growth factor receptor (EGFR) and activates downstream mitogen activated protein kinases (MAPK). Activated MAPK results in a crosstalk with growth factors or insulin like growth factor-1 (IGF-1) cascades, encouraging endometrial proliferation. Estrogen also regulates Calbindin-D28k, an intracellular calcium-binding protein, which is involved in the regulation of endometrial receptivity by

modifying the concentration of intracellular calcium ions (49). The expression of Calbindin-D28k is high during the proliferative phase and at the time of sexual maturity. The calbindin-D28k plays an important role in endometrial receptivity. Another intracellular calcium-binding protein, Calmodulin (CaM) play a major role in the contraction of myometrial smooth muscles. It also plays essential role in the variety of cell proliferation. Estrogens together with chorionic gonadotropin increase the expression of decidualization marker-Notch1. Notch 1 plays important role in cell survival and differentiation. Lack of Notch-1 promotes the apoptosis of stromal fibroblasts cells and uterine sloughing which lead to decidualization defects affecting the pregnancy (50).

1.1.9.2. Progesterone

Progesterone is secreted from luteinised theca granulose cells of the corpus leuteum. It is the key hormone that drives endometrial receptivity and interruption of luteal phase progesterone using anti-progestins makes the endometrium non receptive. The progesterone receptor knockout mice instigated the role of progesterone in continuation of pregnancy and maintenance of endometrial receptivity (51).

Progesterone intercedes its actions in the target organs by progesterone receptors (PR). Progesterone act on genital tract and on the breast provided that they are sensitized by estrogen. Glucocorticoids can bind to PR and at high concentrations progesterone binds to androgen receptors and glucocorticoid receptors (52).

PRs exist in two isoforms PRA and PRB. the ratio of these two isoforms varies constantly in endometrium during menstrual cycle. Isoform PRC also exists in trace amount. The transcriptional activity of PRA is

cell and gene specific and PRB is a stronger transcriptional activator(51). progesteron produces myohyperplasia and diminishes the contractility of myometrium. Progesteron increases the secretion of the glycogen rich glands and enhance the secretory activity in the endometrium. It however decreases the tone of the circular muscle fibers at the isthmus. Inhibition of decidualization is seen in PRA knockout mice, suggesting an important role of PRA in decidualization whereas uterine responses to progesterone are not affected in PRB knockout mice. Progesteron has negative feedback on the Hypothalamo-pitutary axis, primarily upon the midcycle gonadotrophin surge and it is accountable for short duration(53).

1.1.9.3. Human chorionic gonadotropin

Human chorionic gonadotropin (hCG) is the confirmed marker of trophoblast cells. It is composed of a and β subunits that are non-covalently linked together. It is secreted by cytotrophoblast cells and induces extravillous cytotrophoblasts proliferation and invasion by inhibiting TGF-β receptors, thus preventing the apoptosis of trophoblast cells. Presence of hCG is the prime detection method in the pregnancy confirmation tests. hCG maintains the pregnancy, until the placenta takes over. hCG acts through luteinizing hormone/choriogonadotropin receptor that is present on the corpus luteum. Apart from this, hCG plays an important role in angiogenesis, decidualization, immune modulation and remodeling of extracellular matrix in the endometrium(54).

1.1.9.4. Corticotropin releasing hormone (CRH)

CRH is a neuropeptide that is a principal regulator of Hypothalamo-Pituitary Axis (HPA) and is secreted from the hypothalamus in response to stress reaction. The characteristics of acute

inflammatory response from the invading semi-allograft blastocyst can be seen in the endometrium during blastocyst implantation; however once implantation is successful, the embryo suppresses this reaction. CRH also plays a critical role in the mother's immune system to prevent graft vs. host reaction by slaying of activated T cells through Fas-Fas Ligand interactions. Blockage of CRH receptors by an antagonist antalarmin, reduces the chances of implantation by 70% in animal models. On the other hand, in in-vitro, CRH receptor blockade increases the trophoblast invasion by 60% suggesting a role in regulation of trophoblast invasion (55).

A defective CRH/CRH receptor system is usually seen in recurrent implantation failure (RIF) patients, placental defects and preeclampsia conditions. Intrauterine administration of autologous CRH-treated peripheral blood mononuclear cells increased the chances of positive implantation by 44% in RIF patients, suggesting a potential role for CRH in treating the RIF patients (56).

1.1.9.5. Calcitonin

Calcitonin/Thyrocalcitonin is a known potential regulator of implantation and a marker of endometrial receptivity(57). It plays a critical role in calcium homeostasis in the body by reducing serum calcium concentration in response to hypercalcemia and also acts counter to the parathyroid hormone.

During WOI a high concentration of calcitonin in endometrium is seen and inhibition of calcitonin synthesis by antagonists in mice reduces the implantation rates by 50-80%. It is hypothesized that calcitonin upregulates the expression of integrin β3 in endometrial epithelial cells facilitating implantation (58). In vitro incubation of blastocysts with 10nM calcitonin has been shown to accelerate differentiation of blastocyst cells suggesting a role in embryonic

development. Progesterone regulates the expression of calcitonin and through calcitonin it modulates the expression of E-Cadherins(59).

1.2.1. Endometrial receptivity

The term endometrial receptivity refers to the duration when luminal epithelium is favorable for blastocyst implantation. This limited-time period also called as implantation window, is restricted to 19 to 24 days approximately of a menstrual cycle of 28 days (60). This corresponds to the time when the embryo hatches, day 6 after the LH surge, and it is subsequently ready for implantation within the following 24 hours (61).

The development in the receptivity of an endometrium depends on the adequate secretory transformation of the estrogen-primed endometrium in response to progesterone. Significant developmental changes can be observed in luminal epithelium, glandular epithelium as well as in endometrial stroma. Many molecules have been identified to be involved in this process, such as integrins and their ligands (e.g. osteopontin), mucins, growth factors (HB-EGF), cytokines (LIF, leptin, IL-1, IL-11), homeobox transcription factors (HOXA gene products), lipids and other molecules (62).

The availability of new methods for investigating human endometrium has provided deeper knowledge in the molecular regulation of endometrial receptivity. Gene expression in microarrays allows one to study the expression levels of thousands of genes simultaneously. In recent years, the global gene expression analysis has been successfully applied in several endometrium transcriptome studies and distinct regulation of hundreds of genes in different menstrual cycle phases has been demonstrated (63).

Although each study has revealed many candidate genes for endometrial receptivity, the number of common genes distinguished is

relatively limited (64). Opening of the implantation window is characterized by remarkable ultrastructural changes in endometrial epithelial cell morphology. In several studies, it has been observed that the time-frame of implantation coincides with the presence of endometrial pinopodes. Endometrial pinopodes are nothing but the cytoplasmic protrusions of the endometrial surface, which arise from the apical surface of the epithelial cells and extend into the uterine cavity. Although some groups have questioned the correlation between pinopodes and endometrial receptivity (65), the timed correlation of pinopode expression, period of blastocyst hatching, and preference of human blastocyst to attach to pinopodes suggest that pinopodes are the structural markers of a receptive endometrium. Moreover, pinopode formation and maintenance seem to be hormone-dependent, where progesterone plays a crucial role in their appearance, while estrogen interferes with the formation or induces regression (66).

Besides, the co-expression of pinopodes and other markers of endometrial receptivity have been demonstrated, such as integrin avβ3, osteopontin, glycodelin, progesterone receptors. Relatively, the lack of clinical investigation of the endometrium partly depends on the short of objective tools for examining endometrial receptivity in a clinical setting. As a result, the most current therapeutic interventions aimed at modulating the endometrial receptivity are empirical, with scarce evidence and data to support their use (67).

Nevertheless, in recent times, the developments in understanding the molecular regulation of endometrial receptivity are offering novel insights into the role of the endometrium in determining whether or not implantation could be successful. Indeed, studies comparing endometrium at the pre-receptive and receptive phases in fertile women and women with poor reproductive success demonstrate that

implantation failure is, at least in part, due to a failure of the endometrium to differentiate into a receptive state (68).

1.2.2. Implantation

Blastocyst implantation occurs within a short period known as the "window of implantation". Implantation window is defined as the limited-time period when the uterus is ready to accept the blastocyst. The ovarian steroids including E and P regulate implantation. Communication between the blastocyst and the receptive uterus is crucial for successful implantation. Implantation can be divided into three phases: apposition, adhesion (attachment) and invasion (69).

Following the invasion, trophoblasts invade into the endometrial stroma. Early pregnancy loss is associated with problems that occur prior to, during or after implantation in which the embryo is not competent or the uterus is not in the receptive state. Fertilization occurs in the outer third of the fallopian tube 24 hours after ovulation (70).

Following fusion of sperm and ovum, cell divisions occur and at the same time, the embryo moves down the fallopian tube towards the uterine cavity. The zygote undergoes mitotic divisions to form a morula, which is a cluster of blastomere. The morula then enters the uterine lumen, which then transformed into blastocyst stage that contains a cavity known as blastocoel. The blastocyst consists of two different types of cells, the inner cell mass and trophectoderm. Trophectoderm is the outer layer of the blastocyst that becomes the progenitor for the future trophoblast cells (71).

The blastocyst remains free floating within the uterine luminal fluid for 1–3 days before escapes from the zona pellucida and gain implantation competency (72). The blastocysts further differentiate and possess three different cell types that include primitive endoderm, outer epithelial trophectoderm and inner cell mass. The trophectoderm starts to make first

physical contact with the endometrium to initiate the implantation process.

1.2.2.1. Stages of implantation

1.2.2.1.1. Apposition

In rats, the developing blastocyst enters the uterine cavity on day 5 of pregnancy. Once blastocyst hatches from zona pellucida, it is ready to initiate implantation with the first stage being apposition. Apposition is a transient and dynamic process. Uterine contraction and mucin, which lines the uterine lumen, involve in propelling the blastocyst within the cavity.

This leads to intimate but unstable contact between the trophectoderm and the receptive epithelium. Meanwhile, the loss of fluid in the uterine lumen at the time of blastocyst attachment was also thought to assist in bringing the blastocyst close to the luminal epithelium (73). Uterine closure sandwiches the blastocyst between the opposing uterine walls. The embryonic pole where the inner cell mass of the blastocyst is located makes the first contact with the receptive endometrium. Pinopodes are also thought to be involved in embryo apposition (74).

1.2.2.1.2. Adhesion

Paracrine signaling between the blastocyst and endometrium initiates a more stable adhesion following the establishment of apposition (75). Adhesion is associated with localized increase in stromal vascular permeability at the site of blastocyst attachment.

Trophoblast surface interdigitates with the apical surface results in blastocyst closely attached to the endometrium. The adhesion stage is regulated by cell adhesion molecules which include integrins, selectins, lectins and cadherins (76). The establishment of firm adhesion prevents

blastocyst from being flushed away from the uterine cavity and in case it is forcefully removed, this will result in damage to the luminal epithelium (77).

1.2.2.1.3. Invasion

In primates and rodents, the final step of implantation requires trophoblast to invade into maternal blood vessels leading to the formation of hemochorial placenta. The placenta serves as a site of fetal-maternal exchange during pregnancy (78). In mice and rats, the invasion process is initiated with apoptosis of the luminal epithelial cells. Following that, trophectoderm will invade between these cells and penetrate the basal lamina (79).

Molecular interactions between the embryo and the endometrium are needed to secure normal invasion and survival of the embryo. Under the regulation of P, the endometrial stroma undergoes decidualization where stromal cells and ECM transform into the decidua. Decidua is an important structural and biochemical tissue that connects maternal tissue to the embryo. The implantation process is completed when the blastocyst is embedded into the surface endometrium (80).

1.2.3. Cell adhesion molecules

The cell adhesion molecule (CAM) family is composed of cadherin, integrin, selectin and immunoglobulins. These glycoproteins act as surface ligands mediate neighboring cell adhesion. Their classical functions include tissue integration, morphogenic movements, and cellular migrations and so on. Adhesion molecules, endometrial growth factors, endometrial immune markers, endometrial cytokines are the biochemical markers of endometrial receptivity(81).

Intercellular junctions mediate adhesion and communication between adjoining epithelial cells. Synchrony of adhesive signaling systems

between the blastocyst and the endometrium is required in successful implantation of an embryo. The search for potential informative methods to assess the endometrial receptivity is ongoing (82,83). Adhesion junctions shows extreme plasticity that allows the cell changes in cell division or cell movement and changes in cell shape during differentiation (84).The complex sequences of cell adhesion molecules which acts as a molecular mediators in endometrium interactions are incompletely understood (85). Numerous glycoproteins and carbohydrate ligands and their receptors are expressed in trophectoderm and luminal epithelium at the time of implantation (86,87).

1.2.3.1. Tight junction

Tight junctions are located at the uppermost part of the lateral cell membrane forming selective barrier in between the adjacent cells. It regulates paracellular transport between the adjacent cells and maintains the strict organization of epithelial cells.

A huge number of proteins have been recognized in junctional complex. The transmembrane tetraspanins Claudins and occludin are the key players in building up the fence functions. They form the core of the tight junction and are linked with cytoplasmic plaque proteins including ZO-1, -2, and -3 to the actin-cytoskeleton, and other junctional complex (figure 1.2)(88). Human endometrial epithelial cells during the proliferative phase of the menstrual cycle show a typical polarized phenotype. This polarization is controlled by 17β-estradiol (E2) in proliferative phase. During the secretory phase, it is predominantly governed by progesterone (P4), when the endometrial cells become receptive for implantation(89). These polarized junctions in endometrial cells and its redistribution are observed during the menstrual cycle (90).

The nuclear receptors mainly estrogen receptor (ER) and progesterone receptor (PR) primarily mediates the action of E2 and P4 which activate them to act as transcription factors(91).

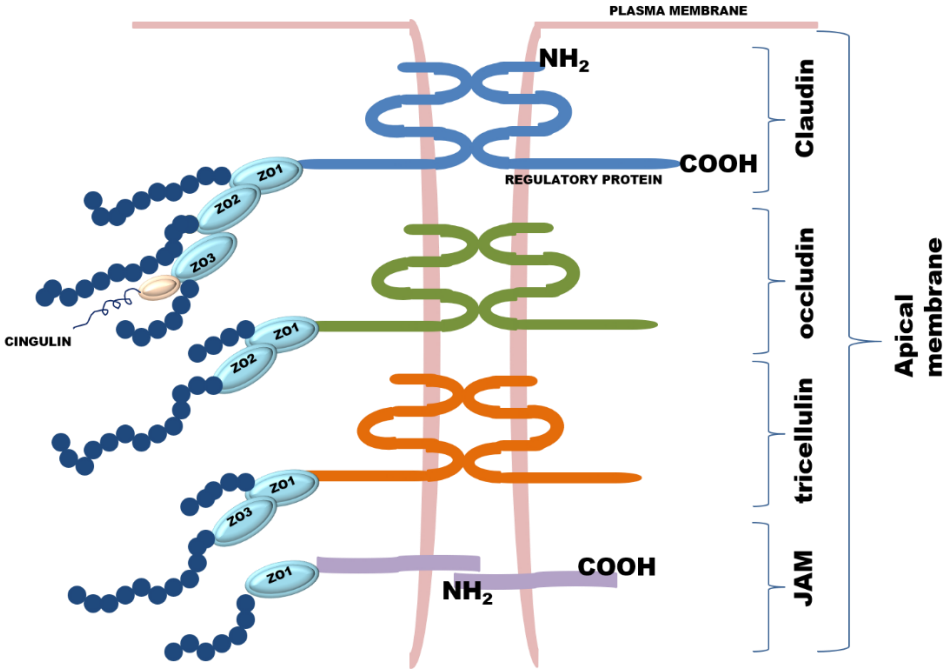

Figure 1.2: Schematic representation of tight junctions in adjacent endometrial cells.

1.2.3.2. Occludin

In 1993, Occludin was the first transmembrane TJ protein identified. Occludin protein is first identified and isolated from the chicken liver an antigen for monoclonal antibodies (mAbs) raised against a junctional fraction. Occludin is an integral membrane protein of 65-KD. Along with MarvelD2 (tricellulin) and MarvelD3, Occludin belongs to the TJ-associated marvel protein family [14].

Cloning and sequencing of the corresponding cDNAs revealed that it comprises a tetraspanin protein with 4 transmembrane domains, 2 extracellular loops, and 1 intracellular loop, a long C-terminal and short N-terminal cytoplasmic domains (92).

The extracellular loops of occludin forms homophilic interaction with the adjacent cells and create a barrier against macromolecules but

not against small ions (93). The first extracellular loop is characterized by tyrosine and glycine residues (60%). The C-terminal domain interacts with intracellular ZO proteins, thus link occludin to the actin filaments. The ZO proteins are multi-domain proteins holding 3 PDZ domains, a Src homology-3 (SH3) and a region of homology to GUK at N-terminus. These structures endow with an intracellular scaffold and are required for regulation and maintenance of tight junction. TJ proteins bind to the N-terminal while the C-terminal interacts with the actin cytoskeleton and cytoskeleton-associated proteins. Claudins bind to the first PDZ domains of ZO-1, -2, and -3, JAM binds to the 3rd PDZ domain, and occludin binds to the GUK domain of ZO-1. The 2nd PDZ domains are used for interactions between ZO proteins (Figure 1.3).

Occludin has diverse functions. The numerous studies indicate that occludin has crucial role in the TJ structure and permeability in the epithelia (94,95). Overexpression of occludin reduces permeability in Madin-Darby canine kidney (MDCK) epithelial cells which increase the electrical resistance across a monolayer of cells. It serves as an indicator of monolayer integrity and permeability(96,97).

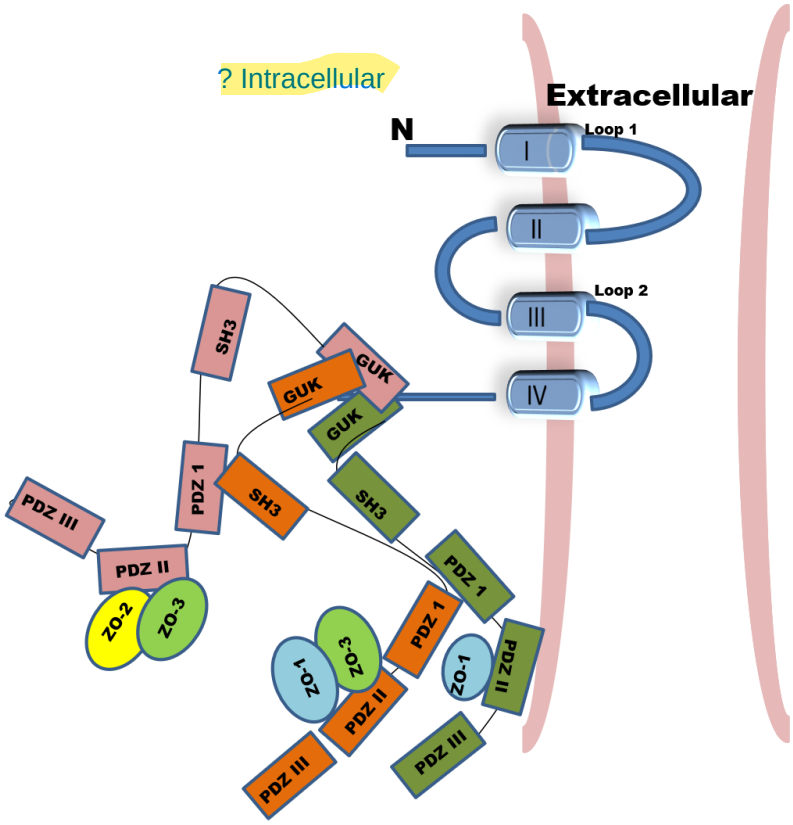

Figure 1.3: Interaction of occludin with TJ associated proteins.

In embryonic stem cells, occludin depletion did not show any changes in differentiation into polarized epithelial cells or TJ assembly. Likewise occludin knockout mice showed equivalent density and organization of TJs in the intestinal epithelium to that of wild-type mice (98). The intestinal barrier function and ion transport in the knockout mice showed electrophysiologically normal functions(99).

The deletion of the occludin C-terminal leads to impaired fence function in epithelial cells. These studies demonstrated that knockout occludin mice did not show any abnormal changes in morphological TJ assembly and barrier integrity, but histological and functional abnormalities are noted in several tissues. It showed major postnatal growth retardation including various histological abnormalities. The occludin knockout mice showed chronic inflammation and

hyperplasia of the gastric epithelium, loss of cytoplasmic granules in striated duct cells of the salivary gland, testicular atrophy suggesting more complex occludin function (100). The synthetic peptides disrupt TJs, by inhibiting the interaction of extracellular domains occludin with adjacent cells, and raise paracellular permeability to macromolecules. Thus occludin plays a role in the maintenance of TJs (93). Occludin is associated with cellular proteins that can be amended by signaling pathways. Occludin serves as an initial receptor for GTPase signaling(101). Additionally, occludin is regulated by ubiquitination as E3 ubiquitin-protein ligase bind to the N-terminus of occludin (102).

The Ras/rapidly accelerated fibro sarcoma/mitogen-activated protein kinase/extracellular signal-regulated kinase (Ras-Raf-MEK-ERK) signaling pathway is also essential in occludin function. Overexpression of Raf-1 reduces the expression of occludin and disrupts junctional proteins and stimulates cell stratification by displacing localization of the actin cytoskeleton. All in all, these studies propose that occludin is related with multiple cellular proteins and is modulated by signaling pathways. The phosphorylation plays major role in the continuance and assemblage of the TJ structure. The protein kinase C and casein kinase I and II, are responsible for the phosphorylation. Occludin dephosphorylation causes TJ disruption. The occludin is phosphorylated on the Ser and Thr residues. C-terminal domain of occludin possess the phosphorylation sites, T403, T404, 424, and/or T436 Which are targeted by protein kinase C. The mutation of threonine residues to alanine, prevents the phosphorylation and hinders occludin assembly. In vitro studies reveal that phosphorylation of occludin is regulated by the balance between kinases and phosphatases and controls its localization and permeability (103). Protein phosphatase (PP)1 and 2A directly intermingle with occludin to dephosphorylate it at Ser and Thr

residues. PP1 dephosphorylates occludin on Ser residues, and PP2A dephosphorylates at the Thr residues. The extracellular calcium depletion and calcium repletion is responsible for occludin disassembly and reassembly of TJs respectively (104).

The intensity of tyrosine-phosphorylated occludin is low in the intact epithelium. Tyrosine phosphorylation is caused during disassembly by various stimuli. Hydrogen peroxide interrupt tyrosine kinase-dependent mechanism, phosphorylate Y398 and Y402 in the C-terminal c-Src tyrosin kinase and hence induces TJ disassembly.

In similar way, acetaldehyde induces TJ disruption in an occludin tyrosine phosphorylation-dependent manner by directly inhibiting protein tyrosine phosphatase (105). The main reason of junctional dissociation is tyrosine phosphorylation of occludin that attenuates the interaction with Zona Occludin-1 (ZO-1).

These pathways affect cell growth, proliferation, apoptosis, signal transduction, and other critical factors. It further suggests that occludin may play a significant role in the endometrial receptivity.

Zonula occludens are cytoplasmic membrane proteins that belong to membrane associated guanylate kinase (GUK) homologs family. It include three members, ZO-1 (~220 kDa), ZO-2 (~160 kDa), and ZO-3 (~130 kDa) that links to the actin cytoskeleton (106). The Zona Occludin-1 (ZO-1) was the first identified protein of TJ family. ZO-1 is an essential transmembrane component of the epithelial and endothelial junctional complexes (107). It seals the epithelial cells at their apices and act as primary barrier for diffusion of solutes via the paracellular pathway. It also acts as a fence to maintain cell polarity by regulating the distribution of apical and basolateral plasma membrane proteins(108,109).

It is important for creating a paracellular barrier(76). ZOs anchor transmembrane proteins, claudins, occludin, and JAMs, to the actin cytoskeleton. ZO-1 and ZO-2 are expressed in both the epithelium and the endothelium and ZO-3 is expressed only in the epithelium (110). ZO-1 is localized in the apical region, but was not present in the lower two thirds of the lateral plasma membrane. It is localized in the cytoplasmic surface of plasma membranes with a molecular mass of 220 kDa (111). Cingulin (140 kDa) and Occludin (65 kDa) is directly associated with ZO-1. The ZO-1 is also crucial for uterine receptivity development and can be detected during the receptive period. The functions of ZOs have been observed using deletion strategies. Deficiency of ZO-1 in mouse epithelial cells of mammary gland leads to delayed recruitment of claudins and occludin and delayed barrier establishment (112).

In the mouse uterus, the Zona occludin-1 (ZO-1) expression is associated with epithelial cell junctions during the peri-implantation period. It is present only on days 6 and 7 of pregnancy. Occludin appears only during uterine receptivity for implantation (113). At the initiation of implantation and as trophoblast invades, ZO-1 is expressed in uterine stromal cells of the primary decidual zone (PDZ). It suggests that expression of this tight junction molecule in the PDZ acts as permeability barrier. It regulates the access of immunologically competent maternal cells and molecules to the embryo. This provide homotypic guidance of trophoblast cells in the endometrium (114,115). ZO-1 communicates with Claudin and regulates the paracellular permeability by development of an intramembranous diffusion barrier that averts fusion of apical and basolateral membranes. It manages the composition and amount of the uterine luminal fluid. The paracellular diffusion depends on the composition of ZO-1 since it is the main component that regulates opening and closing of paracellular pathway(116). Jalali

et al., (117) evaluated the expression of tight junction in the endometrium of estrus cycle and pregnancy.

The OCLN and ZO-1 gene expression remained unchanged during porcine pregnancy. The Western blot analysis also did not reveal significant differences in the expression of occludin from Day 10 and Day 13 of estrous cycle and pregnancy. The expression of ZO-1 on Day 16 of pregnancy was decreased A significantly as compared to corresponding day of estrous cycle. Immunoreactivity of OCLN and ZO-1 was observed in the endometrial luminal and glandular epithelium on all days of estrous cycle and pregnancy. ZO-2 knockout mice show reduced proliferation, improved apoptosis, and paracellular permeability, which indicate a disturbed barrier function. ZO-2 knockout causes damaged blood-testis barrier (118).

ZO-3 knockout mice revealed no phenotypic alterations, indicating that it is potentially a dispensable component of TJs (119). The ZOs, shuttle between the cytoplasm and the nucleus, mainly in response to stress. ZO-2 accumulates in the nucleus of MDCK epithelial cells following heat shock or chemical insult (120). These studies prove that the functions of ZOs reveal multiple possibilities that remain to be deciphered regarding their involvement in cell regeneration. All ZOs interact with nuclear proteins, transcription factors, to modulate the growth and proliferation of epithelial and endothelial cells, which are essential to the endometrial regeneration process. It reveals the important role of ZOs in cell signaling in the recruitment and attaching of other TJ proteins and overall integrity of the TJs.

1.2.3.3. Claudin

Claudins are family of small proteins of 21–34 kDa that create the strength of the TJs, with at least 26 components reported in

humans(121). Claudins constitute the backbone of tight-junction strands. It is transmembrane proteins distributed at the apical region of the lateral plasma membrane and diffused throughout the cytoplasm. It plays an essential role in tight junctions to form physical barriers and regulate paracellular transportation. They establish the barrier properties of cell–cell contact between the plasma membranes of neighboring cells. It tightens the paracellular cleft for solutes but form paracellular ion pores. Claudin proteins include a short cytoplasmic N-terminal region, four transmembrane domains with two extracellular loops and cytoplasmic C-terminal tail. It is presumed that extracellular loops stipulate these Claudin functions. The larger first extracellular loop is significant for determining the paracellular tightness and the selective ion permeability. It contains charged amino acids to regulate paracellular ion selectivity. This loop forms disulfide bond with highly conserved cysteine residues to increase the protein stability. The second shorter extracellular domain form dimers with claudins on opposing cells through the hydrophobic interactions. It cause narrowing of the paracellular cleft, participates in adhesion and holds opposing cell membranes, works as a receptor for bacterial toxin (122). The carboxy-terminal end comprises PDZ-domain-binding motif, with a site of many post-translational modifications, thus differs in sequence. All Claudins, (Figure 1.4) except claudin-12, have a PDZ motif that interact with cytoplasmic-scaffolding proteins, such as the ZO 1, MUPP1, PATJ and MAGUKs family of adapters which is essential for strand organization in cells and allow interaction with other proteins (123). The motif is responsible for post-translational modifications that affect the function of these proteins. Cytoplasmic tail contains amino-acid residues that are sites for post-translational modification, such as serine/threonine phosphorylation, SUMOylation, tyrosine

phosphorylation, and palmitoylation, which influence the function of claudins. In association with claudins and occludin, tricellulin consists of two long tails, with the C-terminal tail containing an occludin–ELL domain that interacts with the GUK domain of ZO proteins (figure 1.2).

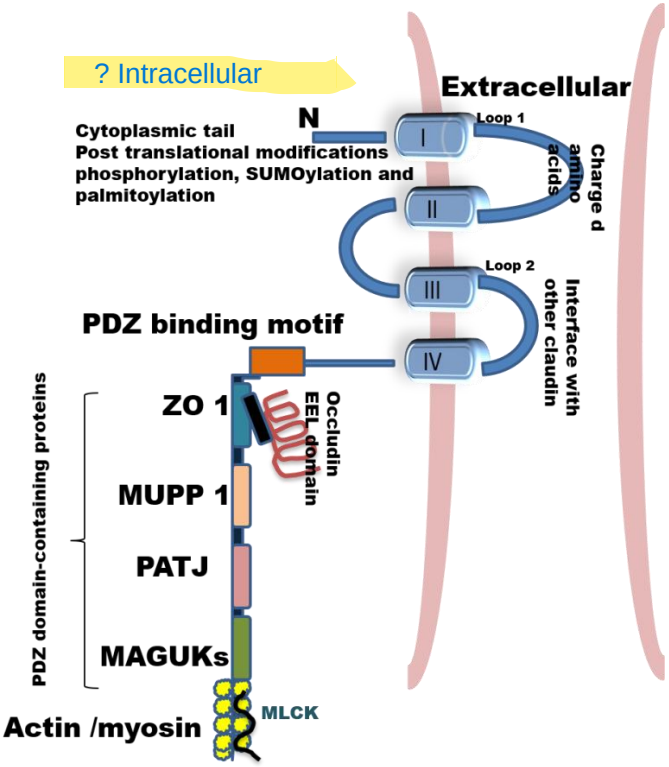

Figure 1.4: Domain Structure of claudin.

Claudin-1 is distributed at the apical region of the lateral plasma membrane and diffused throughout the cytoplasm. Occludin was present only on days 6 and 7 of pregnancy. Occludin appears only during uterine receptivity for implantation (113). The regulation of TJs in endometrial cells was investigated by isolating and culturing epithelial and stromal cells from human endometrium. Claudin-1,3,4,7, and occludin was detected together with ERa and PR (124).

The epithelial cell polarity is maintained by the adhesiveness of apical plasma membrane of uterine epithelial cells which is utmost requirement for embryo implantation. This fence function plays a

crucial role in embryo implantation. All TJ proteins, including claudin-3 and -4, were upregulated by P4 and the upregulation was inhibited by E2. The barrier function in endometrial cells was downregulated by P4, which also inhibited the proliferation of endometrial epithelial cells. The formation of stress fibers, demonstrated by F-actin staining, was induced by P4. P4 increases stress fiber formation via PAR1 which results in a decrease in the barrier function(125). The decrease in the barrier function in normal endometrial cells may contribute to cytoskeletal remodeling. These finding may be associated with the maintenance of embryo implantation.

Phosphorylation of claudin-1 by mitogen-activated protein kinase (MAPK) promotes barrier function and protein kinase A (PKA) phosphorylation of claudin-16 exacerbates Mg2+ transport(126). According to the sequence similarity, Claudins has differentiated into two groups, classic Claudins and non-classic Claudins (127). Claudins are encoded by CLDN genes with four introns and small genes. Part of them acquire a similar sequence and closely located on chromosome 16. It suggests that they have been generated by duplication and, their regulation might be linked (128). The expression level and distribution of claudin-1, -2, -4, and -5 in equine tissues were assessed by Western blotting and immunohistochemistry methods. Claudin-1 was mainly identified in lung, duodenum, and uterus. Claudin-2 was evenly observed in equine tissues. Claudin-4 was abundantly detected in liver, kidney and uterus, and claudin-5 was strongly expressed in lung, duodenum, ovary, and uterus, as determined by the Western blotting method (129,130).

Claudins are crucial components of TJs, cofunction with occludin to establish TJs selectivity. Claudins have permeability and biophysical properties for permeable molecules to coordinate the gate function of

paracellular TJs(131,132). Prior studies in a mouse lacking of Claudin from TJs showed reduced number of TJs strand and decreased TJs barrier function. Claudin 1, 3, 4 and 5 were expressed in the uterus in estrus cycle and in early stage of pregnancy (133). Claudin 4 illustrate specific localization and strong expression during implantation(134).

To date, 24 members of the Claudin family have been identified in mouse and human. Immuno-replica electron microscopy determined that Claudins are specially localized on tight-junction strands. Exogenously expressed Claudins form large network of structures that look like tight-junction strands. The expression of Claudins varies considerably among tissues.

Claudin 5 is expressed primarily in endothelial cells of blood vessels and claudin-11 is expressed in oligodendrocytes and Sertoli cells. The majority cell types express more than two claudin to constitute tight junction strands within individual single strands. Claudin are polymerized together to form heteropolymers. Claudins adhere with each other to make homophilic and heterophilic interactions with adjacent cells to create barriers against or pores for the selective molecules passage in the paracellular pathways (76,135,136).

Mutations in claudin are associated with many diseases. It plays a crucial role in enterotoxin and viral infections like hepatitis C virus and HCV. Changed expression and disruption of claudins and the induction of epithelial to mesenchymal transition (EMT) is linked with carcinogenesis. The expression of claudins is regulated on transcriptional, posttranscriptional, or epigenetic regulations (miRNA, DNA methylation, and histone modifications). In neoplasia, the expression of claudins was downregulated and upregulated. Claudin-3 and claudin-4 are highly expressed in ovarian, breast, and prostate cancers. Claudin-1 was downregulated in breast cancer, but other

studies found it to be upregulated. The differences might be associated with type of tumors, its localization, invasiveness, and stage. The discrepancies in these results point out the importance of knowing the role of claudins in physiological and pathophysiological processes (137).

Rat vagina showed the downregulation of claudin expression and is modulated by estrogen as estrogen might modulate epithelial permeability. In endometrium, expression of claudins is different and depends on the day of the menstrual cycle. Claudin-4 expression is enhanced in the luteal phase of the menstrual cycle. An interaction between embryo and endometrium is associated with numerous structural and morphological changes among TJs and is necessary for proper implantation. Claudin-4 expression in the endometrium increases during implantation and is regulated by ovarian hormones (101,138,139).

In idiopathic infertility and with eutopic endometrium, the expression of claudin-4 was upregulated as compared to the fertile group(140) Alternatively, a decreased expression of claudin-3 and claudin-4 is correlated with endometriosis which has significant contribution in infertility (141).

Claudin-7 is also participating in fertilization. In the pregnant rats, expression of claudin-7 significantly decreased and changes its location during implantation(142). Other studies in rats illustrated that biseptol-A, which is used to treat various respiratory, gastrointestinal, and urinary infections in early pregnancy, affects the Claudin expression in the endometrium, which changes the susceptibility of the endometrium to implantation, as a result reduces fertility(143). The expression of claudins was also reported in the luminal epithelium of the fallopian tube. The disturbance in the junction structure of epithelial

cells in the fallopian tube is responsible for ectopic pregnancies. The hydrocortisone regulates the expression of claudin-3 and claudin-4. The increasing expression of claudins contributes to strengthening intercellular TJs in the fallopian tube(144,145). Mokhtar et al., (146) hypothesized that, administration of testosterone caused failure of tight junction complexity and downregulated expression of claudin-4 and occludin in the endometrium. Reduced endometrial tight junction complexity and claudin-4 and occludin expression in the endometrium during implantation window by testosterone may hinder with embryo attachment and successive implantation. In mice pregnancy, the placenta showed increased expression of claudins 1–7, claudin-11, claudin-12, and claudin-23 indicating their function in paracellular transportation during exchange of nutrients, ions between mother and fetus.

Claudins participate in the proper functioning of the placenta. The expression of claudin-4 and claudin-7 is observed in human amnion during late pregnancy. Amniotic fluid volume is controlled by absorption across the amniotic epithelial cells (147–149). These findings provide knowledge about the expression patterns and localization of Claudins, and valuable information to understand tight junction-related diseases according to tissue specificity and function of Claudins. Several studies have specified that Claudins might play a vital role in both physiology and pathogenesis and triggered an expectation to use it as diagnostic or prognostic markers.

1.2.3.4. Adherent Junction Cadherins

Cadherins (CADs) are calcium dependent adhesion molecules. These transmembrane glycoproteins possess the sequence repeats of 110 amino acids and shows homophilic or heterophilic Binding. Most of the

members of the Cadherin superfamily mediate cell-cell interaction and have been expressed within a single mammalian species.

Classical CADs, such as E- (epithelial) and P- (placental) CAD, are integral membrane glycoproteins that share the sequential repeats of amino acid which generally promote/mediate cell adhesion through homophilic interactions. They regulate epithelial, endothelial and neural cell adhesion, with different CADs expressed on different cell types (150–152). Cadherins comprises five extracellular (C1/C2/C3/C4/C5) domains bound by Ca2+ binding and one intracellular domain. Classical cadherin connects to β-catenin, which attaches to a-catenin linking to the actin cytoskeleton, as well as p120 catenin, regulating GTPases -Rho, Rac, and Cdc42. Cadherin regulates cellular proliferation, migration and stem cell differentiation (Figure 1.5).

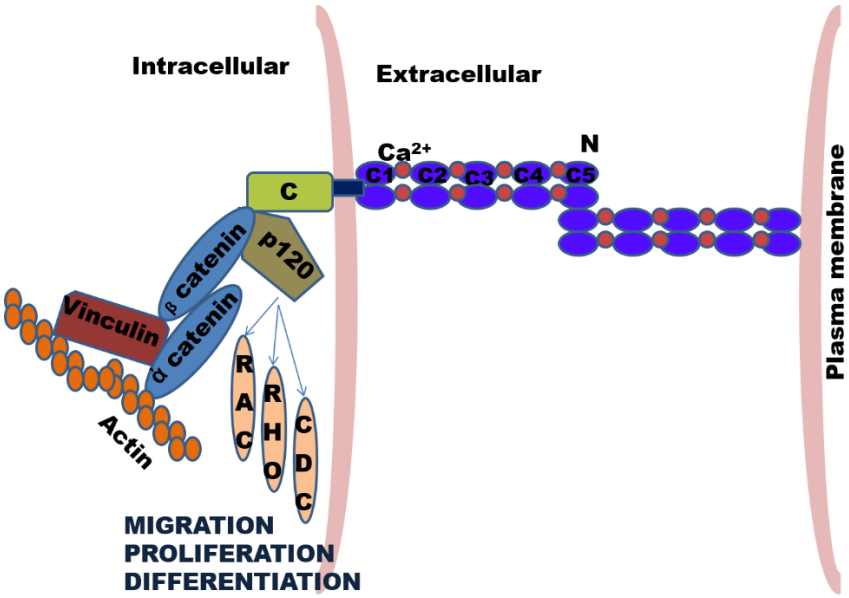

Figure 1.5: Diagrammatic representation of Cadherin.

They are identified and highly expressed on the endometrial epithelium throughout the menstrual cycle and mediate during implantation. Likewise other epithelial cells, the uterine luminal epithelial cells are attached by adherence junctions. These junctions are connected to

cytoskeletal filaments with a dense 10-to 30-nm-thick cytoplasmic plaque. These the adherence junctions usually coexist in the subapical regions of juxtaposed cells (153–156).

In the Adherence Junctions, E-Cadherin (E-CAD) is one of the dominant molecules. However, little data is available regarding the E-CAD and its role during implantation in humans. It has previously been implicated in rat uterine receptivity. The apical membranes of mouse uterine epithelial cells showed enriched E-CAD expression. E-CAD gene mutations in mice resulted in defective embryo implantation. Embryo implantation models have proved that E-CAD conveys adhesive properties to the uterine epithelium. Calcium dependent cell–cell adhesion, E- and P-CAD are found in the luminal and glandular endometrial epithelium throughout the menstrual cycle (157).

E-CAD expression is up-regulated by progesterone. It is found on the trophoblast and it may be involved in the initial attachment of the embryo. E-CAD in the mouse as transgenic mice lacking the gene fails to form trophectoderm which shows its important role in implantation (158). However, P-CAD expressions has not been shown any relation with progesterone level and is not detected on human trophoblasts. As a result, the role of P-CAD in the human remains unclear. P-CAD null mouse, the uterus is unaffected(159).

K- (kidney) CADs and OB-(Osteoblast) CAD are atypical CADs. During the proliferative phase, K-CADs is strongly expressed in the glandular epithelium and decreases after ovulation. Throughout the all phases of menstrual cycle, OB-CAD expression in the glandular epithelium remains unchanged. In the stromal cells, OB- CAD expression showed that it is inversely proportional to that of K-CAD(160). The expression of Cadherin and Catenin is observed in mice by Western blotting and immunocytochemical analysis during embryo implantation. It revealed

the expression of E-Cadherin in the endometrial epithelial cells, at apical region at peri-implantation stage at the implantation sites. Progesterone regulates the E-Cadherin expression(161).

Trophoblast exhibits a down regulation of Cadherin, Catenin and Ca2+ at peri-implanting stage. It suggest that the trophoblast exhibit an invasive phenotype and endometrial epithelium display an adhesive phenotype during the window of implantation(162). The E-Cadherin-null mouse shows defective embryo development and implantation failure. In endometrium, E-Cadherin, type 1 N-Cadherin, P-Cadherin, type 2 cadherin-6 (K-Cadherin) is located at the lateral epithelial plasma membrane for the establishment and maintenance of adherens junctions(163,164).

E-Cadherin is found on luminal epithelium and involved in the initial attachment of the embryo. Its expression at the cell surface is required for epithelial continuity and Cadherin-mediated adhesion may maintain the continuity of luminal cell layer at the implantation site to enable blastocyst invasion to permit a interstitial implantation (75,165).

Collectively E-CAD regulates uterine function, endometrial differentiation and gland development. It is unsure that ablation of E-CAD causes abnormal stromal and glandular differentiation. Further investigations of endometrial cells of luminal, glandular and stromal interactions in the uterus are warranted.

1.2.3.5. Gap junction

Gap junctions connect neighboring cells via channels. Gap junction form channels to facilitate the cellular cross talk between the cells. The molecular changes take place by passive diffusion. They exist between two cells and are relatively nonspecific. Gap junctions form plaques by grouping twelve connexons. It is composed of two extracellular loops, four transmembrane-spanning domains, one cytoplasmic loop, one

amino-(N) terminus and one carboxy-(C) terminus tail region. Each extracellular loop contains three conserved cysteines (Figure 1.6).

The gap junctional channels allow the passage of molecules smaller than 1000 Da depending upon apparent selectivity principally on molecular size(166).

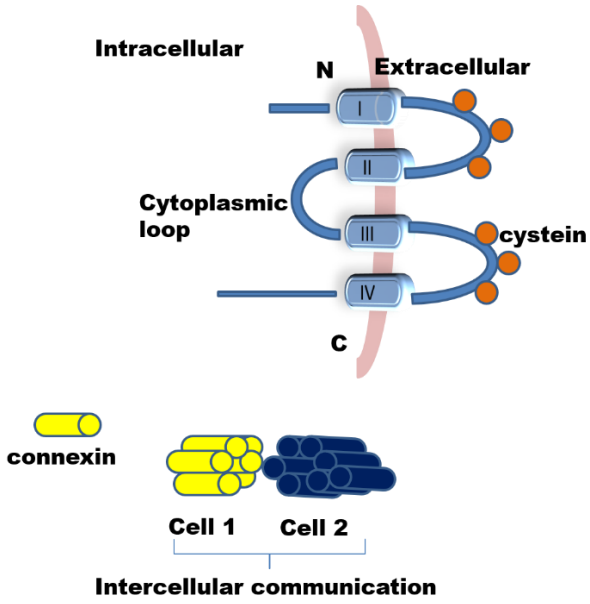

Figure 1.6: Diagrammatic representation of Gap junction.

Small molecules, such as certain morphogens directly transmitted between cells via gap junctions but prevent the transmission of proteins and nucleic acid. This direct cell-cell communication is an important for regulating the cell mechanism during regulation of trophoblast invasion and in the embryogenesis. Successful implantation requires a close interaction between the embryo and the uterine cells. The implantation period, the receptive phase into uterine tissue is restricted to a limited time period. The appropriate differentiation of the receptive endometrium is under the control of ovarian steroid hormones. The embryonic signals modulate the endometrial environment before trophoblast invasion. The gap junction protein expression is directly

related to endometrial changes. The identifying and characterizing a multigene family that codes the gap junction proteins is connexins (Cx) (167).

Cx play vital role in female reproductive health and its disturbed expression are correlated with different female reproductive disorders like polycystic ovarian syndrome, recurrent miscarriage, pre-term birth and endometriosis(168). The role of Cx may play important role in improving female fertility by assisting the processes of uterine receptivity, enhancing the endometrial quality.

1.2.3.6. Connexin

Connexin expression seems to play important role in endometrial receptivity and defined as a cell biological marker as well as an indicator of the early contact between conceptus and uterine luminal epithelium which is necessary for implantation. In the rat, preimplantation stage showed suppressive effect of gap junctional proteins connexin 26 and cx43 under the influence of progesterone. The highly regulated connexin expression in the endometrium and trophoblast suggests a key role of different intercellular pathways in regulating the trophoblast invasion into endometrium.

Grummer et al., (169) stated that during preimplantation phase, endometrial tissue express downregulation of cx26 mRNA and cx43 mRNA in response to the rise in secretion of progesteron (P). The estradiol-171 (E2) and P differentially regulate the expression of cx26 and cx43 in the rat endometrium. Administration of E2 increased mRNA for cx26 and cx43 enhancing configuration of gap junctions containing these proteins. In contrast, administration of P decreased mRNA for both cx26 and cx43 and supress gap junction formation. Administration of both P and E2 simultaneously also suppress the connexin expression.

The E2 resulted in an increase of connexin transcripts and P appears to inhibit the stimulation of connexin expression.

In rodents and rabbit, the receptive endometrium is distinguished by a lack of cell-to-cell communication channels. The rat endometrium showed suppressed expression cx26 and Cx 43 in the epithelium and stromal compartment respectively by maternal progesterone. Experimental studies revealed that these connexin genes react very responsively to progesterone and estrogen. In rat and rabbit, implanting blastocyst revealed connexin expression in the endometrium(170).

The downregulation of connexin expression in the endometrium is cell biological indication for receptivity in different species. The induction of Cx in response to blastocyst signals, seems to be a precondition for implantation(171).

Cx are involved in appropriate development and functioning of female reproductive system and plays a vital role in endometrium during implantation and in development of placenta. These Cx play essential role in reproductive problems related to infertility, pregnancy complications and changes in endometrial physiology. Altered expressions of Cx43 reduce the oocyte quality causing human female infertility. Cx37 is also influences the women's fertility. Its expression is reduced in type 1 diabetes (172). Downregulation of Cx37 also responsible for infertility in diabetic women. Cx26 is sensitive to estrogen and play a major role in endometrial function. Cx43 is expressed in stromal decidualization in secretary phase of menstrual cycle. The reduced level of Cx26, Cx32, Cx43 expression in endometrial stroma causes early pregnancy loss, reproductive impairments, intra uterine growth retardation and pre-eclampsia (173). The reproductive

disorders are associated with downregulation Cx expression and decreased intercellular cell communication. The establishment of connexins expression and hence the gap junction intercellular communication can be considered as a therapeutic strategy for treating the reproductive disorder.

1.3. Role of stem cells and growth factors in endometrial regeneration

Human endometrial mucosa undergoes cyclical morphologic and functional changes with dynamically remodeling tissue in response to fluctuating hormones during a woman's reproductive life. The regeneration of functionalis layer of endometrium suggests that endometrial stem cells may rejuvenate the basalis stroma (174).

Stem Cell-based therapy is actively being investigated as a new potential treatment for endometrial regeneration. Various types of cells, including embryonic stem cells, mesenchymal stem cells (MSCs), very small embryonic like stem cells (VSELSCs), bone marrow derived mononuclear cells (BM-MNCs) and adipose stem cells, have been found to improve endometrial degeneration(175).

The recent identification of different stem cells - mesenchymal stem cells (MSCs), side population (SP) cells, label-retaining cells (LRCs), menstrual blood-derived stem cells (MenSCs), have been detected in the endometrium (176,177). The scope of stem cell therapy is quickly developing and, to date, clinical trials have begun to investigate the use of stem/progenitor cells in the therapy of degenerative and damaged tissues(178).

Stem cells are undifferentiated cells that have the self-renewal and multi-lineage differentiation potential. Considering these qualities, stem cell therapy can be useful in the treatment of endometrial regeneration. Stem cells potentially substitute damaged cells in the endometrium. Cell replacement strategies have been studied in some animal models. In

addition to multipotency and pluripotency, stem cells have exhibited beneficial paracrine effects and can decrease cell death and provide growth of host cells. The transplanted stem cells provide morphological and functional benefits through trophic support, cell regeneration, anti-inflammation, stimulation of regulatory interactions with host cells (179). Congenital anomalies and acquired intrauterine adhesions caused by infections, curettage or previous caesarean section scar can affect the normal uterine morphology and affects uterine contractility, leading to obstetrical complications like uterine factor infertility, recurrent miscarriage, Dysfunctional uterine bleeding (DUB) (180).

One of the increased risk factors for pregnancy complications is impaired endometrium. Endometrial abnormalities can result in thin dysfunctional endometrium with complete loss or dysfunction of endometrial stem cells (181). Thin endometrium is related to lower implantation and pregnancy rates. Many treatments have been tried to improve endometrial development, but none has been authenticated up to now.

In 2004, bone marrow recipients who had received bone marrow mesenchymal stem cell (BM-MSC) transplant for leukemia were first detected donor-derived endometrial epithelial and stromal cells in their endometrial samples. This proved that BM-MSCs have the potential to repair endometrial cells and are associated with endometrial regeneration (182). Stem cell-based therapies hold promise for coming use in restoring poor endometrium. Reconstructing normal endometrial structure is the purpose of intrauterine stem cell therapy. The delivery of exogenous stem cells able to promote endometrial regeneration is considered as a hopeful method for restoring endometrium. In our study, ordinary circumstances, the transplanted stem cells followed by infusion of activated Platelet rich Plasma (aPRP) will adhere to ECM that provides a suitable milieu for growth and regeneration.

The implantation is governed by ovarian steroids progesterone and oestrogen whose actions are mediated by number of cytokines and growth factors. The cytokines and growth factors are chemical messengers that mediate intercellular communication whose biological actions are mediated locally by specific receptors. They correlate with many body functions including injury, inflammation, immune response and implantation. Pro-inflammatory mediators are observed in response to inflammation and during implantation are similar(183). The cytokines and growth factor playing crucial roles in implantation include insulin-like growth factor, leukemia inhibitory factor and many non-adhesive molecules such as mucin(24). Platelet-rich plasma (PRP) excrete several growth factors and cytokines that are associated with the healing and regeneration process (184).

The scope of PRP application has been continuously expanded in various fields of surgery as it accelerates the healing and regeneration of soft and hard tissues (185). The activated platelets in PRP plays significant role in the healing, aggregating rapidly at the injured site and releasing a multiple growth factors and cytokines that are associated with regenerating soft and hard tissues (186). The growth factors that are secreted by the activation of platelets include platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), vascular-endothelial growth factor (VEGF), insulin-like growth factor (IGF), and epidermal growth factor (EGF) (187).

Based on these theoretical acquaintances, PRP is used as a source for growth factors in various treatments, such as implant placement and bone grafting, chronic wound cure, and fat transplantation. Thus, to enable its effective clinical application and to enhance bioactivity level of the growth factors of PRP, there is necessity of activating PRP by using thrombin(184).

In this study, the cytokines and growth factors (GFs) secreted by PRP and PRP activated by using thrombin (aPRP) were determined. The levels and specific roles played by of C-C motif ligand 2 (CCL2), tumor necrosis factor-alpha (TNF- α), interleukin (IL)-1 β, C-X-C motif ligand (CXCL)-8, CXCL10, IL2, IL4, IL-6 IL-10, IL-12, IL-17A, TGF- β, interferon-GAMMA(IFN-γ) in the PRP and aPRP are expatiated. The cytokines and GFs regulate cell migration, proliferation, attachment, differentiation and promote endometrial cell regeneration(188).

They facilitate attachment of protein molecules on endometrial luminal epithelial cells and trophectoderm to their respective receptors or vice versa. Thus, they function as signaling molecules and enhance communication between cells playing as vital aspect while expanding strategies to improve fertility. For fruitful implantation, the endometrium must be implantation competent. Identification of suitable implantation markers and mediators of implantation to the receptive phase is yet to be identified across board. The implantation failure associated with embryo transfer in assisted techniques may possibly be due to absence of some of these cytokines/growth factors. The clear understanding of roles of cytokines and growth factors in implantation will motivate further research on their roles in implantation to improve fertility or investigating infertility.

1.4. Junction proteins in implantation and implantation failure

The endometrial activity in the implantation has been extensively studied. It is known that appropriate endometrial development is essential for normal implantation. The embryo interacts with endometrium that produces suitable intercellular adhesion molecules. However, little is known about the mechanisms whereby junction proteins influence embryo implantation. Further studies are in urgent

need to clarify the role of junction proteins expression in successful implantation.

The presence or absence of specific cell-adhesion junction proteins such as the ZO, E-Cad, integrins, metalloprotease domain containing (ADAM) proteins and communicating Gap junctions CX and their respective ligands postulate possible molecular mechanisms for blastocyst adhesion. Cell- cell junction proteins have plays important roles since from the early events of reproduction including gamete transport, sperm–oocyte interaction, embryonic development, and implantation. Winterhager E et al., (189) studied the CX expressions in mice. They demonstrated that several connexins are involved in female reproduction. Lack of CX 43 causes reduced growth of granulose cells, cumulus cells and oocyte deficiency, impairing fertility. Thus, expressions of CX 43 are determinant of embryo quality and correlated with fertility outcome. Connexins are important in placentation. Low expressions of CX37, cease oocyte growth. Reduced CX26 in endometrium causes disrupted implantation and reduced expressions of CX43 in the uterine stromal epithelium is responsible for impaired decidualization in mice and humans.

The families of adhesion molecules engaged in sperm–oocyte interaction include ADAMs, cadherins and integrins. Both sperm and oocyte express integrins that are involved in the initial interaction. The sperm adhere to the oocyte before penetration. The predominant concept is that ADAMs located on sperm head attach to integrins on oocytes.

The integrins interaction with cell surface protein includes tetraspanin CD9. The CD9 fundamentally induce a redistribution of adhesion molecules on the oocyte surface that precedes sperm–oocyte attachment and fertilization (Figure 1.7A)(190).

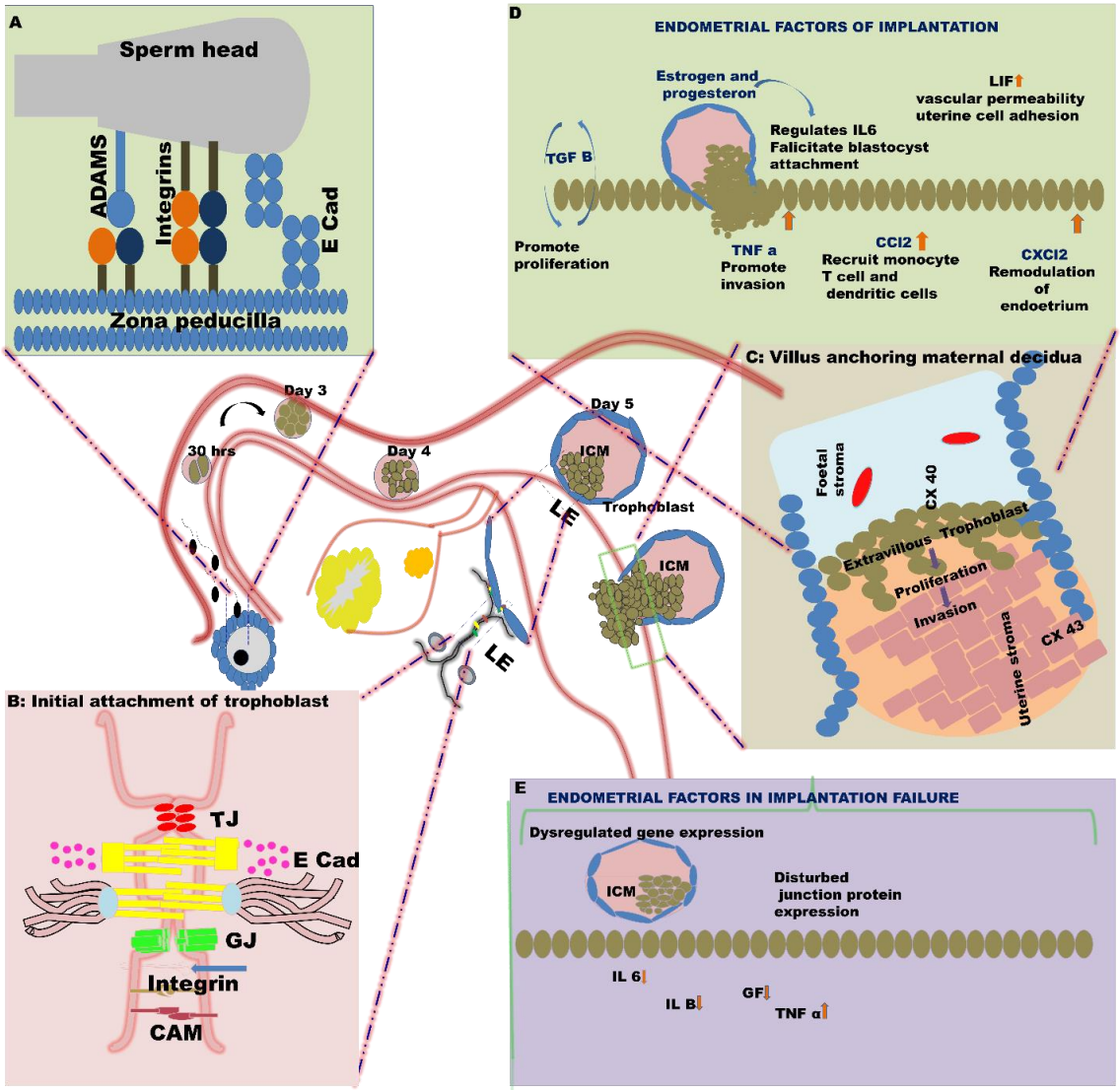

Figure 1.7: Junction proteins in implantation and implantation failure.

Cadherins organize the inner cell mass and trophectoderm. The trophoblast-endometrial epithelium and adjacent endometrial luminal cells variously express cadherins, zona occludin, integrins, trophinin, connexins and selectin. The cell adhesion molecules help in early reproductive events. Early embryos undergo compaction, figuring inner cell mass (ICM) and trophectoderm which forms embryo and extra-embryonic structures including the placenta respectively (191).

Adhesion molecules are involved from stages of compaction to implantation, and in embryonic development (Figure 1.7B)(192). E-cadherin and N-cadherin are involved for the growth of embryos, while P-cadherin is not required for early embryogenesis. The first adhesion reliant differentiation in the embryogenesis is trophectoderm influenced by E-cadherin (193). E-cadherin knockout mouse embryos lacked Ca2+ binding and had a disorganized ICM and failed to form a trophectoderm. Such embryos are unable to implant (194).

Embryos with altered expression of E-cadherin in oocytes had showed poor development and had a low pregnancy rate in sheep. It was put forwarded that the normal expression of E-cadherin is required for embryo development (195). The trophoblast moves within the luminal epithelium to achieve initial apposition. Adhesion between the endometrium and the trophectoderm is a prerequisite for implantation which involves adhesion molecules (89,196). ZO-1 is expressed in luminal and glandular epithelial cells in endometrium. ZO-1 is localized at the interface between the trophectoderm and the luminal epithelium at the time of the blastocyst attachment. The luminal area near the blastocyst attachment showed apical ZO-1 expression, suggesting that the rest of the luminal epithelial cells are closed by tight junctions. ZO-1 is expressed at apical side with extension into the lateral borders of luminal epithelium cells(115). The integrins predominant on the trophectoderm interacts to integrins expressed by the uterine epithelium to reduce the MUC–1 at the point of trophoblast apposition with the endometrium. The Osteopontin link the integrin avβ6 on the trophectoderm to avβ6 on luminal epithelium (73,197).

Interestingly, preimplantation embryos express connexin genes in ICM and the trophectoderm(198). Preimplantation embryos utilize of different gap junction channels, but connexin genes knockout mouse

has not yet revealed necessary role for connexin in preimplantation or implantation. The double knockout of CX31 and CX43 had not any effect on the preimplantation embryos, entailing that GJ is not essential during this very early developmental period. It remains a secrecy that multiple connexins are expressed in preimplantation(199,200).

The distribution of connexins may have a role in establishing positional information for cell differentiation(201). The reduced expression of E-cadherin expressions lowers the infiltration of the trophoblast during implantation. During the placenta formation, elongated trophoblast cells form two-layered syncytiotrophoblast along with chorionic invasion and embryonic blood vessels formation (Figure 1.7C).

Decreased expressions of CX26, CX31 and CX31.1 are responsible for impaired transport of metabolites across the syncytial trophoblast causing reduced glucose uptake by the embryo resulting in an early embryonic death. Both knockouts influences on trophoblast lineage differentiation (202,203). Cytokines and growth factors play multifaceted roles in embryo implantation. The alteration of the cell adhesion junction proteins expression and cytokines, including GFs, could lead to infertility and implantation complications. Cytokines are multifunctional glycoproteins. Its actions are mediated by specific cell surface receptors. It gives potent intercellular signals that regulate functions of endometrial cells and trophoblast–luminal epithelium interactions. The trophoblast and endometrial epithelium produce cytokines that modulate the endometrial receptivity by regulating the expression of various adhesion molecules (Figure 1.7D) (204). Disturbed expression of cytokines and their signaling leads to an absolute or partial failure of implantation and abnormal placental formation. Cytokines are known to be crucial and indispensable for the embryo

implantation. The disruption of these junction proteins, GFs and cytokines can lead to implantation failure. Initial attachment of the blastocyst may entail adhesion between exposed surface receptors and ligands on the endometrial epithelium. The luminal epithelium appears to act as a barrier through rest of the menstrual cycle, but provides an opportunity during a brief window of implantation. The importance of junction proteins and cytokines in implantation has been acknowledged by several previous studies (205).

It is ethically and practically not possible to study human implantation in vivo. There is a lack of an ideal model to study embryo implantation in humans. Animal models do provide important information regarding the process regulating implantation, but the process varies across species. These results cannot always be extrapolated to humans.

Previous studies on the implantation process in humans or animal studies have focused exclusively on early dialogue between endometrium with implanting embryo. As a novel step in identifying the junction proteins in pre-implantation stage at implantation window, our study compared cell adhesion and cell communicating junction protein expression pattern between endometrial cells of female wistar rat. The several cytokines (CCL2, TNF-a, IL-1β, CXCL-8, CXCL10, IL2, IL4, IL-6 IL-10, IL-12, IL-17A, TGF- β, IFN-γ) were highly expressed in the endometrium. Also, tight junctions (ZO-1, Cla-1), adhesion molecules (E-Cad), cell communication molecules (CX-40)) were identified in the endometrium. Stable adhesion through these junction proteins expressed in the endometrium are required for implantation. Disruption of these above explained factors appears to have consequences on fertility.

The argument is made that a deeper understanding of junction proteins and implantation will inform new strategies that improve endometrial receptivity and increase the efficiency of embryo implantation and assisted reproduction.

1.5. Conclusions

The changes that occur during endometrium at the time of implantation include expression of cell adhesion molecule receptors and ligands that may be involved in implantation. The specialized endometrial proteins expressed are responsible for the developmental changes either directly or indirectly on epithelial and stromal cells. Disruption of any one of these factors is studied in animal models suggest that receptivity defects result when the function of these proteins is hampered. Future studies will examine techniques to use this information to treat endometrial disturbances and related infertility. It will develop novel forms that will target the disturbed endometrium and will support receptivity.

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Chapter – 2

Activated Platelet-Rich Plasma Accelerate Endometrial Regeneration and Improve Pregnancy Outcomes in Murine Model Of Disturbed Endometrium

2.1. Introduction

Regenerative Medicine is considered as advanced technology that utilizes stem cells for healing and regeneration of diseased organs. However, the prevailing concern for autologous stem cells is requirement of additional procedures related to isolation of cells and allogenic stem cells have chances of immunological reaction. In such scenario, autologous PRP offers promising direction as it non immunogenic and able to induce activation of stem cells, remodeling of extracellular matrix (ECM), and new blood vessels formation.

The endometrium is a dynamic organ, undergoes cyclic rejuvenation and breakdown during the reproductive phase. Endometrial receptivity is a significant factor in implantation. Dysregulated endometrium is a major cause of female infertility. It is usually exemplified by deprived glandular epithelial growth, scattered stroma, reduced vascular development and altered expression of cell adhesion molecules. In vitro fertilization (IVF) treatments require need of proper endometrial thickness to improve the pregnancy rates. The correlation between implantation and junction proteins has been mentioned in various studies (1–4). Several treatments have been attempted to restore endometrial function including estrogen, aspirin, vitamin E, and pentoxifylline. However, outcomes are still poor. Recent studies have reported that intrauterine infusion of granulocyte colony-stimulating factor (GCSF), bone marrow-derived stem cells (BM-SCs) are used for regeneration of damaged endometrium in murine models. However, there are some limitations and some concerned issues that are still unexplained with respect to the usability including immune reactions (5).

In recent years some studies have stated that intrauterine infusion of platelet rich plasma (PRP) may be effective in patients with thin and unresponsive endometrium to traditional treatments. However, the scientific basis for the PRP effect on endometrium has not been elucidate, which impedes the

acceptance of PRP therapy. Jang et al., 2017 (6) validated molecular biological trials to quantify the endometrial regeneration by PRP treatment in a rat model, but did not elucidated functional analysis of PRP. PRP is a rich source of platelet concentration including growth factors (GFs). The GFs regulate normal tissue structure, and promote rapid healing of tissue damage. PRP contains platelet-derived growth factor (PDGF), Interleukin (IL), transforming growth factor-beta (TGF-β), epidermal growth factor (EGF), fibroblast growth factor (FGF), Chemokine C-C motif ligand (CCL2), tumor necrosis factor-alpha (TNF- α), chemokine C-X-c motif ligand (CXCL) 8, CXCL 10, interferon-GAMMA (IFN-γ). These growth factors play a role in endometrial cell proliferation and differentiation. PRP may have beneficial regenerative effects on damaged endometrium (7). Activation of platelet might influence the release of bioactive molecules exuded by platelet α-granules are influencing stem cell trafficking, proliferation, and differentiation with a complex effect on pro-inflammatory processes.

In the present study, we explored whether activated PRP (aPRP) treatment can have a beneficial effect compared with a rat model endometrium disturbed by 95% ethanol treated with aPRP. The aPRP infusion in disturbed endometrium (DE) can promote endometrial regeneration. To evaluate the potential use of aPRP in the endometrial regeneration, we developed a rat model of DE and conducted histological, biological, and functional analysis to investigate whether PRP administration could restore endometrial function and improve pregnancy outcomes.

2.2. Materials and methods

2.2.1. Preparation of PRP

Male (n=10) adult Wistar rats and female adult Wistar rats (n=72), weighing approximately 220 g (range, 190-250g) each were used. Out of 10 male rats, five male rats were used for blood collection to isolate PRP and remaining male rats (n=5) were used further for mating. Male rats (n=5) were

anesthetized with subcutaneous injection of Thiosol 1 g/vial, (Neon laboratories Ltd.) using insulin syringe at a dose of 12 units/gm of body weight. 5 ml of blood was drawn into a sodium citrate tube via cardiac puncture and postcava to collect a good amount of blood from the experimental animals. A needle of 25G was inserted into ventricle and posterior vena cava slowly. The same procedure was repeated three to four times to collect more blood samples. The blood samples from jugular vein puncture were collected. The centrifugation of blood was carried out at 1200 rpm for 10 minutes in a cooling centrifuge. After the centrifugation process, blood gets separated into three layers viz. plasma, buffy coat, and erythrocytes. The top most layer, plasma, was then aspirated and stored at -4°C. Then the middle buffy coat layer was extracted and transferred into a new sterile tube. This layer underwent another 10 minutes of centrifugation. The platelets were allowed to sediment at the bottom of the tube. While the upper part of the platelet-poor plasma layer was discarded, the lower half of the PRP was carefully collected using a pipette. The entire procedure was performed in highly sterile conditions (Figure 2.1).

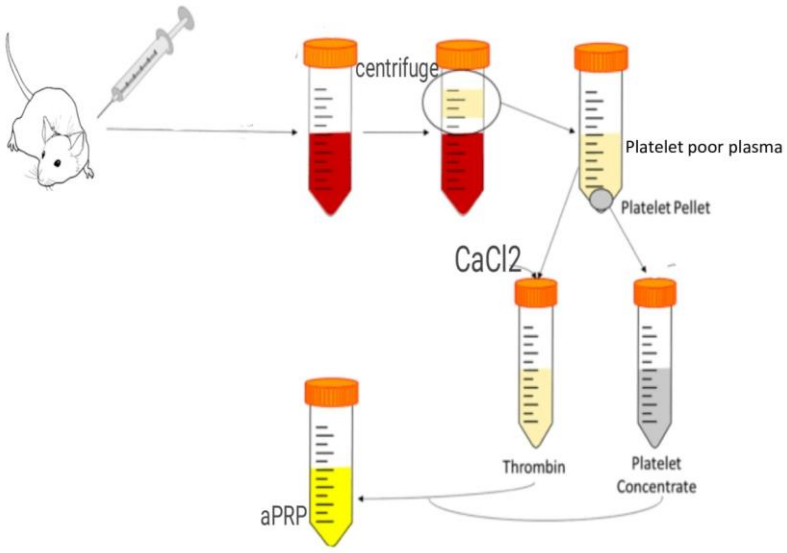

Figure 2.1: Schematic representation of preparation of PRP, thrombin and aPRP

2.2.2. Preparation of thrombin

The plasma of 1000µl concentration was diluted 10 times with distilled water, to make a 10 ml solution. The pH was adjusted to 5.3 by adding 100µl of 1% acetic acid. As thrombin precipitates at pH ≤ 5, it resulted in precipitate formation. It was then centrifuged at 2000 rpm for 5 minutes and later was kept still for 30 min. The precipitate was then resuspended in normal saline to make a volume of 10 ml solution. The pH was adjusted to approximately 7. This solution was later placed in 37°C water bath followed by an addition of 0.1 ml of 0.1M calcium chloride (C5670, Sigma-Aldrich) to it. A clot was formed which was removed in 5 minutes. Then a water clear form of thrombin was obtained and stored in at

-4°C.

PRP Activation

In the United States, activation of PRP is done by commercially available thrombin, which is derived from bovine plasma (8). The platelets in the PRP contain platelet growth factor (PGF). Whereas the a-granules of the nonactivated PRP contain nonfunctional PGF, because they are in contact with the tissue or not released yet. To stimulate the release of these growth factors, platelets must be activated. Thrombin being a potent platelet activator, facilitates immediate PGF release from the PRP (9,10). The 0.5 ml of thrombin was added into 0.5ml of PRP, to activate it, which changed into a semi-solid, jelly-like structure in 3 mins.

2.2.3. Cytokine and chemokine profiling of PRP

It is known that thrombin activated PRP (aPRP) produces high levels of various cytokines and chemokines. We have analyzed the supernatants from aPRP for these factors. Relative quantitation of cytokines and chemokines was carried out by using LEGENDplex Rat Th Cytokine Panel (13-plex) (Biolegend, San Diego, CA) Kit. Cytokine levels of CCL2, TNF- a, IL-1β, CXCL8, CXCL10, IL2,

IL4, IL-6 IL-10, IL-12, IL-17A, TGF- β, IFN-γ were measured with flow cytometry, by following the manufacturer's protocol.

2.2.4. Experimental animals

The study was approved by Institutional Animal ethical Committee (DYPMCK/IAEC/2020/JAN/01). Out of 10 male rats, five male rats were used for PRP isolation and remaining male adult Wistar rats (n=5) were used for further study. Female adult Wistar rats (n=72), weighing approximately 220 g (range, 190-250g) each, were used. Two rats were housed together in a standard animal cage of size 420×270×180 mm. Rats were housed in a light-controlled room with free access to tap water and chow food/pellets, with 12 hours of light and 12 hours of darkness, and were maintained at a temperature approximately at 24°C.

2.2.5. Animal study groups and treatments

The animals were anesthetized for the surgical procedures with subcutaneous, extra-peritoneal injection of Thiosol 1 g/vial, (Neon laboratories Ltd.) at a dose of 12 units/250 gm of body weight. The 72 female Wistar rats were allocated into three groups: (I) control group (II) Disrupted endometrium (DE) group and (III) activated PRP (aPRP) treated group. The aPRP treated group was mated with 5 male rats at 15 days after experiment initiation. In Control group (n=24), the uterine horns were exteriorized and clamped just above the cervix with small curved hemostatic forceps. The 0.25 ml intrauterine infusion of normal physiological saline was subjected to bilateral intrauterine horns. Similarly DE group (n=48) was injected by 95% ethanol into the uterine horns to disrupt the endometrial lining (figure 2.2). Out of which n=24 were used for DE study and further n=24 animals were used for aPRP study. After one day of endometrial disruption, n=24 in the DE group was administered with aPRP. The aPRP-treated group (n=24) subjected to administration of 0.5ml thrombin activated PRP into both uterine

cavities as the same manner initially. The three animals from each group were sacrificed at Day 1 (D1), Day 3 (D3), Day 6 (D6) and Day 9 (D9) interval from each group. Remaining three animals were used to confirm pregnancy outcome. Mid uterine horns were excised immediately after the animals were sacrificed and placed into 10% neutral buffer formalin for further research. Animals were sacrificed post experiment by cervical dislocation. A yellow-colored category No.2 biomedical waste management bangs were used for seal sacrificed rats. It was given to central incineration facility in Animal House of D. Y. Patil Medical College, Kasaba Bawada, Kolhapur.

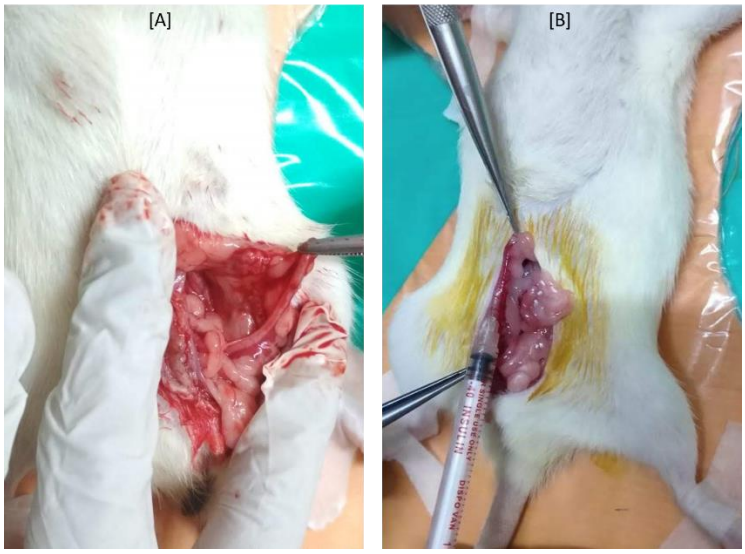

Figure 2.2: Endometrial Disruption of endometrium and intrauterine infusion of aPRP: The experiments were performed to establish DE rat model. [A] Bilateral uterine horns were extraperitonised for endometrial damage. [B] endometrial damage by 95% ethanol and infusion of aPRP after DE, is carried out.

2.2.6. Hematoxylin-eosin staining analysis

The uterine specimens were fixed in 10% formalin for 24 hours, embedded in paraffin, sectioned into 4-µm thick sections and allowed to stain with hematoxylin-eosin (H&E). Endometrial morphology was analyzed by H&E staining, and images were captured using the bright-field microscope (Nikon) at magnifications of ×20. To determine the area of the endometrium (µm),

each slide was analyzed in a double-blinded manner by two experts using Image J image analysis software.

2.2.7. IHC analysis

The intensification of epithelial, stromal, and vascular cells was assessed by immunohistochemical (IHC) for alpha smooth muscle actin (a-SMA), Cytokeratin (CK)18, CK 19, Connexin-40 (Cx-40), E-Cadherin (E-Cad), Claudin-1(Cla-1), Zona Occludin-1 (ZO-1). Tight junctions comprise ZO-1 and Cla-1, adherence junction include E-Cad and gap junctions include Cx-40. CK 18 and CK 19 are epithelial markers whereas a-SMA is smooth muscle cell marker. After deparaffinization and rehydration, antigen retrieval was carried out with 0.01 M sodium citrate buffer (pH 6.0) in a pressure cooker at 100°C for 20 minutes followed by 20 min cooling time. The tissue sections were incubated and nonspecific binding was blocked with respective serum for 45 min at room temperature. Sections were then incubated with a-SMA(mouse monoclonal;14-9760-82; Invitrogen; USA), CK 18 (mouse monoclonal; ab7753; Abcam, UK), CK 19 (mouse monoclonal; ab7753, ABD serotech; UK), E-Cad (Mab anti human: Invitrogen, USA), Cla-1 (Rabbit anti human: Invitrogen, USA), ZO-1 (Mab anti human: Invitrogen, USA) at a dilution of 1:200, in BSA (Hi Media) in humid chamber followed by washing with DW containing 0.05% Tween 20. This was followed by incubation of sections for 60 min at room temperature with a secondary antibody labeled with Alexa 488 (Molecular Probe) in dark according to the manufacturer's instructions.

After washing with D/W containing 0.05% tween 20, sections were counterstained with DAPI (Invitrogen). Sections were mounted in fluorescent mounting medium (Dako). Negative controls were stained without primary antibodies. Stained sections were examined under fluorescence microscope (NIKON, Japan). This assessment was successively analyzed in a double-blind manner by two investigators with Image J software as previously described. For quantitative assessment of protein expressions of IHC staining, five

randomly selected section fields of endometrial tissue slides were scanned into a TIFF image file at a magnification of ×20. Immunostaining is assessed using H score. H score =Σ Pi(i +1), where i is intensity of staining (11,12). The a-SMA, CK-18, CK 19, E-Cad, Cla-1, ZO-1, Connexin-40 positive samples were defined as having low positive intensity (1), medium positive intensity (2) or high positive intensity (3) signal and Pi is the percentage of stained epithelial cells ranging from 0% to 100%. The H-score ranges from 0 to 4.

2.2.8. Treatment of aPRP improves birth rate of live pups

Female rats in aPRP treated group (n=3) were mated with male rats (n=5) at fifteen days after initiation of experimental study. Pregnancy outcomes included the time to conceive and live-birth rate.

2.3. Results

2.3.1. aPRP released cytokines and chemokines

The Cytokines and Chemokines release profile is summarized in Table 2.1 and Figure 2.3.

No detectable levels of IL-β, IL 4 and IL 12 were found in activated samples. Significantly lower amounts of TNF-a, IL 6, IL 10 and IL 17 were detected in activated PRP as compared to the non-activated PRP (p < 0.05). Activated PRP produced significantly higher amount of CCl2, CXCL 10, IL-2, TGF-β with respect to that of TNF-a, IL 6, IL 10 and IL 17 (p < 0.05). CXCL-8 and IFN γ induced a significantly high Cytokine and Chemokine release with respect to that of IL-β, IL 4 and IL 12 (p < 0.05)(Figure 2.3).

Table 2.1: Cytokine and Chemokine release kinetics according to the activation method

Cytokines & Chemokines	PRP(pg/ml)	aPRP (pg/ml)
CCL2	254±4.24***	323.5±17.67***
CXCL 10	58±1.41**	165.5±23.33**

IL-2	50±1.41**	152.5±2.12**
TGF-β	60±00**	103.5±2.12**
TNF α	52.5±0.7**	54±00**
IL-β	51.5±0.7**	52±00**
IL-4	49±2.82**	51±1.41**
CXCL-8	59.5±0.7**	69.5±0.7**
IL-12	53±00**	53.5±0.7**
IFN γ	51.5±0.7**	57±2.82**
IL-10	51±1.41**	54±2.82**
IL-6	52.5±0.7**	56.5±0.7**
IL-17	51.5±0.7**	53.5±0.7**

Table 2.1: Statistical significance: *- p<0.05; **- p<0.01; ***-p<0.001 (highly significant***; significant**)

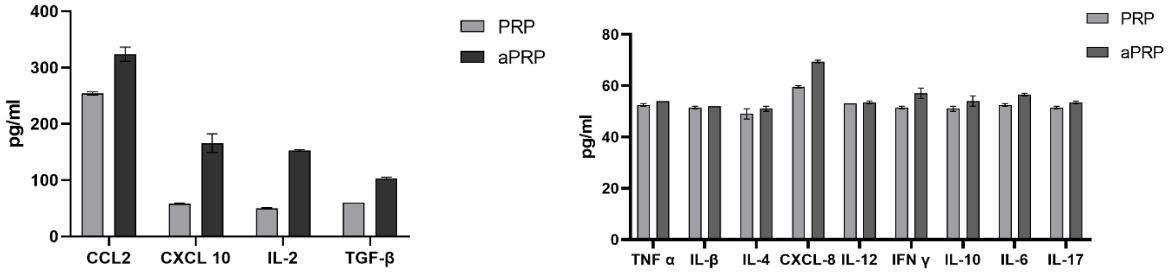

Figure 2.3: Cytokine activity of PRP and aPRP: Detection of cytokines and chemokines in PRP and activated PRP. The levels of common cytokines and chemokines CCL2, TNF- a, IL-1 BETA, CXCL8, CXCL10, IL2, IL4, IL-6 IL-10, IL-12, IL-17A, TGF-BETA, IFN-γ in rat PRP were assessed using LEGENDplex Rat Th Cytokine Panel (13-plex) (Biolegend, San Diego, CA) Kit. It showed high levels of IL-6 and IL-8, while low levels of IL-1a, IL-1β and GM-CSF were detected.

2.3.2. Intrauterine Infusion of aPRP restores endometrial epithelium in a DE rat Model

The effect of aPRP was assessed on DE at 1,3,6,9 days after aPRP treatment. For this purpose, we conducted hematoxylin and eosin staining (HE). In tissue sections from DE that did not undergo PRP treatment (DE group), HE revealed thin endometrial atrophic columnar epithelium lining, with degenerative changes and discrete stroma (Figure 2.4, middle panel). However, well

organized proliferated glandular and endometrial stromal cells were observed in the aPRP treated group (Figure 2.4, right panel) as compared to control (Figure 2.4, Left panel). Epithelial thickness in aPRP treated D1, D3, D6 and D9 revealed an increase of 1.51 fold, 2.30 fold, 1.52 fold and 2.62 fold, respectively. As compared to control and aPRP treated group, DE group showed much decline in epithelial thickness. These results specified that aPRP treatment on DE encouraged the recovery of endometrial structure in disturbed endometrium (Figure 2.5). Evaluation of epithelial thickness was calculated by using image J software (Table 2.2).

Table 2.2: Estimation of epithelial thickening of endometrium

Variables	Control (µm)	DE (µm)	aPRP treated (µm)
Day 1	165.06±17.37***	42.87±10.69**	250.25±16.73***
Day 3	120.85±16.49***	50.19±17.30**	276.42±26.73***
Day 6	103.06±23.15***	78.15±23.97**	157.26±30.95***
Day 9	81.55±29.11***	96.71±26.57**	213.05±24.98***

Statistical significance: Statistical significance: *- p<0.05; **- p<0.01; ***-p<0.001 (highly significant***; significant**)

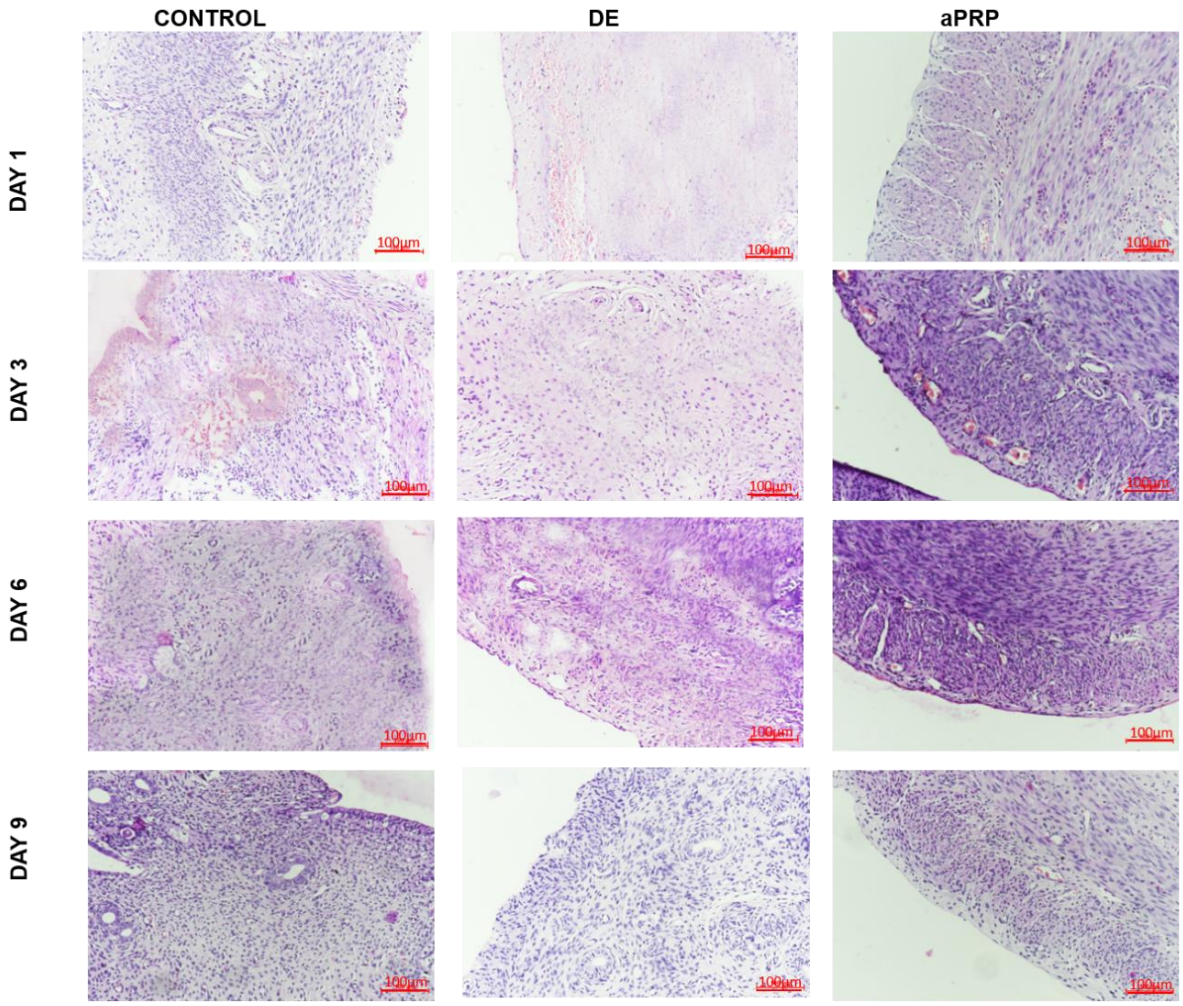

Figure 2.4: Hematoxylin and eosin analysis: The aPRP infusion improves regeneration of the DE. Endometrial tissue staining evaluated morphologic structures. Control (left panel) showed normal morphology of epithelial cells. DE (middle panel) evaluated thin and discrete epithelial morphology. The endometrial lining was significantly thicker in the experimental group than in the DE group (P<0.1). The aPRP-treated (right panel) revealed normal epithelial morphology similar to control group. There was no significant difference between the experimental group and the normal group. (Magnification 20X).

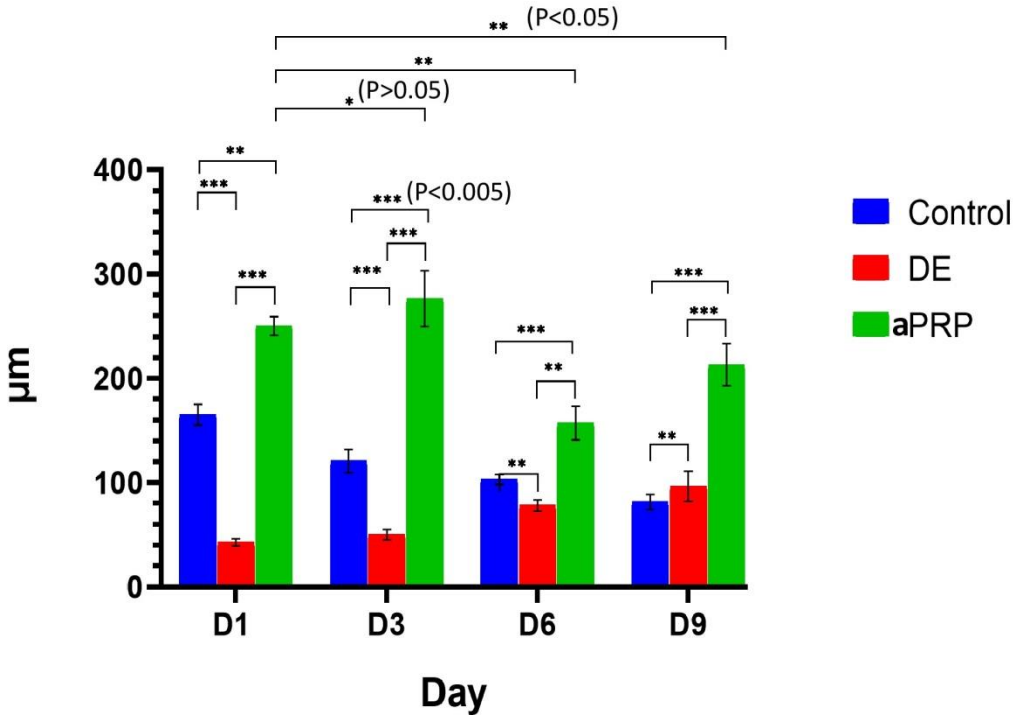

Figure 2.5: Evaluation of Epithelial thickness: The thickness of the endometrial epithelium was measured by image-j software, wherein the thickness decreased significantly after the disruption of the endometrium. Increase in thickness was observed in aPRP treated group. Statistical significance: Statistical significance: *- p<0.05; **- p<0.01; ***- p<0.001(highly significant***; significant**)

2.3.3. Immunohistochemical evaluation

Positive nuclei to a-SMA, CK18, CK 19, Cx-40, E-Cad, Cla-1, ZO-1 were fluorescent green stained. The immunohistochemical expressions were evaluated in the surface epithelial cells, glandular epithelium and stromal cells. These expressions from aPRP treated group were compared with control and DE group. Colon cancer samples were taken as positive control. The colon cancer tissue sections are known to express the above protein of interest. A positive expression from positive control confirms that the IHC protocol is working and optimized. Treated animals showed the highest expressions in surface epithelium, stromal region and glandular epithelium at D1, D3, D6 and D9 compared to DE group (P<0.05). The control animals attained high expression score in surface epithelium and stromal cells that is not statistically different compared to treated D1group (P>0.05).

CK was mainly localized in the endometrial epithelial cells and endometrial glands; whereas, the expressions of Cx-40, E-Cad, Cla-1 and ZO-1 were observed in the endometrial stromal cells and endometrial epithelial cells. IHC nuclear staining for a-SMA was observed nearly similar to control group in the endometrial cells. Compared to the control group, quantitative comparison of IHC staining in the DE group showed significantly decreased expressions of a-SMA, CK18, CK 19, Cx-40, E-Cad, Cla-1 and ZO-1 in all days group. The expression of these factors was significantly higher in the activated PRP treated group, compared to the DE group. In addition, there was no significant difference in the expression of these factors between the PRP-treated group and the control group (Figure 2.6).

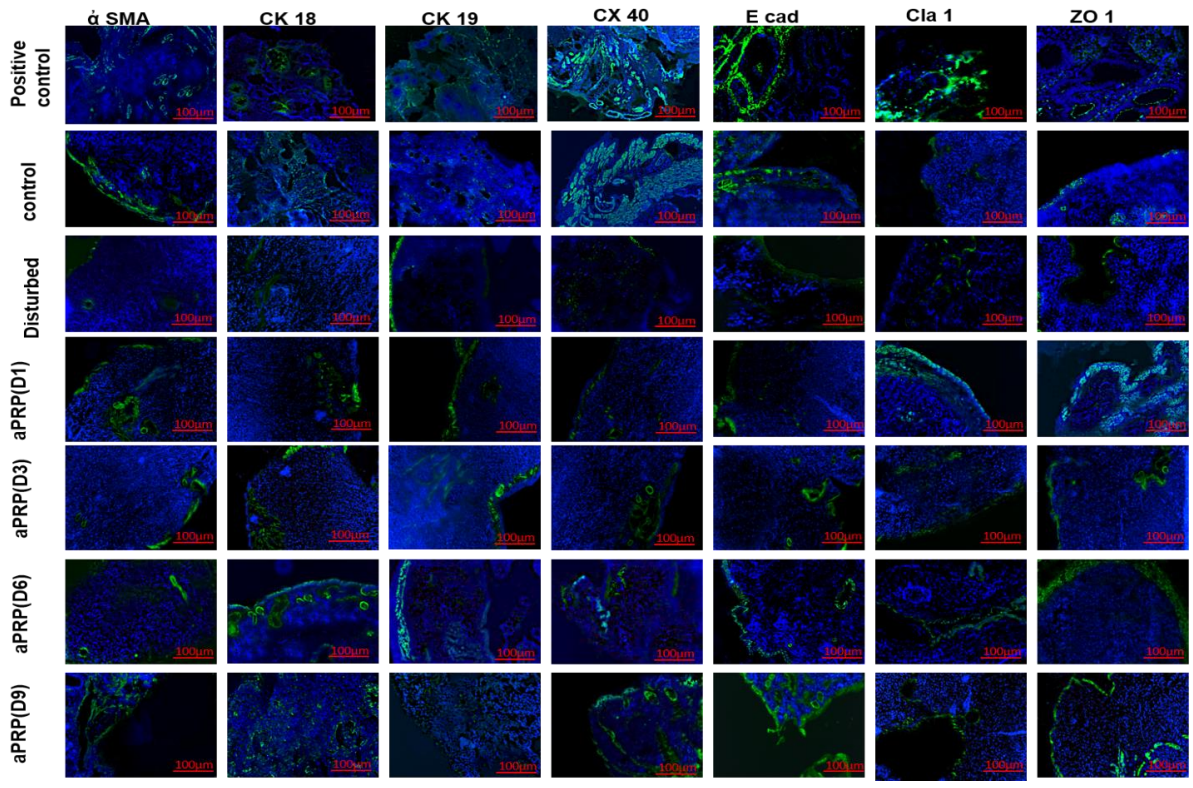

Figure 2.6: Immunohistochemistry of aPRP: Immunofluorescence staining (magnification 20X) of a-SMA, CK18, CK 19, Cx-40, E-Cad, Cla-1 and ZO-1 in the aPRP-treated group. Fluorescent Green expressions indicates positive signal.

2.3.4. Treatment of Activated PRP in DE Improves Live pups in Wistar rat

Wistar rats took an average of three days to conceive, as confirmed by vaginal plug smears.

The female rat was mated with a fertile male. All rats from control group gave birth to live pups. DE group did not give birth to any live pups. All rats from aPRP treated groups delivered pups. Control group delivered average 8 live pups while aPRP treated group gave birth to 13 pups (table 2.3). Though the average days required to conceive for aPRP treated group was little longer, the number of live pups indicates that aPRP treatment improves better receptivity. These results indicate that endometrial disruption caused by endometrial injury could be restored by aPRP treatment. The aPRP treatment clearly improved the rate of live-births since DE group failed to deliver.

Table 2.3: Activated PRP treatment in DE Improves live birth

Variables	Control group	DE group	aPRP treated group
Live births	8	-	13
average days to conceive	3 days		6 days

2.4. Discussion

This study proposes that intrauterine administration of autologous PRP exerts morphological proliferative effects on damaged endometrium. PRP is known to restrain growth factors and cytokines that accelerate cell proliferation, vascular angiogenesis and cell migration resulting in speedy healing and regeneration(13).

Till date, there have been no specific studies evaluating the effects of thrombin activated PRP on the endometrial regeneration in murine models. This study revealed that CCL2, TNF- α, IL-1 β, CXCL8, CXCL10, IL2, IL4, IL-6 IL-10,

IL-12, IL-17A, TGF- β, IFN-γ play critical roles in cellular proliferation within the endometrium. These cytokines and chemokines produced by aPRP potentially exhibited an anti-inflammatory potential. Since PRP was activated and lysed by the action of thrombin, PRP was capable to release cytokines. Thereby, making it an easier and more effective strategy to deliver platelet bioactive molecules.

In the present study, using cytokine and chemokine profiling, we detected the expression of CCL2, TNF- α, IL-1β, CXCL8, CXCL10, IL2, IL4, IL-6 IL-10, IL-12, IL-17A, TGF-β, IFN-γ. It revealed significantly increased expression of CCl2, CXCL 10, IL-2, TGF-β and negligible amounts of TNF-α, IL 6, IL 10 and IL 17 but no significant change in the expressions of IL- β, IL 4 and IL 12 is noted in aPRP as compared to the non-activated PRP ($p < 0.05$). The aPRP secrete elevated levels of IL 6, IL 10 and IL 17 as an anti-inflammatory cytokine, which is mediated through inhibitory effects on TNF-α.

Thus, the high levels of IL 6, IL 10 and IL 17 produced by aPRP may counterbalance the disturbed endometrium induced inflammation caused by pro-inflammatory cytokines. CCL2 regulates cellular adhesion through activation of β integrin. TGF-β and CXCL 8 and CXCL10 is involved in wound healing cascade. CCl2 and TGF-β both are engaged in normal progression of the healing processes. These cytokines and chemokines are involved in modulation of endometrial cell growth, proliferation and implantation.

Endometrial epithelial lining and junction proteins are studied by HE and IHC analysis, respectively. Treatment of thrombin activated PRP on DE rat model illustrated the enhancement of epithelial proliferation in endometrium. Moreover, treatment with PRP may suggest decrease of fibrotic lesions in disturbed endometrium. These results provided correlative evidence for the possible use of activated PRP in achieving implantation with improvement in uterine vascularization and endometrial receptivity. Immunohistochemical analysis on the expression of α-SMA, CK18, CK 19, Cx-40, E-Cad, Cla-1 and

ZO-1 showed significant differences between the DE group and aPRP-treated groups. In the present study, an increased expressions of above-mentioned proteins were noted in the aPRP-treated group, compared to the DE group which indicates cellular proliferation in the endometrium.

Additionally, a-SMA is known to play a central role in remodeling of cellular functions and healing process. The intensity of CK 18 and CK 19 was significantly strong in the aPRP-treated group, compared to the DE group located in the epithelial lining and columnar epithelium. CK is cellular marker for the epithelium. Thus, aPRP may accelerate induction of endometrial epithelial differentiation. Our analysis revealed the expressions of Cx 40 and E-Cad at stromal endometrium and epithelial lining regulating cell adhesion and proper exchange of molecules within cells. Cla-1, ZO-1 along with Cx 40 and E-Cad involved in regulating endometrial function and development, along with endometrial receptivity for establishing the necessary conditions for implantation in rats.

The expressions of junction proteins identified as cell surface specific markers of stem cells in the human endometrium. These results assisted us to consider that endometrial stem cells may be involved in the proliferative and effects of aPRP on disturbed endometrium. The aPRP may stimulate the endometrial stem cells and promote regeneration. Hence the combined use of aPRP and stem cells may improve the outcome of treatment for disturbed endometrium.

On the other hand, functional analysis of PRP infusion effectiveness is clarified. The intrauterine infusion of aPRP can promote endometrial regeneration after endometrial damage and improve endometrial receptivity in a DE rat model by restoring histological structure. Furthermore, aPRP treatment augmented receptivity outcomes. The rats carried full term pregnancy and delivered healthy pups, thereby, ascertaining the clinical use of aPRP in disrupted endometrium. The results suggest that aPRP infusion may

be a productive approach for treating impaired endometrium in the clinical setting.

Another significant task is to determine which PRP elements improve which p athways and to investigate the underlying mechanisms. The PRP infusion affects the pro-inflammatory factors and endometrial proliferation, emphasizing the valuable effect of aPRP treatment in the murine model.

We demonstrated that aPRP helps to restore endometrial functional morphology and improve implantation outcomes following endometrial disturbance in rats, enabling full term delivery and live-births.

These promoting findings set up the theoretical basis for the capacity of aPRP to promote endometrial regeneration and improve implantation outcomes. It will support the clinical application of aPRP treatment with compromised endometrial pathologies.

However, it is unclear whether the rat model accurately represents human phenotypes. In that manner, further studies are required for evaluating the usefulness of aPRP in the humans.

2.5. Conclusions

This study showed positive effects of aPRP as a potential treatment for disturbed unresponsive endometrium. It restores functional endometrial morphology to improve receptivity. The clinical application of aPRP and its effects on compromised endometrium are still at a preliminary stage. Further explorations of study and clinical trials are necessitated for the optimization of aPRP application along with a larger randomized study to determine the effect of PRP in implantation failure in human.

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Chapter – 3

Bone marrow-derived mononuclear cells (MNCs) followed by activated PRP in Murine Model of Disturbed Endometrium

3.1. Introduction

The physiological angiogenesis in endometrium tends to the dynamic nature of its regeneration. For successful implantation of developing embryo, the endometrium undergoes cyclic growth making it receptive in nature. Various growth factors, cytokines and ovarian steroid hormones, play an important role in regulating the cyclic growth of endometrial cells (1). Generally, it is observed that the receptivity of endometrium is compromised in case of infertility and recurrent pregnancy loss. Till date no reliable method has been established to assess the endometrial receptivity (2). Despite the use of various treatments such as, exogenous estrogen, aspirin, vitamin E and sildenafil citrate, granulocyte colony stimulation factor (G-CSF), the endometrium remains unresponsive, thereby posing a major challenge (3). The endometrial receptivity is governed by the expression of different proteins that can serve as markers of endometrial receptivity. These junction proteins have been used to achieve a therapeutic approach for implantation failure. The use of protein markers promises to promote our understanding of implantation while providing clues for infertility causes. Understanding the junction proteomics in detail will likely improve endometrial receptivity and provide new insights into targeting the disturbed endometrium (4–7). The establishment of reliable biomarkers for the detection of disturbed endometrial receptivity has been remains an elusive target. Stem cell based potential therapeutic approaches have recently attracted much attention. Bone marrow derived mononuclear cells (BM MNCs) include populations of endothelial progenitor cells, lymphocytes, hematopoietic and mesenchymal stem cells. They can be easily isolated from the bone marrow. Experimental studies of MNC treatment have demonstrated the beneficial effects of MNCs are due to its effect of angiogenesis, modulation of inflammation, neurogenesis and arteriogenesis. Several clinical studies have shown the safety and efficacy of MNCs in

patients with ischemic stroke (8,9). Several animal studies and clinical studies have proposed that MNCs are a potential treatment for myocardial infarction, stroke, cerebral ischemia, limb ischemia. Autologous transplantation of use of safety and efficacy of MNCs in patients with ischemic stroke have demonstrated the beneficial effects (10–14). The cardiovascular outcomes of stem cell therapy after acute myocardial infarction revealed that BM MNCs transplantation (BM MNCs Tx) may improve outcome for heart failure. Also, it was found that, transplantation of BM-MNCs can avert depression or anxiety in chronic mild stress. It prevent increased high-mobility group box 1 and IL-1β expression in the hippocampus and increased brain-derived neurotrophic factor expression in brain associated with inflammatory and neurogenic condition (15). Bone marrow stem cells also contribute to regeneration of the endometrium (16). There are evidence that BM-MNCs promote endometrial regeneration and restoration of in an Asherman syndrome rodent model (17). On the basis of these facts, animal models of disturbed endometrium (DE) have been made by injecting 95% ethanol in intrauterine cavity of female Wistar rat. BM-MNCs isolated from the femoral bone marrow of rat were used to evaluate the regeneration of DE.

3.2. Materials and methods

3.2.1. Isolation of BM MNCs

All the animal experiments were carried out after obtaining the approval from Institutional Animal Ethics Committee (DYPMCK/IAEC/2020/JAN/01). MNCs were obtained from the tibia and femur of the five 4–6 wk old male Wistar rats (n=5) using the flashing method. The rats were anesthetized by injecting Thiosol (1gm/10 ml, 5units/100 g of body wt) intraperitoneally. The rats were shaved and disinfected the skin with alcohol [70% (v/v)] and transferred in a laminar flow cabinet. Adherent muscles, tissue, fat surrounding the tibia and femur were stripped off gently to expose the bone and knee end. Bones were cut in middle leaving both condyli intact. A

needle was inserted into the bone to aspirate the cells. The bones were subjected to several flushes using a 1 ml syringe filled with 500 units of Heparin and HES 6% in 1:1 proportion, until all the bone marrow was flushed out and continued flushing until the bone appears white. The cells were collected in Lymphoprep. The collected cells were minced and digested. The digestion was centrifuged on 2200g×20min. Removed the upper layer very carefully, leaving 1 ml on top of the buffy coat. The buffy coat was transferred in 15-mL conical tube and washed with Phosphate buffered saline (PBS). The pellet thus obtained was dissolved in 0.5ml FBS with 20% DMSO (Figure 3.1). The cell count was done in a Neubauer chamber and tested cell viability by the Trypan Blue dye exclusion test. The cells were stored in LN_2 for further studies.

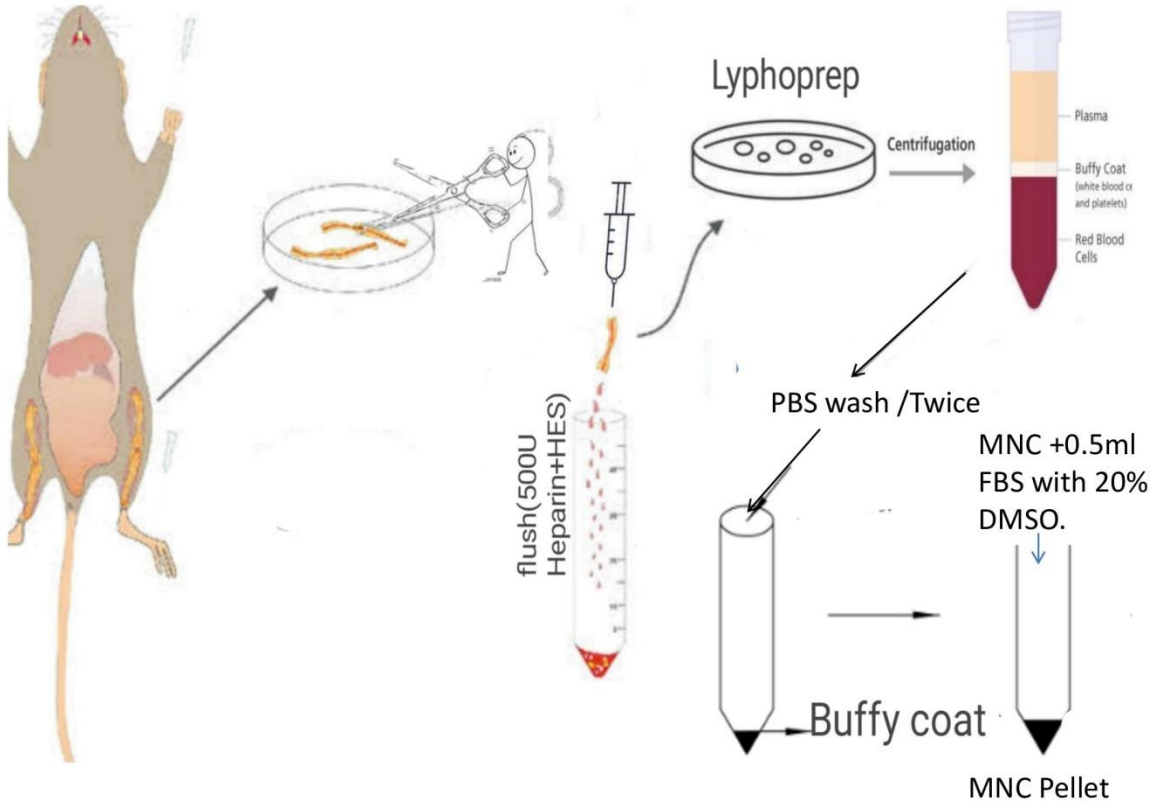

Figure 3.1: Schematic representation of isolation of BM-MNCs

3.2.2. Characterization of MNCs

Surface CD markers of the MNCs were analyzed for characterizing the MNC phenotype by flow cytometry. Mononuclear cells were acquired as described above. MNCs were thawed for five minutes at room temperature. The cells were counted in a Neubauer Chamber. A cell suspension of 1x 10^4 cells in 500ul were incubated with primary antibody for 45 min at room temperature with saturating concentrations of monoclonal antibodies Stage specific embryonic antigen – 1 (SSEA-1), Cluster of Differentiation (CD) 34, TEK tyrosine kinase (Tie-2), T cell marker (Thy), CD 133, CD 90, Delta like Homolog (DLK) (5ul each). After incubation, the cells were washed and centrifuged at 200 g, five minutes for two times in PBS. After that, they were labelled with secondary antibody Alexa 488 with dilution 1:100 for 30 minutes in the dark at room temperature followed by three PBS wash of 5min each and resuspended in 100ul PBS. Cell fluorescence was analyzed by flow cytometry in an FACS Caliber instrument (Becton Dickinson) and the data were analyzed using Cell Quest software (Becton Dickinson).

3.2.3. Cell labelling

Stock solution A is prepared by adding 1 ml of sterile PBS into the salt. Stock solution B is prepared by adding 10 ul of stock solution A with 1 ml of cell culture media. Working solution is prepared by adding 100 ul of Stock solution B to 1 ml cell culture media covering the cells. The 1×10^6 BM-MNCs were incubated in a green fluorescent salt dye as mentioned above at 37°C for 20 min and used for transplantation study. We used a green fluorescent dye to track the number and engraftment of the infused cells. To perform local intrauterine transplantation of cells, an insulin syringe with a 30-gauge needle containing the labelled cells was inserted into the middle of the right and left horns and injected into luminal cavity of the horn.

3.2.4. Experimental protocol

Herein, MNCs were harvested from the male Wistar rats and Phenotypic analysis was done by flow cytometry. We compared the effects of local intrauterine infusion of BM-MNCs on the DE in a rat model (Figure 3.2). After induction, endometrial junction expressions were evaluated at day 3, day 6 and day 9 using immunohistochemistry.

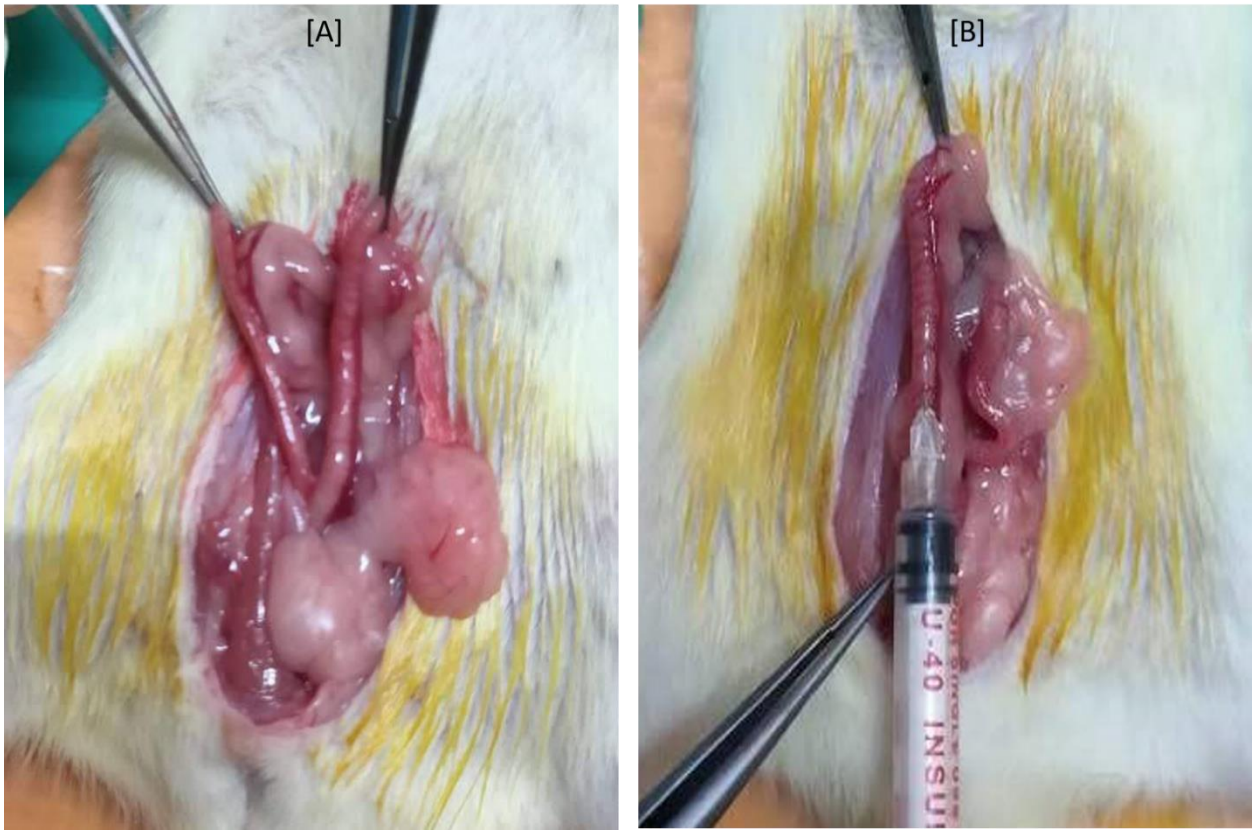

Figure 3.2: Endometrial disruption of endometrium and intrauterine infusion of MNCs: Representative photographs of uterine horns of female Rats (A) infusing 95% ethanol in bilateral horns (B) BM MNCs-aPRP Tx in bilateral uterine horns.

3.2.5. MNCs transplantation-Animal study for endometrial regeneration

Both male (n=10) and female wistar rats (n=72), weighing approximately 200-250g, were procured from the animal house. All animals were subjected to standard 12 hr light and dark cycles while maintaining temperature approximately at 24°C and relative humidity and providing free access to water and chow ad libitum. Vaginal cytology was carried out to evaluate

the stage of estrous cycle. In total 82 rats were procured, where in ten of them were male and the rest were female. The five male rats were used for isolation of MNC while five male rats were used for mating. The 48 female Wistar rats were subjected to disturb the endometrium. Out of which 24 rats were used for DE study and remaining 24 rats were subjected to MNCs-aPRP. The female rats divided into the following three groups: [1] Control group (n=24): Intrauterine horns were subjected to Normal saline. [2] DE group (n=24): Briefly, intrauterine horns were subjected to 95% Ethanol. [3] BM MNCs-aPRP group (n =24): After 24 hrs of DE, the uterus is infused with activated PRP followed by transplantation of BM-MNC (BM MNC-aPRP Tx) in intrauterine horns. The three female rats from each group were sacrificed at Day 1 (D1), Day 3 (D3), Day 6 (D6) and Day 9 (D9) interval from each group. Remaining three animals were used to confirm pregnancy outcome. Experimental DE and BM MNC-aPRP Tx group at day 1 (D1), day 3 (D3), day 6 (D6) and day 9 (D9) is compared with respective control group to analyse the results.

3.2.6. Surgical procedure

All the animals were anesthetized via intra-peritoneal administration of 12 units thiosol (1gm/10 ml, Neon laboratories Ltd.) per gram of body weight (15). In the control group, using a small curved hemostatic forceps, both the uterine horns were externalised and clamped over the cervix and was subjected to intrauterine infusion of 0.5ml normal saline. As shown in Figure 3.2. A, n=48 rats in experimental group were subjected to endometrial disruption by intrauterine infusion of 0.5ml 95% Ethanol. After the disruption of endometrium, n=24 rats were used for further transplantation study. The PRP preparation and its activation by thrombin was conducted as described in previous chapter 2. The BM-MNCs group received intrauterine infusion of activated PRP followed by transplantation of labelled 1×10^6 BM-MNCs (1:1),

as described above. After D1, D3, D6 and D9 day of transplantation, three rats from each group were sacrificed and the uterine horns were excised. The mid part of uterine horns were excised. It immediately snap frozen and cryosectioned to ensure the engraftment of BM-MNCs and bit of excised portions were fixed in 10% neutral buffer formalin for further histological and immunohistochemical analysis. Animals were sacrificed post experiment by cervical dislocation. A yellow-colored category No.2 biomedical waste management bangs were used for seal sacrificed rats. It was given to central incineration facility in Animal House of D. Y. Patil Medical College, Kasaba Bawada, Kolhapur.

3.2.7. Quantification of engrafted BM-MNCs

To quantify the number of engrafted BM-MNCs, 100 serial sections of 5 µm thickness were sectioned from the frozen tissue. Transplanted cells were already stained with green fluorescent dye. Tissue Sections were counterstained with a nuclear stain DAPI. The numbers of engrafted BM MNCs were established by enumerating green fluorescent dye-stained BM MNCs in endometrium recruited from experimental transplanted groups. At a 20X magnification, the visual field of the Nikon camera corresponds to an area of 0.12mm^2. The central regions of such visual fields on each section were counted, corresponding to a surface area of 0.1mm2/section. The number of engrafted cells on a 1000µm^2 section was concluded from the number of cells/0.1mm^2 by multiplying by a factor of 3.33. This number was again multiplied by 200 to get the total number of cells/mm^2. The cells on every section were calculated twice the same examiner.

3.2.8. Hematoxylin-eosin staining analysis

The paraffin embedded uterine specimens were sectioned into 4-µm thick sections to stain with hematoxylin-eosin (H&E). Endometrial morphology was

analyzed by H&E staining, and images were captured using the fluorescence microscope (Nikon; Japan). Images were captured at magnifications of ×20. To determine the area of the endometrium (µm), each slide was analyzed in a double-blinded manner by two experts using Image J image analysis software.

3.2.9. Immunohistochemical staining

The uterine horns excised from each group on days 1, 3, 6 and 9, were fixed in 10% neutral buffer formalin. The fixed tissued were embedded in paraffin and 4µm thick sections were processed for immunohistochemical staining. The sections were subjected to 60ºC in oven for 10 minutes and treated with xylene for deparaffinization. After deparaffinization, the sections were rehydrated in a descending series of alcohol grades and finally placing them in 0.05% Tween-20 (Sigma-Aldrich) in distilled water. The antigen retrieval was performed for α-SMA (mouse monoclonal: AbDSerotec), CK-18 (mouse monoclonal: AbDSerotec) and CK-19 (mouse monoclonal: AbDSerotec), Connexin (CX)-40 (mouse monoclonal: AbDSerotec), E-Cad (Mab anti human: Invitrogen), Cla-1 (Rabbit anti human: Invitrogen), ZO-1 (Mab anti human: Invitrogen), by placing the slides in 10 mmol trisodium citrate buffer (pH-6.0) and subjecting them 100°C in pressure cooker for 20 min followed by a cooling period of 20 minutes. The sections were treated with goat serum for 45 minutes at room temperature in order to reduce nonspecific binding of antibodies. The sections were then treated with primary antibodies E-Cad, Cla-1, ZO-1, CK-18 with dilution 1:100 and CK 19, CX-40, with dilution 1:200 in BSA (Hi Media), and incubated overnight in a humidified chamber followed by couple of washes in 0.05% Tween-20 in distilled water. The sections were further treated with Alexa 488 (molecular probe) labelled secondary antibodies in dark for 60 mins at room temperature. The slides were washed again in 0.05% Tween-20 in distilled water, then counter stained using DAPI

(Invitogen) and mounted in fluorescent mounting media (Dako). Tight junctions encompass ZO-1 and Cla-1, adherence junction comprises E-Cad and gap junctions contain Cx-40. CK 18 and CK 19 are epithelial markers. The a-SMA is smooth muscle cell marker. The sections were visualized under fluorescent microscope (NIKON) while two investigators graded a-SMA, CK-18, CK 19, Connexin-40, E-Cad, Cla-1 and ZO-1 expression in a blinded fashion and assessed immunostaining using H score. H score =Σ Pi (i +1), where i is intensity of staining (16,17). Intensity of staining was assessed by blinded investigators as no E-Cad, Cla-1, ZO-1, CK-18, CK 19, and Connexin-40 and a-SMA signal (0). E-Cad, Cla-1, ZO-1, CK-18, CK 19, Connexin-40 and a-SMA positive samples were defined as having weak (1), moderate (2) or strong (3) signal and Pi is the percentage of stained epithelial cells ranging from 0% to 100%. The H-score ranges from 0 to 4.

3.2.10. BM MNCs-aPRP Tx improves live birth rate in transplant group

Female rats in BM MNCs-aPRP Tx group were mated with healthy males at seven days after after BM MNCs-aPRP treatment. Pregnancy outcomes included the time to conceive, live-birth rate and litter size.

Statistical Analysis

All values represent means ± SD. Statistical analyses were performed using the Tukey's multiple comparisons test for more than two groups. Values of $p < 0.0001$ were considered to denote statistical significance. SAVanalytics software was used for statistical analysis.

3.3. Results

3.3.1. Verification of BM MNCs with surface markers and flow cytometry

Our flow cytometry data showed that BM MNCs were SSEA-1, CD 34, Tie-2, Thy, CD 133, CD 90, DLK positive. BM MNCs express typical MNC markers were

characterized by flow cytometry using monoclonal antibodies specific for several surface markers. BM MNCs were strongly positive for CD133 and CD 90. They were also positive for CD 34, Tie 2, Thy, DLK. BM- MNCs showed moderate expression of Tie 2, Thy, DLK (Figure 3.3)

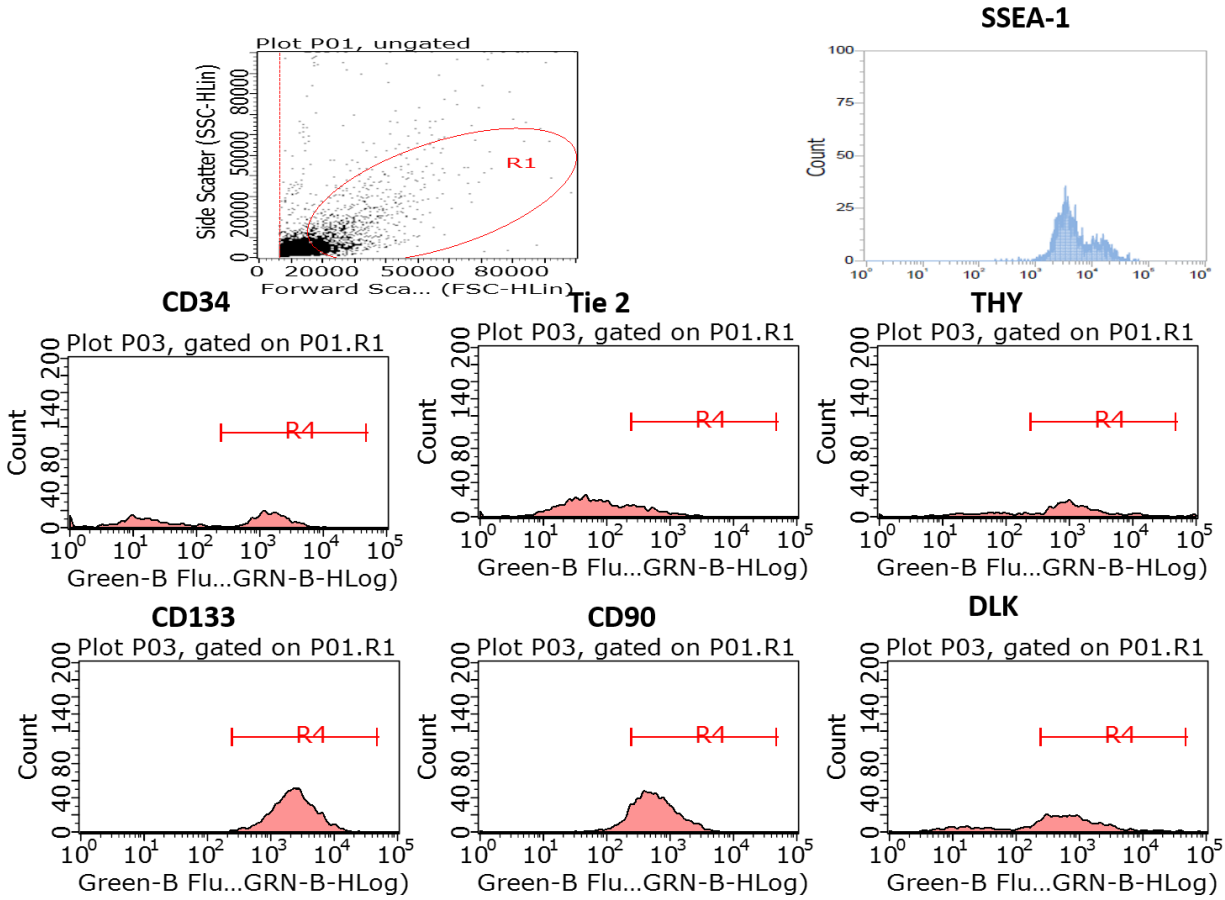

Figure 3.3: Verification of BM- MNCs with surface markers: BM MNCs express the typical MNC specific markers SSEA-1, CD 34, Tie-2, Thy, CD 133, CD 90, DLK. MNCs were stained with monoclonal antibodies specific for MNCs and examined by flow cytometry by forward and side scatter properties (top left panel). Mean fluorescence intensity is shown in each sample. Data are representative of three replicate experiments

3.3.2. Quantification of BM MNCs

As shown in Figure 3.4, in all DE groups, some of the infused cells were localized in the uterus that appears in fluorescent. Local administration of BM MNCs resulted in the recruitment of cells to the DE area ($p<0.001$). Engraftment of BM MNCs in disturbed endometrium was verified by the use of

nanoparticle labelled BM MNCs (Figure 3.4)followed by activated PRP (refer Figure 2.2 and Table 2.1). Scattered labelled BM MNCs were detected between the stromal regions of endometrium in rat at Day 1, Day 3, Day 6 and Day 9. The number of labelled BM MNCs in Day 1, Day 3 and Day 6 group was 37,296±666 cells/mm^2 (P<0.001), 22,644±650cells/mm^2 (P<0.001), 16,100±507.44 mm^2 (P<0.001) respectively (figure 3.5). Endometrial sections from Day 9 did not show any engrafted BM- MNCs. Endometrial sections from Day 1 showed clustered cells while Day 3 and 6 showed scattered cells. Endometrial sections from Day 9 group revealed no cells positive for labelled cells. The Day 1 group had higher numbers of labelled cells compared with other groups.

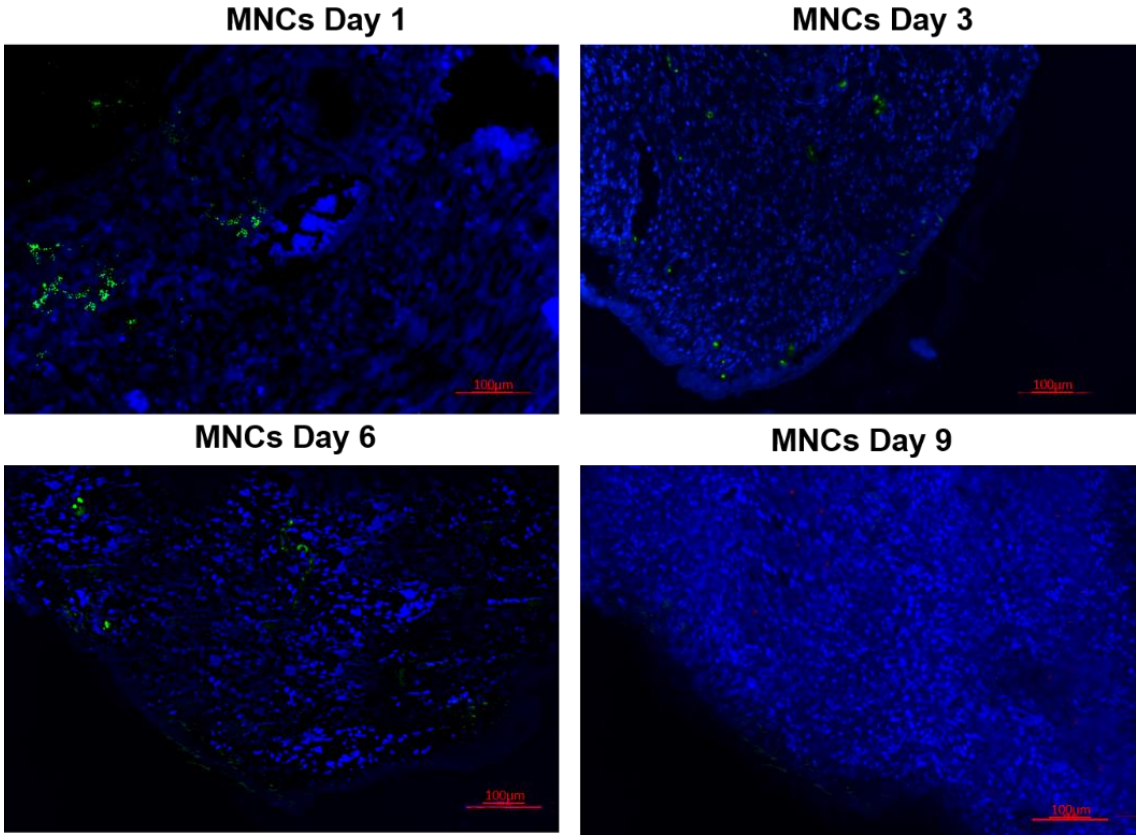

Figure 3.4: Estimation of transplanted MNC's: (Magnification 20X) The DE group received 1×10^6 BM-MNCs. Cells were transplanted into the disrupted endometrial cells. Paraffin-embedded sections stained with nanoparticles showed the presence of transplanted BM MNCs (fluorescent signals). Nuclei were counterstained with DAPI (blue).

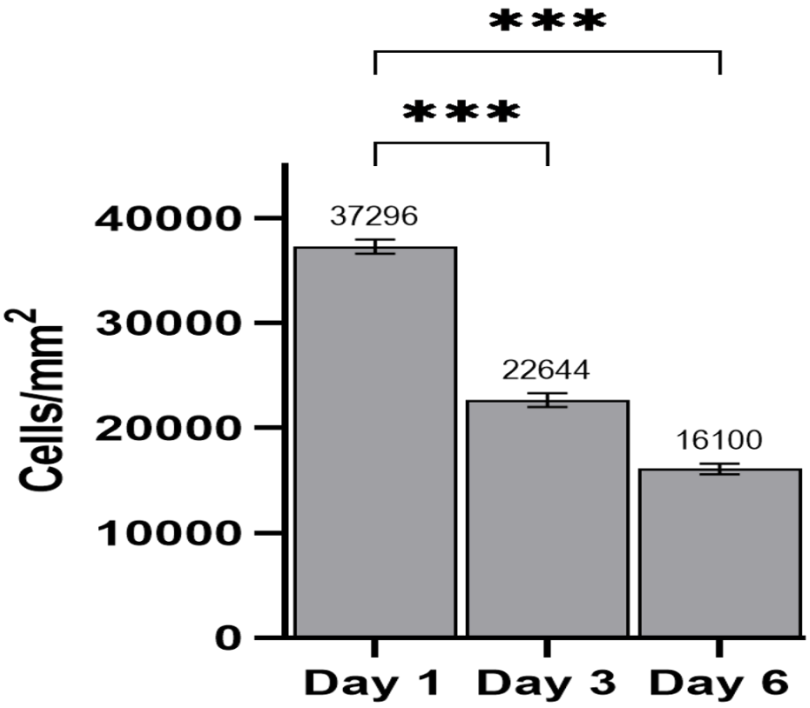

Figure 3.5: Quantification of transplanted MNC: Engrafted cells in the BM MNCs-aPRP group were counted manually from two different endometrial sites and presented in cells in cm/mm^2. Endometrial sections from Day 9 did not show any engrafted BM- MNCs. Endometrial sections from Day 9 group revealed no cells positive for nanoparticles-labelled cells whereas Day 1 group had higher numbers of nanoparticle-labelled cells compared with other groups.

3.3.3. Hematoxylin & eosin staining by evaluation of epithelial thickness

Endometrial thickness was evaluated using hematoxylin eosin staining (Figure 3.6). The data analysis showed that control group has greatest endometrial thickness ($p < 0.001$). There was no significant difference in endometrial thickness between Day 1, Day 3 and Day 6 of DE group. DE Day 9 showed efficient endometrial thickness. Transplantation of BM MNCs remarkably increased endometrial thickness as compared to DE group ($p< 0.001$). This confirms BM MNCs-aPRP is effective in improving endometrial thickness by regenerating endometrial cells.

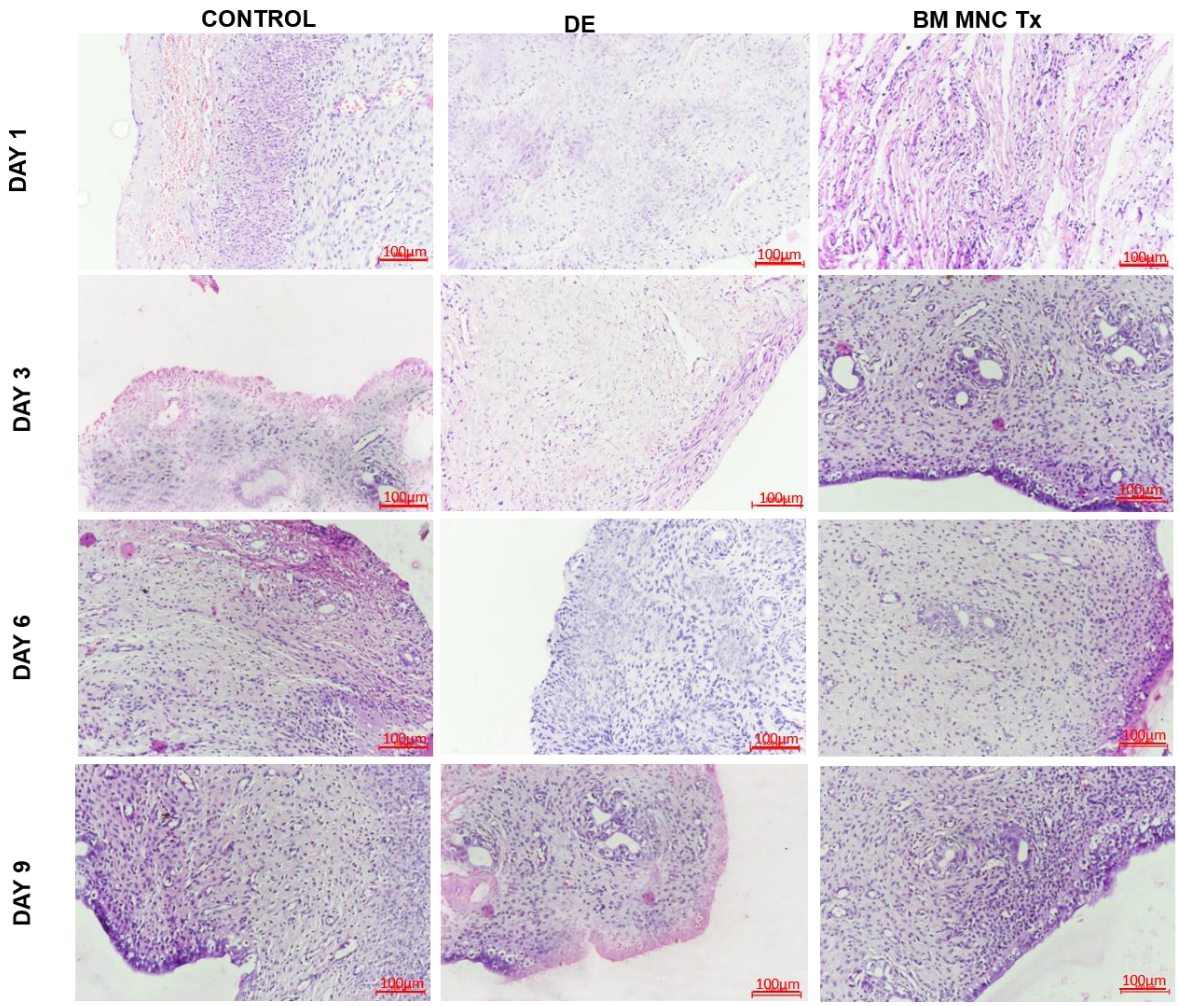

Figure 3.6: Hematoxylin and eosin analysis: Evaluation of epithelial lining in control group has greatest endometrial thickness ($p < 0.001$). Transplantation of BM MNCs remarkably increased endometrial thickness as compared to DE group ($p< 0.001$).

As compared to DE group, epithelial thickening in BM MNCs-aPRP Tx group is elevated (Table 3.1). Difference in endometrial thickness was significant between the control and BM MNCs-aPRP Tx group as compared to DE group ($p< 0.001$) (180.07±10.27 µm in control group and 78.57±14.16 µm in BM MNCs-aPRP Tx group) on day 1. Endometrial thickness on the day 3 of BM MNCs-aPRP Tx was 14.10±13.89 µm in control group and 67.54±14.16 µm in BM MNCs-aPRP Tx group which was significantly different ($p < 0.001$) from DE group. Endometrial lining on the day 6 of BM MNCs-aPRP Tx was 40.98±25.20 µm in control group and 43.67±20.63 µm in BM MNCs-aPRP Tx group which

was significantly different ($p < 0.001$) from DE group. Likewise, day 9 revealed 44.91±30.17 µm and 71.37±26.91 µm in control and transplanted group respectively (Figure 3.7).

Table 3.1: **Evaluation of epithelial thickness**

Variables	Control (µm)	DE (µm)	BM MNCs-aPRP Tx(µm)
D1	180.07±10.27***	306.15±9.81**	78.57±14.16*
D3	14.10±13.89**	25.37±18.07***	67.54±14.78***
D6	40.98±25.20**	46.66±19.32**	43.67±20.63*
D9	44.91±30.17**	9.40±71.37**	71.37±26.91**

Table 3.1: Statistical significance: *- p<0.0481; **- p<0.0019; ***-p<0.0003; BM MNCs-aPRP Tx effects on the DE (n=24), compared with control (n=24) and transplant group (n=24). (Highly significant***; significant**)

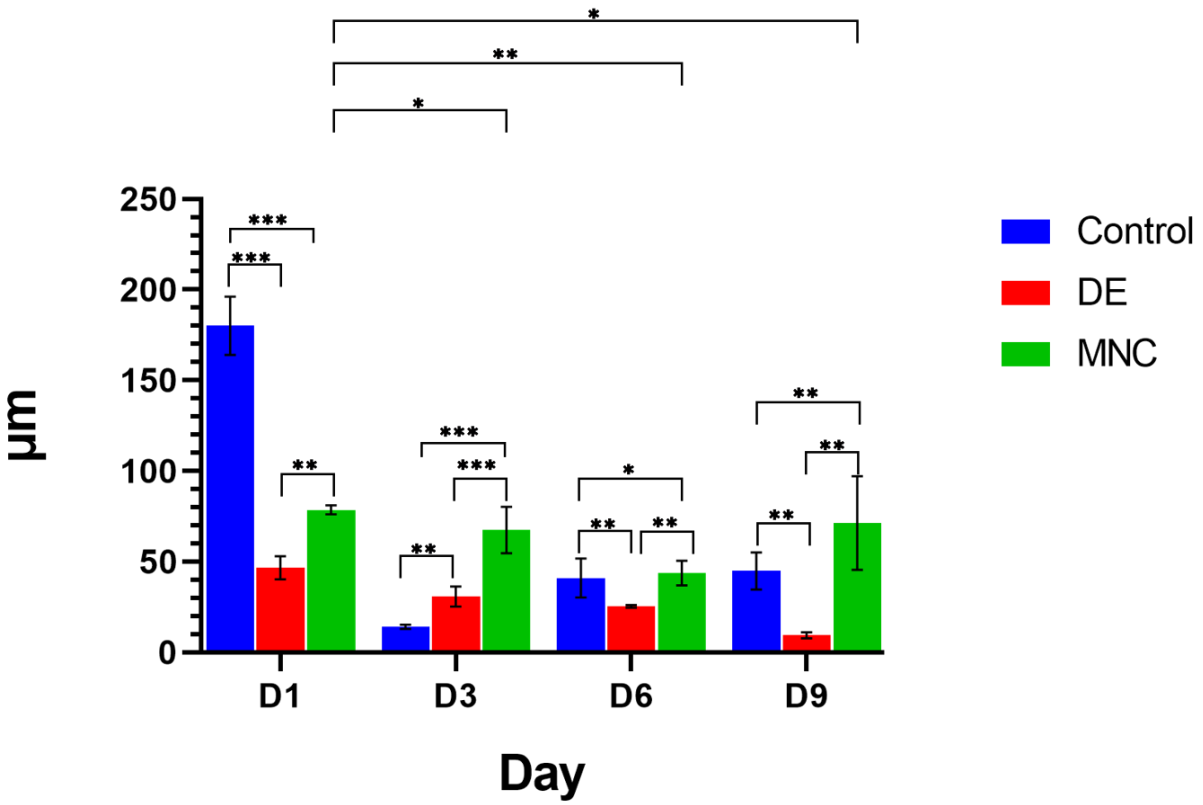

Figure 3.7: Evaluation of epithelial thickness. Statistical significance: *- p<0.05; **- p<0.01; ***- p<0.001(highly significant***; significant**)

3.3.4. Immunohistochemical analysis to ensure the regeneration of DE

Immunohistochemical analysis of the tissue samples allowed an objective evaluation of the tissue expression of a-SMA, CK 18, CK 19, Cx 40, E cad, Cla-1 and ZO-1. We explored the effects of BM MNCs transplant (BM MNCs-aPRP Tx) in disturbed endometrium. We infused the activated PRP followed by BM MNCs Tx into disturbed uterine cavities of rat and compared the results at Day 1, Day 3, Day 6 and Day 9 with respective control and disturbed endometrium. Engraftment of BM MNCs Tx was verified by the use of antibodies specific for a-SMA, CK 18, CK 19, Cx 40, E cad, Cla-1 and ZO-1. There was a significant decrease in the expression of a-SMA, CK 18, CK 19, Cx 40, E cad, Cla-1 and ZO-1 in the DE compared with the control group and BM-MNCs Tx. However, there was no much difference in the tissue expression of DE group comparing DE Day 1, 3,6 and 9 with each other. Scattered expressions were detected between the stromal regions of disturbed endometrium at Day 3, Day 6, Day 9. DE at Day 1 did not show any expressions. In BM MNCs-aPRP Tx, endometrial sections of Day 3 and Day 6 group revealed extremely few expressions as compared to Day 9 group. IHC expression was mainly localized in the endometrial glandular and stromal region. The expressions of a-SMA, CK 18, CK 19, E cad and Cla-1 were detected in the endometrial stromal cells whereas ZO-1 in stratum functionalis region. The DE at Day 9 group had weak expressions of a-SMA, CK 19 Cla-1 in stratum functionalis cells. BM MNCs-aPRP Tx group was obviously showed increased expressions of these factors as compared with disturbed endometrium group. The BM MNCs-aPRP Tx treated group at Day 6, 9 demonstrated comparatively strong expressions of a-SMA, CK 19, Cx 40 and E-cad while moderate expressions of CK 18, and Cla-1 and comparatively less expressions of CK-19 and ZO-1. The BM MNCs-aPRP Tx treated group had higher expressions of a-SMA and E-cad (Figure 3.8).

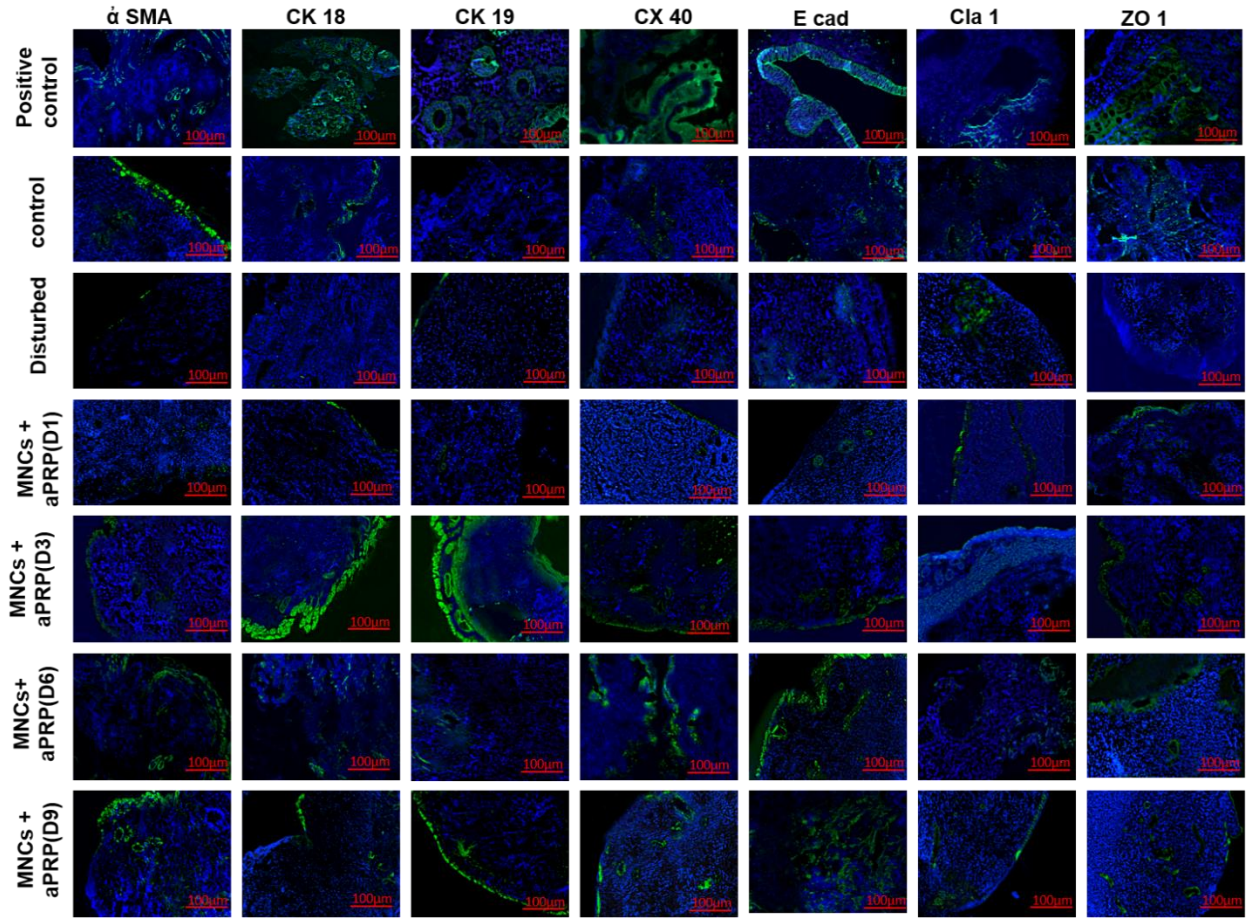

Figure 3.8: Immunohistochemistry of MNCs transplanted endometrium.

3.3.5. BM MNCs-aPRP Tx improves live birth rate in female rats

In BM MNCs-aPRP Tx group, the treated female rats were mated with a fertile male. Vaginal plug smears conducted daily starting on the morning after the female was mated with a fertile male to confirm whether they are concived or not. The results indicate that implantation was hamperd by disturbed endometrium but restored by BM MNCs-aPRP Tx group. While all control group gave birth to live pups, DE group did not give birth to any live pups and BM MNCs-aPRP Tx group delivered healthy pups. The BM MNCs-aPRP Tx clearly improved the rate of live-births since DE group failed to conceive (Table 3.2).

Table 3.2: Rate of pregnancy in control, DE and BM MNCs aPRP Tx groups

Variables	Control group	DE group	BM MNCs-aPRP Tx group
Pregnancy rate	5.4%**	-	10.3%***

Statistical significance: **-($p < 0.001$), ***- ($p < 0.001$). Pregnancy rate was 5.4% in control group and 10.3% in Tx group which was significant ($p < 0.001$).

3.4. Discussion

Prianishnikov was the first to discover the endometrial adult stem cells (ASCs) present in the basalis layer, with their differentiation acquired by functional changes in hormonal receptivity(18). Endometrium regenerates the new upper functionalis layer from the basalis layer after menstruation. The epithelial cells proliferate from gland stumps to reconstruct the surface epithelium. It remains possible, however, that the stratum basalis harbors stem cells or progenitor cells which plays crucial role in endometrial re-epithelisation. Endometrial side population exhibited several endothelial cell markers expression compared to endometrial main population cells(19).

Indeed, BM MNCs population, which presumably includes endothelial cell markers expression of SSEA-1, CD 34, Tie 2, Thy, CD 133, CD 90 and DLK having stem cell-like properties. SSEA-1express endometrial epithelial ASCs and represent the endometrial stem/progenitor cell compartment playing an important role in homing stem cells. It also support the existence of epithelial stem cells in the endometrium that are involved in glandular regeneration (20). CD34 has been linked to facilitation of adhesion, cell proliferation, and regulation of differentiation. The CD34 population of cells shows a more pronounced differentiation capacity (21). Also, Thy is expressed on epithelial cells and variety of other stem cells. It has speculated role in cell-cell and cell-matrix interaction. plays major role in cell growth, development and accelerates cell proliferation. Cells isolated by using CD133 antibodies are

being able to enhance angiogenesis, and functional recovery(22,23). CD 90 and Dlk1, a member of the Epidermal Growth Factor family, is expressed in multiple tissues during development(24). Above mentioned positive expressions suggest that recruitment of BM MNCs at DE will regenerate and re epithelize the endothelium of both the functionalis and basalis layers of the endometrium. In BM MNCs cell transplantation, engraftment of these cells in the DE may be influenced by deficiency of nutrients, inflammatory response, fibrosis formation, damaged vasculature and hypoxia. To keep the infused cells intact and to optimize the transplantation conditions to withstand the unfavorable conditions, we transplanted BM MNCs with thrombin activated PRPs. We found that BM MNCs transplant followed by thrombin activated PRP forms gel layer which hold the cells intact. Joshi et al.,(25) explained that the MSC produces elevated levels of IL-6 and IL-8 to counterbalance the hepatocyte transplantation-induced liver inflammation. Likewise, the different chemokines and cytokines release reported by thrombin activated PRP. It is essential to optimize whether the BM MNCs transplantation withstands these conditions after cell infusion. As cells get trapped in PRP gel, the transplanted cells will get selective expansion advantage into the disturbed endometrial environment and the cells will get chance to acclimatize and expand. However, there have been no studies to evaluate the recruitment capacity of these cells to the endometrium. During transplant, we labelled the cells with quantum dots.

The Tx group showed the total cells engrafted per mm^3 was 37,296±666. 22,644±650 and 16,100±507.44 at transplant day 1, day 3 and Day 6 respectively. It showed 40%, 56.84% decline in cell count at Day 3 and day 6 as compared to Day 1 ($p \leq 0.001$). The labelled cell studies confirm evidence that cells get transplanted to the DE and got engraftment. These cells regenerated the disrupted endometrial cells. To confirm this, we followed immunohistochemistry.

Our study showed that local transplantation of BM MNCs in intrauterine horns resulted in endometrial regeneration by enhancing epithelial thickness and a-SMA, CK 18, CK 19, Cx 40, E cad, Cla-1 and ZO-1expression. The epithelial thickness is evaluated by HE staining. The DE group revealed discrete stroma and absent or irregular epithelial lining as compared to control group. In contrast, Tx group showed well organized thickened epithelial lining, glandular epithelium and intact stromal region. We studied the cell morphology upto day 9. The estrous cycle in rat lasts within 5 days. The endometrial lining reconstructs automatically in every estrus cycle. But in DE group, the endometrial lining is disrupted till next estrus cycle at day 9 as compared to transplant group. Transplant group revealed efficient epithelial cell organization with glandular and stromal regeneration. The immunohistochemistry revealed that junction proteins maintained the cell morphology, proliferation, migration, and differentiation. The DE showed decreased expressions leads to an alteration in the epithelial lining and junction protein expressions of endometrial cells which is responsible for the degeneration of the endometrium by reducing cell proliferation, vasoconstriction of the endometrial vessels, along with lacking trophic support.

Domnina et al., (26) explained the intravenous and local administration of bone marrow and endometrial MSC to an AS model to exhibit optimistic effects on fertility. They also detected that both intravenous and local transplantation routes are effective in the conception rate. The female rats from all three groups were placed into breeding trial. The pregnancy outcomes in all group females, we found that litter sizes are comparatively lower in control than transplant groups. DE group did not conceive in next two cycles. It may indicate the endometrial damage remain as it is for quite long time. It suggests that endometrium remained affected. However, an evaluation of endometrial histology from DE animals identified extensive

endometrial disruption and disorganized stromal-glandular architecture indicative of severe endometrial damage, a condition that causes infertility. In contrast, pregnancy outcomes in control group were significantly inferior as compared to Tx group. Control group gave birth to only 8 number of litter and Tx group gave birth to 12 litters. Collectively, these findings suggest that BM MNCs plays an essential role in endometrial regeneration following parturition and tissue remodeling that accompanies fertility outcome.

Our results suggested that transplanted cells recruited well due to stable gel layer of activated PRP. Further these activated PRPs have beneficial effects on endometrial cells due to growth factors (GFs). Activated PRP release immediate GFs into the uterine horns and promote the functionality of transplanted BM MNCs and regenerative changes in DE. A comparative study showed higher expressions of a-SMA, CK 18, CK 19, Cx 40, E cad, Cla-1 and ZO-1 till Day 9. As compared with control group, all DE group revealed weak expressions. BM MNCs-aPRP Tx group showed strong expressions till Day 9. Though estrus cycle repeats after every day 5, the BM MNCs Tx group revealed remarkably strong expressions. BM MNCs-aPRP Tx can generate functional endometrium comprising glandular, stromal, immune cells and angiogenic vasculature. Endometrial epithelial regeneration by BM MNCs-aPRP Tx takes place as a consequence of cellular differentiation. These findings collectively suggest that BM MNCs are one of the most likely candidates for endometrial stem/progenitor cells differentiation.

3.5. Conclusions

This study demonstrated encouraging effects of BM MNCs transplantation not only on endometrial regeneration but also on its receptivity. Rats were subjected to 95% ethanol in the uterine cavity to induce DE. BM MNCs followed by aPRP were transplanted directly into the uterus; normal saline was injected into the control. The expression of a-SMA, CK 18, CK 19, Cx 40, E

cad, Cla 1 and Zo-1 was significantly greater in rats that received BM MNCs compared to those that did not. BM MNCs can be successfully applied for DE treatment in the rat model results in significantly thicker endometrium. We suggest that improved pregnancy outcome and litter size in rats with BM MNCs Tx was higher than in DE group and control group. It proposes possible application of BM MNCs Tx for endometrial regeneration.

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Chapter – 4

Application of Bone Marrow-Derived Very small embryonic like Stem Cells in Murine Model of Disturbed Endometrium

4.1. Introduction

The endometrium undergoes dynamic remodeling over 400 cycles of endometrial shedding, differentiation and during the reproductive period (1,2). The endometrium is physiologically regulated by the sex steroid hormones and other biological molecules, and is essential for implantation and pregnancy. The transfer of Bone marrow (BM) stem cells from the BM into the endometrium, contribute to uterine reconstitution and endometrial repair by releasing cytokines (3). Disturbed endometrium (DE) could cause infertility and pregnancy abnormalities, ultimately leading to abnormal implantation.

Nowadays, there are still no efficient therapeutic methods to indulge the disturbed endometrium owning to the unknown mechanism (4,5). Bone marrow stem cells are one type of adult stem cells with the uniqueness of self-renewal, autotransplantation, multipotential differentiation, without any immune rejection (6–8). Evidence indicates that BM contains heterogeneous populations of hematopoietic stem cells (HSCs) and non-HSCs. The BM constitute a potential source of mesenchymal stem cells (MSCs) (9,10); precursors of germ cells (GCs) (11,12); endothelial progenitor cells (EPCs) (13); multipotent adult stem cells (MASCs) (14); marrow isolated adult multilineage inducible (MIAMI) cells and multipotent adult progenitor cells (MAPCs) (15). All these non-HSCs residing in the BM perform a crucial role in homeostasis and, if needed, could be assembled from the BM into circulation during tissue injury and stress, thereby facilitating the regeneration of damaged organs (16–18). It is a broad concern to treat the injured cells by using bone marrow stem cells. Bone marrow stem cells can migrate and accumulate in endometrial tissue after transplantation. Clinical research implicated that intrauterine transplantation of autologous bone marrow derived mesenchymal stem cells exert beneficial effects for the regeneration of injured endometrium (19). Moreover, BM VSELs transplantation has been exploited as a definitive therapy for hematological malignancies in clinical

practice. Therefore, BM VSELs have become an attractive and important resource in regenerative medicine. A small fraction of BM RBC consists of VSELs which are considered as primordial germ cells that participate in organogenesis and are present throughout life. VSELs are considered to be the true and elusive stem cells in endometrium and differentiate in to lumen epithelial cells and glands during the endometrial remodeling.

In this work, we evaluated the potential of BM VSELs in 95% ethanol induced DE in reserving it to the normal functional and morphological endometrial physiology. We isolated VSELs from the male murine BM which showed positive expression of Stage-specific embryonic antigen 1 marker ($SSEA1^{+}$) (20). The fluorescent labelled BM VSELs followed by thrombin activated platelet rich plasma (BM VSELs-aPRP TX) were transplanted into DE murine model. Present study provides considerable evidence of the regenerative potential of VSELs in DE endometrium. Additionally, it confirmed the improvement in fertility by the number of live births of pups.

4.2. Materials and Methods

4.2.1. Animals

All the animal experiments were carried out after obtaining the permission of Institutional Animal Ethics Committee (DYPMCK/IAEC/2020/JAN/01). Female Wistar rats of 6-10 weeks with body weight 250 ± 20 g (n=72) and male Wistar rats (n=10) were obtained from Animal House. All the rats were maintained in a specific-pathogen free environment with the temperature of 20 ± 2°C, humidity of 50%-60% and 12 h/12 h dark/light.

4.2.2. Isolation of BM VSELs from male Wistar rats

VSELs were acquired from the tibia and femur of the 6-10 wk male Wistar rats (n=5) while five male rats were used for mating afterwards. The rats were anesthetized by injecting Thiosol (1gm/10 ml, 5units/100 g of body wt) extraperitoneal. The rats were transferred to a laminar flow cabinet by shaving and disinfecting the skin with alcohol [70% (v/v)]. The bilateral bone

shaft and knee end were exposed by gently stripping off the adherent muscles and fat. Bilateral bones were cut in middle leaving both condyli intact. Cells were harvested by flushing the bone marrow cavity with 500 units of Heparin and HES 6% in 1:1 proportion. The cells were collected in Lymphoprep and digested by mincing. The suspended cells were centrifuged on 2200g×20min. Removed the upper plasma layer and middle buffy coat. The flushed bone marrow was collected in Lymphoprep and subjected to centrifugation at 2200g×20min. After centrifuge, the upper plasma layer along with the buffy coat was removed, while the red blood cell (RBCs) pellet was resuspended in RBC lysis buffer, washed on shaker and briefly centrifuged. The RBC lysis was repeated twice and finally obtained pellet was washed twice with PBC. The cells obtained in the final pellet were VSELs. The cell count was obtained in a Neubauer chamber and live cells were distinguished by Trypan Blue dye exclusion test. The cells were suspended in 0.5ml FBS serum containing 20% DMSO (Figure 4.1). Further it was transferred to -20°C and -40°C for overnight.

After that the cells were stored in LN2 for further studies.

Lymphoprep

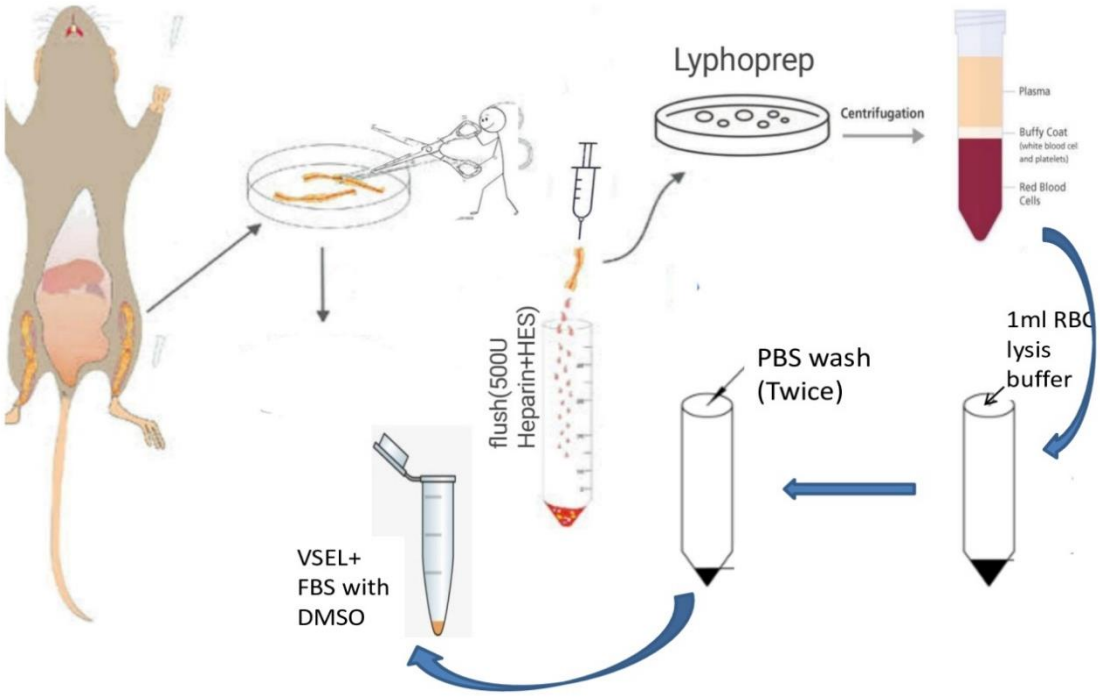

Figure 4.1: Schematic representation of isolation of BM VSELs

4.2.3. Flow Cytometry Analysis

The cells were distinguished by flow cytometry with the antibodies of SSEA. A cell suspension of 1×10^4 cells in 500µl PBS and were incubated with primary antibody for 45 min at room temperature with saturating concentrations of monoclonal antibodies Stage specific embryonic antigen-1 (SSEA-1). The cells were washed twice with PBS and centrifuged at 200 g after incubation. The cells were labelled with secondary antibody Alexa 488 (1:100) for 30 minutes in the dark at room temperature followed by three PBS wash of 5min each and resuspended in 100µl PBS. Cell fluorescence was analysed by flow cytometry in an FACS Calibur instrument (Becton Dickinson) and the data were analyzed using Cell Quest software (Becton Dickinson).

4.2.4. Cell labeling

The 1×10^6 BM VSELs were incubated in a green fluorescent salt dye as mentioned below at 37°C for 20 min and used for transplantation study to track the number and engraftment of the infused cells. 1ml of sterile PBS was added into salt to prepare stock A from which 10µl was mixed with 1ml of cell culture media to prepare stock B. The working solution was prepared by adding 100µl of Stock B to 1ml cell culture media covering the cells. An insulin syringe containing the labelled cells was inserted into the bilateral right and left horns and injected into luminal cavity of the horn to perform local intrauterine transplantation of cells.

4.2.5. Preparation of PRP and thrombin and PRP Activation

Two rats were anesthetized and 5 ml of blood was withdrawn into a sodium citrate tube via cardiac puncture and posterior vena cava to collect a good quality blood from the experimental animals. The PRP was then extracted and transferred to a new sterile tube. The 1000µl plasma was diluted with distilled water and 100µl of 1% acetic acid was added to make the pH 5.3 followed by centrifugation at 2000 rpm for 5 minutes. 10ml normal saline is added to this precipitate and pH adjusted to 7. The clot is formed, by adding 0.1 ml of 0.1 M Calcium Chloride in 5 minutes, which was removed and water clear thrombin solution is obtained. The thrombin was stored in at -4°C. To activate the PRP, 0.5 ml of thrombin was added into the 0.5ml PRP, which in turn changed into a semi-solid, jelly-like structure within 3min.

4.2.6. Cytokine and chemokine profiling of PRP

PRP activated with thrombin (aPRP) produce high levels of various cytokines and chemokines. We analyzed relative quantitation of cytokines and chemokines from aPRP supernatants using a LEGENDplex Rat Th Cytokine Panel (13-plex) (Biolegend, San Diego, CA) Kit. Cytokine levels CCL2, tumor necrosis factor-alpha (TNF- a), IL-1 BETA, CXCL10, IL2, IL4, IL-6 IL-10, IL-12, IL-

17A, TGF-BETA, CXCL8, interferon-GAMMA (IFN-γ), in supernatants were measured with flow cytometry according to the manufacturer's protocol.

4.2.7. Establishment of disturbed endometrium model

Rats in experimental group were subjected to endometrial disruption by intrauterine infusion of 0.5ml 95% Ethanol. In total 82 rats were procured, where in ten of them were male and the rest were female. The five male rats were used for isolation of VSELs while five male rats were procured for mating. Therefore, in this study, 72 female rats were randomly divided into three groups: control, DE, BM VSELs-aPRP transplant (BM VSELs-aPRP Tx) (n=24 in each group). Briefly, all the animals were anesthetized via extra-peritoneal administration of 12 units thiosol (1gm/10 ml, Neon laboratories Ltd.) per gram of body weight. The uterine horns were externalized and clamped over the cervix. The control rats were subjected to normal saline in intrauterine horns. Rats in DE group received mechanical damage in intrauterine horns by 95% Ethanol. After 24 hrs of DE, BM VSELs-aPRP Tx group received transplantation of labelled 1×10^6 BM VSELs-aPRP Tx in intrauterine horns (Figure 4.2). Six rats were sacrificed at time day 1, day 3, day 6 and day 9. Bilateral excised mid part of uterine horns was immediately snap frozen and cryosectioned to make sure the engraftment of BM VSELs. The samples were fixed in 10% neutral buffer formalin for further hematoxylin-eosin staining (HE) and immunohistochemistry (IHC). Animals were sacrificed post experiment by cervical dislocation. A yellow-colored category No.2 biomedical waste management bangs were used for seal sacrificed rats. It was given to central incineration facility in Animal House of D. Y. Patil Medical College, Kasaba Bawada, Kolhapur.

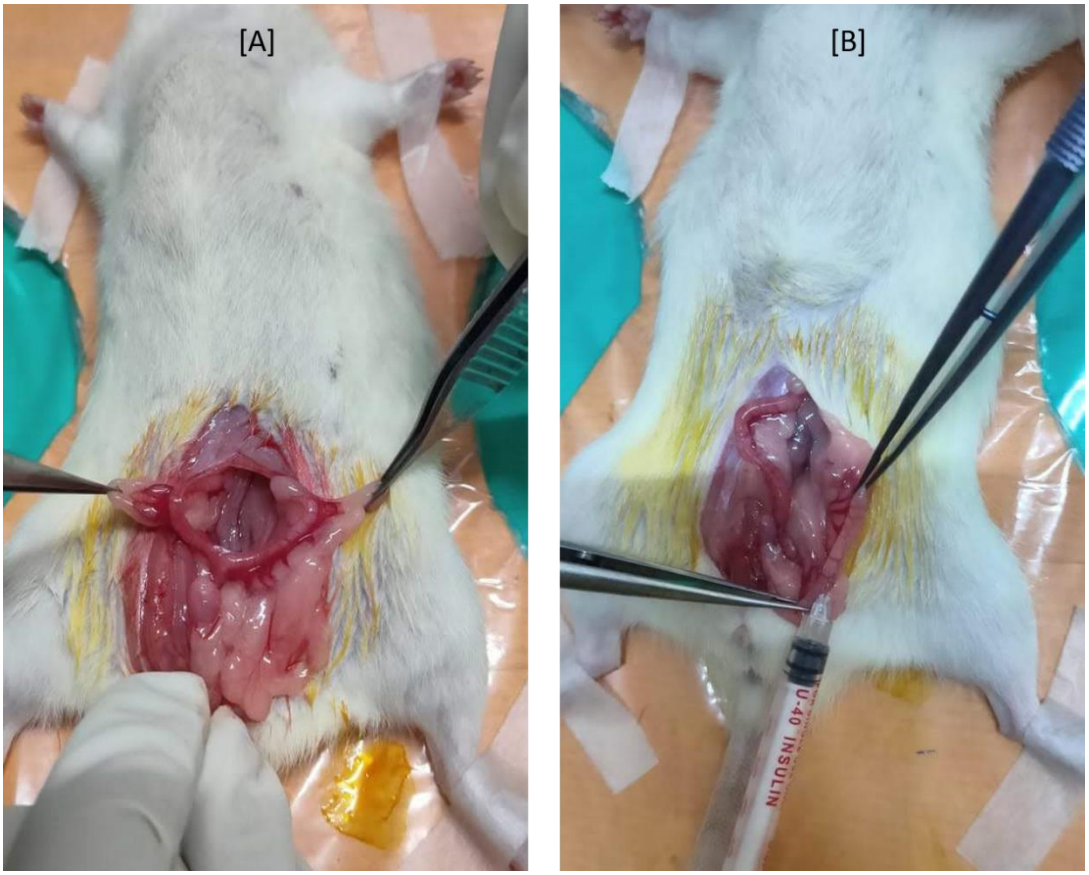

Figure 4.2: Endometrial Disruption of endometrium and intrauterine infusion of VSELSCs.

4.2.8. Quantification of engrafted BM VSELs

To quantify the number of engrafted BM VSELs, frozen tissue was used for 100 serial sections of 5 µm thickness. Transplanted cells which are already stained with green fluorescent dye were counterstained with a nuclear stain DAPI. The number of engrafted BM VSELs were ascertained by enumerating green fluorescent dye in endometrium. At a 20X magnification, the visual field of the Nikon camera corresponds to an area of 0.12mm^2. The central regions of visual fields corresponding to a surface area of 0.1mm2/section were counted. To get the total number of cells/mm^2, the number of engrafted cells on a 1000µm^2 section was counted from the number of cells/0.1mm^2 multiplying by a factor of 3.33 and again multiplied by 200. The cells on every section were calculated twice the two different investigators.

4.2.9. HE staining

The tissues underwent regular paraffin embedding, sectioning (4 µm) and HE staining. The morphological changes were observed under the fluorescence microscope (Nikon; Japan). Images were captured at magnifications of ×20.

4.2.10. Immunohistochemistry

The immunohistochemistry of a-SMA, Cytokeratin (CK)-18, CK-19, Connexin (CX)-40, E cadherin (E-Cad), Claudin -1 (Cla-1) and Zona Occludin (ZO-1) expressions were conducted. The paraffin sections were subjected to 60ºC for 10 minutes and treated with xylene for deparaffinization following rehydration of alcohol grades. The antigen retrieval was performed for a-SMA (mouse monoclonal: AbDSerotec), CK-18 (mouse monoclonal: AbDSerotec) and CK-19 (mouse monoclonal: AbDSerotec), Connexin (CX)-40 (mouse monoclonal: AbDSerotec), E-Cad (Mab anti human: Invitrogen), Cla-1 (Rabbit anti human: Invitrogen), ZO-1 (Mab anti human: Invitrogen), by placing the slides in 10 mmol trisodium citrate buffer (pH-6.0) following incubation with goat serum for 45 minutes at room temperature in order to reduce nonspecific binding of antibodies. The sections were then treated with primary antibodies E-Cad, Cla-1, ZO-1, CK-18 with dilution 1:100 and CK 19, CX-40, with dilution 1:200 in BSA (Hi Media), and further treated with Alexa 488 (molecular probe) labelled secondary antibodies. After staining, the samples were monitored under microscope. Fluorescent green color was supposed as the positive expression. Five fields from each image were selected for quantification. Based upon the density of staining, the expression of a-SMA (Smooth muscle marker), CK-18 and CK-19 (epithelial cell marker), CX-40 (gap junction protein marker), E-Cad (adherence junction protein marker), Cla-1 and ZO-1 (tight junction protein marker) were calculated. The expressions were divided into strong, medium and weak grades. Different scores (0,1,2,3) were attributed to negative, weak, medium and high expression. The scores calculated and averaged based upon two methods.

Finally, 0-1 was supposed as negative expression (-), 1-2 as weak expression (±), 2-3 as medium expression (+) and 3-4 as high expression (++).

4.2.11. BM VSELs-aPRP Tx improves live birth rate in transplant group

Female rats in BM VSELs-aPRP Tx group were mated with five healthy males at seven days after BM VSELs-aPRP Tx treatment. Pregnancy outcomes included the time to conceive, live-birth rate and litter size.

Statistical analysis

All the data were presented as mean and standard deviation (SD) and analyzed by Tukey's multiple comparisons test for more than two groups. $P < 0.001$ was considered as statistical significance.

4.3. Results

4.3.1. BM VSELs Phenotype

The BM VSELs obtained from male rat bone marrow aspirates were analysed by flowcytometry. With FACS analysis, BM VSELs expressed SSEA. In FACS analysis, the cells obtained from bone marrow aspirate showed positive expression of SSEA while did not show any expression of CD 34, Tie-2, Thy, CD 133, CD 90, DLK. Thereby, confirming that the population of cells obtained from bone marrow were phenotypically identified to be VSELs. We conducted it for confirmation purpose (Figure 4.3).

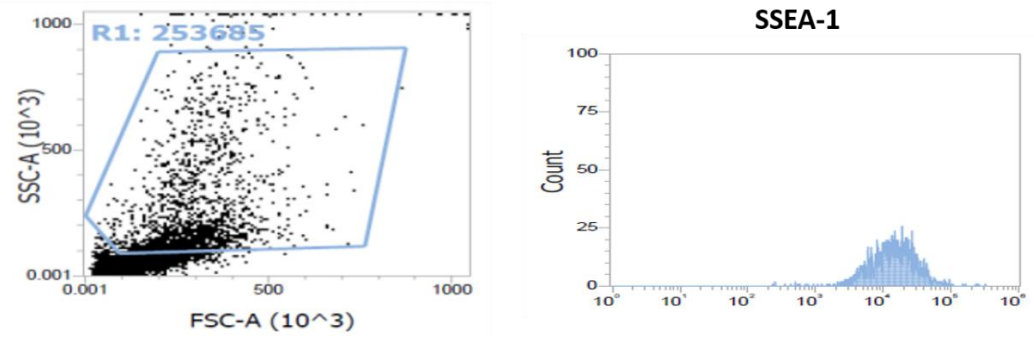

Figure 4.3: Phenotypic confirmation of VSELs by Flowcytometry.

4.3.2. Quantification of BM VSELs

As shown in Figure 4.4, engraftment of BM VSELs in DE was verified by the use of green fluorescent dyed BM VSELs. Scattered labelled BM VSELs were detected between the stromal regions of endometrium in rat at Day 1, Day 3, Day 6 and Day 9. The number of labelled BM VSELs in Day 1, Day 3 and Day 6 group was 1,14,552±1332 cells/mm^2 (P<0.001), 29,970±666 cells/ mm^2 (P<0.001), 13,942±601.20 mm^2 (P<0.001) respectively. Endometrial sections from Day 9 did not show any engrafted BM VSELs. Local administration of BM VSELs resulted in the recruitment of cells to the DE area (p<0.001). Endometrial sections from Day 1 showed both scattered and clustered cells while Day 3 and 6 showed scattered cells. Endometrial sections from Day 9 group revealed no cells positive for labelled cells. The Day 1 group had higher numbers of labelled cells compared with other groups. The number of cells recruited at days 3 and 6 showed an extreme decline (Figure 4.5). It may suggest that transplanted cells did not adhere properly to a large extent.

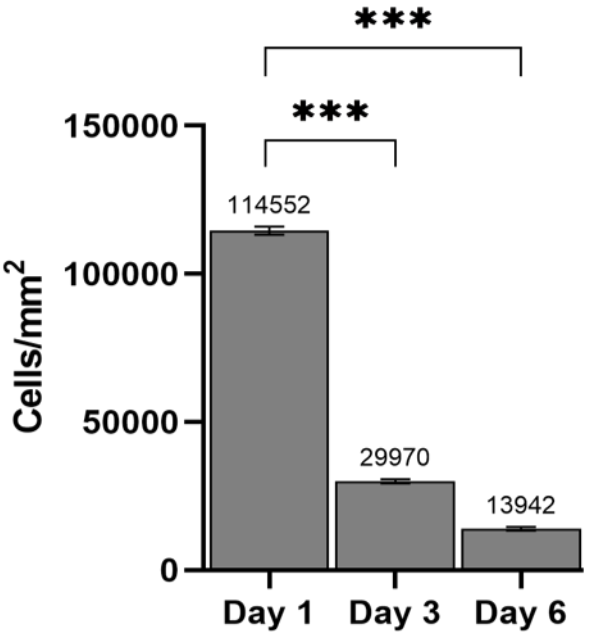

Figure 4.4: Quantification of transplanted BM VSELSCs-aPRP.

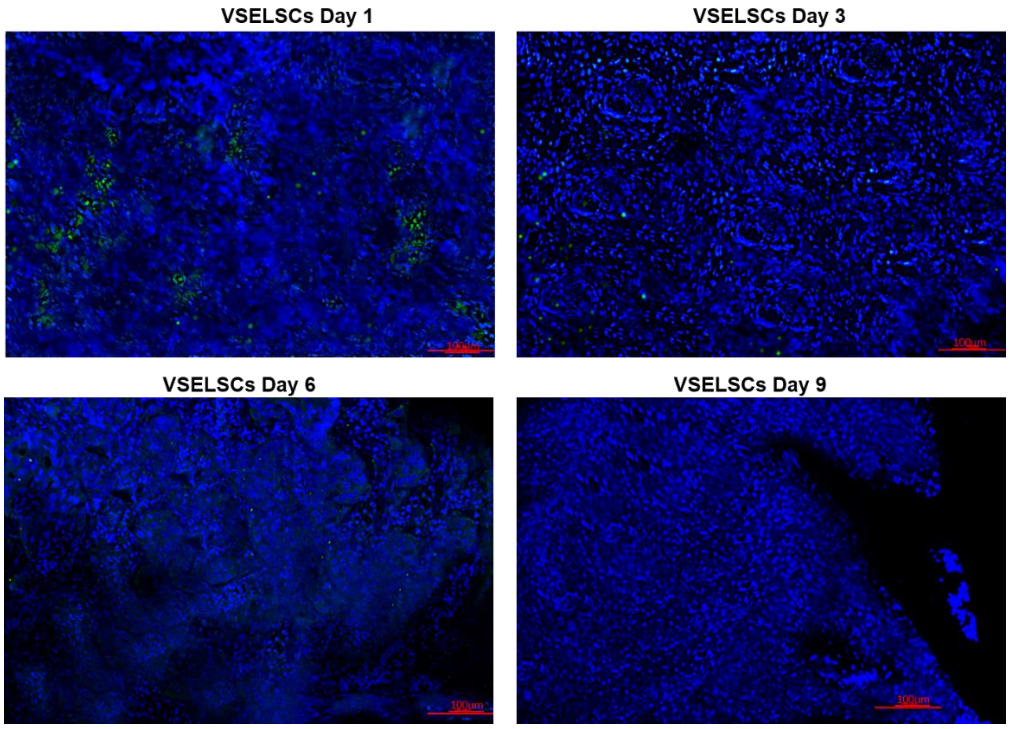

Figure 4.5: Quantification of transplanted VSELSCs.

4.3.3. Cytokine and chemokine levels in aPRP

The cytokines and chemokines-CCL2, TNF α, IL-β, CXCL 10, IL-2, IL-4, TGF-β, CXCL-8, IL-12, IFN γ, IL-10, IL-6, IL-17 were analyzed by the array and summarized in Table 2.1 and figure 2.3 in previous chapter 2.

4.3.4. Histopathological observations by HE

Endometrial thickness, superficial epithelium of the endometrium, and endometrial glands presented a significant difference in rats with BM VSELs transplantation compared with the control group. Histologic evaluation of the endometrium in the BM VSELs Tx group showed an intact endometrial layer with an increased endometrial thickness and endometrial glands ($p < 0.001$). We observed the changes for two consecutive cycles. The endometrial lining in the DE group was destroyed with extensive fibrosis in the endometrium layer and even no healthy tissue (Figure 4.6). The endometrial thickness of the control, DE, and BM VSELs Tx group were summarized in Table 4.1.

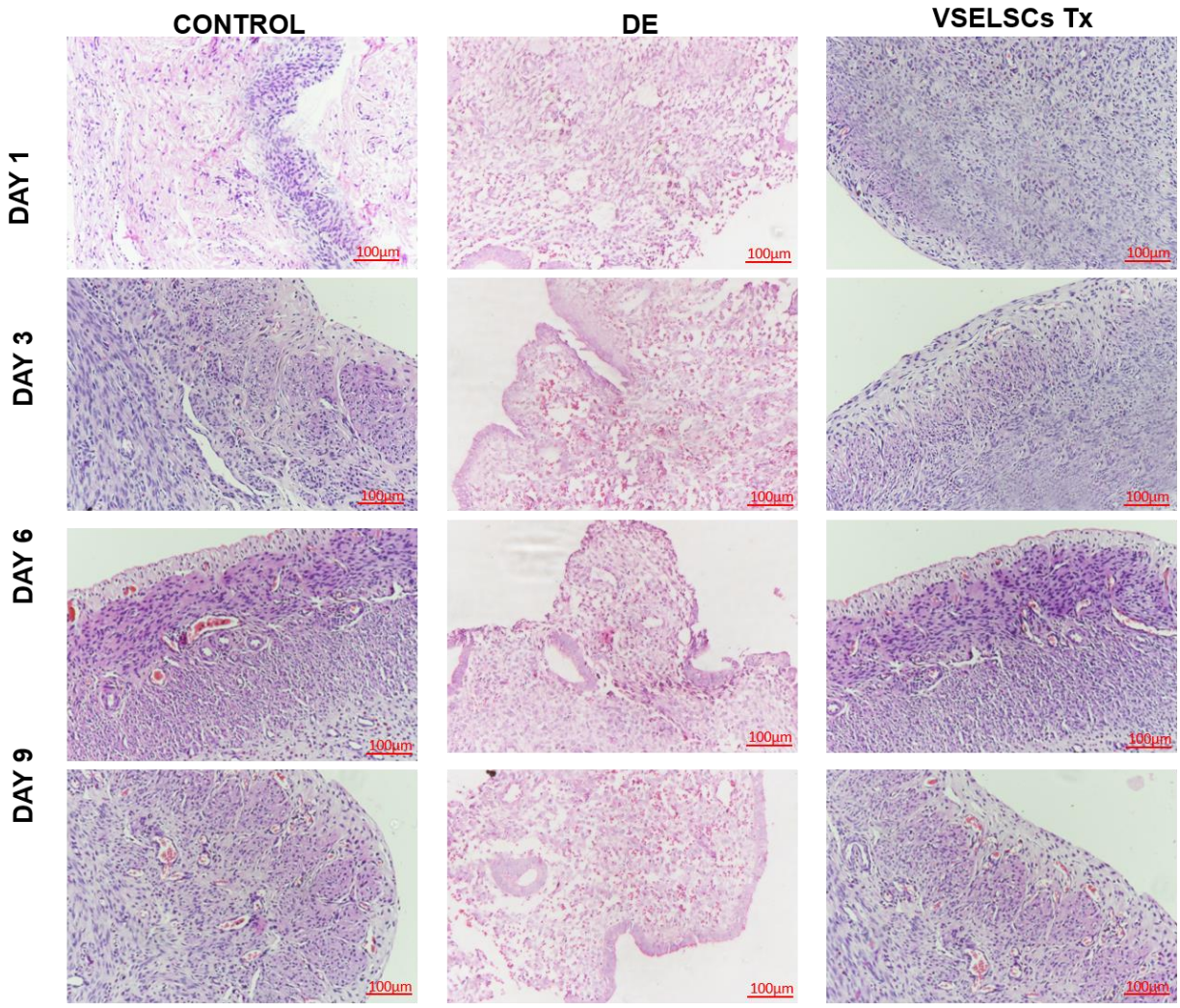

Figure 4.6: Hematoxylin and eosin analysis.

A significantly thicker endometrial lining was observed in the experimental group compared with that of the control group ($P<0.01$). Whereas no significant difference was observed between the transplant group and the control group ($P>0.05$). There was no significant endometrial thickness between Day 1, Day 3 and Day 6 of the DE group. DE Day 9 showed efficient endometrial thickness. Transplantation of BM VSELs astonishingly increased endometrial thickness compared to the DE group ($p< 0.001$). This confirms that BM VSELs-aPRP, effectively improves endometrial thickness by regenerating endometrial cells. Endometrial thickness on day 1 of BM VSELs - aPRP Tx was 122.06±16.67 µm in the control group and 197.99±25.94 µm in BM

VSELs -aPRP Tx group which was significantly different ($p < 0.001$) from DE group. The endometrial lining on the day 6 of BM VSELs -aPRP Tx was 290.71±17.93µm in the control group and 294.43±13.54 µm in BM VSELs-aPRP Tx group which was significantly different ($p < 0.001$) from DE group. Likewise, endometrial thickness was significantly different between the control and the BM VSELs-aPRP Tx group as compared to the DE group ($p< 0.001$) (285.91±21.23 µm in the control group and 296.50±20.69µm in the BM VSELs-aPRP Tx group) on day 9 (Figure 4.7).

Table 4.1: Evaluation of epithelial thickness

Variables	control	DE	BM VSELs-aPRP Tx
Day 1	122.06±16.67***	79.90±22.39*	197.99±25.94**
Day 3	288.69±26.26***	47.079.45*	298.21±25.2***
Day 6	290.71±17.93***	41.24±15.64*	294.43±13.54**
Day 9	285.91±21.23***	37.51±10.34*	296.50±20.69***

Statistical significance: *- p<0.0481; **- p<0.0019; ***-p<0.0003; BM MNCs-aPRP Tx effects on the DE (n=24), compared with control (n=24) and transplant group (n=24).

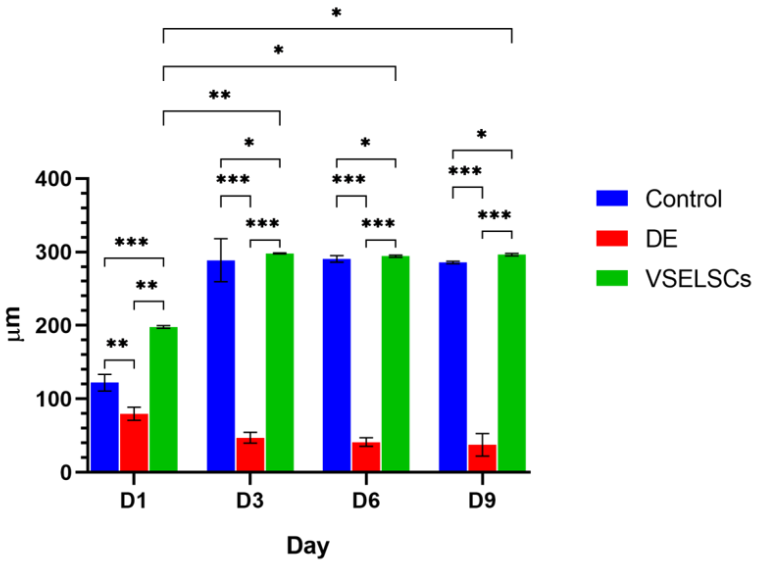

Figure 4.7: Evaluation of epithelial lining.

4.3.5. Treatment of BM VSELs Promotes the Regeneration of Endometrial Cells

To explore the effect of BM VSELs transplantation on disturbed endometrium, we assessed the expression of a-SMA, CK 18, CK 19, Cx 40, E cad, Cla-1, and ZO-1 with immunohistochemical staining. Immunohistochemical staining showed that a-SMA, CK 18, CK 19, and ZO-1 expression was mainly localized in the endometrial epithelium in the stratum functional region, and Cx 40, E cad, Cla-1 staining was mainly expressed in the endometrial stromal cells. The expression of a-SMA, CK 18, CK 19, Cx 40, E cad, Cla-1, and ZO-1 in the experimental group was significantly more substantial than that of DE groups ($P<.01$), slightly weaker than that of the control group ($P>.05$). In the DE group, there was a significant decrease in all expression of a-SMA, CK 18, CK 19, Cx 40, E cad, Cla-1 and ZO-1 compared with the control group and BM VSELs Tx. However, there was not much difference in the tissue expression of DE day 1, 3, 6, and 9 with each other. Scattered expressions were detected between the stromal regions of disturbed endometrium on Day 3, Day 6, Day 9. DE on Day 1 did not show any expressions. In BM VSELs-aPRP Tx, endometrial sections of Day 3 and Day 6 group revealed extremely few expressions as compared to Day 9 groups. IHC expression was mainly localized in the endometrial glandular and stromal region. The DE at Day 9 group had weak expressions of a-SMA, CK 19, Cla-1 in stratum functionalis cells. The BM VSELs group showed increased expressions as compared with the DE group. The BM VSELs-aPRP Tx treated group at Day 6, 9 demonstrated comparatively strong expressions of a-SMA, CK 18, E-cad, and Cla-1 while moderate CK 19, and Cx-40. The BM VSELs-aPRP Tx treated group had higher expressions of a-SMA, E-Cad and Cla-1 (Figure 4.8).

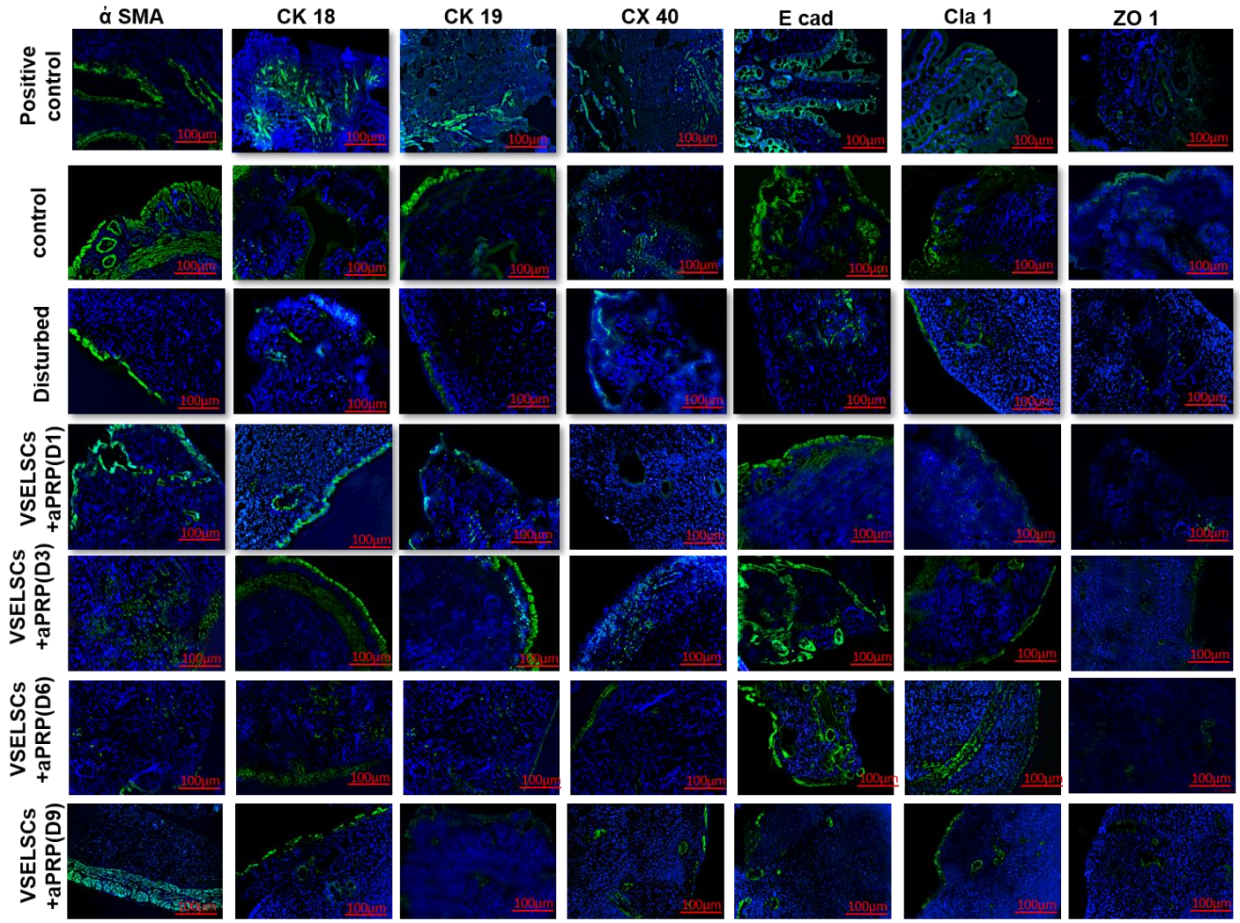

Figure 4.8: Immunohistochemistry of BM VSELSCs-aPRP transplanted endometrium.

4.3.6. BM VSELs-aPRP Tx improves endometrial functionality

The treated female rats were mated with a fertile male in the BM VSELS-aPRP Tx group. VSELS Vaginal plug smears were conducted to confirm whether they were conceived or not. The results indicate that implantation was hampered by disturbed endometrium but restored by BM MNCs-aPRP Tx group. While all control groups gave birth to live pups, the DE group did not give birth to any live pups, and the BM MNCs-aPRP Tx group delivered healthy pups. The control group conceived within 5 days, but the transplant group took 8 to 10 days. The BM VSELs-aPRP Tx improved the rate of live births since the DE group failed to conceive (Table 4.2).

Table 4.2: Rate of pregnancy in control, DE and BM MNCs-aPRP Tx groups

Variables	Control group	DE group	BM MNCs-aPRP Tx group
Pregnancy rate	8.4%**	-	9.3%***

Statistical significance: **-($p < 0.001$), ***- ($p < 0.001$). The pregnancy rate was 5.4% in the control group and 10.3% in Tx group which was significant ($p < 0.001$).

5.4 Discussion

Any endometrial pathology affects implantation and reproduction. Endometrium pathology includes cavity adhesion, fibrosis, ischemic injury, sparsely glandular, inactive dilatation, thin endometrium that indicate disturbed endometrium. Approximately 50% of the disturbed endometrium display infertility. Therefore, more attention has been compensated to this condition. Bone marrow stem cells have the advantages of convenient harvest and abundant supplements. Moreover, the application of Bone marrow stem cells could avoid self-immune rejection. Bone Marrow mesenchymal stem cells (MSCs) were famous for their ability of self-renewal and multi-differentiation. Bone marrow derived cells were supposed to migrate in endometrial tissue to thicken the endometrium and improve endometrial receptivity.

Therefore, BM VSELs transplantation was thought to have the potential to treat disturbed endometrium. However, no study explains the BM VSELs transplantation to treat DE for assisted reproductive technology (ART). Our strategy for transplantation was to transplant VSELs followed by aPRP, which showed significant engraftment. We believe aPRP stabilized VSELs at the site of injury, which facilitates better engraftment. Indeed, BM VSELs population, expressing SSEA-1 participates in endometrial regeneration by supporting the existence of epithelial stem cells in the endometrium involved in glandular regeneration.

PRP is activated by commercially available thrombin derived from bovine plasma and is the most potent platelet activator (21). It stimulates immediate PGF release from the PRP (22,23). The a-granules of the nonactivated PRP contain nonfunctional PGF, because they are not released or in contact with the tissue. The PRP activated by thrombin release pro inflammatory cytokine and chemokines CCL2, TNF- a, IL-1β, CXCL8, CXCL10, IL2, IL4, IL-6 IL-10, IL-12, IL-17A, TGF-β, IFN-γ. To begin releasing these growth factors, we activated platelets with thrombin. PRP has beneficial therapeutic effects in regenerative medicines due to the growth factors stored in platelet. After activation with thrombin, PRP forms a gel like consistency. The transplant of cells followed by aPRP will get trapped in PRP gel surface. It will prevent cell loss, and cells will interact appropriately with disturbed endometrial cells. It will assist in cellular events like adhesion, migration, and differentiation. Further aPRP releases above mentioned proinflammatory factors that enhances the functions of transplanted BM VSELs. The elevated levels of IL 6, IL 10, and IL 17 suggest that this therapy may encourage endometrial regeneration and improve endometrial receptivity. These chemokines exert a potent inhibitory action on endometrial cell necrocytosis induced by ethanol infusion. The immunomodulatory mechanisms of BM VSELs-aPRP Tx, underlay therapeutic effects.

We have reported in our earlier work about the importance of tropical growth factor support increase engraftment potential of cells (24). This tropical support of MSC was by the production of high levels of IL-6 and IL-8 that may counterbalance the hepatocyte engraftment. Likewise, chemokines and cytokines released by thrombin activated PRP are essential to optimize whether the BM VSELs transplantation withstands these conditions after cell infusion.

However, there have been no studies to evaluate the recruitment capacity of these cells to the endometrium. No data compares local intrauterine

administration for BM VSELs via intraluminal uterine injection. This indicated that intrauterine infusion of BM VSELs followed by aPRP had a therapeutic effect of promoting the endometrial regeneration in disturbed endometrium by exerting cytokines and chemokines as a protective pro inflammatory action against endometrial damage after ethanol infusion. The labeled cell studies evidence that transplanted cells get engrafted to the DE and initiate endometrial regeneration. Our data show morphologically normal and functional epithelial lining, stronger expression of cell junction proteins by HE and IHC. The expression of a-SMA, CK 18, CK 19, Cx 40, E cad, Cla-1 and ZO-1 in the transplant group was nearly similar to that in the control group. The a-SMA, CK 18, CK 19, Cx 40, E cad, Cla-1 is believed to be the markers for endometrial receptivity and have an important role in implantation.

We demonstrated that the rats in the transplant group had a thicker epithelial endometrium as compared to control. However, an evaluation of endometrial histology from DE animals identified extensive endometrial disruption and disorganized stromal-glandular architecture indicative of severe endometrial damage, a condition that causes infertility.

The pregnancy outcomes in all group's females, litter sizes are comparatively lower in control than in transplant groups. DE group did not conceive in the next two cycles which indicates the endometrial cell remained affected and damage remained for quite a long time. In contrast, pregnancy outcomes in the control group were significantly inferior as compared to Tx group. The control group gave birth to only 8 litter, and Tx group gave birth to 10 litter. Collectively, these findings suggest that BM VSELs-aPRP Tx plays an essential role in endometrial regeneration following parturition and tissue remodeling that accompanies fertility outcome. The VSELs-aPRP Tx holds incredible potential to stimulate and provide sufficient cells for treating DE. Use of aPRP along with cell transplant is likely to have enhanced therapeutic benefits. Our

research on the effects of BM VSELs strongly supports the BM VSELs Tx potential and its clinical use.

It reports the development of a novel strategy for augmenting BM VSELs engraftment co-transplanting with aPRP. This approach is promising and advances this experimental study closer to clinical endometrial pathology to assist in ART.

4.5. Conclusions:

The conclusion of the study is that transplantation of BM VSELs followed by aPRP could be an innovative strategy for improving the engraftment potential of cells. VSELs-aPRP Tx holds the potential to stimulate the regeneration of endometrial cells and improve the fertility rate. This could be a new strategy to treat various endometrial pathological conditions.

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Chapter – 5

Transplantation of Human Placenta Derived Mitochondria Promotes Cell Communication in Endometrium in a Murine Model of Disturbed Endometrium

5.1 Introduction

An endometrium with disturbed and altered junction protein has been a challenging problem in infertility management. Several treatment approaches have been developed to enhance the implantation rate by improving endometrial receptivity, embryo qualities, and treating co-morbidities with exogenous estrogen, aspirin, clomifene citrate, vitamin E, and pentoxifylline (1). However clinical success rates are still poor. The appropriate endometrial receptivity plays a crucial role in improved outcomes of fertilization. Implantation is an intricate biological interaction between the embryo and the endometrial cells which is influenced by various hormonal and cytokine pathways. The Endometrial receptivity decreases with impaired endometrial quality, maternal aging, increased reproductive tract defect, decreased follicle and altered reproductive endocrinology (2). In general, the endometrial junction protein has received much less attention compared with the blastocyst-endometrium junction proteomics at the time of embryo implantation in reproductive research. Among the factors affecting embryo-endometrium competence, endometrial proteomics plays an important role in receptivity. The correlation between endometrial junction protein and receptivity has been mentioned in our previous studies (3). We documented low expressions of E Cadherin (E-CAD), Zona Occludin-1 (ZO-1), Vascular Angiogenic Precursor (VAP), and Claudin-1 (Cla-1) in human endometrial cells at implantation window may hamper the endometrial receptivity. Identifying factors that impact disrupted endometrium in the implantation window is important for the fertility treatment approaches (4). Sarvi et al., proved that intrauterine infusion of granulocyte colony-stimulating factor improves cytokines and growth factors in thin endometrium patients. The intrauterine infusion of Platelet rich plasma

(PRP) and granulocyte-colony stimulating factor (G-CSF) implemented good results in endometrial rejuvenation in patients with a thin endometrium (5,6). Therapeutic trials of PRP and G-CSF targeting Asherman's syndrome revealed temporary changes in endometrial restoration. However, a detailed study of the cell action is needed. The intrauterine use of G-CSF in infertile women did not increase endometrial thickness in all women. There was no difference in endometrial thickness between parous and infertile women (7,8). Chang et al., stated the use of intrauterine infusion of PRP to improve the endometrial thickness in women with thin endometrium (9). In spite of these treatments, only minor modifications in the endometrium thickness were recorded and have not been validated so far. Bone marrow derived mesenchymal stem cells (BMMSCs) were the first mesenchymal stem cells that have been widely researched.

Most of the studies have used BMMSCs in regenerative medicine. Autologous BMMSCs have less immune rejection. However, BMMSCs have their own limitations. The isolation process of bone marrow is painful, hazardous, and generates adverse psychological effects for the patients.

Though all these therapeutics, dysregulated endometrium remains a challenge and requires future enormous investigations for further management of inadequate endometrium. Nowadays, autologous mitochondria transfer for oocyte rejuvenation has been widely applied in different clinical scenarios to improve fertilization (10). Some groups investigated the autologous mitochondrial injection along with intracytoplasmic sperm injection to improve oocyte quality in women with multiple IVF failures. Dalton et al., investigated mitochondrial inheritance during the meiotic divisions of the mouse oocyte. The synthesis of mitochondrial adenosine triphosphate is increased

provides the energy required for the germinal vesicle rupture and resumption of meiosis (11). During the last decades, growing attention in mitochondrial research has developed. All the initial phases of fertilization necessitate high energy consumption. Mitochondria are the primary source of ATP for cell growth, transport, and hormone synthesis. It is essential to the continued existence of cells in terms of energy production. It has been suggested that mitochondria play an important role in the manifestation of cell rejuvenation. However to date, little is known about the endometrial remodelling using hMTx in the endometrium with disturbed junction proteomics.

5.2 Materials and methods

5.2.1 Isolation of Mitochondria from human term placenta

The use of human full-term placenta for Mitochondria isolation was approved by Institutional Ethics Committee (Ref.-outward no. DMCK/73/2017). Human term placenta was obtained at the time of elective lower segment caesarian section (L.S.C.S.) immediately under sterile conditions with informed consent. The donor had negative serologic tests and all routine blood investigations were normal. Placenta was collected, transferred and processed at 0-4°C. Under sterile conditions, the membranes were separated by blunt dissection. Cotyledons were separated and washed repeatedly with chilled 0.9% normal saline under a laminar air hood to drain the blood. The, cotyledons minced into small pieces and passed through a meat grinder. The resulting mince was washed by medium A (0.25 M sucrose containing 10mM tris HCl adjusted to pH 7 with 1 M HCl). Approximately 100 g of washed mince were suspended to 30 ml Medium A and homogenized with Teflon pestle driven at 1200 rev/min. The homogenate was centrifuged at 250xg for

10 min and the pellet discarded. The supernatant was transferred to another tube and centrifuged at 1000xg for next 10 min. The pellet containing nuclei was discarded and supernatant is again centrifuged at 10000xg for 10 min. The pellet containing mitochondria was obtained (Figure 5.1). Mitochondria is resuspended in FBS containing 20% DMSO in 1:1 ratio. Then it is shifted to -40°C for overnight and stored in LN_2 till further study. The waste from the remaining study was disposed in Yellow Colored bag No.2 and handovered to Central Biowaste Management facility of Dr. D. Y. Patil Medical College, Hospital and Research center, Kadamwadi, Kolhapur.

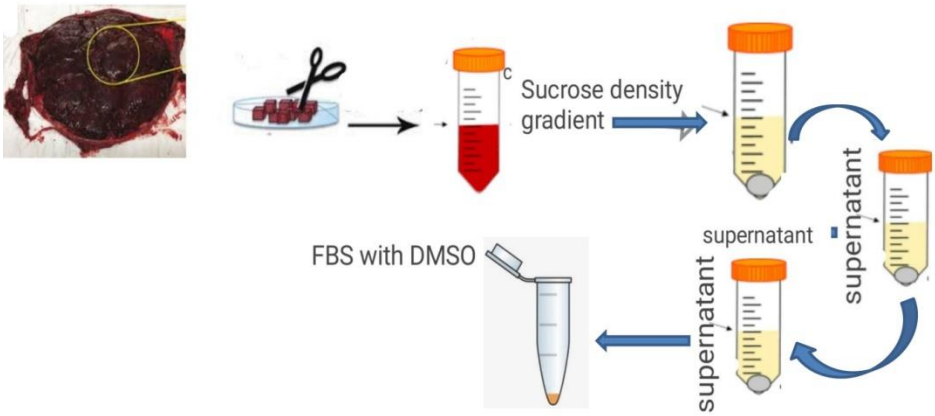

Figure 5.1: Schematic representation of isolation of mitochondria from human term placenta.

5.2.2 Labeling and detection of Mitochondria with Rhodamine B by flow cytometry

Rhodamine B is mitochondrial specific fluorescent dye and specifically concentrated in mitochondria by its transmembrane potential in living cells. Stock solution of 1 mg/ml Rhodamine B (Sigma-Aldrich, R8004) is prepared in D/W. The solution is protected from light and stored at 4°C. Rhodamine B solution is diluted at 100-fold dilution at a concentration of 10 µg/ml in PBS.

Mitochondria (1 x 10^6) were incubated in 1 ml of buffer solution containing 40 nM Rhodamine B in the dark at 4°C for 30 min. This is the minimal concentration of 40 nM Rhodamine B, as its intracellular accumulation would not disturb the function of the mitochondria. It provides a significant change in fluorescence intensity, which can be detected by flow cytometry (7). After incubation, Mitochondria were placed in ice-bath and assayed for flow cytometry and animal study. Viability of mitochondria is quantified by flow cytometry. Determining viability of mitochondria is an important step when evaluating a cell response on disturbed endometrium. It is necessary to distinguish viability of Mitochondria to avoid the non-specific staining. Data were stored onto the hard disk as list-mode files of BD FACS Aria (Becton Dickinson, Stockholm) and analyzed with software CellQuest supplied by the manufacturer.

5.2.3 Protein determination

The total protein content in a pellet containing mitochondria was determined by Lowry method of using bovine serum albumin (BSA) as standard. Based on the formation of a protein-Cu++ Complex and reaction of phosphomolybdate phosphotungstate reagent (Folin-ciocalteau phenol reagent) by tyrosine and tryptophan residues of protein a colored product is formed. The absorbance of which is measured at 660 nm. 10 µl of tissue homogenate was diluted to 1000 µl with distilled water and mixed with 5 ml of alkaline copper sulphate solution in a test tube. Mixed well and allowed to stand for 10 min. Then added 0.5 ml of Folin-ciocalteau reagent (1:1 diluted with water) and incubated at room temperature in the dark for 30 min. The blue color developed was read at 660 nm. A standard graph was

plotted with BSA and calculated the amount of protein in the sample.

5.2.4 Experimental protocol

Mitochondria were isolated from Human term placenta by sucrose density gradient. Flow cytometry is used to determine the purity of the mitochondria. Enzymatic bioassays were conducted for NADH-cytochrome c reductase, NADPH-cytochrome c reductase, Cytochrome b5, Cytochrome P450, complex I, complex II, complex III, complex IV activity. Protein concentration in mitochondria was determined by Lowry's method using bovine serum albumin (BSA) as standard (1). Animal study is conducted using female Wistar rat model of disturbed endometrium. The effect of intrauterine administration of hMTx-aPRP on disturbed endometrium was studied

5.2.5 Determination of Functionality of Mitochondria

The mitochondria were washed twice with phosphate buffer to remove the sucrose. Then it is suspended in 50 mmol/l phosphate buffer (pH 7.0) at a concentration of 3-5 mg/ml. The suspended mitochondria were frozen and thawed 3 to 5 times. It was used for the determination of Spectrophotometric assays

5.2.5.1 Spectrophotometric assays

The assessment of mitochondrial enzymatic activities is essential for investigating mitochondrial function. We described a simple step-by-step protocol for assessment of the enzymatic function for Mitochondria by using a UV spectrophotometer. An efficient mitochondrial pellet and the choice of specific buffers, substrates allow maximal sensitivity, specificity for each assay.

5.2.5.2 Bioassay of NADH-cytochrome c reductase

NADH dehydrogenases interact with cytochrome c as electron acceptor. The NADH-cytochrome c reductase (NADH cyt C red) is characterized by high affinity for NADH and cytochrome c and sensitivity to inhibition by rotenone and antimycin A. The increased absorbance of cytochrome C was measured at 550nm.The 2ml final volume of working solution containing10 mM Tris-HCL (pH 7.6), 1 mM KCN, 60 µM cytochrome c and 0.3 mM of NADH is incubated. Adding up of mitochondria to the preincubated mixture, the increasing absorbance of cyt c was measured at 550 nm. The activity was calculated using an extinction coefficient of 19.6 mM^{-1} cm^{-1}(2).

5.2.5.3 Bioassay of NADPH-cytochrome c reductase

NADPH-cytochrome c reductase (NADPH-cyt c red) activity was determined by measuring the increasing absorbance of cytochrome c essentially as described by Shimakata et al. The 2ml reaction mixture of 50 mM Tris HCl (PH 7.6), 1 mM KCN; 60 uM Cytochrome C and 0.3 mM of NADPH is preincubated following addition of mitochondria sample. The increasing absorbance of Cyt c was quantified at 550 nm. The activity was calculated using an extinction coefficient of 19.6 $mM^{-1}cm^{-1}$(2).

5.2.5.4 Bioassay of Cytochrome b5

The reaction mixture containing 100 µl of 100 mM Tris HCl (pH 7.6), 50 µl of mitochondria and 800 µl of distilled water was preincubated for a minute. Absorbance is measured at 500 nm. The Cytochrome b5 (Cyt b5) was measured by the reduction of NADH. The reaction mixture was reduced by

adding 50 µl of NADH and again rescanned. Cyt b5 was quantified using an extinction coefficient of 185 $mM^{-1}cm^{-1}$(3).

5.2.5.5 Bioassay of Cytochrome P450

Total Cytochrome P450 (Cyt P450) content were measured in 100 mM Tris HCl (pH 7.6), by the carbon-monoxide difference spectrum of sodium dithionite reduced sample, using a sample volume of 300µland an extinction coefficient of 91 mM^{-1} cm^{-1}. Two ml of 100 mM Tris HCl (pH 7.6) buffer was mixed with 300 µl of mitochondria and 1.70 ml of distilled water. The cuvette was bubbled with carbon monoxide and placed in sample beam of spectrophotometer. Baseline was recorded between 500 to 400 nm. Cyt P450 was quantified using an extinction coefficient of 91 mM^{-1} cm^{-1} (3).

5.2.5.6 Determination of complex I activity

In complex I assay, DCPIP is used as a terminal electron acceptor. The Complex I activity was verified from the rate of reduction of DCPIP as the alteration in absorbance at 600 nm after the addition of complex I inhibitor rotenone. The 1 µmol/l antimycin A, 3 mg BSA, 2 mM/l KCN, 5 mM/l MgCl2, 65 µmol/l decylubiquinone, 80 µmol/l DCPIP and approximately 40 µg mitochondria were mixed with phosphate buffer (25 mM pH 7.2) in a net volume of 1 ml. Complex I oxidizes NADH, and the produced electrons reduce the artificial substrate decylubiquinone that consequently delivers the electrons to DCPIP. Absorbance at 600 nm was monitored at room temperature after the addition of 0.2 mM/l NADH. After 2 min, 1 µmol/l rotenone was added and the absorbance was measured again. The activity was expressed as µmoles of

DCPIP reduced/ min/mg protein with extinction coefficient 19.1mM^{-1} cm^{-1}(4).

5.2.5.7 Determination of complex II activity

The Complex II activity was determined from the rate of reduction of DCPIP as the change in absorbance at 600 nm after the addition of antimycin. One ml of the reaction mixture included 3 mg/ml BSA, 2 millimol/l EDTA, 2 millimol/l KCN, 1 µmol/l antimycin A, 1 µmol/l rotenone, 20 millimol/l sodium succinate, 65 µmol/l decyl ubiquinone, approximately 40 µg mitochondria and 50 millimol/l phosphate buffer (pH 7.2). The reaction was initiated with the addition of 60 µmol/l DCPIP and observed at 600 nm spectrophotometrically. The activity was expressed as micromoles of DCPIP reduced/min/mg protein with extinction coefficient of DCPIP is 19.1mM^{-1} cm $^{-1}$ (4).

5.2.5.8 Determination of complex III activity

The complex III activities were determined spectrophotometrically. Incubations included 40 µg mitochondrial protein mixed with approximately 20 µg Mitochondria was mixed with 100 µmol/l EDTA, 2 mg BSA, 3 mM/l sodium azide, 60 µmol/l ferricytochrome-C, decylubiquinol and 50 mM/l phosphate buffer (pH 8) in a final volume of 1 ml. Decylubiquinol was prepared by mixing 1.3 mM/l decylubiquinone with a few grains of sodium dithionate and vortexed vigorously. The resultant solution was centrifuged at 12,000g for 10 min. The supernatant containing decylubiquinol was used for the assay. The reaction was monitored at 550 nm after the addition of 1 µmol/l of antimycin A. The activity was calculated from the extinction

coefficient of ferricytochrome C ($21mM^{-1}cm^{-1}$). Activity of complex III was expressed as micromoles of ferricytochrome C reduced/min/mg protein(5).

5.2.5.9 Determination of complex IV activity

Ferricytochrome-C was mixed with a few grains of sodium dithionate in 30 mM phosphate buffer (pH 7.4) for 10 to 20 min in the dark. The prepared solution was centrifuged at 12,000 g for 10 min. The supernatant containing ferrocytochrome C was used for the complex IV assay. One ml of ferrocytochrome C was mixed with approximately 10 µg mitochondria and 1 ml phosphate buffer. The reaction was started by the addition of enzyme source and was monitored at 550 nm. Complex IV activity was expressed as µmoles of ferrocytochrome-C oxidized/min/mg protein using the extinction coefficient 21 mM^{-1} cm^{-1}(6).

5.2.6 Preparation of PRP, thrombin and PRP activation

Preparation of PRP, thrombin and PRP activation (8-10) is carried out as per the methods described in chapter 2.

5.2.7 cytokine and chemokine profiling of PRP

We analyzed relative quantitation of cytokines and chemokines using a LEGENDplex Rat Th Cytokine Panel (13-plex) (Biolegend, San Diego, CA) Kit. Cytokine levels CCL2, tumor necrosis factor-alpha (TNF- a), IL-1 BETA, CXCL10, IL2, IL4, IL-6 IL-10, IL-12, IL-17A, TGF-BETA, CXCL8, interferon-GAMMA (IFN-γ), in supernatants were measured with flow cytometry according to the manufacturer 's protocol.

5.2.8 Mitochondria transplantation study for endometrial regeneration

Mitochondria play pivotal roles in embryogenesis. We explored whether mitochondria transplantation in disturbed endometrium

show regeneration as compared to control group. Use of activated PRP before Mitochondria transplant will facilitate the gel like matrix that will help Mitochondria to adhere properly within disturbed endometrium. We studied if hMTx-aPRP could be a regenerative resource to improve disturbed endometrium in experimental group.

5.2.8.1 Experimental Animal Groups and treatments

The study was approved by Institutional Animal Ethics Committee (DYPMCK/IAEC/2020/JAN/01). Animal handling and use were carried out in accordance with Institutional Animal ethical Committee guidelines. Female, Wistar rats were assigned into control and experimental groups. Female, adult Wistar rats of approximately 5 to 6 months age weighing ~ 250-300 gm were used. The female Wistar rats (n=54) were housed in plastic cages. Two rats were housed together in each animal cage (420×270×180 mm) with a 12 h light/dark. Animals received tap water and chaw food pellets ad libitum and room temp was maintained at 24°C. Vaginal smearing was used to confirm normal cycling and to detect the stage of the estrous cycle (11). The animals were divided into groups: Control group and Experimental group.

In control group, 18 rats were anesthetized with subcutaneous, extra-peritoneal injection of Thiosol 1 g/vial, (Neon laboratories Ltd.) at a dose of 12 units/gm of body weight (12). The uterine horns were exteriorized and clamped just above the cervix with small curved hemostatic forceps. The 0.25 ml intrauterine infusion of normal saline is administered in uterine horns. While 36 rats in the experimental groups were administered 0.25 ml of 95% ethanol into uterine horns with

insulin syringe with a 30-gauge needle to disturb the endometrial lining. In experimental group, rats were examined for disturbed endometrium. Experimental group is again subgrouped into disturbed endometrium (DE) group and hMTx-aPRP group. After 24 hrs of endometrial disruption, the DE lining of each rat in hMTx-aPRP group were administered with intrauterine infusion of activated PRP followed by 1×10^6 Rhodamine B labelled mitochondria (1:1) i.e., 0.25 ml hMTx-aPRP into both uterine cavities as the same manner by the surgical procedure described above initially (Figure 5.2). The three animals from each group were sacrificed at 24hr, 48hr and 72hr interval. The mid-part of uterine horns was excised and placed into 10% neutral buffer formalin for further study of immunohistochemistry and bioassay.

Animals were sacrificed post experiment by cervical dislocation. A yellow-colored category No.2 biomedical waste management bangs were used for seal sacrificed rats. It was given to central incineration facility in Animal House of D. Y. Patil Medical College, Kasaba Bawada, Kolhapur.

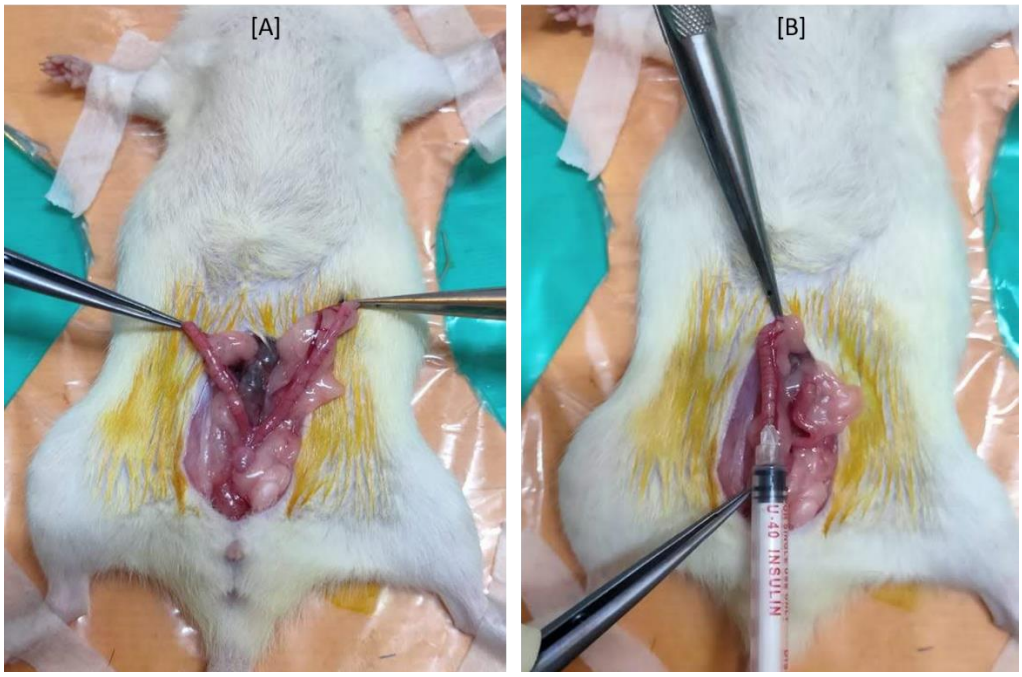

Figure 5.2: Intrauterine infusion of hMTx-aPRP.

5.2.9 Verification and Quantification of engrafted Mitochondria

To quantify the number of engrafted Mitochondria, 200 serial sections of 5 µm thickness were sectioned from the frozen tissue. Sections were stained with mitochondria-specific fluorescent Rhodamine B stain and counterstained with a nuclear stain DAPI. The numbers of engrafted mitochondria were established by enumerating Rhodamine B labelled Mitochondria in endometrium recruited from experimental transplanted groups. At a 20X magnification, the visual field of the Nikon camera corresponds to an area of 0.12mm^2. The central regions of such visual fields on each section were counted, corresponding to a surface area of 0.1mm^2/section (Figure 5.4). The number of engrafted mitochondria on a 1000µm^2 section was concluded from the number of mitochondria /0.1mm^2 by multiplying by a factor of 3.33. This number was again multiplied by 200 to get the total number of mitochondria /mm^2.

5.2.10 cDNA amplification and gene specific PCR

A frozen hMTx-aPRP transplanted endometrial tissue was cut in laminar airflow as per user instruction. It was washed using 1X PBS and added 0.25 % trypsin on it. The tissue was chopped using micro scissor. The suspension is washed using minimum volume of culture media (<0.5 µl to 4.5 µl) containing FBS. Then centrifuged it at 1500 rpm for 5 min. and discarded supernatant. One ml trizol reagent was added into pellet and homogenized in trizol using vortex. The homogenized sample was incubated at room temperature for 5 minutes, to which 250µl chloroform was added per ml of trizol used and was mixed vigorously by hand for 30 seconds. The sample was kept at room temperature for 5 min. After that the sample was centrifuged at 12000g for 15 minutes at 4°C. Carefully removed the upper aqueous layer using pipette in fresh sterile tube leaving behind some of the aqueous phase about 1 mm above DNA layer to prevent DNA contamination. Then 550µl of Isopropanol was mixed gently to the aqueous phase and kept at room temperature for 5 min followed by incubation in -20° for 10 to 15 min for better precipitation. It is then centrifuged at 12000g for 30 minutes at 4°C and removed the supernatant from tube obtaining only RNA in pellet. The pellet was washed with 500 µl of 75% ethanol. Vortex the tube briefly then centrifuged to pellet the RNA at 7500g for 5 minutes at 4°C. Discarded the wash and air dried the RNA pellet at room temperature for 5-10 minutes. It was resuspended in 50 µl of nuclease free water for 15 minutes for total solubilization of RNA into suspension and stored it at -80°C till further procedure. 500ng of RNA was used for cDNA synthesis. The High-capacity cDNA Reverse Transcription kit (Applied

biosystem - 4368814) is used for assay. All reagents were thawed on ice. A non-template control (NTC) and a non-enzyme control (NEC) were added. The additions in reaction mixture were 5X cDNA synthesis buffer 4µl, dNTP Mix 2µl, RNA Primer 1µl, RT Enhancer 1µl, Verso Enzyme Mix 1µl, RNA template 1µl adjusting the final volume of 20µl by Nuclease free water. The cDNA synthesis reaction was incubated at 42°C for 30 min and inactivated at 95°C for 2 min for 1 cycle. This step polyadenylates the first strand cDNA allowing subsequent global amplification of cDNA using a MT ATP-6, MT ATP-8, MT COX-1, MT COX -2, MT COX -3 primer. After the run, the tubes were stored carefully at -80^{0}C. All primers used were obtained from Eurofins. The forward and reverse sequence of oligonucleotides primers is

MT ATP-6	**(f: GAAGCGCCACCCTAGCAATA; R: GCTTGGATTAAGGCGACAGC)**
MT ATP-8	(f: TACTACCGTATGGCCCACCA; R: GCTTTGGTGAGGGAGGTAGG)
MT COX-1	(f: CGTTGTAGCCCACTTCCACT; R: GGCGTAGGTTTGGTCTAGGG)
MT COX -2	(f: CCGTCTGAACTATCCTGCCC ; R:GAGGGATCGTTGACCTCGTC)
MT COX -3	(f: ACCCTCCTACAAGCCTCAGA; R: GACGTGAAGTCCGTGGAAG)

Primary amplification products were again subjected to a further amplification. PCR was performed for each sample in duplicate and each gene with Negative control. The reaction was performed using 2X Power SYBR™ Green PCR Master MixCat.#4367659) containing SYBR® Green 1 Dye, AmpliTaq Gold DNA Polymerase LD, dNTPs with dUTP/dTTP blend and optimized buffer components. Amplification was carried out using the following cycling profile: initial denaturation at 95°C for 5 min followed by Denaturation at 95°C for 2 min, Annealing 40s at 55°C and 1min Extension at 72°C. Melting Curve Study was conducted at 95°C-60°C. Serial dilutions of secondary amplification products were prepared and used as the template in a PCR reaction to amplify β actin. The cDNA pools were

discarded if no β actin signal was detected. In subsequent PCR experiments to probe cDNAs for the presence of test genes, 1 ul of a 10-fold concentration of the cDNA dilution at which β actin amplification products were first detected was used as template. Human placenta derived mitochondria were processed as controls using the protocols described. All primer pairs successfully amplified DNA fragments of the appropriate size.

5.2.11 Hematoxylin-eosin Analysis

The uterine horn specimens were fixed for 24 hours in 10% neutral buffer formalin and embedded in paraffin. The 4 µm sections were taken and stained with hematoxylin-eosin (H&E). Endometrial morphology was explored by H&E staining. Images were captured using fluorescence microscope (NIKON). Images were captured at magnifications of 20X. Each slide was examined in a double blinded manner by using Image J software (MD, USA).

5.2.12 Immunohistochemistry analysis

The epithelial, stromal, and vascular cell growth was evaluated in disturbed endometrium following mitochondria transplantation by immunohistochemistry (IHC) for α-SMA (smooth muscle marker),CK-18 and CK-19 (epithelial cell markers), E Cadherin (adherence junction protein marker), Claudin-1and Zona Occludin (tight junction protein marker), and Connexin-40 (gap junction protein marker). Samples were fixed with 10% (w/v) neutral-buffered formalin .The 4µ tissue sections were deparaffinized by warming the slides at 60º C in oven for 10 minutes. Then slides were transferred in two changes of xylene for five minutes each. Sections were rehydrated through alcohol

grades and finally placed in D/W containing 0.05% tween 20 (Sigma-Aldrich). Antigen retrieval was done using 10 mmol citrate buffer (pH 6.0) for E-Cad (Mab anti human: Invitrogen), Cla-1 (Rabbit anti human: Invitrogen), ZO-1 (Mab anti human: Invitrogen) a-SMA (mouse monoclonal: AbDSerotec), CK-18 (mouse monoclonal: AbDSerotec) and CK-19 (mouse monoclonal: AbDSerotec) in a pressure cooker at 100°C for 20 min followed by 20 min cooling time and nonspecific binding was blocked with respective serum for 45 min at room temperature. The slides were incubated overnight at 4°C with primary antibodies E-Cad, Cla-1, ZO-1, CK-18with dilution 1:100 and CK 19, Connexin-40, a-SMA with dilution 1:200 in BSA (Hi Media) in humid chamber followed by washing with DW containing 0.05% Tween 20. Sections were further incubated for 60 min at room temperature with a secondary antibody labeled with Alexa 488 (Molecular Probe) in dark. After washing with D/W containing 0.05% tween 20, sections were counterstained with DAPI (Invitrogen). Sections were mounted in fluorescent mounting medium (Dako). Negative controls were stained without primary antibodies. Stained sections were examined under fluorescence microscope (NIKON). Two investigators graded E-Cad, Cla-1, ZO-1, CK-18, CK 19, Connexin-40 and a-SMA expression in a blinded fashion and assessed immunostaining using H score. H score =Σ Pi(i +1), where i is intensity of staining (13,14). Intensity of staining was assessed by blinded investigators as no E-Cad, Cla-1, ZO-1, CK-18, CK 19, and Connexin-40 and a-SMA signal (0). E-Cad, Cla-1, ZO-1, CK-18, CK 19, Connexin-40 and a-SMA positive samples were defined as having weak (1), moderate (2) or strong (3) signal and Pi is the

percentage of stained epithelial cells ranging from 0% to 100%. The H-score ranges from 0 to 4.

5.2.13 Treatment of aPRP improves birth rate of live pups

Female rats in aPRP treated group (n=3) were mated with male rats (n=5) at fifteen days after initiation of experimental study. Pregnancy outcomes included the time to conceive and live-birth rate.

Statistical analysis

Data are presented as mean values with standard deviations. Statistical analysis of mitochondria count of Rhodamine B stained transplanted Mitochondria was done using a two-tailed Student's t-test with P as the criterion of significance (P values: $P < 0.001$- difference highly significant; $P < 0.01$ - difference significant; $P < 0.05$-difference is said to be almost significant, $P > 0.05$- Not significant). Statistical differences in the numbers of transplanted mitochondria at 12hrs, 24 hrs and 48hrs were assessed.

5.3 Results

5.3.1 Preparation of mitochondria

Using the procedures described under Materials and methods, human placenta derived mitochondria were prepared. Approximately 41.8µg mitochondrial protein/ml is estimated from 50 g of placenta.

5.3.2 Mitochondria viability

we evaluated viability by flowcytometric analysis. Mitochondria viability is calculated by flowcytometer as 89.15% viability (Figure 5.3).

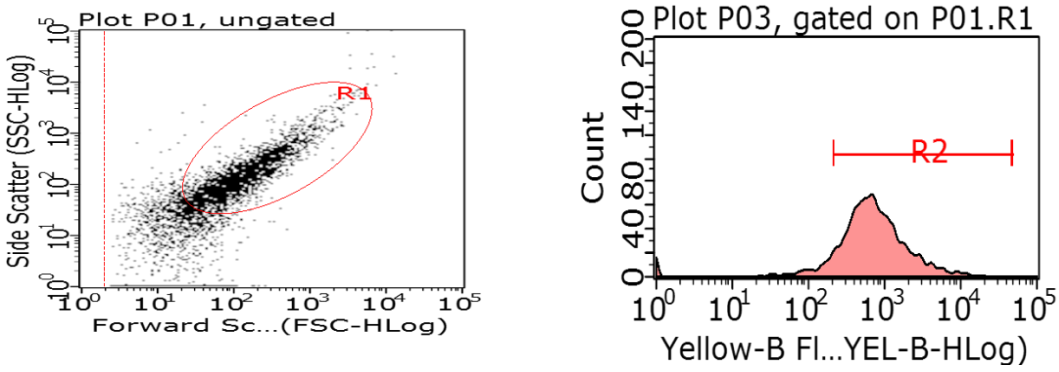

Figure 5.3: Mitochondria viability by Flowcytometry.

5.3.3 Quantification of Mitochondria engraft in endometrium of Rat

Engraftment of Mitochondria in rat endometrium was verified using Rhodamine B (Figure 5.4). We found that the magnitude of endometrial cells with regard to Rhodamine B stained mitochondria was highest in the 12 hr and 24 hr rat group. Scattered stained Mitochondria were detected between the stromal regions of endometrium in rat at 12hr, 24hr, 48hr. Endometrium sections at 72 hrs did not showed Rhodamine B stained mitochondria. Staining of endometrium sections from animals at 48 hrs revealed extremely few Rhodamine B positive mitochondria. The 12 hr and 24 hr group had higher numbers of mitochondria compared with 48 hr group. The number of stained mitochondria in 12 hr group was 63300±4666.9 mitochondria/mm^2 compared with 24 hr group 54607±2818.52 mitochondria/mm^2 ($P<0.01$) and 42828±3999.39 mitochondria/mm^2 in the 48 hr group ($P<0.01$). At 72 hrs, stained mitochondria were negligible in the endometrium of mice, suggesting that transplanted mitochondria may started to proliferate to a large extent or Rhodamine B stain get fade up (Figure 5.5). In particular, the immunohistochemistry of 24 hr

group and 48 hr group had showed very few proliferation markers while endometrium sections from 72 hr group show the presence of proliferation markers to larger extent, so we can conclude that transplanted mitochondria get started to differentiate into endometrial cells.

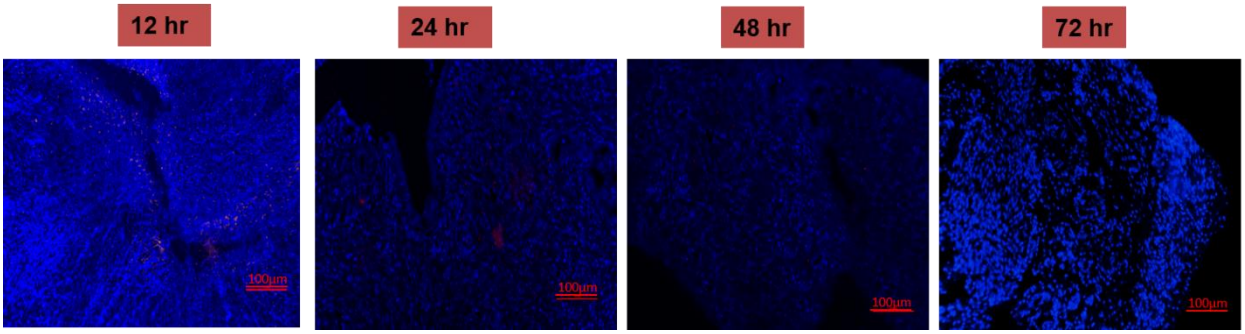

Figure 5.4: Engraftment of transplanted Mitochondria.

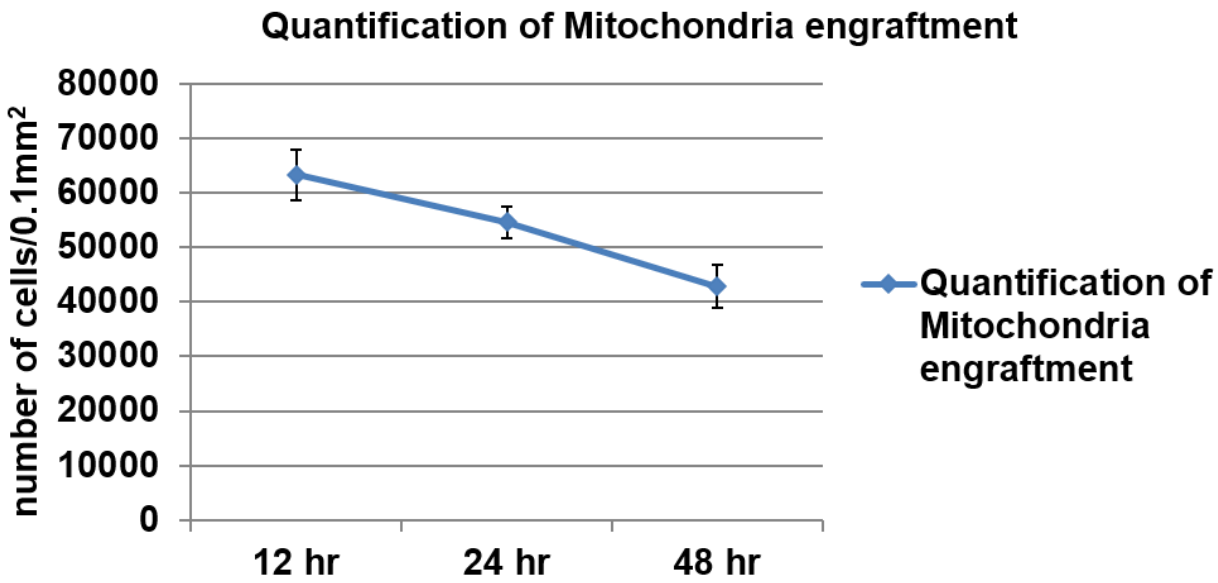

Figure 5.5: Quantification of transplanted Mitochondria.

5.3.4 Determination of specific activities of enzymes in mitochondria

In mitochondria, the specific activities of enzymes in µmoles/min/mg protein were tabulated in Table 5.1 and represented in figure 5.6. The activity of Cyt B5 (150.96±3.95), Cyt P450 (320.69±9.09) and Complex IV (836.40±15.39) in the

mitochondria were low as compared to NADH-cyt c red (1,174.46±5.04), NADPH-cyt c red (1,132.41±4.16), Complex I (1,039.55±11.41), Complex II (1,043.77±10.45), and Complex III (1,089.15±19.28). The maximal activity was observed in the NADH-cyt c red.

Table 5.1: Determination of specific activities of enzymes

Specific activity (µmoles/min/ mgprotein)	NADH-cyt C red.	NADPH-cytC red	CytB5	CytP450	Complex I	Complex II	Complex III	Complex IV
Mitochondria	1,174.46 ±5.04	1,132.41 ±4.16	150.96 ± 3.95	320.69 ±9.09	1,039.55 ± 11.41	1,043.77 ±10.45	1,089.15 ±19.28	836.40 ±15.39
Control Endometrium	1456.10 ±5.36	1190.66 ±8.00	135.72 ± 5.13	257.57 ±7.97	1836.09 ±3.70	1458.56 ±7.10	1639.18 ±8.85	1140.41± 13.22
DE 24hrs	128.53 ±5.27	137.21 ±10.50	23.68 ±1.52	163.91 ±3.33	139.86 ±10.23	109.74 ±9.67	176.31 ±5.06	232.37 ±17.11
hMTx -aPRP 24hrs	148.80 ±48.70	137.21 ± 10.50	114.51 ±2.64	41.08 ±5.27	130.51 ±8.79	162.16 ±4.09	736.22 ±7.13	271.92 ±6.69
DE 48 hrs	165.94 ±5.01	273.45 ±4.93	43.44 ±6.80	165.18 ±4.56	135.35 ±4.57	115.93 ±6.01	173.63 ±4.89	284.68 ±4.60
hMTx -aPRP 48 hrs	1229 ±12.85	1572.91 ±15.27	114.44 ± 3.51	274.58 ±2.80	170.66 ±7.86	184.09 ±3.21	1672 ±8.49	1084.36 ±14.68
DE 72 hrs	169.27 9.01±	464.57 ±2.51	63.77 ±3.60	170.66 ±5.97	128.96 ±11.29	118.20 ±8.63	217.49 ±9.99	272.35 ±10.00
hMTx -aPRP 72 hr	1237.86 ±8.08	1374.58 ± 3.21	135.05 ±5.03	366.12 ± 11.97	269.13 ±5.68	119.55 ±10.00	1436.45 ±9.61	1436.65 ±9.61

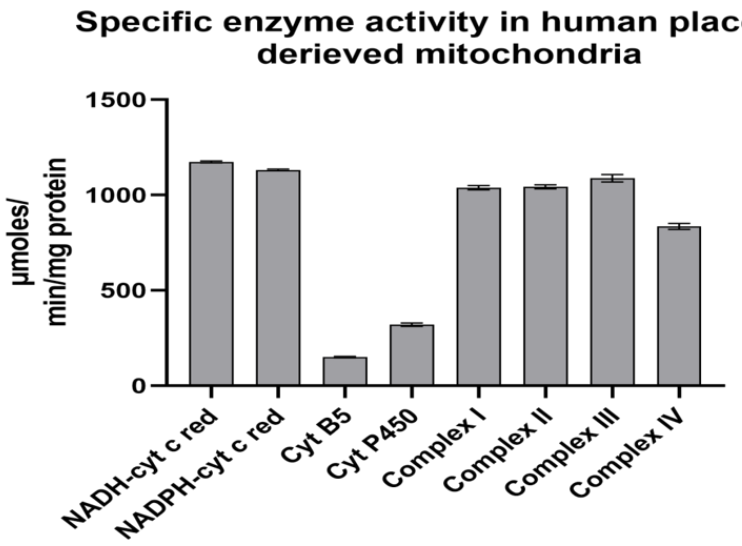

Figure 5.6: Enzyme activities of mitochondria derived from human placenta.

Results are mean ±SD (n=6)

Values of specific activities of all above mentioned enzymes were determined in µmoles/min/mg protein.

The specific activities of NADH cyt C red, NADPH cyt C red, Cyt B5, Cyt P450, Complex I, Complex II, Complex III and Complex IV are summarized in Table 5.1. The graphical representation of quantified NADH-cyt C red activity in Mitochondria, control endometrium, disturbed endometrium (DE) 24hrs, DE 48 hrs, DE 72hrs, hMTx-aPRP 24 hrs group, hMTx-aPRP 48 hrs, hMTx-aPRP group 72 hrs is summarized in Figure 5.7A. When compared to control endometrium (1257.79±135.01) with DE 24 hrs (128.53±5.27), DE 48 hrs (165.94±5.01), DE 72 hrs (169.27±9.01), hMTx-aPRP 24 hr (148.80±48.70), hMTx-aPRP 48 hrs (1229.00±12.85), hMTx-aPRP 72 hrs (1237.86±8.08), maximal activity of NADH-cyt c red activity was observed in hMTx-aPRP 72 hrs group. As compared to control group, the specific activities of NADH-cyt c reductase were declined by 89.78% in DE 24 hrs group, 86.81% in DE 48 hrs group and 86.55% in DE 72 hrs group (p->0.05). NADH-cyt c red

activity was increased by 1.15 fold (p->0.05) in hMTx-aPRP hrs group, 7.31 fold (p->0.05)in hMTx-aPRP hrs group and 7.41fold in hMTx-aPRP 72 hrs group as compared with DE 24 hrs, DE 48 hrs and DE 72 hrs group. In experimental group, it is clearly showed that the activities were highest in hMTx-aPRP 72 hrs group by 7.41 fold than that of hMTx-aPRP 24 hrs group and hMTx-aPRP 48 hrs group (Figure 5.7A).

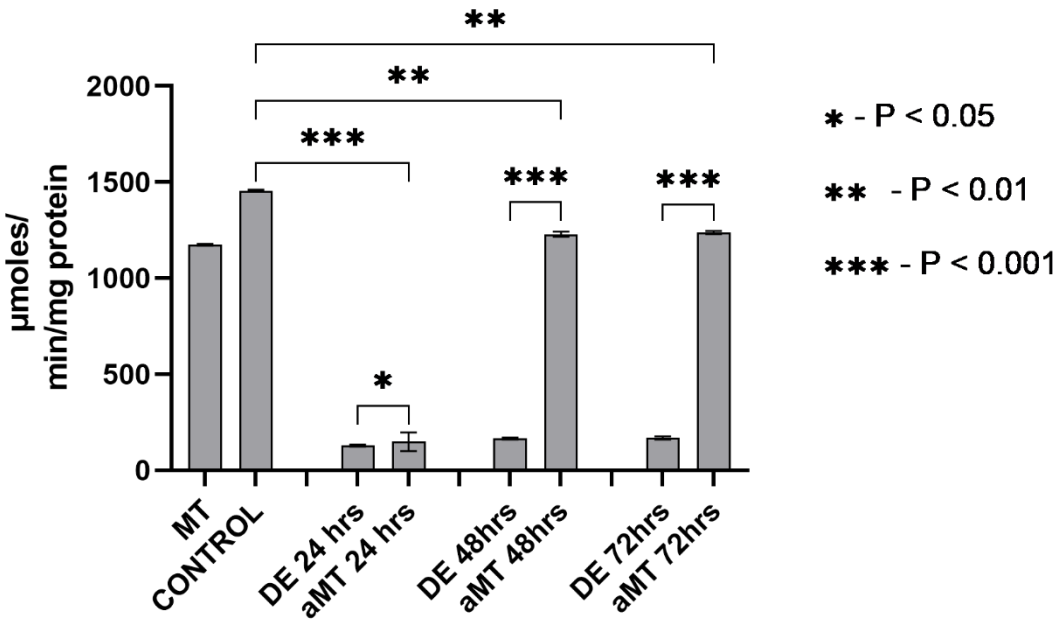

Figure 5.7A: Specific activity of NADH-cyt c red.

The specific activity of NADPH cyt C red detected in mitochondria is 1132.41±4.16 while in control, it is 1190.66±8.00. In DE 24hrs, DE 48 hrs and DE 72hrs, it is 137.21±10.50, 273.45±4.93, 464.57±2.51 respectively. When endometrium is disturbed with ethanol, NADPH cyt C red concentration in endometrium was reduced by 88.48 % in DE 24hrs as compared to control (P - > 0.05). In hMTx-aPRP 48 hrs group, the endometrial tissue represented higher specific activity (1572.91±15.27) than that of hMTx-aPRP 24hrs group (137.21± 10.50) and hMTx-aPRP 72hrs group (1374.58±3.21). This enhanced activity observed in hMTx-

aPRP 48 hrs was statistically significant (P - < 0.05). It was increased by 1.3 fold than control (Figure 5.7B).

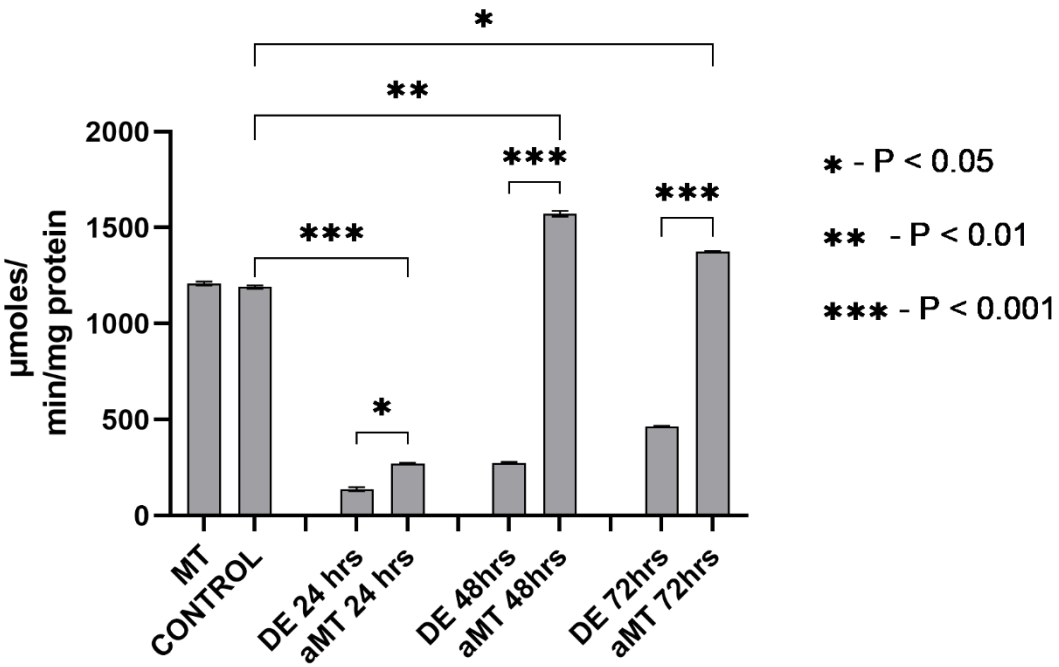

Figure 5.7B: Specific activity of NADPH-cyt c red.

In mitochondria, the Cyt B5 content was high (150.96±3.95) when compared to control (135.72± 5.13). The higher amount in the hMTx-aPRP was statistically significant. The transplantation of mitochondria in disturbed endometrium showed significantly enhanced Cyt B5 level at hMTx-aPRP 72hrs group (135.05±5.03) and reduced Cyt B5 level at hMTx-aPRP 24hrs group (114.51±2.64) and hMTx-aPRP 48 hrs (114.44±3.51) in the rats. The hMTx-aPRP 48 hrs (114.44±3.51) showed decrease of 12.35% as compared to control. There was approximately 4.83, 2.63 and 2.14 fold increases (p->0.05) for Cyt B5 level in hMTx-aPRP 24hrs, hMTx-aPRP 48hrs and hMTx-aPRP 72hrs as compared to DE 24hrs, DE 48hrs and DE 72hrs respectively ((Figure 5.7C).

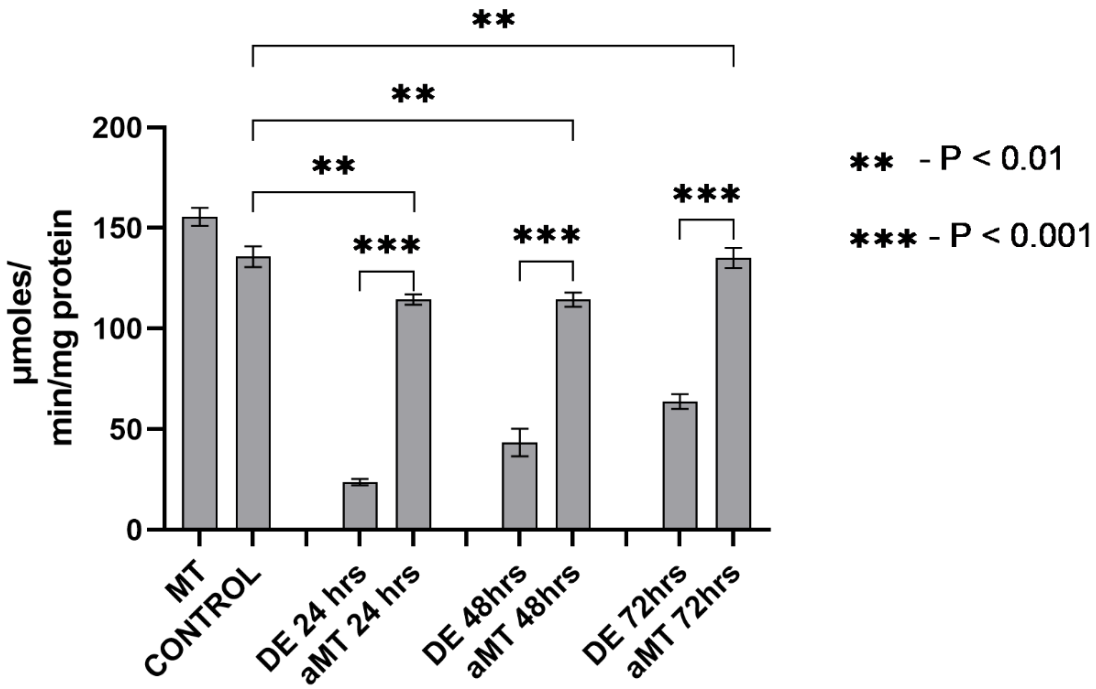

Figure 5.7C: Specific activity of Cyt B5.

In mitochondria, Cyt P450 revealed 320.69±9.09. Control endometrium exhibited 257.57±7.97. Endometrial disturbance by ethanol treatment decreased the activities of CytP450 in endometrial tissue by approximately 76.4 % (P - < 0.001). Simultaneous treatments of hMTx-aPRP on disturbed endometrium elevated the activities by 4.56 fold (P -> 0.05) and 6.19 fold (P -< 0.001) in hMTx-aPRP 48 hrs group (274.8±2.80) and hMTx-aPRP 72 hrs group (P -< 0.001) respectively but hMTx-aPRP 24hrs group (41.08±5.527) showed reduced enzyme activities by 39% (P - < 0.05) (Figure 5.7D).

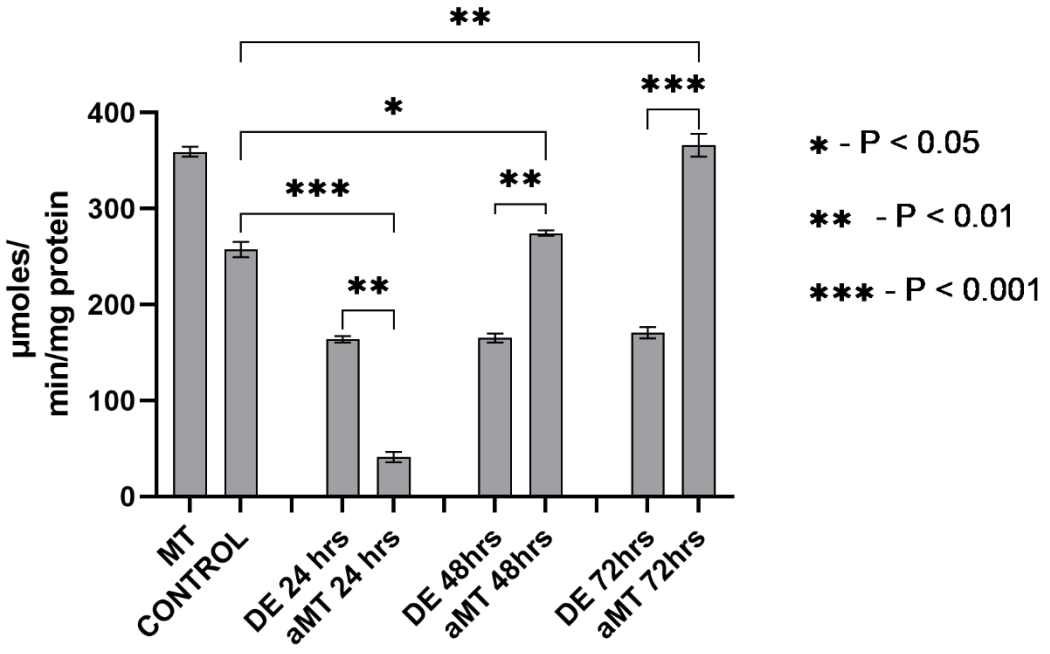

Figure 5.7D: Specific activity of cyt P450.

In mitochondria, the specific activity of Complex I was found to be 1039.55±11.41. Control (1836.09±3.70) revealed approximately 1.66 fold increase than mitochondria and disturbed endometrium analysed decline of approximately 92.44% as compared to control (P->0.05). When scrutinizing this enzyme level at different experimental groups, it is declined in hMTx-aPRP 24hrs group (130.51±8.79) and hMTx-aPRP 48 hrs group (170.66±7.86) by 92.82% and 90.63% respectively (P-<0.05). The highest activity was measured in hMTx-aPRP 72hrs group (269.13±5.68) is magnified by 1.18 fold (P->0.05) (Figure 5.7E).

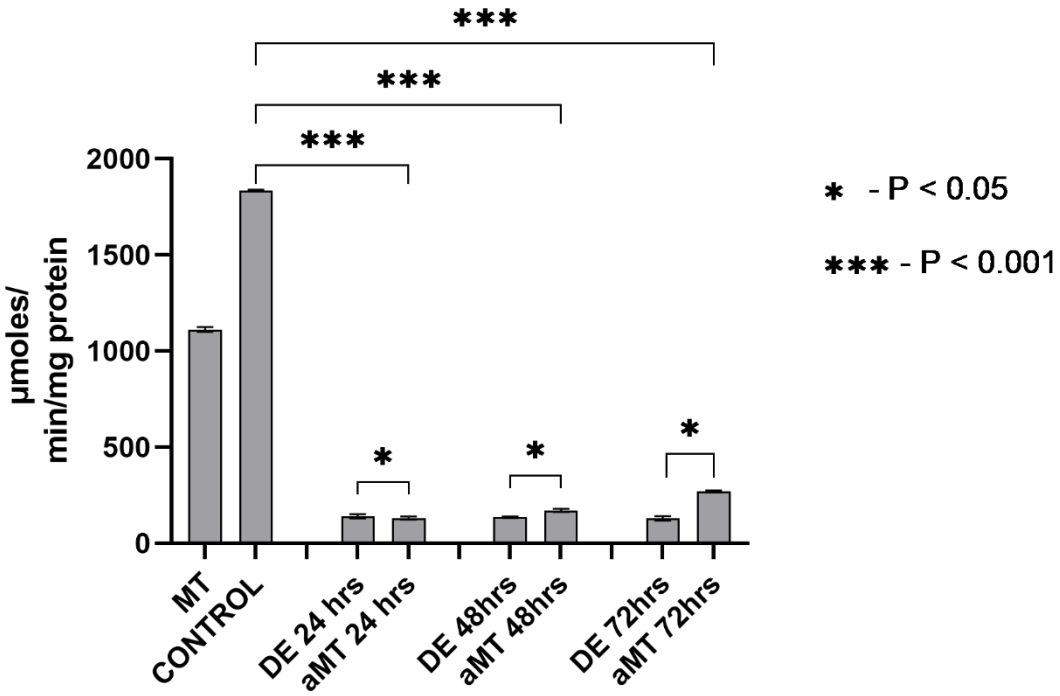

Figure 5.7E: Specific activity if Complex I.

The specific activities of Complex II were depicted in Figure 5.7F. Both mitochondria (1,043.77±10.45) and control (1458.56±7.10) exert significantly higher activity (P-<0.05) and all disturbed endometrium showed decline of approximately 86.96% than control (P->0.05). This enhanced activity of Complex II were very high in hMTx-aPRP 48 hrs group (184.09±3.21; P->0.05) when compared to hMTx-aPRP 24 hrs group (162.16±4.09; P->0.05) and hMTx-aPRP 72hrs group (119.55±10.00; P->0.05). The hMTx-aPRP 24hrs group, hMTx-aPRP 48hrs group and hMTx-aPRP 72hrs group showed increase of 1.01 fold, 1.21 fold 4.27 fold, 1.37 fold (P->0.05) respectively as compared to disturbed endometrium.

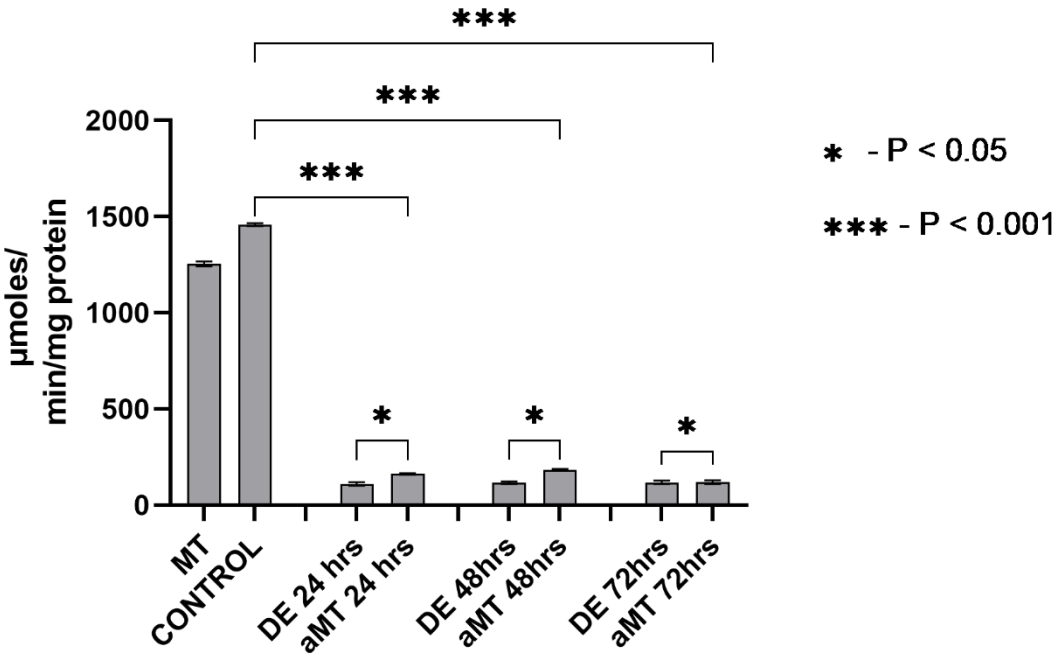

Figure 5.7F: Specific activity of Complex II.

In mitochondria, the Complex III content was 1089.15±19.28. Control showed significantly highest activity of 1639.18±8.85 (p-<0.05). Disturbed endometrium demonstrated lowest values (p->0.05). The transplantation of mitochondria in disturbed endometrium showed significantly gradually enhanced Complex III level at hMTx-aPRP 24hrs group (736.22±7.13), hMTx-aPRP 48hrs group (1672 ±8.41) and hMTx-aPRP 72hrs group (1436.45±9.61) in the rats (Figure.5.7G) (p-<0.05). Disturbed endometrium showed decline of 89.4 % than control. The hMTx-aPRP 24hrs group, hMTx-aPRP 48hrs group and hMTx-aPRP 72hrs group showed gradual increase of 4.27 fold, 8.30 fold and 9.45 fold respectively as compared to disturbed endometrium (Figure 5.7G).

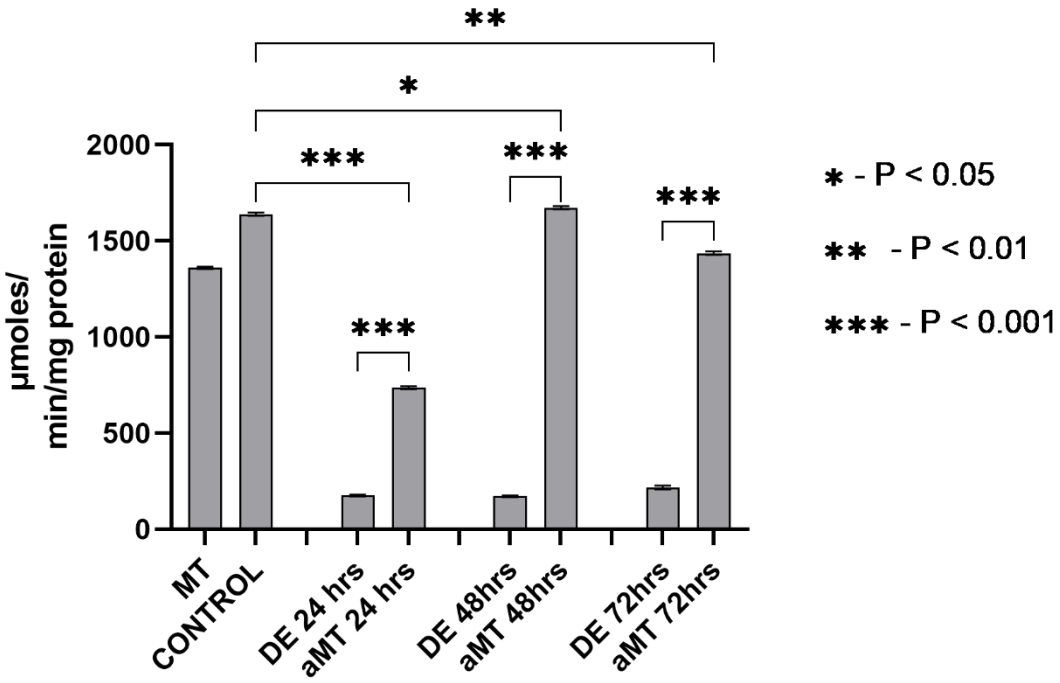

Figure 5.7G: Specific activity of Complex III.

The quantified Complex IV activity was represented in Figure 5.7H. The mitochondria showed 836.40±15.39 of activity. Control revealed 1140.41±13.22µmoles/min/mg protein (p->0.05). Disturbed endometrium showed decline of 80.38% than control. In hMTx-aPRP 24hrs group (271.92±6.69), hMTx-aPRP 48hrs group (1084.36±14.68) and hMTx-aPRP 72hrs group (1436.65±9.61), a significantly enhanced level of Complex IV activity was observed (p-<0.05).

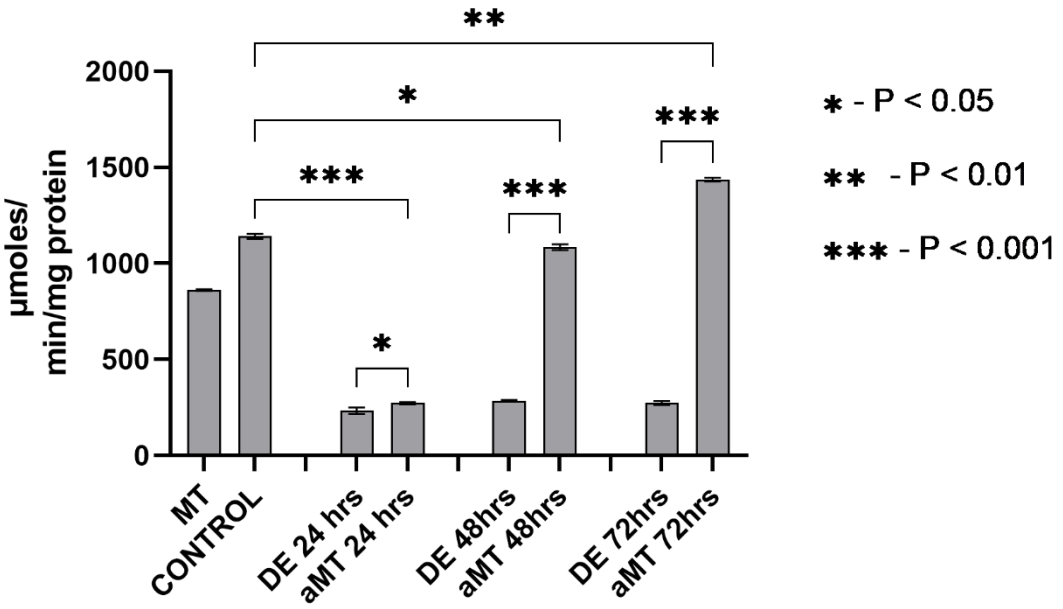

Figure 5.7H: Specific activity of Complex IV.

5.3.5 Detection of human-specific mitochondria mRNA gene expression in recipient Rat uterus using PCR

A cDNAs was successfully amplified from lysed uterine sample harvested at 24 hr, 48 hrs and 72 hrs after hMTx-aPRP transplant. The uterine sample expressed β actin. Analysis of human-specific mitochondria mRNA gene expression in these amplified cDNAs was undertaken and these data are shown in Figure 5.8. Expression of MT ATP-6, MT ATP-8, MT COX-1, MT COX-2, MT COX-3 was constitutive. Detection of human specific mitochondria mRNA in recipient rat uterus using PCR was performed to detect the presence of human MT ATP-6, MT ATP-8, MT COX-1, MT COX -2, MT COX -3 mRNA. The real-time PCR results of rat uterus showed significant up-regulation of MT ATP-8, MT COX-1, MT COX -3, MT COX -2, MT ATP-6 (p=0.009) in the hMTx-aPRP treated group compared to the mitochondria as a control group.

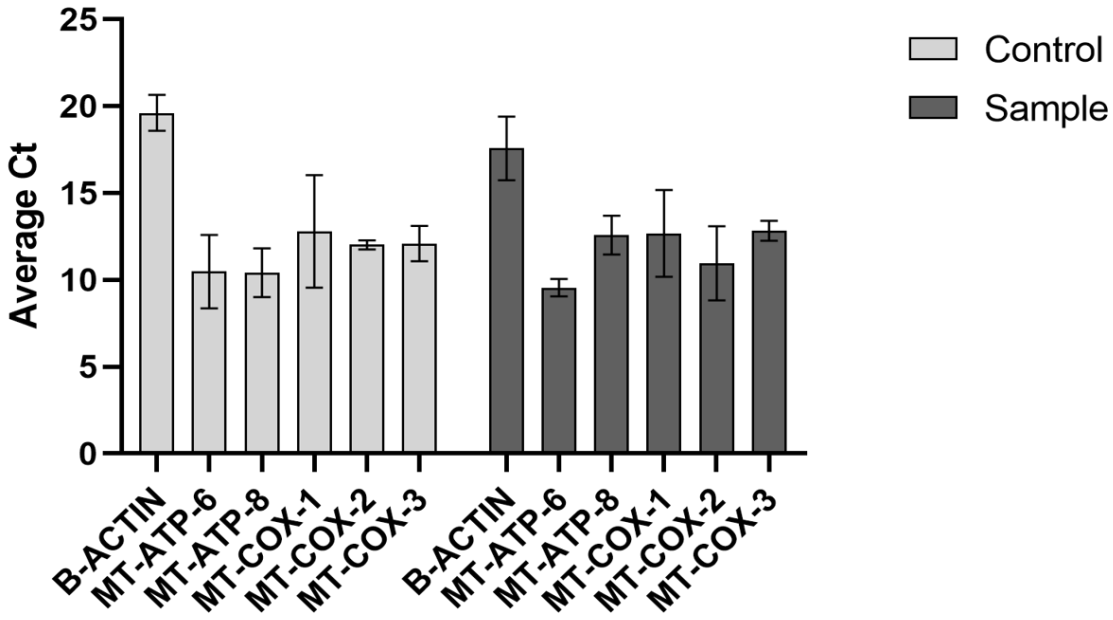

Figure 5.8: Real time-PCR gene expression in rat endometrium.

5.3.6 H&E staining

To evaluate endometrial damage and the effect of hMTx-aPRP in our rat model of damaged endometrium, H&E staining were performed. Section from the uterus of rats in control group, DE group and hMTx-aPRP group were analyzed (Figure 5.9). The DE group showed narrowed endometrial lumen lined by atrophic columnar epithelium with degenerative changes and loss of endometrial glands. However, in the hMTx-aPRP treated group, we found that basalis layer was distinguishingly increased with prominent nucleoli, proliferated glands and stromal cells, compared to the DE group.

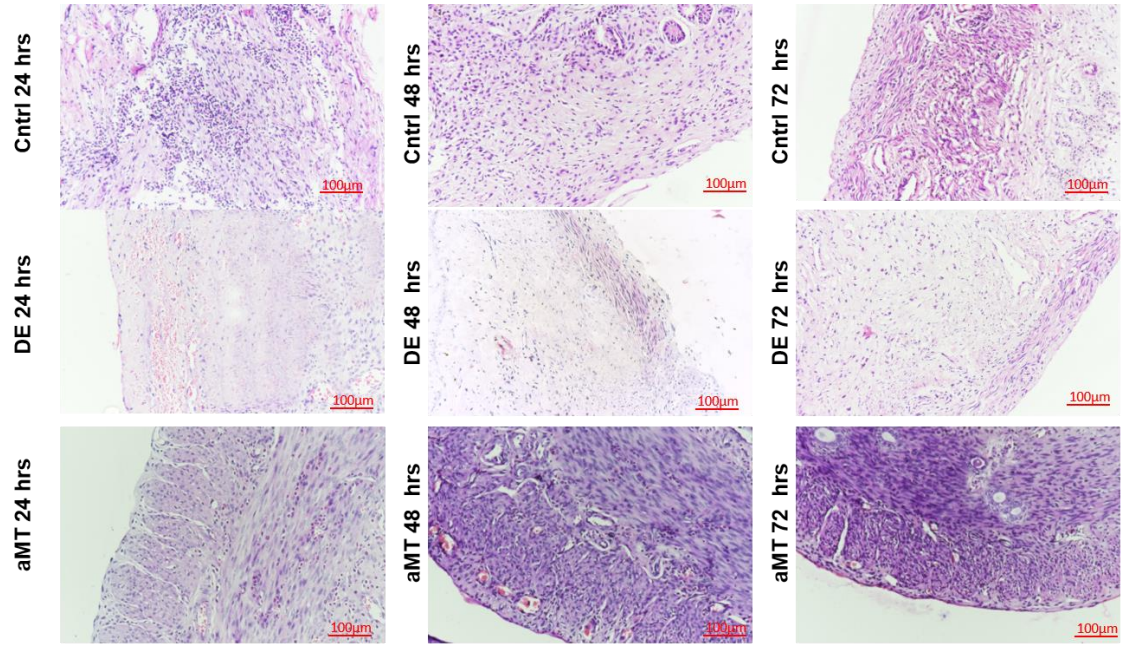

Figure 5.9: Hematoxylin and eosin analysis.

5.3.7 IHC staining analysis to ensure the hMTx-aPRP engraft in disturbed endometrium

We explored the effects of hMTx-aPRP transfer in disturbed endometrium. We microinjected the activated PRP followed by hMTx into disturbed uterine cavities of rat and compared the results at 24h, 48h and 72h with control and disturbed endometrium. Engraftment of hMTx-aPRP in rat endometrium was verified by the use of antibodies specific for a-sma, CK 18, CK 19, Cx 40, E cad, Cla-1 and ZO-1. Scattered expressions were detected between the stromal regions of disturbed endometrium at 48hr and 72 hr. Disturbed endometrium at 24 hr did not showed any expressions. Endometrial sections of 48 hr group from hMTx-aPRP animals revealed extremely few expressions as compared to hMTx-aPRP 72 hr group.

In the control group, IHC results showed that the expression of Cx 40 was mainly localized in the endometrial epithelial cells of stratum basalis while other expressions were observed in the endometrial glandular and stromal region. The expressions of a-sma, CK 18, CK 19, E cad and Cla-1

were detected in the endometrial stromal cells whereas ZO-1 in stratum functionalis region. Compared to the positive control and control group, IHC staining in the disturbed endometrium group showed significantly decreased expressions of a-sma, CK 18, CK 19, E cad, Cla-1, Cx 40 and ZO-1. The disturbed endometrium at 24hrs and 48hrs did not show expressions of any antibodies. The disturbed endometrium at 72 hrs had weak expressions of a-sma at stratum basalis cells and Cla-1 in stratum functionalis cells. We found that the microinjected hMTx-aPRP group was obviously showed increased expressions of these factors as compared with disturbed endometrium group at any hrs. The expression of these factors was significantly higher in the hMTx-aPRP treated group at 48 hrs compared with disturbed endometrium groups. In addition, the hMTx-aPRP treated group at 72 hrs demonstrated strong expressions of CK 18 and E-cad and moderate expressions of a-sma, Cx-40, Cla-1 and comparatively less expressions of CK-19 and ZO-1. There was no significant difference in the expression of CK-18 between the hMTx-aPRP treated group and the control group. The hMTx-aPRP treated group had higher expressions of E-cad compared with control (Figure 5.10A, 5.10B, 5.10C).

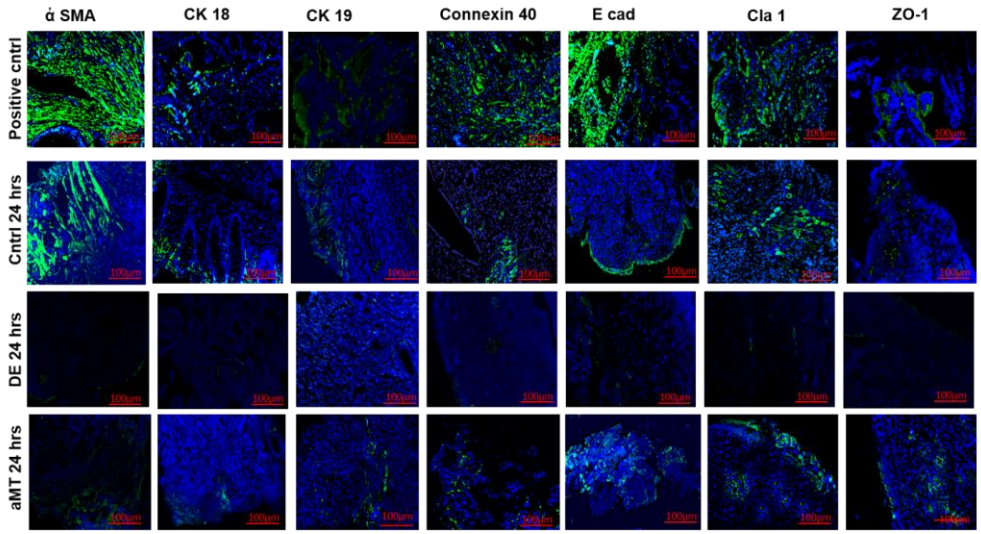

Figure 5.10A: Immunohistochemistry of Rat endometrium 24hr after mitochondrial

transplant.

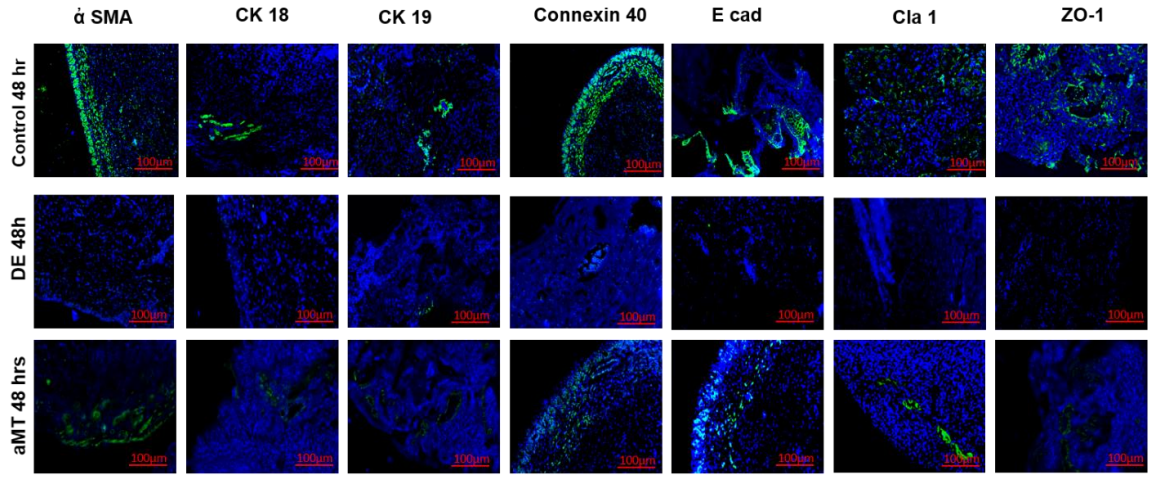

Figure 5.10B: Immunohistochemistry of Rat endometrium 48hr after mitochondrial transplant.

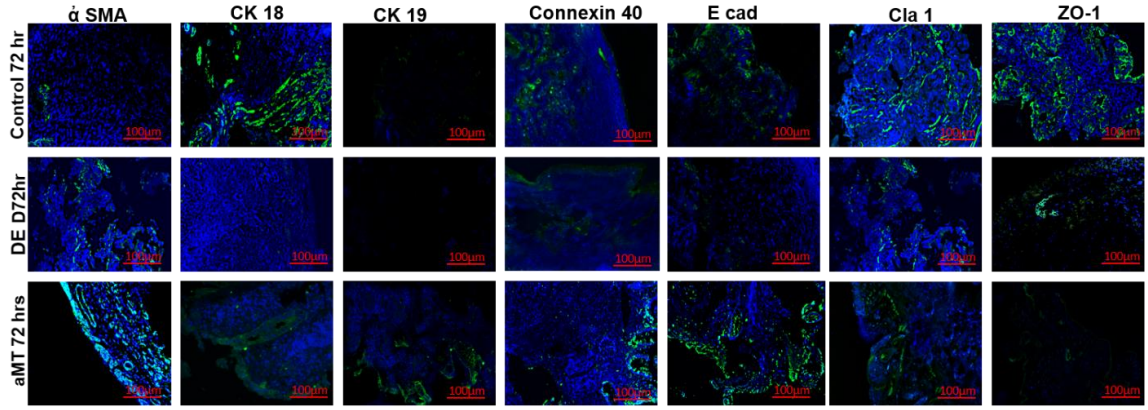

Figure 5.10C: Immunohistochemistry of Rat endometrium 72hr after mitochondrial transplant.

5.3.8 Treatment of hMTx-aPRP in DE Improves Live pups in Wistar rats

The female rat was mated with a fertile male. All rats from control group gave birth to average 7 live pups. DE group did not give birth to any live pups. All rats from aPRP treated groups gave birth to 12 pups. The average days required to conceive for hMTx-aPRP treated group was 5 days. The hMTx-aPRP treatment clearly improved the rate of live-births since DE group failed to deliver (Table 5.2).

Table 5.2: hMTx-aPRP Tx in DE Improves live birth

Variables	Control group	DE group	aPRP treated group
Live births	7	-	12
average days to conceive	3 days		5 days

5.4 Discussion

Mitochondria transplantation therapy has attracted considerable interest as an innovative therapeutic strategy for treatment purposes. But several critical points regarding Mitochondria transplant remain unexplained. There are a number of critical issues regarding mitochondria transplants that need to be overcome. Mitochondria contain their own DNA that encodes the subunits of the oxidative phosphorylation system. Mitochondria are a source of energy production and synthesis. Most of the energy needs of the body cell are derived through the electron transport chain and oxidative phosphorylation. If we augment this powerhouse of energy source to damaged or degenerated parts, then degenerated cells will regain their regenerative potential by rescuing cell degeneration. We can speculate that mitochondria transplanted into the damaged region would provide a nutrient supplement to allow cell recovery. Mitochondrial uptake by vascular delivery would be very complex. The direct delivery of mitochondria to the affected area would be a better option with very few complications. The only drawback of local transplant is that the cells may fail to intact with the site and get expelled. Disturbed endometrium involves a damaged or disordered ECM that must be remodelled and repaired. To keep the transplanted mitochondria intact at disturbed endometrium (DE), we used a strategy to transplant hMTx-aPRP for stable gel layer formation. The gel layer of aPRP will assist in the stabilization of transplanted mitochondria. It will hold transplanted mitochondria in place

by preventing their loss. Further aPRP will promote the functionality of transplanted mitochondria and regenerative changes in DE as aPRP contains numerous growth factors. This layer will act like a path that mitochondria will interact with endometrial cells to conduct cellular events like adhesion, migration, differentiation. In hMTx, engraftment of these cells in the disturbed endometrium is influenced by many factors, including Hypoxia-inducible factor, inflammatory response, impact on chemokines and cytokines, deficit nutrients, and free oxygen radicals. The disturbed endometrium may cause reduced cell proliferation, vasoconstriction of the endometrial vessels, along with lacking trophic support. Joshi et al., stated that the MSC produces high levels of IL-6 and IL-8 that may counterbalance the hepatocyte transplantation-induced liver inflammation (15). Likewise, the different chemokines and cytokines release reported by thrombin activated PRP. It is essential to optimize whether the mitochondria transplantation withstands these conditions after cell infusion. However, there have been no studies to evaluate the recruitment capacity of these mitochondria to the endometrium. There is no data comparing local intrauterine administration for hMTx via intraluminal uterine injection either.

The measurement of specific activities of NADH cyt C red, NADPH cyt C red, Cyt B5, Cyt P450, Complex I, Complex II, Complex III, and Complex IV enzymes demonstrated that, in general, experimental hMTx-aPRP treated endometrial cells had significantly higher values compared with disturbed endometrium indicating the regeneration of damaged endometrial cells.

Mitochondria enzyme serves link between the Krebs cycle and the electron transport chain. The ATPs are produced in process of converting succinate to fumarate by oxidative phosphorylation. NADH cyt C red performs DNA repair functions. NADPH cyt C red has several essential roles in cell metabolism. It acts as a coenzyme in redox reactions. It acts as a

donor of ADP-ribose moieties in ADP-ribosylation reactions. It donates electrons during the process of cellular respiration and helps in the oxidation of glucose. They accept the energized electrons and pass this energy to power the electron transport chain reaction. Cyt b5 reductase has involvement in many oxidation and reduction reactions. It is involved in the transmission of reducing equivalents from the physiological electron donor, NADH, through a FAD domain to cytochrome b5. Mitochondrial complex I, II, III, IV enzymes utilize most of the energy released from the breakdown of nutrients to synthesize ATP. This ATP provides the energy required for cell reactions.

We verified the presence of differentiation of hMTx-aPRP by immunohistochemistry and Real time-PCR. Real time -PCR analysis showed expressions of the human mitochondria genes MT ATP-6, MT ATP-8, MT COX-1, MT COX -2, MT COX -3 in the hMTx-aPRP treated endometrial cells. Immunohistochemical analysis showed that the endometrium of experimental animals had an elevated number of cells positive for a-SMA, CK18, and CK19, Cx40, Cla-1, ZO-1. The expression of these factors in the regenerated rat endometrial cells provides useful information that regeneration of disturbed endometrium involves a vital role of hMTx-aPRP.

McCully and coworkers stated the transplant of mitochondria isolated from the intact tissue of the same patient in Ischemia. Masuzawa et al. studied transplantation of pectoral major muscle derived autologous mitochondria from the rabbit model of ischemic Cardiomyopathy (27,28). These studies do not show any significant increase in various inflammatory markers after mitochondrial transplantation. Immune responses in mitochondrial transplantation have not been discussed in prior studies. A few studies discussed immune responses during mitochondrial transplantation to date. They also transplanted mitochondria showed good engraftment and proliferative results without any adverse immune

rejection. Autologous transplantation of mitochondria is not possible in every diseased condition. Understanding immune response during mitochondria transplant would be of worth in reducing the risk associated with allogeneic mitochondria transplant. Allogeneic mitochondrial transplantation may be the next line of treatment. It will be very interesting from a scientific standpoint, and further research will likely be carried out in the future.

5.5 Conclusions

This study reports the novel strategy for boosting human placenta derived mitochondria engraftment by co-transplanting it with thrombin activated PRP. This approach is promising so we could see the obvious effect of hMTx-aPRP on endometrial improvement. This study adds valuable knowledge on relatively unexplored protein modifications in endometrial cells by hMTx-aPRP. It would ultimately be reflected in higher energy production at the endometrial site. Mitochondria transplant as therapeutic molecules appears to have very promising in the coming future. The hMTx-aPRP promises an important adaptation of this therapy. This study will accelerate research related to innovative medicines based on mitochondrial transplantation in murine models. However, conducting a study in women undergoing IVF with a larger population and eliminating the confounding factors of implantation failure are recommended to achieve higher clinical pregnancy rates.

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Chapter 6

Disturbances in tight and adherence junction in the endometrial cells among women with unexplained infertility

6.1 Introduction:

The infertility management has several significant advances due to the new diagnostic tests to identify an underlying cause. The treatment preferences depend on the etiology recognized on standard tests during the infertility assessment. The couples who fail to conceive despite all investigations being reported normal is termed unexplained infertility. (1,2). The prevalence of unexplained infertility ranges from 8% to 37% (3). The reason of infertility could be caused by female factor (over one-third) or by male factor (over one-third) or by a combination of problems in both partners or is unexplained (approximately 20%) (4). Unexplained infertility is an idiopathic infertility in which causes remains unknown and investigations like semen analysis, tests of ovulation and tubal patency have failed to detect any gross abnormalities and cannot be detected by the routine infertility evaluation (5,6). Women in couples with infertility of unknown cause have normal ovulatory cycles and hormonal profiles, no organ pathology and their partners show no evidence of semen quality problems (7). Women with unexplained infertility is an attractive study group in the search for target junctions concerned with endometrial receptivity and implantation (8). Assessment of the endometrium beyond its appearance on ultrasound examination is limited due to lack of clinically useful tests of receptivity (9). Implantation depends on quality of embryo and receptivity of endometrium. Furthermore, in ARTs, impaired uterine receptivity is major reason behind embryo implantation failure (10–12). The junctional regulation of endometrium is a target for fertility regulation that influences embryo attachment (13–15). The cell–cell communication between the luminal epithelium modifies and alters polarity at post implantation stage. The attachment of luminal epithelial

cells makes contact at their apical borders where the trophoblast cells invade and penetrate the luminal epithelium and reach the basal lamina (16,17). Claudin-1 (Cla-1), Zona occludin-1 (ZO-1) and E-Cadherin (E-Cad) are with strong homophilic binding activity important for cell sorting and guidance that control lumen membrane changes in the period of implantation (18). It usually coexists in the subapical regions localized in the cytoplasmic surface of plasma membranes which seal the epithelial cells at their apices to create a primary barrier (19). It also functions polar distribution of plasma membrane proteins (20). In the current study, we investigated endometrial Tight junctions (TJs), Adherent junctions (AJs) and Vascular angiogenic precursor (VAP) expression profiles in women with unexplained infertility in comparison with fertile controls at the time of implantation window. In present study, immunohistochemical methods were used to investigate how the key AJs and TJs proteins like E-Cad, Cla-1, ZO-1 and VAP contribute to the morphological alterations during implantation. In addition to providing new insights into the complex process of endometrial receptivity, identification of TJs, AJs and VAP of receptive endometrium could lead to improvement in the diagnosis and treatment of infertility and promote the treatment of implantation-based infertility.

6.2 Materials and methods:

The use of specimens from human subjects was approved by Institutional Review Board of D. Y. Patil Medical College, Hospital and Research Institute Kadamwadi Kolhapur. The patients (n=5) diagnosed with unexplained infertility by hormonal assay, blood investigations, ultrasonography (USG), hysterosalpingography (HSG) and diagnostic laparoscopy at Dr. D. Y. Patil Medical College, Hospital and Research

Institute Kadamwadi Kolhapur were enrolled for the study. The endometrial biopsy from women who experienced first full term normal delivery is taken as control (n=5). All patients were within reproductive age ranging 30.5±2.5 years. All patients were nulliparous and had experienced at least two to three years of infertility. All women abstained from intercourse or used barrier methods of contraception to avoid pregnancy for the period between last menses and sample collection. Informed consent was obtained from all individual participants included in the study.

6.2.1 Ultrasonography:

It allows accurate and noninvasive evaluation of fibroids, cysts, polyps, congenital abnormalities, endometriosis or polycystic ovarian syndrome (PCOS), intrafollicular structures, cyclic uterine endometrial changes and demonstration of growing ovarian follicles (number and size). USG of patient was performed on SonoAce R7, China.

6.2.2 Hysterosalpingography:

Hysterosalpingography (HSG) suggests the opening of the fallopian tubes at the distal end and no blockage at the junction of the tube and uterus (proximal). HSG carried out by using MDX 100R, India.

6.2.3 Diagnostic Laparoscopy:

Diagnostic laparoscopy was done by using laparoscope (NPF Krylo, Russia) with a small incision in the abdominal wall. Images of pelvic cavity were taken with the help of high intensity light and high-resolution camera.

6.2.4 Endometrial fluid sample:

As a source of endometrial cells, aspirated endometrial fluid from the uterine cavity was collected. Samples were collected during the post-ovulatory secretory phase of the menstrual cycle by introducing an

intrauterine insemination cannula (Surginova, Mumbai, India) into the uterine cavity connected to a 10 ml syringe. Endometrial cavity was flushed by 1ml normal saline and aspirated. Sample extraction was performed by vacuum application with the syringe manually. Aspirate volumes varied from 0.6 to 1 ml. Neither analgesic nor local anesthetics were used during sample collection and only mild discomfort was reported in patients. Additional normal saline was added into an aspirated material to increase the volume. This aspirated saline was centrifuged (Remi RM-12C) at 1500 rpm for 5 minutes to obtain a pellet. These cells were used for the preparation of smear on positively charged glass slides (Pathnsitu Biotechnologies, Hyderabad, India). Smears were allowed to air dry. These smears were fixed by chilled 1:1 methenol-acetone (HiMedia, Mumbai, India) and stored in - 40^0C until further use.

6.2.5 Endometrial brush smear:

Endometrial cells were collected by rotating endometrial brush to traverse the uterine cavity. Cytological brush (Liang et al. 1994)(21) was passed up in the uterine cavity. Following the gentle probing, the brush was rotated twice or thrice to traverse the uterine cavity thoroughly. Rotating of the brush removes the endometrial cells by gentle scrapping. Smear of these cells were taken on the positively charged slides and subjected to Hematoxylin-Eosin (HE) staining and IHC.

6.2.6 Endometrial biopsy samples

Endometrial biopsies were performed in the luteal phase of the menstrual cycle (day 18–21 of menses) at Dr. D. Y. Patil Medical College, Hospital and Research Institute Kadamwadi Kolhapur. Endometrial biopsies were fixed in 10% neutral buffered formaldehyde/distilled water (v/v) at 4°C for 24 hrs and then

dehydrated in a series of alcohol grades, cleared in xylene, and embedded in paraffin. Serial sections (4µ) were taken by using microtome (Leica RM 2235, Nussloch, Germany). Sections were mounted on positively charged slides and subjected to HE staining and used for IHC.

6.2.7 HE staining of endometrial biopsy:

The sections of 4µ were taken by using high profile blades (Thermoscientific, US) with microtome. The sections were placed on albumin-coated slides. The sections were de-waxed in xylene and allowed to air dry. The sections were rehydrated in alcohol grades followed by D/W wash. Slides were stained with hematoxylin stain (Hi Media laboratories, Mumbai, India) for 2-3 minutes. After washing under running tap water, slides were dipped in acid alcohol. Again, slides were washed under running water for 15 minutes. Then counterstained with eosin (HiMedia laboratories, Mumbai, India) for 2 minutes and dehydrated in alcohol grades. The slides were air dried and treated with xylene. Slides were mounted by using DPX (Merk, India).

6.2.8 Immunohistochemistry analysis:

The 4µ tissue sections were deparaffinized by warming the slides at 60º C in oven for 10 minutes. The tight junction proteins markers (ZO-1 and Cla-1), adherence junction proteins marker (E-Cad) and angiogenic precursor marker (VAP) were evaluated by IHC. Then slides were transferred in two changes of xylene for five minutes each. Sections were rehydrated through alcohol grades and finally placed in D/W containing 0.05% tween 20 (Sigma-Aldrich, USA). Antigen retrieval was done using 10 mmol citrate buffer (pH 6.0) for E-Cad (Mab anti human: Invitrogen, USA), Cla-1 (Rabbit anti human: Invitrogen, USA), ZO-1 (Mab

anti human: Invitrogen, USA) and VAP-1 (santa cruz biotechnology, CA, USA) in a pressure cooker at 100°C for 20 min followed by 20 min cooling time and nonspecific binding was blocked with respective serum for 45 min at room temperature. The slides were incubated overnight at 4°C with primary antibodies Cla-1, ZO-1, E-Cad and VAP (dilution 1:100) in BSA (Hi Media, India) in humid chamber followed by washing with DW containing 0.05% Tween 20. Sections were further incubated for 60 min at room temperature with a secondary antibody labeled with Alexa 488 (Molecular Probe, Eugene, USA) in dark. After washing with D/W containing 0.05% tween 20, sections were counterstained with DAPI (Invitrogen, CA, USA). Sections were mounted in fluorescent mounting medium (Dako, Denmark). Negative controls were stained without primary antibodies. Stained sections were examined under fluorescence microscope (Meji Technolologies, Japan with progRes software, Germany). Two investigators graded Cla-1, ZO-1, E-Cad, and VAP expression in a blinded fashion. Negative Cla-1, ZO-1, E-Cad, and VAP expression was graded as no Cla-1, ZO-1, E-Cad and VAP signal (−). Cla-1, ZO-1, E-Cad and VAP positive samples were defined as having weak (+), moderate (++) or strong (+++) signal.

6.3 Results:

6.3.1 Clinico-pathological evaluation:

Clinico-pathological evaluation of control and infertility patients was presented in Table 6.1.

Table 6.1: Clinico-pathological characteristics of control and infertility patient

Variables	Control	Patient
Sample size (n)	5	5
Age (yrs)	30 (±3)	30 (±3)
Menstrual history (Days)	2-3 /28-32	2-3 /28-32

T3 (ng/dl)	58(50-86)	68 (50-86)
T4 (ng/dl)	6.59 (5-6.9)	5.95 (5-6.9)
TSH (mIU/ml)	1.68 (1.50-1.77)	1.63 (1.50-1.77)
FSH (mIU/ml)	1.63 (1.50-1.77)	1.63 (1.50-1.77)
LH (mIU/ml)	5.7-7.9	5.7-7.5
USG		
Control (n=5)	Normal	Normal
Patients (n=5)	Normal	Normal
Endometrium thickness (mm)	7 to 9	7.5-8.5
Right/left ovary	Normal	Normal
HSG (Tubal patency)		
Control (n=5)	Patent	Patent
Patients (n=5)	Patent	Patent
Laproscopy Morphology of pelvic organs.		
Control (n=5)	NAD	NAD
Patients (n=5)	NAD	NAD

T3-Tri-idothyroxine, T4-Thyroxine, TSH-Thyroid stimulating hormone, FSH- Follicle Stimulating Hormone, LH-Luteinizing Hormone, USG- Ultrasonography, HSG-hysterosalphingography, NAD- No Any Deformity

The hormonal analysis, USG, HSG, and laparoscopy of patients were compared with control. Both control and patients group showed normal hormonal profiles, USG, HSG and laparoscopy.

6.3.2 Ultrasonography:

USG (abdominal and Transvaginal) of infertility patients showed no any gross abnormalities. Both control and patients showed good uterine endometrial thickness with almost triple line pattern consisting of central hyperechoic line surrounded by two hyperechoic layers (white arrow) with moderate thickness ~ 9mm to 14mm and growing ovarian follicles with follicle size 15 to 18mm (figure 6.1).

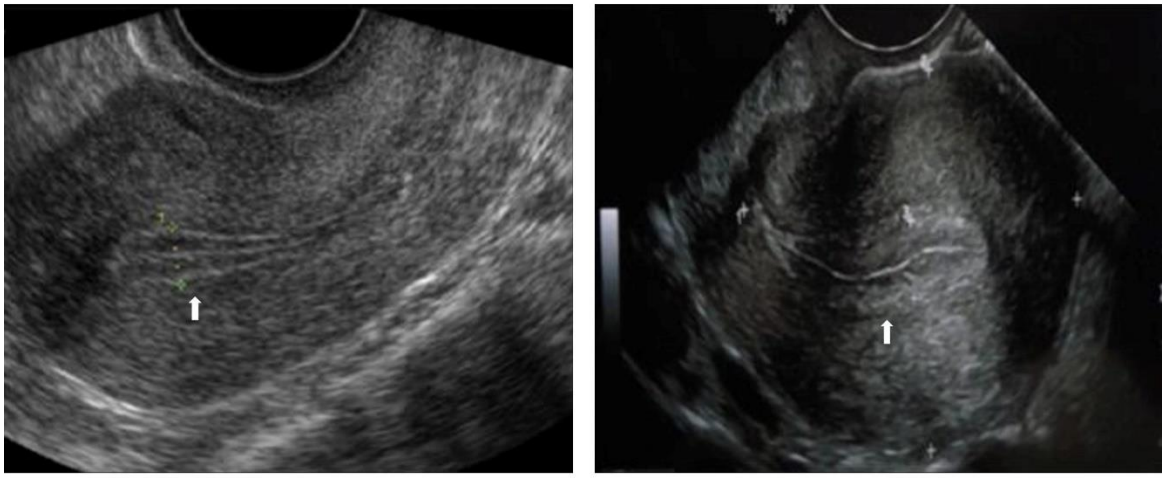

Figure 6.1: Ultrasonography study: USG findings showed endometrium with moderate thickness consisting of central hyperechoic line (white arrowhead) surrounded by two hyperechoic layers with moderate thickness ~8mm to 10mm.in both control and infertility patient.

6.3.3 Hysterosalpingography:

HSG of infertile patients showed the tubal patency on both sides and dye spilled into the abdominal cavity (figure 6.2).

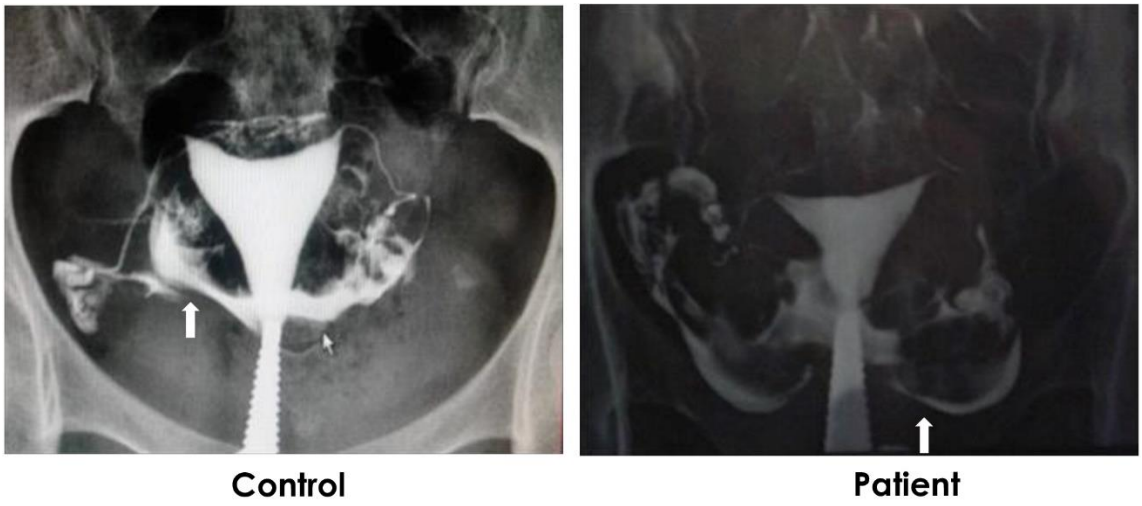

Figure 6.2: Hysterosalpingography evaluation: HSG of control and infertile patients (white arrowheads) showed the dye filled the fallopian tubes and spilled into the abdominal cavity.

6.3.4 Laproscopy

Laparoscopy of infertile patients showed the normal structures of pelvic organs like uterus, fallopian tubes and fimbriae. Both control and patients did not show any adhesion, tissue mass, ovarian cyst, endometriosis or any abnormal growth outside the uterine cavity (figure 6.3).

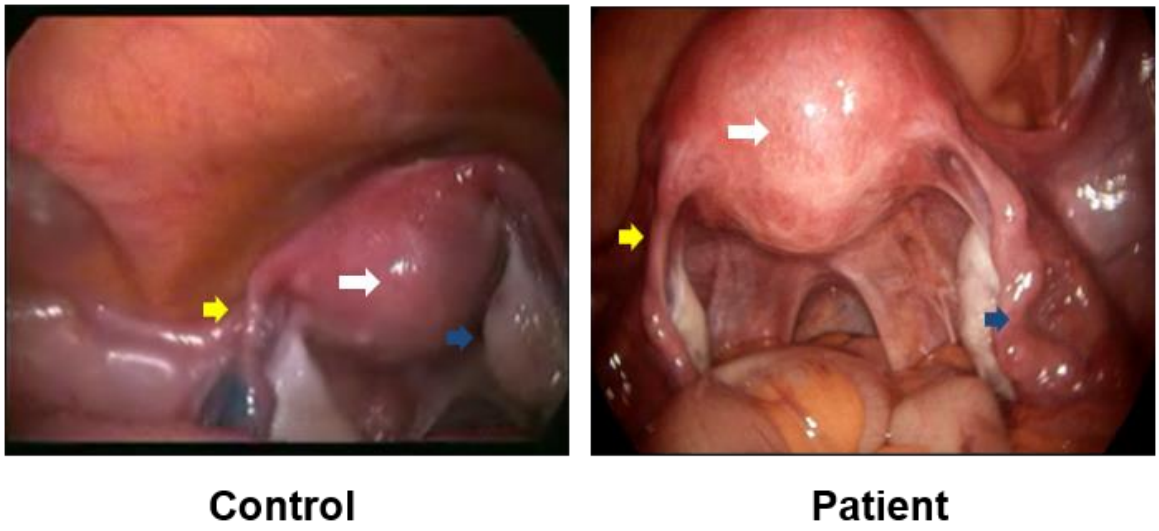

Figure 6.3: laparoscopic analysis: Laparoscopy showed the normal structures of uterus (white arrowhead), fallopian tube (yellow arrowhead) and ovaries (blue arrowhead) with no adhesion, tissue mass, ovarian cyst, endometriosis or no any abnormal growth outside the uterine cavity in control and patient.

6.3.5 Endometrial fluid sample:

Smears taken by endometrial fluid aspirates showed very few numbers of cells. Some cells were polygonal cells with eosinophilic cytoplasm and are scattered. So, it was difficult to study expression on these samples (Figure 6.4).

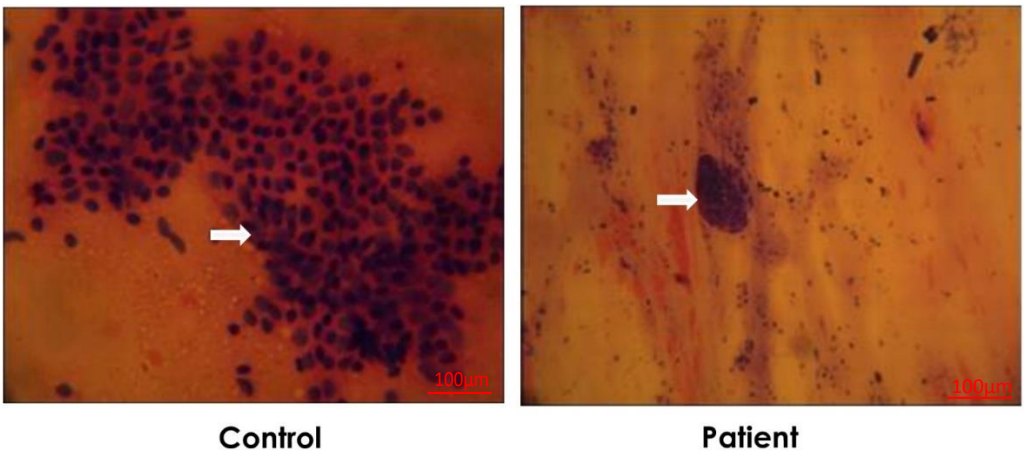

Figure 6.4: Hematoxylin and eosin staining of endometrial fluid sample: HE staining of endometrial aspirate showed overlapping cells in control and patient (White arrowhead).

6.3.6 Endometrial brush smear:

HE staining of endometrial samples collected by cytological brush showed cells of honeycomb pattern. Cells were in clusters with bits of endo-cervical tissues. Cells were overlapped, in clusters, polygonal and squamous with small nucleus. Endo-cervical cells also seen in honeycomb pattern (figure 6.5). Endometrial smear obtained by using endometrial flush shows lesser number of cells as compared to the endometrial smear obtained by using cervical cytology brush by rotating it in uterine cavity.

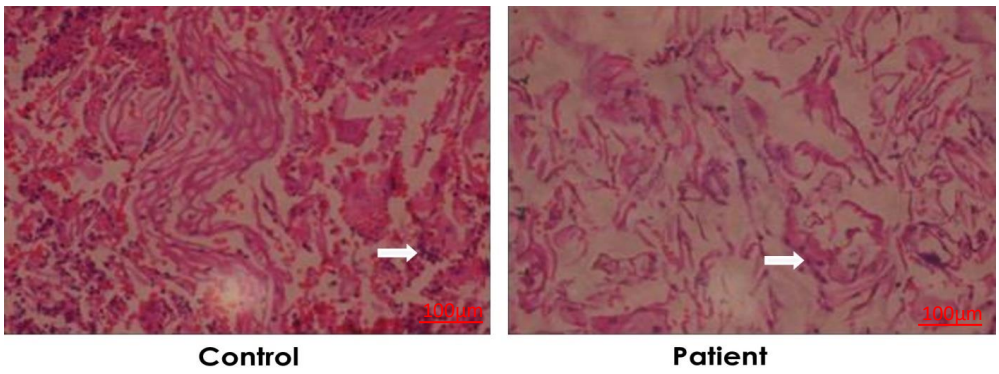

Figure 6.5: Hematoxylin and eosin staining of endometria samples by brush smear: HE staining of endometrial brush smear showed overlapping cells with bits of endo-cervical tissues, in clusters, polygonal and squamous with small nucleus (white arrowhead).

6.3.7 HE staining of endometrial biopsy:

HE staining showed the morphological changes and integrity of the epithelial and stromal cells. HE staining of endometrial biopsy of control group showed compact stroma with glands lined by tall stratified columnar epithelial cells whereas infertility group showed the loose and edematous stroma with glands lined by tall stratified columnar epithelial cells (figure 6.6).

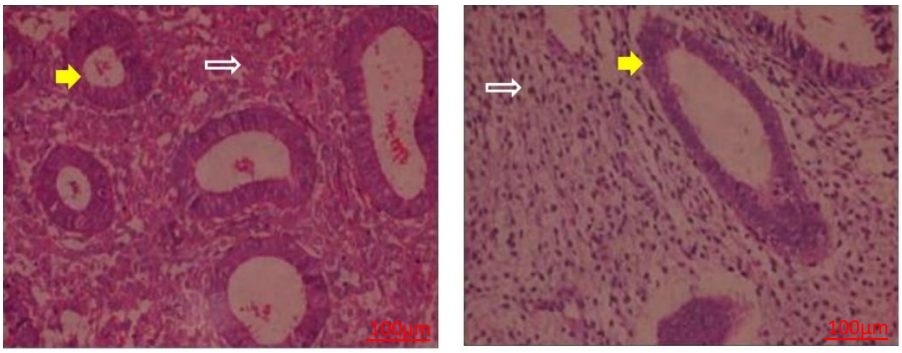

Figure 6.6: Hematoxylin and eosin staining of endometrial biopsy: HE staining of endometrial biopsy showed the morphological changes and integrity of the epithelial and stromal cells. Control endometrial biopsy showed compact stroma (white arrowhead) with glands lined by tall stratified columnar epithelial cells (yellow arrowhead) whereas patient group showed the loose and edematous stroma (white arrowhead) with glands lined by tall stratified columnar epithelial cells (yellow arrowhead).

6.3.8 Immunohistochemistry:

Endometrial biopsy samples from 5 patients with infertility and 5 samples of normal endometrium were analyzed for Cla-1, ZO-1, E-cad, and VAP expression by IHC. Normal endometrium revealed a strong specific fluorescent green signal of Cla-1, ZO-1, E-cad, and VAP. Comparison of expression of TJs and AJs in endometrial biopsy samples of infertility patients showed weak expressions of ZO-1 while expressions of E-Cad and Cla-1 are absent in all infertility patients. Infertility patients group showed strong expressions of VAP and no expressions of E-Cad

and Cla-1. Low levels of ZO-1 were detected in stromal and glandular epithelium of infertility patients. E-cad and Cla-1 did not exhibit any positive signals in any infertility cases (figure 6.7).

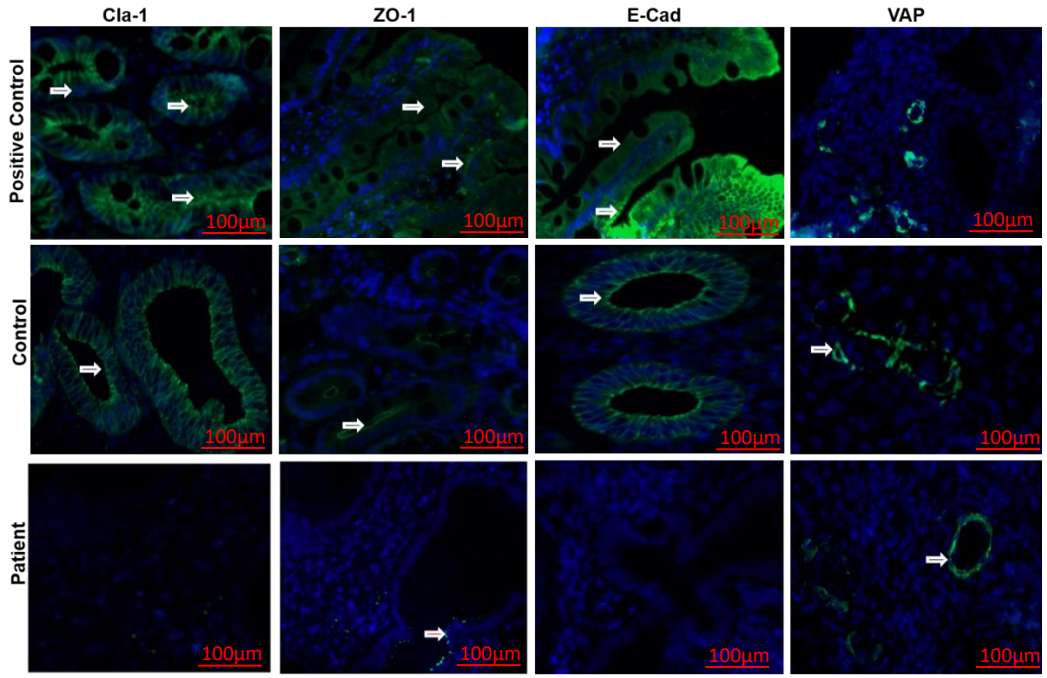

Figure 6.7: Tight and Andherence junction expression in endometrium: IHC demonstrated that positive control showed strong expression of Cla-1, ZO-1, E-Cad and VAP (Alexa 488: Green Florescence) (White arrowhead). Control group showed moderate to strong expressions of the same protein junctions (White arrowhead). Infertility patients showed markly decreased expressions of ZO-1. Expression of E-Cad and Cla-1 did not exhibit any expressions, but VAP showed strong expressions (White arrowhead). Nuclei were staining with 4,6-diamidino-2-phenylindole (DAPI: blue fluorescence).

6.4 Discussion

Infertility can cause stress for a couples involved in complex infertility treatment such as In Vitro Fertilization (IVF) and Intracytoplasmic Sperm Injection (ICSI). Till date TJs, AJs and VAP analysis has not been proven to confirm receptivity of endometrium. To our knowledge, none of tests have been successfully applied in practice. Most commonly, diagnostic procedure for infertility includes routine blood investigations for hormonal assay, USG, HSG and endometrial curettage to rule out

genital TB as a possible cause of infertility. As endometrial curettage is an invasive process, we tried non-invasive processes to study endometrial changes by taking endometrial fluid aspirate and endometrial smears by cytological brush. But the endometrial flush aspirates showed clogging of endometrial cells. The number of cells obtained by cytological brush was abundant as compared to aspirate samples, but smear showed clumps of overlapped cells. These noninvasive methods showed poor quality of IHC staining; thus results were not included in the present investigation. HE staining of endometrial biopsy of control group showed compact stroma with glands lined by tall stratified columnar epithelial cells as compared to infertility group. The stroma of infertility group showed loose and edematous stroma with glands lined by tall stratified columnar epithelial cells. Receptive endometrium is crucial for blastocyst implantation. In fact, failure of endometrium to establish proper junctional mechanism might be a considerable cause of infertility. Junctional complexes have been identified as important factor for receptive endometrium. Interaction of both the trophectoderm and the luminal epithelium are responsible for implantation. The present study proves that major components of TJs and AJs. ZO-1, E-cad, Cla-1 play major role in implantation as the two endometrial epithelial tissues attach, interact, and participate in interactive invasion. As normal fertile women show the high expression of TJs and AJs as compared to the infertility patients, it may suggest that epithelial changes with respect to the distribution ZO-1, E-cad and Cla-1 is important factor for endometrial receptivity which is altered in the infertile patients. Low expressions of TJs and AJs in the implantation window period may be

responsible for the loss of epithelial cell polarity results in implantation failure.

We observed E-Cad, ZO-1 and Cla-1 expression in luminal epithelial cells prior to implantation in fertile women. This suggests that E-Cad, ZO-1 and Cla-1 positive stromal cells behave like a guide in trophoblast invasion into the stroma. Expressions of these TJs and AJs may be required for successful uterine anchorage of the embryo. We demonstrate that the endometrial TJs and AJs expression pattern at the time of implantation period in women with unexplained infertility is different from that in fertile women. As these women have no specific reason for infertility, there might be disturbances in uterine receptivity for embryo implantation. Endometrial biopsy analysis by histology is traditional parameter to determine luteal-phase defects. The patients with unexplained infertility who have exhausted from all conventional treatment options, identifying TJs and AJs complex proteins might be a effective clinical diagnostic test, allowing to investigate the causative factor of unexplained infertility. The diagnosis of TJs and AJs expression in endometrium will predict the success of implantation. The knowledge acquired from this research, will surely assist in the development of specific therapeutics that will optimize receptivity of endometrium for successful embryo implantation.

6.5 Conclusions

These findings suggest that impaired down-regulation of E-Cad, ZO-1 and Cla-1 expression in luminal epithelial cells, during the window of implantation, might be one of the potential molecular mechanisms of infertility in patients with disturbed endometrium. We have found that ZO-1 showed weak expressions. E-Cad and Cla-1 are absent in infertility patients. We suggest that ZO-1, E-Cad and Cla-1 may be especially

necessary at the time of implantation so that the uterine luminal environment is more tightly controlled.

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Chapter – 7

General Discussion

7.1. GENERAL DISCUSSION:

Although the fact that the endometrium is a critical tissue for reproduction, little is known about the mechanisms that ensure tissue remodeling, and that are altered in endometrial pathogenesis. A most important reason is the lack of study models that reliably mimic endometrial biology. Many treatment strategies have so far been put forwarded for treating thin endometrium either without or with the endometrial pathologies. Unpredictability in the endometrial improvement is a limitation of traditional therapies including sildenafil, tamoxifen citrate, clomifene citrate pentoxifylline and tocopherol, human chorionic gonadotropin during endometrial therapy. The implantation of embryo is facilitated by endometrial cells. Endometrial cells are synchronized with appropriate distribution of tight junction, adherent junction and gap junctions. Most of the time, women with primary infertility who undergo in vitro fertilization (IVF) and embryo transfer fail to achieve pregnancy despite of normal hormonal profile with no any endometrial pathology including normal male factor investigations.

Recently, cell transplantation therapy has been recommended as an ideal alternative for endometrial regeneration. Based on these studies, we hypothesized that a-SMA, CK-18, CK-19, Connexin-40, E-Cad, Cla-1 and ZO-1 have a causal role in the process of endometrial regeneration.

In this study, in order to restore the disturbed junction proteins, we planned the transplantation of thrombin activated platelet rich plasma (aPRP), Bone marrow derived mononuclear cells (BM MNCs), very small embryonic like stem cells (BM VSELs) and human placenta derived Mitochondria (hMT), were transplanted into disturbed endometrium (DE) rat model as therapeutic agents. To evaluate the potential use of above mentioned theraputics, we developed a rat model of DE by infusing 95% ethanol in bilateral uterine horns with surgical exteriorization of uterine horns by anesthetizing with 1gm Thiosol. We aimed to observe the recruitment and proliferation of these cells

and the morphological changes of endometrial cells, improved expressions of junction proteins and determine the functional mechanism of endometrium. The methods of action of cell therapy include the cytokine and chemokine induction, growth factor production, differentiation of cells. The cell therapy is dynamic, interactive, and specific and could be an effective strategy. Despite its promising nature, further research is required for improving this strategy. These methods and their results are discussed in this article.

The rationale behind using PRP is activated autologous PRP has been important for its safety. It has been employed since the 1970s. The safety of the PRP infusion is attributed to the fact that it is prepared from autologous blood. The preparation procedures are affordable and convenient. Since PRP releases numerous chemokines, cytokines, and growth factors stored in the alpha granules of platelets, it is capable of improving cell regeneration, promoting the proliferation of endometrial stem cells, successively differentiating into endometrial cells. In addition, it could be affirmed that gel surface formed by aPRP stimulated adhesion of transplanted cells and spreading of cells. The released growth factors affected the angiogenesis, the remodeling of the extracellular matrix, differentiation, proliferation, and recruitment of transplanted cells. Therefore, aPRP is applied in regeneration for promoting tissue growth and repair. However, immunogenic reactions followed by transmission of cells could be prevented. The body will take time to digest or react with gel surface, meanwhile the immune rejection will get delayed. Further transplanted cells will get required time to adhere and to react with body tissues. We studied murine disturbed endometrium models infusion of thrombin activated PRP and assessed the capability for endometrial regeneration with the mating outcomes. The treatment with PRP led to the enhanced thickness of the endometrium, elevated rates of pregnancy rates and raised live-birth rates. Nonetheless, in vivo study of

treatment with aPRP resulted in enhanced endometrium regeneration in female rats, which was confirmed by the expression of endometrial cell junction factors, improved epithelial thickness, upregulated expressions of junction proteins. As we conducted this study in murine model, data could not distinctively imply that hormonal profile could exert adverse effects on the therapeutic potential of PRP or not. The PRP is capable of restoring damaged endometrium. The normal morphology of epithelial lining and IHC analysis was used to evaluate the endometrium, followed by exploring the effects of PRP on biological functions. Importantly, the quantification of successful pregnancy and implantation outcomes were evaluated as well.

BM MNCs therapy has been recognized as an efficient therapeutic approach among numerous types of cell therapy. They are capable of dividing into multipotent stem cells. It is feasible to employ BM MNCs therapy as a therapeutic approach for treating DE. Histological evidence concerning epithelial regeneration in the human female proposed that circulating MNCs are direct precursor of the new epithelial regeneration. The colposcopy showed that there is cellular ingrowth from adjacent healthy epithelium (1).

Based on these finding we designed the study of local infusion of BM MNCs followed by aPRP in DE. To observe the above changes, a rat model was prepared after 95% ethanol destruction of the lining of the rat uterus as described above.

We presented evidence to show that the MNCs are the precursor of regenerating epithelium in these findings. Protective effects applied by MNCs following allogeneic transplantation have been reported in several injured models such as damaged, neural, myocardial, hepatic, cartilage, and bone tissues. MNCs, a type of adult stem cells, could be harvested from bone marrow. MNCs are attractive candidates in the emerging research as it has capacity of self-renewal and differentiation potentials for cell therapy in regenerative medicine. Endometrial tissue regeneration has experienced

considerable progress by the application of MNCs. It is rather distinguished that the therapeutic effects of MNCs are due to their immunomodulatory function, along with anti-inflammatory effects of aPRP. Moreover, MNCs are capable of provoking proliferation and aPRP stimulates the secretion of cytokines playing significant roles in the innate defenses. The regulation of the immunomodulatory functions of MNCs is achieved with regard to the inflammatory conditions of their microenvironment. MNCs could be regarded as a feasible and flexible strategy to treat DE (2). Jing et al, (3) demonstrated that bone marrow mesenchymal stem cells (BMSCs) injection into the uterus of rats resulted in a thicker endometrium and the upregulation of vimentin and cytokeratin protein marker of endometrial cells.

Resultantly, directly infusing BM MNCs could protect the disturbed endometrium in rats against cell disruption encouraging endometrial cell regeneration. Post-BM MNCs transplantation, the expression of a-SMA, CK-18, CK-19, Cx-40, E-Cad, Cla-1 and ZO-1 represented a considerable increase. These upregulated markers of the receptivity are considered as the regulators of the endometrial function and possess significant parts in implantation.

The stem cells were suggested to exist in adult tissues are an actively dividing cells defined on the basis of their function. Bone marrow is a reservoir of stem and progenitor cells. Bone marrow-derived cells (BMDCs) transdifferentiate into non-hematopoietic cell lineages including cardiomyocytes and gastrointestinal epithelium (4). BMDCs play a major role in engraftment of the endometrium in rodents and humans by differentiating into epithelial, stromal as well as endothelial cells (5). Du et al.,(6) demonstrated that ischemic injury provides a powerful stimulus for homing and engraftment of BMDCs via systemic administration into the uterus to increase recruitment of BMDCs to the endometrium and improve endometrial receptivity in Asherman's syndrome mouse model. Recently, clinical trials assessed the potential

therapeutic effect of BMDCs in Asherman's syndrome following systemic and intrauterine administration.

However, it is unknown whether local intrauterine injection of BMDCs may result in better stem cell recruitment to the uterus compared with systemic administration(7–9). Further the uterine endometrium undergoes regeneration in each reproductive cycle. The functionalis layer goes through destruction and regeneration with each menstrual cycle for reproductive function. Disruption in endometrium have been implicated in infertility and related complications. Endometrial stem cells reside in the basalis layer as a source of progenitors and differentiate to form endometrial cells to replace shredded cells in each cycle.

We had currently countered this concept to investigating the recruitment of BM VSELs into the endometrium following intrauterine infusion. VSELs are the primitive stem cells, pluripotent and quiescent in nature which give rise to the tissue-committed progenitors. These tissue resident stem/progenitor cells are actively involved in replacing damaged cells. It maintains lifelong tissue regeneration. VSELs in the bone marrow exhibit adult stem cells like asymmetrical, symmetrical cell divisions and clonal expansion so it has best regenerative potential.

VSELs regulate self-renewal, proliferation and differentiation. Herein, we used a strategy to transplant BM VSELs followed by aPRP. The aPRP provide an excellent source of growth factors and cytokines. It regulates proliferation and differentiation of endogenous tissue-resident progenitor cells throughout the body. Transplanting aPRP along with VSELs will provide excellent source of growth factors and cytokines and will have beneficial effects in reproductive tissues. Present study provides a brief introduction to transplant of VSELs followed by aPRP. We postulate that aPRP essentially provide an improved microenvironment to the transplanted VSELs which in turn differentiate into endometrium specific cell thereby restoring endometrial

tissue homeostasis. Local administration route results in vast recruitment of VSELs to the injured endometrium. Furthermore, we demonstrate that VSELs are recruited to the endometrium in higher numbers due to gel surface provided by aPRP. In this study, we used a mouse model consisting of a disturbed endometrial injury, without any unwanted uterine adhesions. But it sufficiently stimulates increased recruitment of VSELs into DE. This model of endometrial injury has been increasingly utilized to improve endometrial receptivity and implantation in rat models.

After cell-based regeneration treatment, we conducted MT transplant for endometrial regeneration of DE. The human placenta derived mitochondria transplantation (hMT Tx) has attracted considerable interest as an innovative therapeutic approach for treatment purposes. Mitochondria are the powerhouses of the cells that produce energy by glucose and oxygen uptake. But in serious consequences of cellular function that demand lots of energy, MT provide energy. As mitochondria play a major role in the homeostasis of body's cells, Mitochondria transplantation is used as theraputics approach in ischemia/reperfusion related disorders. These ischemic alterations are restored, and significantly decreased myocardial ischemia with enhanced cellular function and restoration of blood flow by mitochondria transplant.

To analysis effect of mitochondria on DE, we have used a model of DE. Our studies demonstrate that the transplantation of allogeneic mitochondria, isolated from the human placenta that can be restored at LN2. It can be directly used for DE study. This transplanted MT will provide energy to damaged cells and may augment the function of native mitochondria damaged during endometrial disruption and enhances functional recovery and cellular differentiation. MT is a source of energy production and synthesis derived through the electron transport chain and oxidative phosphorylation. As mitochondria are an organelle, it acts both extracellular and intracellular.

Extracellular, the transplanted mitochondria will enhance high energy synthesis
and cellular ATP stores. Internalized transplanted mitochondria rescue cellular function and replace damaged mitochondria DNA (mtDNA). It has its own DNA that encodes the oxidative phosphorylation subunits. There is less chances of immune or auto-immune reaction as a result of the transplanted organelle, mitochondria. It will augment the energy and nutrient supplement of damaged and degenerated cells and regain their regenerative potential by rescuing cell degeneration.

Our studies demonstrate that hMT Tx can be effective in disturbed endometrium. We explained methodology for the isolation of hMT by sucrose density gradient, determination of specific enzymatic activities to allow for animal study in DE. The direct infusion of mitochondria to the affected area would be a better option than systemic injections with minimal complications. Transplant of hMTx-aPRP form stable gel layer formation to stabilize the transplanted mitochondria. We verified the differentiation of hMTx-aPRP by immunohistochemistry and Real time-PCR. Real time -PCR analysis. It showed expressions of the hMT genes MT ATP-6, MT ATP-8, MT COX-1, MT COX -2, MT COX -3 and immunohistochemical analysis revealed that cells were positive for a-SMA, CK18, and CK19, Cx40, Cla-1, ZO-1. The transplanted mitochondria showed good engraftment and proliferative results without any adverse immune rejection. Autologous transplantation of mitochondria is not possible in every diseased condition so we put forth the idea of allogeneic hMT Tx. Allogeneic mitochondrial transplantation may be very interesting next line of treatment and further research will likely be carried out in the future.

The treatment group with aPRP, BM MNCs, BM VSELs, mitochondria followed by aPRP could stabilize cells. We studied the two consecutive estrus cycles in rats. Even though at 9th day also the epithelial lining and junction expressions

in transplant group showed better recruitment of cells as compared to control. At the same time, activated platelets release tropical GFs support and stabilize transplanted cells. It may suggest that BM MNCs, BM VSELs, MT with aPRP may confer better microenvironment so that regeneration could be possible in second esrus cycle also. This strategy could improve the engraftment potential of cells. It will enhance the implantation and support the live-birth while other DE group failed the live birth of pups in transplant groups. So, we analysed endometrium of primary infertility for junction dynamics in human study. An important outcome of study was the expressions of E Cadherin (E-Cad), Zona Occlidin-1 (ZO-1), Claudin-1 (Cla-1) and vascular angiogenic precursor (VAP) were downregulated in endometrial biopsy of primary infertility patients. So, we come to the decision that one attributed cause could be the malfunctioning of downregulated junction proteins in endometrial cells.

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Chapter – 8

Summary and Conclusions

8.1. Summary and Conclusions:

Endometrial disturbance is frequent related to infertility.

In rats, endometrium can receive implantation during the implantation window likewise in human endometrium. In human endometrium, there have been few experiments investigating the concept of endometrial receptivity. Endometrium is dynamic tissue which constantly undergoes regeneration.

Based on these results we designed the study to demonstrate the effects of transplant of activated platelet rich plasma (aPRP), bone marrow derived mononuclear cells (BM MNCs), Bone marrow derived very small embryonic like stem cells (BM VSELs), human placenta derived mitochondria (hMT) in disturbed endometrium rat model.

For remodulation and regeneration of cells, energy metabolism is required.

So we hypothesized that additional energy will get supplied by mitochondria.

In the current study, the aPRP is major factor in promoting and providing different cytokines and growth factors to injured site. It showed functional regeneration of injured rat endometrium during aPRP transplant (aPRP Tx). We have brought our findings further by demonstrating the potent stimulating effect of aPRP along with transplant of BM MNCs, BM VSELs and hMT. The transplant of BM MNCs, BM VSELs and hMT followed by thrombin activated PRP in turn up-regulated epithelial regeneration of endometrial epithelium, increased stromal cells number and also improved fertility rates in animal models. The cell transplant and hMT transplant followed by aPRP treatment holds great promise in aiding proper recruitment of cells at injured site for treating endometrial damage. The aPRP forms gel surface which acts like scaffold and it will hold the cells intact at desired place.

The effectiveness of BM MNCs, BM VSELs and hMT therapy on disturbed endometrium have been suggested that aPRP and hMT could be a minimally invasive method as compared to BM MNCs, BM VSELs. In the rat model of disturbed endometrium, it has been found that aPRP, BM MNCs, BM VSELs

and hMT could enhance the stromal cells derived from basalis layer to repair endometrial damage. To bring the discoveries further, our current study serves as one novel instance, where aPRP followed by cell transplant exhibits previously unknown synergistic effect on transplanted cells to enhance endometrial regeneration of injured uterus in rats, proposing that the therapeutic usefulness of aPRP, BM MNCs, BM VSELs and hMT Tx could be wider and worth further investigations in even more studies. Moreover, based on results from our experiments, the abovementioned beneficial effects of aPRP, BM MNCs, BM VSELs and hMT Tx are likely mediated by increasing the epithelial regeneration potential and promoting a-SMA, CK 18, CK 19, Cx 40, E-Cad, ZO-1 and Cla-1expression and secretion of essencial growth factors, cytokines and chemokines. In order to provide more solid evidence to support the role of cell transplant treatment, it should be investigated in future study in human.

So, We conducted the study of E-Cadherin, Zona Occludin-1 and Claudin-1 on unexplained infertility patients. The endometrium of infertility patients showed downregulated expressions of adherence junction (E-cadhrin), tight junction (Zona Occludin-1 and Claudin-1) and gap junction (connexin-40) protein. In our previous study, we also identified E-Cadherin was totally absent and may be responsible to hamper the implantation. We found that these junction protein markers revealed deletion in human study.

Infertility affects a huge number of females. Endometrium plays a vital role in implantation and reproduction. The endometrial changes at the time of implantation window include expression of tight junctions, cell adhesion molecule and communicating junction receptors and ligands involved in implantation.

To maintain it's morphological, physiological, and functional structure by eradicating deficiencies and restoring it morphologically and functionally

after injuries are of supreme important factors. The cell therapies have been the subject of many studies due to their effective differentiation and functions. Cell therapy has been proposed to be capable of treating numerous diseases including thin endometrium and uterine adhesions. Therefore, we could assume that stem cell therapy could be commenced for treating damaged and disturbed endometrium as this problem is with limited therapeutic options. To improve the survival and transplant number of transplanted cells, thrombin activated aPRP forms gel surface that can promote stem cell delivery and thus improving the outcomes of transplanted cells. To regenerate the ethanol induced disturbed endometrium (DE), we proposed the transplant of thrombin activated platelet rich plasma (aPRP), bone marrow derived mononuclear cells (BM MNCs), bone marrow derived very small embryonic like stem cells (BM VSELs) and human placenta derived mitochondria (hMT). The hMT seems to be the best clinical option as it is not cell but organelle. It has very negligible immune rejection. As it can be harvested from bio-waste through non-invasive procedures, it is easily accessible. It has rapid self-renewal features and has low immunogenic effects. Despite of the advances in cell transplant therapy, major concerns which are still exist with it can be overruled because it has capability of stimulating angiogenesis through the growth factors (GFs) secretions. The results of our study indicate that transplanted cells and aPRP might play a key role in regeneration of DE by homing to the endometrium, differentiating into cellar phenotypes, and being immunomodulatory. These findings present a potential cell therapy for DE.

We could confirm that these novel therapies might get numerous advantages over the traditional treatments. There still exists room for upgradation, enhancement, and improvement. Much research is required to maximize the potentials of such type of emerging novel therapies. Finally, with the advent of these cell therapies and the potential for endometrial

receptivity treatment, we are looking forward that many of the problems related to disturbed and endometrial pathogenesis will be resolved soon.

Chapter – 9

80 Recommendations

9.1 Recommendations

The cell junction regulating endometrial cell development and the mechanisms by which they are communicated are largely undefined. It has been proposed that intercellular cell junctions may influence aspects of endometrial development. Endometrial receptivity is a complex event demanding contributions from both blastocyst and endometrium. Despite advances in assisted reproduction technologies, defects in endometrial receptivity persist as a barrier to successful implantation in infertility. We demonstrated that downregulated expressions of a-Smooth muscle actin (a-SMA), Cytokeratin-18 (CK-18), Cytokeratin-19 (CK-19), Connexin-40 (Cx-40), E-Cadherin (E-Cad), Zona Occludin-1 (ZO-1), Claudin-1 (Cla-1) in animal model confers defective endometrial receptivity. The strong link between junction proteins and implantation suggested the compelling hypothesis that transplantation of thrombin activated platelet rich plasma (aPRP), Bone marrow derived mononuclear cells followed by aPRP (BM MNCs-aPRP TX), bone marrow derived very small embryonic like stem cells followed by aPRP (BM VSELSCs-aPRP), human placenta derived Mitochondria followed by aPRP (hMT-aPRP Tx) in disturbed endometrium (DE) improve junction protein expressions and thus improves fertility. It ultimately led to better pregnancy outcomes. Here, we demonstrate that intrauterine cell transplant improves the implantation rate and endometrial morphology. The aPRP release tropical GFs support and stabilize transplanted cells to confer better microenvironment. In epithelium, we find that cell transplant accelerates the cell proliferation and that, in stroma and epithelial region, it promotes a-SMA, CK-18, CK-19, Cx-40, E-Cad, ZO-1 and Cla-1 expression. Ultimately, our findings advance our understanding of the contributions of aPRP, BM MNCs-aPRP Tx, BM VSELs-aPRP Tx, hMT-aPRP Tx in improving endometrial receptivity and suggest potential broad use for DE as therapy to encourage implantation.

9.2 Conclusions of the research work

The chapter wise conclusions of the research work carried out and summarized below are

Chapter 1

This is an introductory chapter that provides general information on cell communication in endometrium. the chapter highlights the changes that occur during endometrium during implantation that include expression of cell adhesion molecule receptors and ligands that may be involved in implantation. These endometrial proteins are responsible for the development of epithelial and stromal cells. Disruption of these factors suggests receptivity defects. Further studies will develop novel forms that will target the disturbed endometrium and will support receptivity.

Chapter 2

This chapter focuses on endometrium of unexplained primary infertility patients lack of expressions of tight junction and adherence junction. The findings imply that down-regulation of E-Cad, ZO-1 and Cla-1 expression in endometrial epithelial cells is related to infertility in patients with disturbed endometrium. The ZO-1 showed weak expressions while E-Cad and Cla-1 are absent in infertility. This study suggest that ZO-1, E-Cad and Cla-1 are necessary at the time of implantation so that the endometrial environment is more suitable for receptivity.

Chapter 3

This chapter focuses on the effect of aPRP on endometrial regeneration and how it improves the pregnancy outcomes in rat model of disturbed endometrium. It demonstrated the effects of aPRP as a potential treatment for unresponsive endometrium. It restores functional endometrial morphology. The clinical application of aPRP is still at a preliminary stage. Further explorations of study and clinical trials are required for the

optimization of aPRP application along with a larger randomized study to determine the effect of PRP in implantation failure in human.

Chapter 4

This chapter explains about the transplant of BM MNCs-aPRP for endometrial regeneration in rat model. This study revealed that BM MNCs-aPRP transplant is effective in endometrial regeneration and receptivity. BM MNCs-aPRP Tx were improved the expression of a-SMA, CK 18, CK 19, Cx 40, E cad, Cla 1 and Zo-1. It is significantly greater as compared to those that did not. BM MNCs can be successfully applied for DE treatment in the rat model results in significantly thicker endometrium. The pregnancy outcome and litter size in rats was higher than in DE group and control group. This study focuses the possible application of BM MNCs Tx for endometrial cell regeneration.

Chapter 5

This study focuses the application of BM VSELs in the treatment of disturbed endometrium in rats. The VSELs-aPRP Tx holds incredible potential to stimulate and provides sufficient cells for treating DE. Use of aPRP along with cell transplant is likely to have enhanced therapeutic benefit. Our research on the effects of BM VSELSCs strongly supports the BM VSELs Tx potential and its clinical use. It reports the development of a novel strategy for augmenting BM VSELs engraftment co-transplanting with aPRP. This approach is promising and advances this experimental study closer to clinical endometrial pathology to assist in Assisted Reproductive Technology.

Chapter 6

This chapter describes the transplantation of hMT Tx promotes cell communication in endometrium in rat model of DE. This study states the novel strategy for enhancing hMT engraftment by co-transplanting it with aPRP. This approach is showed promising effect of hMTx-aPRP on endometrial improvement. It put forth the protein modifications in endometrial cells by hMTx-aPRP would be reflected in higher energy production at the

endometrial site. The hMTx-aPRP promises innovative medicines based on mitochondrial transplantation in rat models.

Chapter 7

Chapter 7 encloses the overall discussion of the work by focusing on exploration of tight junctions, adherence junctions and gap junction in endometrium by exploiting the results of transplant of aPRP, BM MNCS, BM VSELs and hMT. Chapter 8 encloses the summary and conclusions of the overall work with regards to the role of tight junction, adherence junction and gap junction in female primary infertility.

9.3 Summary

1. The major purpose of our study was to find out novel forms that will target the disturbed endometrium and will support receptivity.
2. In our first study, we discussed about the endometrium of unexplained primary infertility patients lack of expressions of tight junction and adherence junctions ZO-1, E-Cad and Cla-1 which are necessary for endometrial receptivity.
3. In our second study, we transplanted thrombin activated platelet rich plasma into disturbed endometrium to its effects on DE. The aPRP secretes different Growth factors and cytokines that will boost the regeneration of endometrial cells by forming intact gel like surface that will remain on DE mimicking scaffold like structure.
4. In our third study, we transplanted BM MNCs followed by aPRP. This study exposed that BM MNCs-aPRP transplant is effective in endometrial regeneration and receptivity.
5. In our fourth study, we discussed about the application of BM VSELs in the treatment of disturbed endometrium in rats. The VSELs-aPRP Tx showed incredible potential for treating DE. Our results suggest that BM VSELs-aPRP treatment may become a valuable strategy to promote the regeneration of DE and thus improve implantation and pregnancy rates in rat models.

6. In our fifth study we explored the effect of hMT-aPRP in DE. Our Data provide the first evidence that hMTx followed by thrombin-activated PRP promotes endometrial regeneration in the disturbed endometrium, paving the way for the development of a novel approach for human endometrial regeneration.

7. This overall study will accelerate findings related to innovative medicines based on mitochondrial transplantation in rat models. It will eliminate the confounding factors of implantation failure and to achieve higher clinical pregnancy rates.

9.4 Future Scope

We demonstrated that transplant of aPRP, above mentioned cells and mitochondria helps to restore endometrial function and improve implantation outcomes enabling full term delivery and live-births in rats. This finding promotes the theoretical basis for the cell and organelle transplant to promote endometrial regeneration and improve implantation outcomes. It will support the clinical application of it in compromised endometrial pathologies.

The additional studies are required to evaluate the usefulness of aPRP, BM MNCs, BM VSELs and hMT in the humans.