COMPARISON OF ENZYMATIC AND NON-ENZYMATIC ISOLATION METHODS FOR MENSTRUAL BLOOD MESENCHYMAL STEM CELLS IN MAINTENANCE OF STEMNESS

by
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ABSTRACT

COMPARISON OF ENZYMATIC AND NON-ENZYMATIC ISOLATION METHODS FOR MENSTRUAL BLOOD MESENCHYMAL STEM CELLS IN MAINTENANCE OF STEMNESS

Endometrium is a thin epithelial layer located on the inside of the uterus and provides the ground for the embryo. Endometrium layer becomes suitable for the embryo every month for pregnancy, sheds with menstruation in cases where there is no pregnancy and prepares the endometrial layer for the pregnancy again. Presence of endometrial mesenchymal stem cells (eMSCs) in the endometrial layer brought about the possibility of stem cells in the menstrual blood (MenSCs). Early studies that identified MenSCs stated that these cells showed mesenchymal stem cell characteristics and hence possessed potential to be used in clinical studies due to their ease in isolation. Although different methods were employed in the isolation of MenSCs in the literature, up to now none of these isolation methods have not been compared in terms of preserving the stem cell properties. In this context, the purpose of this thesis is to compare the three different isolation methods including non-enzymatic, collagenase type 1 and dual digestion method and investigate the potential of MSCs isolated from menstrual blood to maintain their stemness until late passages. No difference in cell morphology was observed when the isolation methods were compared. Similarly, analysis of surface markers by flow cytometry showed that stem cell characteristics were preserved in early and late passage MenSCs and there was no difference between the isolation methods. In terms of colony forming feature for MenSCs, cells isolated with dual digestion method demonstrated an advantage compared to the other protocols at early passage. When cell proliferation rate was analysed using WST-1 assay, it was seen that the best isolation method in early passage cells was the non-enzymatic method. However, the decrease in the proliferation rate recorded for the late passage was evident in all MenSCs regardless of the isolation method they were derived from. As a result, for the first time in the literature, isolation methods of MenSCs were compared in terms of stemness characteristics and our results suggested non-enzymatic approach as the best isolation method.

ÖZET

MENSTRÜEL KAN MEZENKİMAL KÖK HÜCRELERDE KÖK HÜCRE OLMA ÖZELLİĞİNİN ENZİMATİK VE ENZİMATİK OLMAYAN İZOLASYON YÖNTEMLERİ İLE KARŞILAŞTIRILMASI

Endometrium, rahmin iç kısmında yer alan ve embriyo için zemin sağlayan ince bir epitel tabakadır. Endometrium tabakası her ay gebelik için embriyoya uygun hale gelir, gebelik olmadığı durumlarda adet ile dökülür ve endometriyal tabakayı tekrar gebeliğe hazırlar. Endometriyal tabakada endometriyal mezenkimal kök hücrelerin (eMKH) varlığı, menstrüel kanda (MK-MKH) kök hücre olasılığını beraberinde getirmiştir. MK-MKH'leri tanımlayan çalışmalar, bu hücrelerin mezenkimal kök hücre özellikleri gösterdiğini ve dolayısıyla izolasyon kolaylığı nedeniyle klinik çalışmalarda kullanılma potansiyeline sahip olduğunu belirtmiştir. Literatürde MK-MKH'lerin izolasyonunda farklı yöntemler kullanılmış olmasına rağmen, bu izolasyon yöntemlerinin hiçbiri kök hücre özelliklerini koruma açısından bugüne kadar karşılaştırılmamıştır. Bu bağlamda, bu tezin amacı, enzimatik olmayan, kolajenaz tip 1 ve ikili sindirim yöntemi olmak üzere üç farklı izolasyon yöntemini karşılaştırmak ve menstrüel kandan izole edilen MKHlerin geç pasajlara kadar kök hücre özelliklerini koruma potansiyelini araştırmaktır. İzolasyon yöntemleri karşılaştırıldığında hücre morfolojisinde herhangi bir farklılık gözlenmemiştir. Benzer şekilde, akış sitometrisi ile yüzey belirteçlerinin analizi, kök hücre özelliklerinin erken ve geç pasaj MK-MKH'lerinde korunduğunu ve izolasyon yöntemleri arasında bir fark olmadığını gösterilmiştir. MK-MKH'lerin koloni oluşturma özelliği açısından, üç izolasyon yöntemi arasında erken pasajda en iyi izolasyon yönteminin ikili sindirim izolasyon yöntemi olduğu belirlenmiştir. WST-1 testi kullanılarak hücre büyüme hızı analiz edildiğinde erken pasaj hücrelerinde en iyi izolasyon yönteminin enzimatik olmayan yöntem olduğu görülmüştür. Bununla birlikte, geç pasaj için kaydedilen çoğalma oranındaki azalma, elde edildikleri izolasyon yönteminden bağımsız olarak tüm MK-MKH'lerde belirgin olarak tespit edilmiştir. Sonuç olarak, literatürde ilk kez, MK-MKH'lerin izolasyon yöntemleri kök hücre özellikleri açısından karşılaştırılmış ve sonuçlarımız enzimatik olmayan yaklaşımın en iyi izolasyon yöntemi olarak önermiştir.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS iv

ABSTRACT v

ÖZET vi

TABLE OF CONTENTS vii

LIST OF FIGURES x

LIST OF TABLES xii

LIST OF SYMBOLS/ABBREVIATIONS xiii

1. INTRODUCTION 1

1.1. ANATOMY OF UTERUS 1

1.1.1. Structure and Function of Uterus 2

1.1.2. Blood, Lymphatic and Nerve Supply in Uterus 3

1.1.3. Layers of Uterus 5

1.1.3.1. *Endometrium* 5

1.1.3.2. *Myometrium* 6

1.1.3.3. *Perimetrium* 6

1.2. ENDOMETRIUM 6

1.2.1. Cell Types in Endometrium 9

1.3. MESENCHYMALS STEM/STROMA CELLS 11

1.3.1. Structure and Function of Mesenchymal Stem Cells 12

1.3.2. Sources for Mesenchymal Stem Cells 13

1.3.3. Cellular Therapies Using Mesenchymal Stem Cells 19

1.4. MENSTRUAL BLOOD MESENCHYMAL STEM CELLS 20

1.5. AIM OF THE THESIS 23

2. MATERIALS 24

2.1. INSTRUMENTS 24

2.2. EQUIPMENT 24

2.3. CHEMICALS 24

3. METHODS 26
3.1. MENSTRUAL BLOOD MESENCHYMAL STEM CELL ISOLATION METHODS 26
3.1.1. Non-Enzymatic Method for MenSCs Isolation 26
3.1.2. Collagenase Type I Digestion Method for MenSCs Isolation 26
3.1.3. Dual Enzyme Digestion Method for MenSCs Isolation 27
3.2. MAINTENANCE OF MENSTRUAL BLOOD MESENCHYMAL STEM CELLS 27
3.2.1. Cell Passaging of MenSCs 27
3.2.2. Determination of Cell Number 28
3.2.3. Cell Thawing 28
3.2.4. Cryopreservation of Menstrual Blood Mesenchymal Stem Cells 28
3.3. CHARACTERIZATION OF MENSTRUAL BLOOD MESENCHYMAL STEM CELLS WITH FLOW CYTOMETRY 28
3.4. COLONY FORMING UNIT ASSAY 29
3.5. CELL PROLIFERATION ASSAY WITH WST-1 30
3.6. STATISTICAL ANALYSIS 30
4. RESULTS 31
4.1. ISOLATION OF MENSTRUAL BLOOD MESENCHYMAL STEM CELLS WITH ISOLATION METHODS 31
4.2. COMPARISON OF STEMNESS CHARACTER MAINTENANCE AMONG DIFFERENT MenSCs ISOLATION METHODS 32
4.2.1. Analysis of Cell Surface Markers at Early and Late Passage MenSCs Isolated by Different Methods 32
4.2.2. Determination of Colony Forming Unit for Menstrual Blood Mesenchymal Stem Cells 41
4.2.3. Characterization of Cell Proliferation in MenSCs via WST-1 47
5. DISCUSSION 51
6. CONCLUSION AND FUTURE PERSPECTIVE 56
REFERENCES 57
APPENDIX A 70

APPENDIX B 71

LIST OF FIGURES

Figure 1.1. Anatomy of the uterus 1

Figure 1.2. Frontal section of the uterus 2

Figure 1.3. Cervix changes during aging 3

Figure 1.4. Lymphatic drainage of the uterus and vagina 4

Figure 1.5. Nerve supply of the uterus 5

Figure 1.6. Histological organization of human endometrium 7

Figure 1.7. Pre-receptivity, receptive and post-receptive stages of endometrium 8

Figure 1.8. Decidualization, menstruation, repair, and regeneration of endometrium 9

Figure 1.9. Possible location of endometrial stem/progenitor cells in endometrium 11

Figure 1.10. Various MSCs sources 14

Figure 4.1. Representative images of MenSCs from three subjects with three isolation methods 31

Figure 4.2. Characterization of MenSCs isolated via non-enzymatic isolation method at Ep_MenSCs by flow cytometry 33

Figure 4.3. Characterization of MenSCs isolated via CL-1 isolation method at Ep_MenSCs by flow cytometry 34

Figure 4.4. Characterization of MenSCs isolated via dual digestion method at Ep_MenSCs by flow cytometry 35

Figure 4.5. Characterization of MenSCs isolated via NE isolation method at Lp_MenSCs by flow cytometry 36

Figure 4.6. Characterization of MenSCs isolated via CL-1 isolation method at Lp_MenSCs by flow cytometry 38

Figure 4.7. Characterization of MenSCs isolated via dual digestion method at Lp_MenSCs by flow cytometry 39

Figure 4.8. Colony formation capacity of MenSCs. Photographs of MenSCs' from three subjects with three isolation methods at Ep_MenSCs were taken at the end of day 14 42

Figure 4.9. Colony formation capacity of MenSCs. Photographs of MenSCs' from three subjects with three isolation methods at Lp_MenSCs were taken at the end of day 14 43

Figure 4.10. Determination of colony number and size in early and late passage MenSCs isolated by NE, CL-1 and dual digestion 44

Figure 4.11. Cell proliferation profile determination of MenSCs 48

LIST OF TABLES

Table 3.1. Mesenchymal Stem Cell Markers with their conjugated dye 29

Table 4.1. Average percentage of stem cell markers of Ep_MenSCs isolated from three different subjects with three isolation methods 40

Table 4.2. Average percentage of stem cell markers of MenSCs isolated from three subjects with three isolation methods at Lp_MenSCs 41

Table 4.3. Efficiency of colony forming unit according to isolation methods at early and late passages 45

Table 4.4. Colony size (mm) according to isolation methods at early and late passages 46

Table A.1. Flow cytometric analysis for Ep_MenSCs from three subjects with non-enzymatic isolation method 71

Table A.2. Flow cytometric analysis for Ep_MenSCs from three subjects with collagenase type 1 isolation method 72

Table A.3. Flow cytometric analysis for Ep_MenSCs from three subjects with dual digestion isolation method 72

Table A.4. Flow cytometric analysis for Lp_MenSCs from three subjects with non-enzymatic isolation method 73

Table A.5. Flow cytometric analysis for Lp_MenSCs from three subjects with collagenase type 1 isolation method 73

Table A.6. Flow cytometric analysis for Lp_MenSCs from three subjects with dual digestion isolation method 74

LIST OF SYMBOLS/ABBREVIATIONS

β	Beta
°C	Degree centigrade
cm	Centimetre
M	Molar
mL	Millilitre
mM	Millimolar
ADMSCs	Adipose derived mesenchymal stem cells
aMSCs	Amniotic mesenchymal stem cells
AS	Asherman's syndrome
BM	Bone marrow
cAMP	Cyclic adenosine mono phosphate
CD	Cluster of differentiation
CFU-F	Colony forming unit fibroblast
CL-1	Collagenase type 1
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
EDT-MSCs	Endometrial decidual tissue mscs
EDTA	Ethylenediamine tetraacetic acid
eMSCs	Endometrial mesenchymal stem cells
Ep_MenSCs	Early passage MenSCs
ERCs	Endometrial regenerative cells
ESC	Endometrium stem cells
FBS	Fetal bovine serum
FCM	Flow cytometry
hBSCs	Human breast milk stem cells
HES	Hydroxyethyl starch
HGF	Hepatocyte growth factor
HSC	Hematopoietic stem cells
iPSCs	Induced pluripotent stem cells
ISCT	International society of cellular therapy

ITGβ1	Integrin beta 1
Lp_MenSCs	Late passage MenSCs
MBMSC	Menstrual blood MSCs
MBPCs	Menstrual blood progenitor cells
MenSCs	Menstrual blood mesenchymal stem cells
MMSCs	Menstrual blood derived MSCs
MSCs	Mesenchymal stem cells
NC	Negative control
NE	Non-enzymatic
NF-$_K$B	Nuclear factor-kappa B
NH_4Cl	Ammonium chloride
Oct-4	Octomer-binding transcription factor 4
OLT	Orthotopic liver transplantation
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGFR	Platelet-derived growth factor receptor
PDSCs	Placenta derived mesenchymal stem cells
PFA	Paraformaldehyde
PLA	Processed lipoaspirate
PSA	Penicillin, streptomycin and amphotericin B
PTGS2	Prostaglandin G/H synthase 2
RBC	Red blood cells
SDMSC	Synovium-derived mesenchymal stem cells
SM	Synovium membrane
SP	Side population
SSEA_4	Stage specific embryo antigen 4
UCB	Umbilical cord blood
UCSCs	Umbilical cord derived stem cells
VEGF	Vascular endothelial growth factor
WST-1	Water soluble tetrazolium

1. INTRODUCTION

1.1. ANATOMY OF UTERUS

Uterus is a female reproductive organ that composed with fallopian tubes and vagina (Figure 1.1.). The uterus is involved in the gestation in which the fertilized egg was accepted and provided a background for development [1]. Uterus is developed from the paramesonephric duct in the human embryo. The shape of the uterus is not symmetric [2]. Therefore, it can be said that; uterus is unequal. The top part of the uterus combines with the fallopian tubes, and the lower part combines with the vagina. The uterus looks like pear-shaped and is 7.5 cm in length [3].

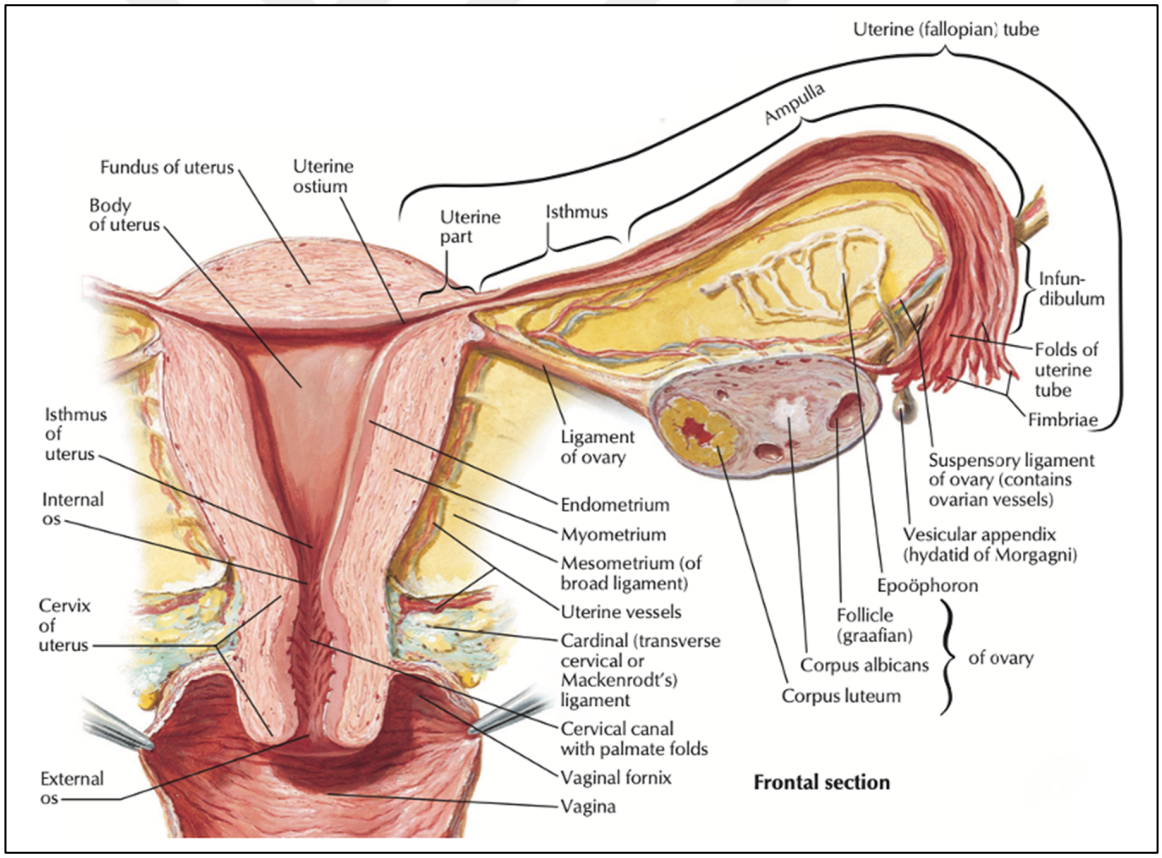


Figure 1.1. Anatomy of the uterus [4]

1.1.1. Structure and Function of Uterus

Uterus is located in the pelvic region and overlies the bladder. It is 7.5 cm in length, 4.5 cm width, and 3.0 cm in thick. The adult and health uterus weights around 60 grams [3]. The uterus is divided into two main parts which are body and cervix. Body part of the uterus is composed of three parts which are fundus, corpus, and isthmus (Figure 1.2.).

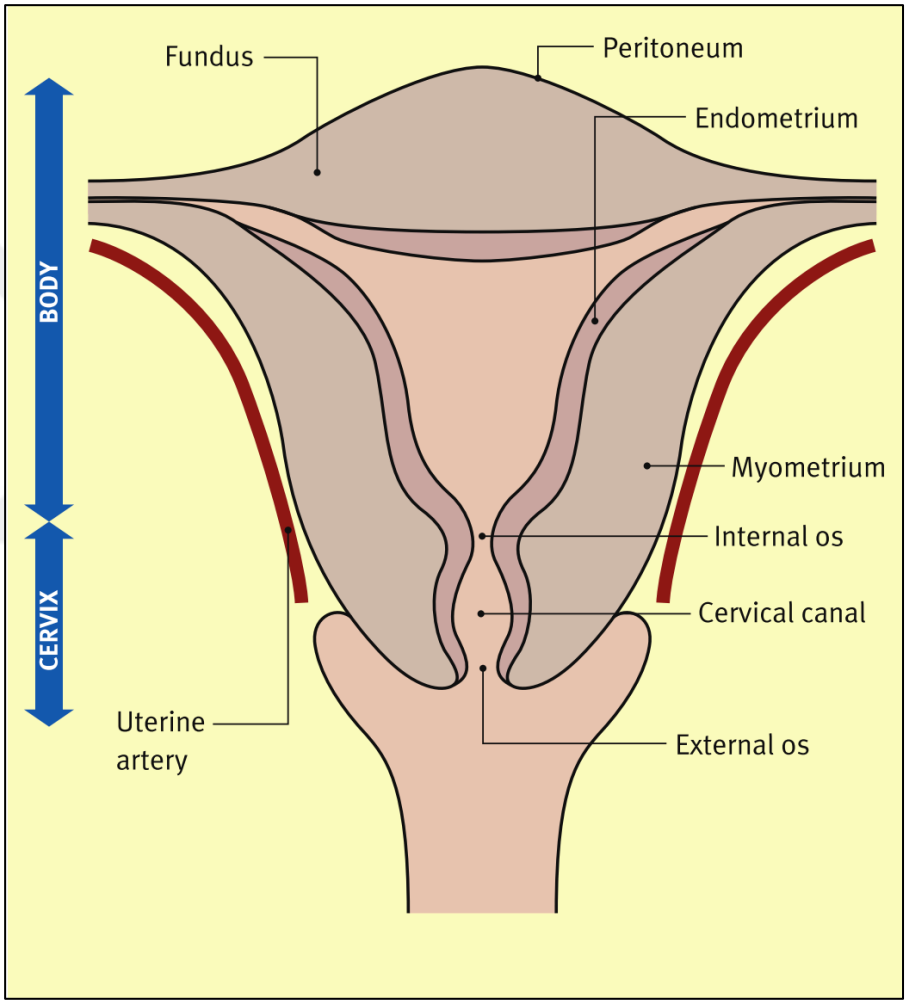


Figure 1.2. Frontal section of the uterus [1]

Fundus or the uppermost part of the uterus is extended through fallopian tubes and connected to each other. Corpus is the main part of the uterus and connected with isthmus at the lower part of the uterus [5]. Isthmus is the lower uterine segment, which becomes lower segment of uterus during pregnancy is 1.5 mm in length [3]. Isthmus communicates with the cervical canal by isthmus os (Figure 1.1.). The last part of the uterus is the cervix. Cervix is connected

with lumen of the uterus and divided into two parts: endocervix and ectocervix. Endocervix which is inner part connects with uterus, while ectocervix is outer part [2]. Cervix size and shape changes during aging. Although in childhood, the size of the cervix is twice as the uterus, in puberty, enlargement in size occurs (Figure 1.3.) [3]. The muscle content of the cervix is different from the other parts of uterus as it only has 10-15 percent muscle. In addition, cervix has collagenous structure. Owing to these properties, the cervix which changes its structure due to pregnancy and childbirth, may return to its original state after birth [1].

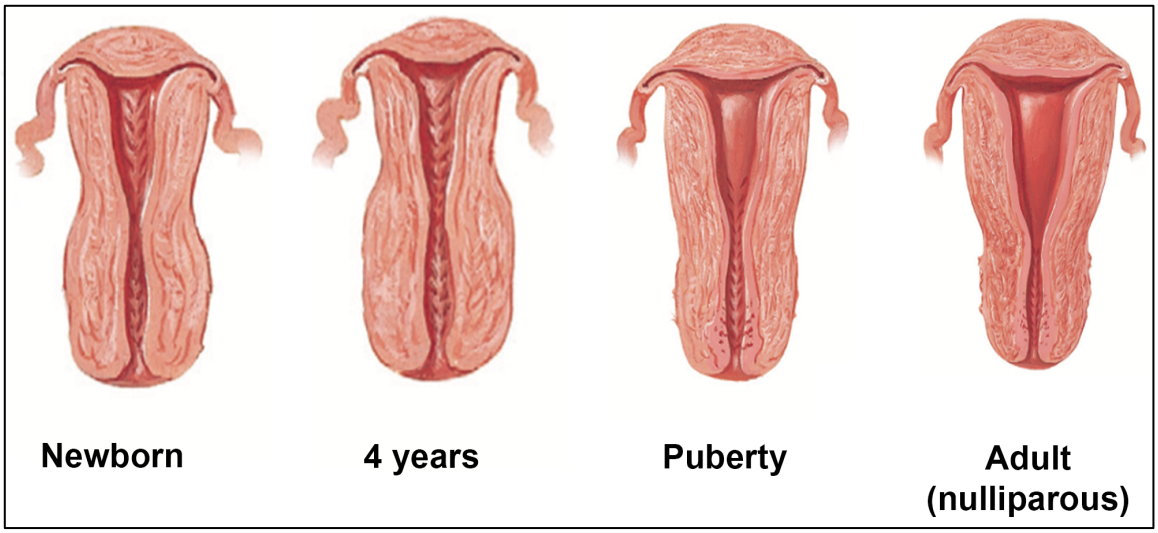


Figure 1.3. Cervix changes during aging [4]

The main purpose of uterus is maintaining the housing for the embryo. When egg is fertilized with sperm in the fallopian tube, zygote moves to uterus from fallopian tube. Zygote then attaches to the uterus layer, called endometrium, prior to its division in multiple times. Until the childbirth, uterus supplies nutrients to the zygote for development. Another function of uterus is the sexual response [5].

1.1.2. Blood, Lymphatic and Nerve Supply in Uterus

Uterus is supplied with the uterine branch. Uterine artery comes from the internal iliac artery and crosses above the ureter and finally reaches to the uterus. The iliac artery connected with the uterus from the isthmus and internal os. Uterine artery gives branches to the upper part

of the uterus and vagina. Uterine vein follows the path of uterine artery, conduct with the ovarian vein, vein of vagina and bladder vein [3].

Fundus, uterine tubes, and ovaries drain together from para-aortic lymph nodes alongside with the ovarian vessels (Figure 1.4.). The cervix drains have three canals. These are external iliac nodes, internal iliac nodes, and sacral lymph nodes [3].

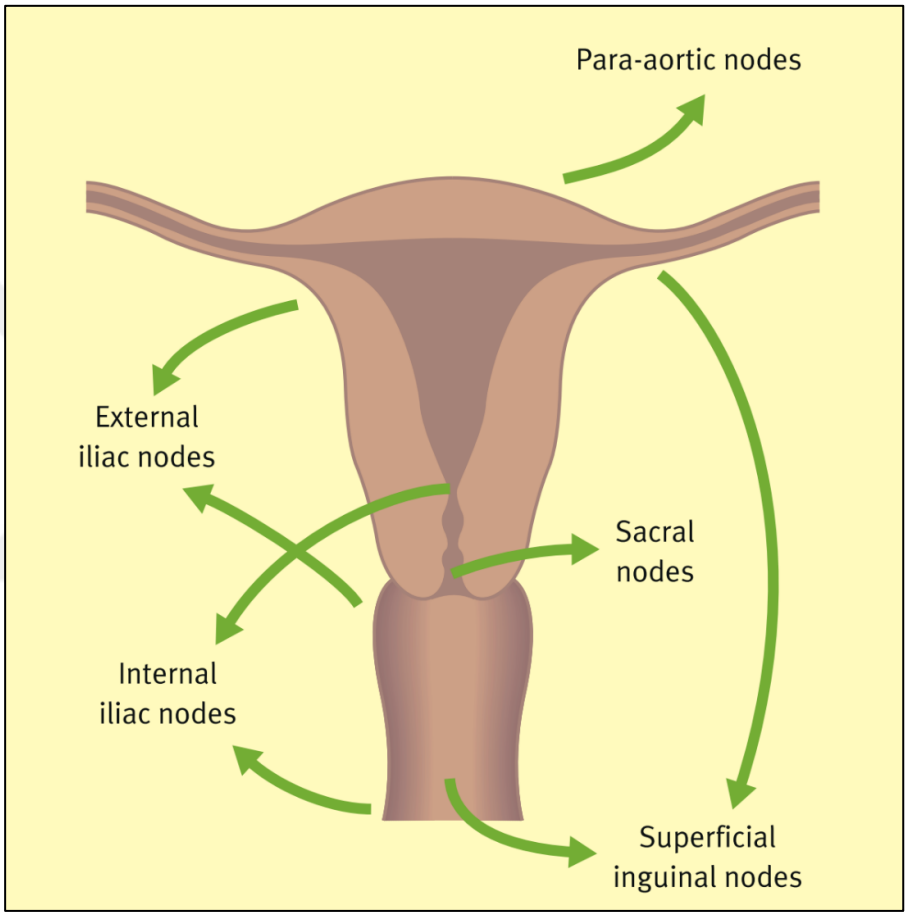


Figure 1.4. Lymphatic drainage of the uterus and vagina [3]

The uterus is supplied by the nerve system through autonomic pathways. The body of uterus is supplied by the sympathetic fibres coming from T10 and L1. The cervix of uterus is supplied with the sacral outflow from S2 and S4 (Figure 1.5.). The uterine is supplied with nerves from the inferior hypogastric plexus and lies in the base of the uterus. Therefore, it is also known as uterovaginal plexus. Uterovaginal plexus passes though uterus and uphill of the uterus artery, which are connected with the ovarian plexus nerves. Uterine nerves make branches, and these branches were compartmentalized into endometrium and myometrium,

and these branches were accompanied with blood vessels. Some uterovaginal plexus nerves passes to the cervix and formed with branches which are renal and aortic plexuses. Ovarian plexus is accompanied with ovarian artery and connected with the ovary and fallopian tubes [3].

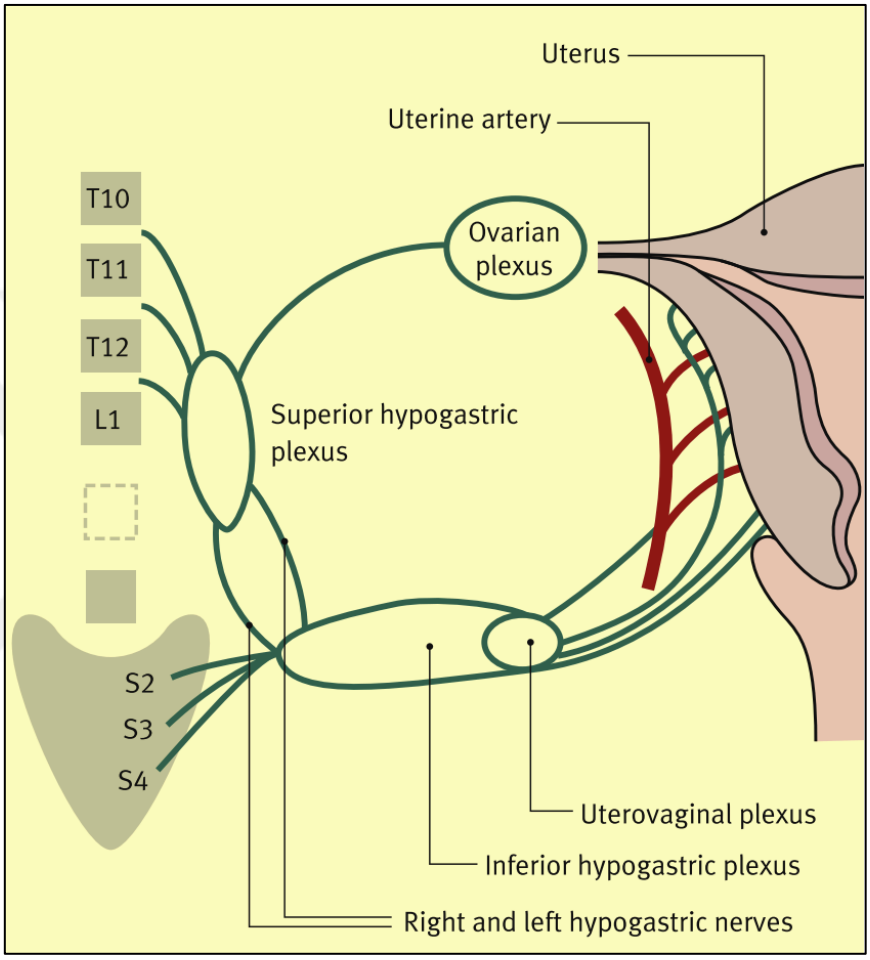


Figure 1.5. Nerve supply of the uterus [3]

1.1.3. Layers of Uterus

1.1.3.1. *Endometrium*

Endometrium is the inner layer of uterus and is a hormone responsive layer [5]. In endometrium layer, tube like glands are present and these tubes are opened to the uterine cavity and mucus secretion [1]. Endometrium layer has a high dynamic capacity for the renewal due to 400 menstrual cycles during woman's life [6].

1.1.3.2. *Myometrium*

Myometrium is the middle layer of the uterus which is placed between endometrium and perimetrium (Figure 1.1.). Myometrium has two main roles. The first role is to protect the fetus from the stresses. The second role is to induce uterine contractions. The function of myometrium is regulated by signals which are in paracrine, endocrine, and mechanical nature. However, the biochemical and mechanical pathways are still investigated [7]. The smooth muscles of myometrium not only have receptors for progesterone and estrogen [8] but also contraction-associated proteins such as gap junction proteins in addition to agonists receptors such as oxytocin, and Na^{+} and Ca^{++} ions to control the myometrium excitability [9].

1.1.3.3. *Perimetrium*

Perimetrium or serosa is the third and outer layer of the uterus. Serosa layer is formed by the peritoneum [1] and can be seen under the ultrasound as a bright colour [5].

1.2. ENDOMETRIUM

Endometrium which is placed in the inner site of uterus has two layers which are functional and basal zone (Figure 1.6.). These two layers thickness depends on the woman's menstrual cycle [10]. Functional zone or functionalis is the upper layer of endometrium. Compact zone and spongy zone are the two layers of functionalis. While the compact zone is associated with the luminal epithelium and contains stroma, the spongy zone located between the compact zone and basalis layer has a dense tortuous gland. Functionalis is re-built after the end of each menstrual cycle. Basal zone or basalis layer is placed between functional zone (upper site of endometriosis) and myometrium layer and supported by the vessels. During menstrual cycle, basalis layer is not shed, and functionalis layer develops on the top of basalis layer [11].

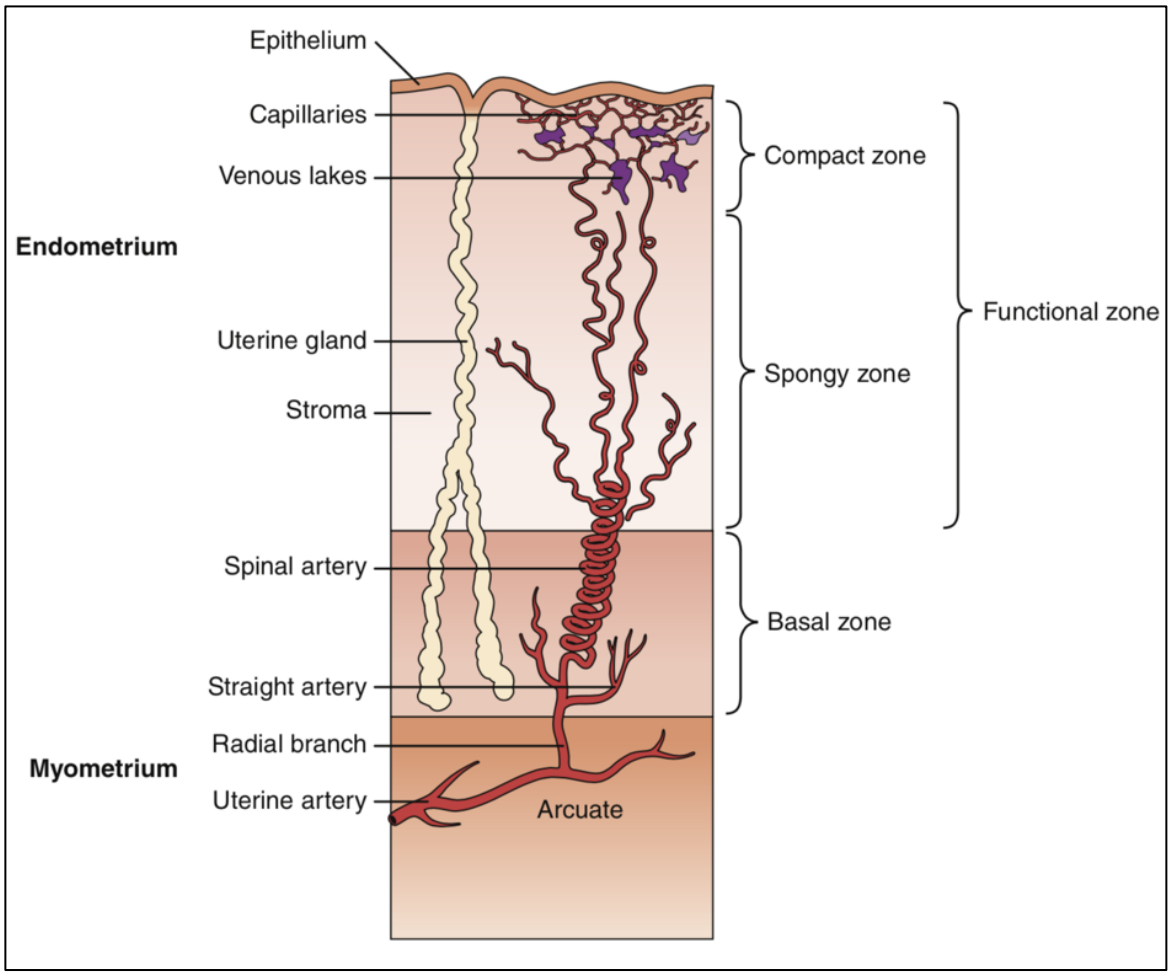


Figure 1.6. Histological organization of human endometrium [12]

Endometrium function is to prevent the adhesion that occurs in the opposite walls of myometrium. During menstrual cycle, endometrium layer grows into a thickness around 6.7 mm. If pregnancy occurs, the endometrium size is further increased and forms the placenta for the embryo [13]. Providing a “fertile ground” is the main function of endometrium. With fertile ground, embryo implantation and development are achieved. In each menstrual cycle, endometrial cells become differentiated. Decidualization is a spontaneous process that occurs in mid-to-late phase of menstrual cycle. If the embryo implantation does not occur, terminally differentiated cells are shed in menstrual cycle. Decidualization is the transformation process of endometrial stromal fibroblasts which is occurred under hormones that oestrogen and progesterone. Under the steroid hormones, the endometrium layer undergoes remodelling and becomes receptive for embryo implantation [14].

Decidualization starts with progesterone induction of stromal cells. Stem-cell like decidualized cells produce high level of cytokines. Chemokines are also involved in the decidualization promotion. cAMP (cyclic Adenosine Mono Phosphate) is required for decidualization as it takes part in Endometrium Stem Cells (ESC) re-programming by inducing expression of cAMP-responsive genes at different stages. Once initiated, local paracrine factors create decidualization waves that which spreads to the endometrium through spiral arterioles (Figure 1.7.) [14].

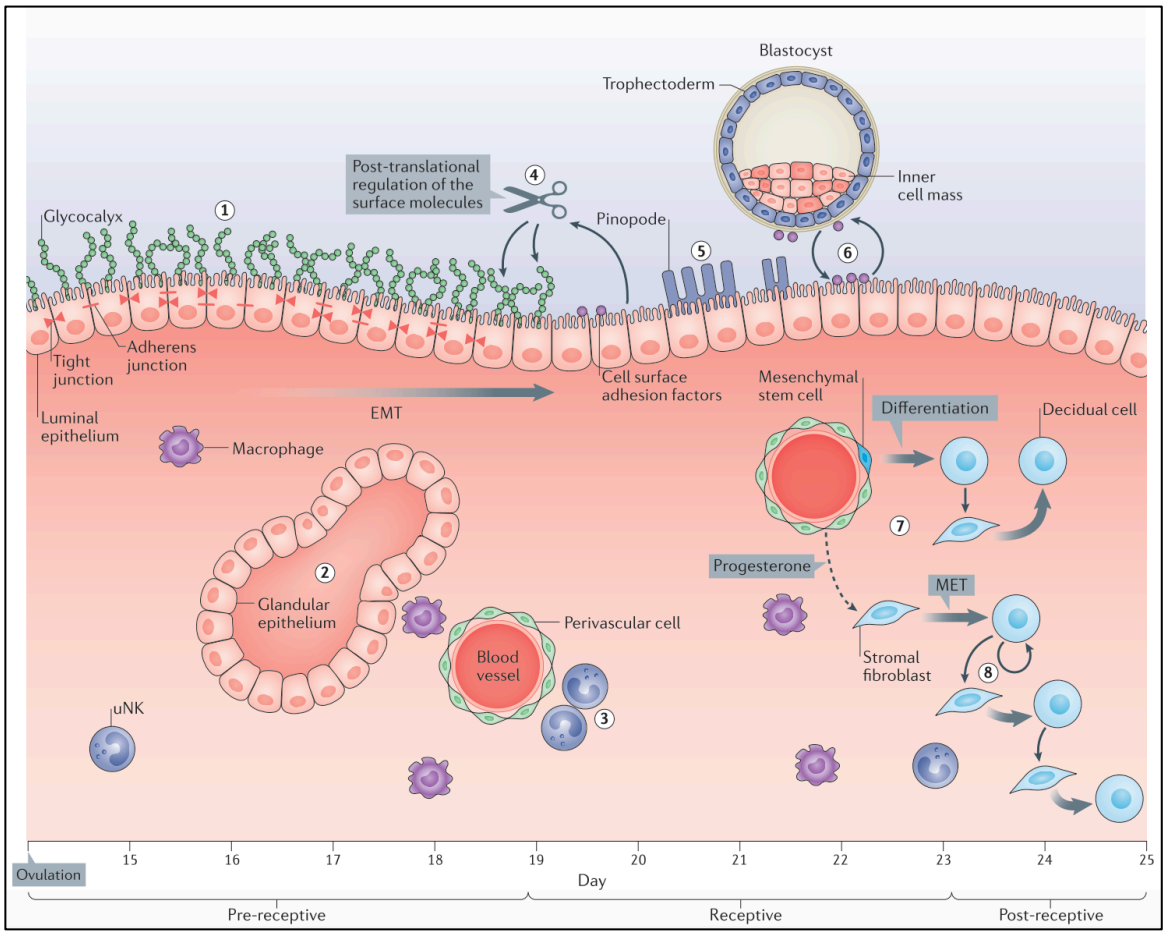


Figure 1.7. Pre-receptivity, receptive and post-receptive stages of endometrium [14]

When oestrogen and progesterone are withdrawn, menstruation was initiated if there is no pregnancy. Destruction of epithelial tissue and re-epithelization occurs at the same time. It was considered that; re-epithelization starts 36 hours after onset of menses and is completed 48 hours later. Decidualize stromal cells are crucial for the endocrine response and paracrine signals transmitting during menstruation. These cells can also express progesterone receptor hence detect the withdrawal of progesterone. When this hormone is withdrawn from

decidualized stromal cells, superoxide dismutase activity is inhibited, and therefore inflammatory reactive oxygen species is increased. Superoxide dismutase induces the activation of nuclear factor KB (NF-KB) and prostaglandin G/H synthase 2 (PTGS2) resulting in the production prostaglandin F2a, one of the inflammatory factors (Figure 1.8.). Hence, the withdrawal of hormone triggers the recruitment of inflammatory cells into perimenstrual endometrium. Although oestrogen is not necessary for endometrium repair, during the re-epithelization oestrogen plays an important role in the stimulation of glandular cells and the regeneration of stromal cells [14].

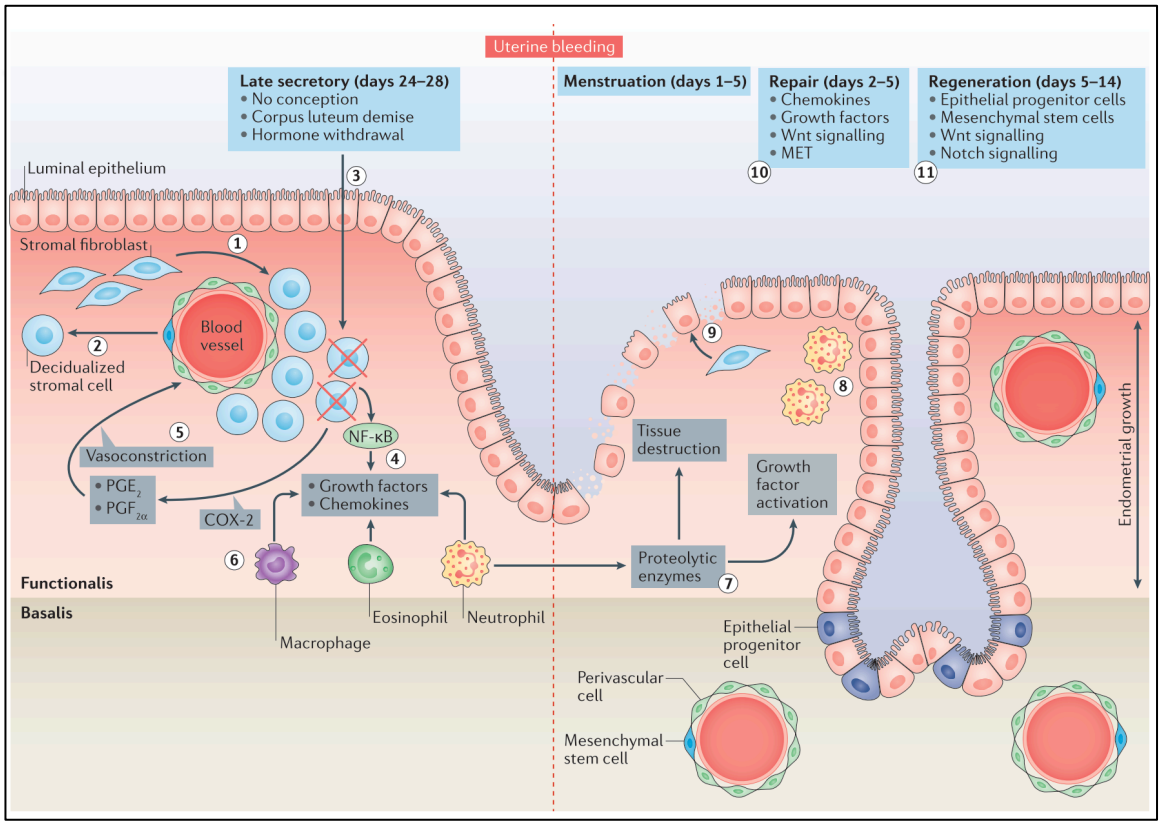


Figure 1.8. Decidualization, menstruation, repair, and regeneration of endometrium [14]

1.2.1. Cell Types in Endometrium

Human endometrium has a dynamic mucosa that changes functionally and morphology 400 to 500 times during reproductive life. Steroid hormones prepare receptive endometrium for implantation. If there is no implantation, functionalis layer of endometrium sheds during menstruation, while basalis layer is remained. New functionalis layer of endometrium grows

over the basalis layer. There are three mechanisms for the replacement of epithelial layer. In the first mechanism, epithelial cells, which were localized at the basal land stump, migrate to the denuded surface. In the second mechanism, stromal cells interact with fibrinous matrix. For the last mechanism, it is suggested that old shedding endometrium fragments were trapped and contribute to synthesis of the new matrix [15]. In addition to all these, 15 years ago, for the first-time adult stem cell population was found in the endometrium layer [16].

Endometrial epithelial progenitor cells are present in the endometrium layer and identified as clonogenic cells (Figure 1.9.) [17]. During menstruation, basal components remained in the basalis layer and epithelial cells were re-epithelized to generate the new functionalis layer [18]. It was stated that basal glands that remained at the basalis layer contains the epithelial progenitor cells [19]. In recent years, epithelial progenitor cells were classified as colony forming unit (CFU) cells and presented as the side population (SP) cells [20] with mesenchymal characteristics. The basic functional features of stem cells are to have the capacity of self-renewal, differentiate and proliferate. Small populations of epithelial progenitor cells have been reported in the human endometrium. In addition, it was concluded that MSC-like cells exist. When endometrial stromal cells are cultured, they differentiate into lineages of endodermal, ectodermal, and mesodermal origin, indicating that endometrial stromal cells are highly plastic [18].

Side Population (SP) cells are universal marker for adult stem cells. It has been reported that, endometrial SP cells have a role in menstrual cycle. SP cells have a mixed cell population and consist of stromal, epithelial, and endothelial cells. SP cells can differ significantly between subjects [21]. Endometrial SP cells are associated with menstrual cycle changes [15]. However, the number of SP cells is higher during the menstrual and proliferative phases. The percentage of SP cells is at the highest level in the early proliferative phase. In the early secretory phase, the percentage is decreased and at the late secretory phase, the percentage is at lowest level [22].

Although rare, endometrial mesenchymal stem/stromal cells (eMSCs) have been identified in the human and mouse endometrium and have a similar phenotypes and properties to MSCs derived from bone marrow and adipose tissue [21]. As a result of studies carried out to determine the position of eMSCs, it was determined that they are in both functionalis and basalis layers of endometrium. This indicates that eMSCs can also be derived from menstrual

blood and may therefore also be referred as menstrual blood-derived stem cells (MenSCs) [23]. Potentially 12 times eMSCs can be obtained from women of reproductive age. Isolation of MSCs is easier for patients compared to bone marrow stem cells since they are taken non-invasively and reduces post-operative discomfort [24]. Moreover, eMSCs have tremendous proliferation properties, and in women aged 30-60 years, the younger the donor, the better the proliferation properties of eMSCs. eMSCs have very strong differentiation properties as well as proliferation properties [25]. Many studies have proven that eMSCs can differentiate into many cell lines under standard culture conditions. In addition, eMSCs can be differentiated into epidermal cells and used as a non-invasive method in dermatological lesions and diseases [26]. When compared with other MSCs, eMSCs appeared to have low tumorigenicity and immunogenicity [23].

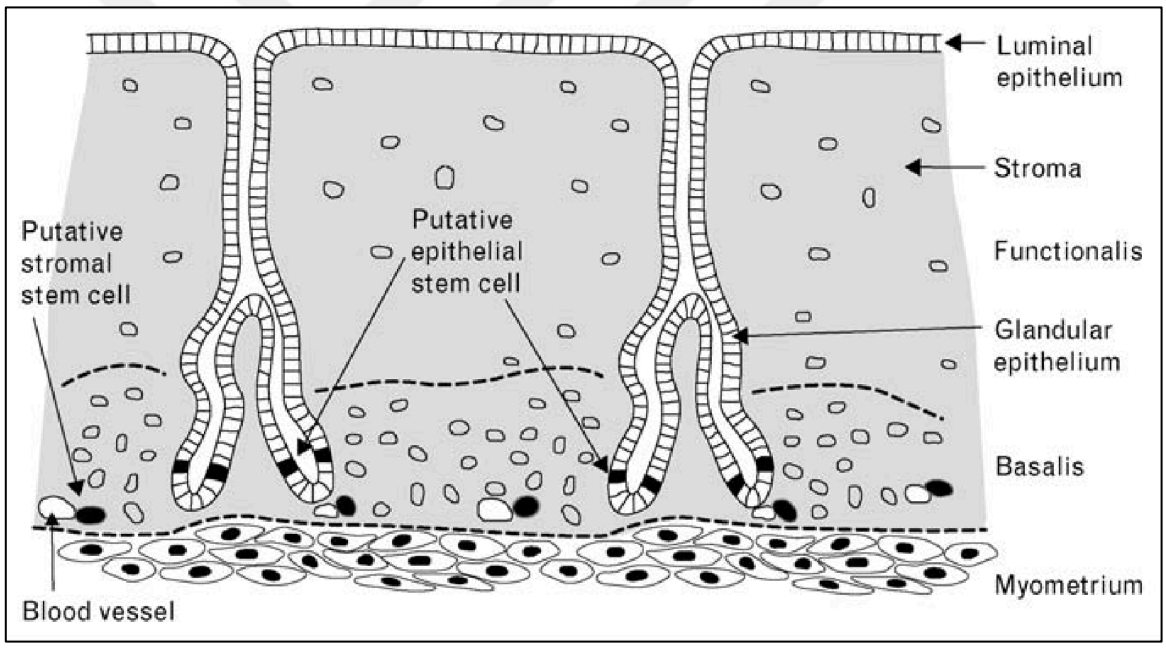


Figure 1.9. Possible location of endometrial stem/progenitor cells in endometrium [11]

1.3. MESENCHYMALS STEM/STROMA CELLS

Stem cells are defined by their multilineage differentiation capacity, self-renewal capacity, and proliferation [27]. When a stem cell is divided into two cells, one cell remains as a stem cell, while another cell differentiates [28]. In the 1970s, Friedenstein and his team were first identified Mesenchymal Stem Cells (MSC) in the bone marrow of guinea pigs [29]. MSCs can be found in humans, mice, rats, canines, and rabbits, and have a capacity for self-renewal

and differentiation [30]. Due to their multipotent differentiation capacity, MSCs can differentiate into osteoblast, chondrocytes, and adipocytes cell lines which originated from the mesoderm *in vivo* and *in vitro*. However, under appropriate *in vitro* conditions, MSCs have been shown to have an unusual ability to differentiate, leading to cells having visceral mesoderm, neuroectoderm and endoderm properties [31]. Because of their differentiation capacity, they currently called mesenchymal stem cells; however, they also called mesenchymal stromal cells due to their appearance found in the bone marrow [32].

Marrow stromal cells can be isolated from other cells in marrow by their tendency to adhere to tissue culture plastic. These cells have many of the characteristics of stem cells for tissues that can roughly be defined as mesenchymal, because they can be differentiated in culture into osteoblasts, chondrocytes, adipocytes, and even myoblasts. Therefore, marrow stromal cells present an intriguing model for examining the differentiation of stem cells. Also, they have several characteristics that make them potentially useful for cell and gene therapy [33].

1.3.1. Structure and Function of Mesenchymal Stem Cells

Bone Marrow (BM) is the first tissue identified as colony-forming fibroblast-like cells when seeded in tissue culture plates [34]. Initially, these cells were described as “stromal cells” and have received much attention in finding a pure cell population that can be used for regenerative purposes and have been the centre of thousands of studies on this subject. In the studies, cells were isolated by different methods and given different names such as MSCs, stromal cells or mesenchymal progenitors [35]. Although, The International Society for Cellular Therapy suggested that these cells be named multipotent mesenchymal stromal cells, most scientists refer to them as MSCs [36]. Although the characterization of MSCs is still controversial, MSCs are generally defined as plastic-adhesive cells with the ability to differentiate into adipogenic, chondrogenic, osteogenic, myogenic, and other cell lines *in vitro* [37,38]. By nature, MSCs proliferate and develop into daughter cells with the same gene expression and phenotype. The two criteria that define MSCs are their potential for regeneration and differentiation [35]. In most studies, MSCs are characterized by examining the differentiation potential of cells or using cell surface antigens. In 2006, three criteria by which human multipotent mesenchymal stromal cells could be identified were proposed by The Mesenchymal and Tissue Stem Cell Committee of the International Society of Cellular

Therapy. MSCs (i) should be plastic-adherent and colony-forming when maintained in standard cell culture conditions; (ii) should express CD73, CD90, and CD105, while lack expression of the surface molecules CD11b, CD14, CD19, CD34, CD45, and CD79a; and (iii) should *in vitro* differentiate into adipocytes, chondrocytes, and osteoblasts [36]. In studies conducted after the isolation of stem cells in the bone marrow, it has been determined that stem cells can be isolated from other adult tissues, and it has been shown that these isolated cells can differentiate into epithelial, endothelial, neural, and hepatic cells [39]. However, cells isolated from various tissues have also been shown to differ significantly in their proliferation and differentiation capacity, morphology, and bone formation *in vivo* [40]. Therefore, MSCs from different tissues may not be the same cell and may contain a heterogeneous population [41]. Many studies have reported that MSCs inhibit immune responses and evade recognition by the immune system [42]. Because of this feature, MSCs facilitate the use of allogeneic clinical applications in both regenerative medicine and immune disease approaches. Therefore, several mechanisms have been proposed to explain how allogenic rejection is prevented by MSCs [43]. Many researchers state that at the molecular level, MSCs migrate to the injury site and secrete various modulatory molecules [44]. In terms of immunomodulation, the timing of MSC treatment is extremely important. There is an appropriate window for treatment to be delivered because the response of MSCs is stimulated by the stimulation of immune cells. The exact way MSCs evade recognition by the immune system has not been fully elucidated [35].

1.3.2. Sources for Mesenchymal Stem Cells

The first MSCs were isolated from BM [45] and shown to differentiate into mesodermal (adipocytes, osteocytes, and chondrocytes) and ectodermal (neurons) cells. For other stem cell sources, the similar potentials have been identified [46]. These sources are adipose tissue [47], amniotic fluid [48], placenta [49], umbilical cord [50], dental pulp [51], breast milk [52], synovium [53], and endometrium [54] (Figure 1.10.). Increasing demands for organ transplantation and the inadequacy of organ donation have prompted further exploration of MSC resources as a source for tissue engineering and as immune modulators. MSCs provide opportunity for autologous transplantation which is good way to avoid tissue rejection [55].

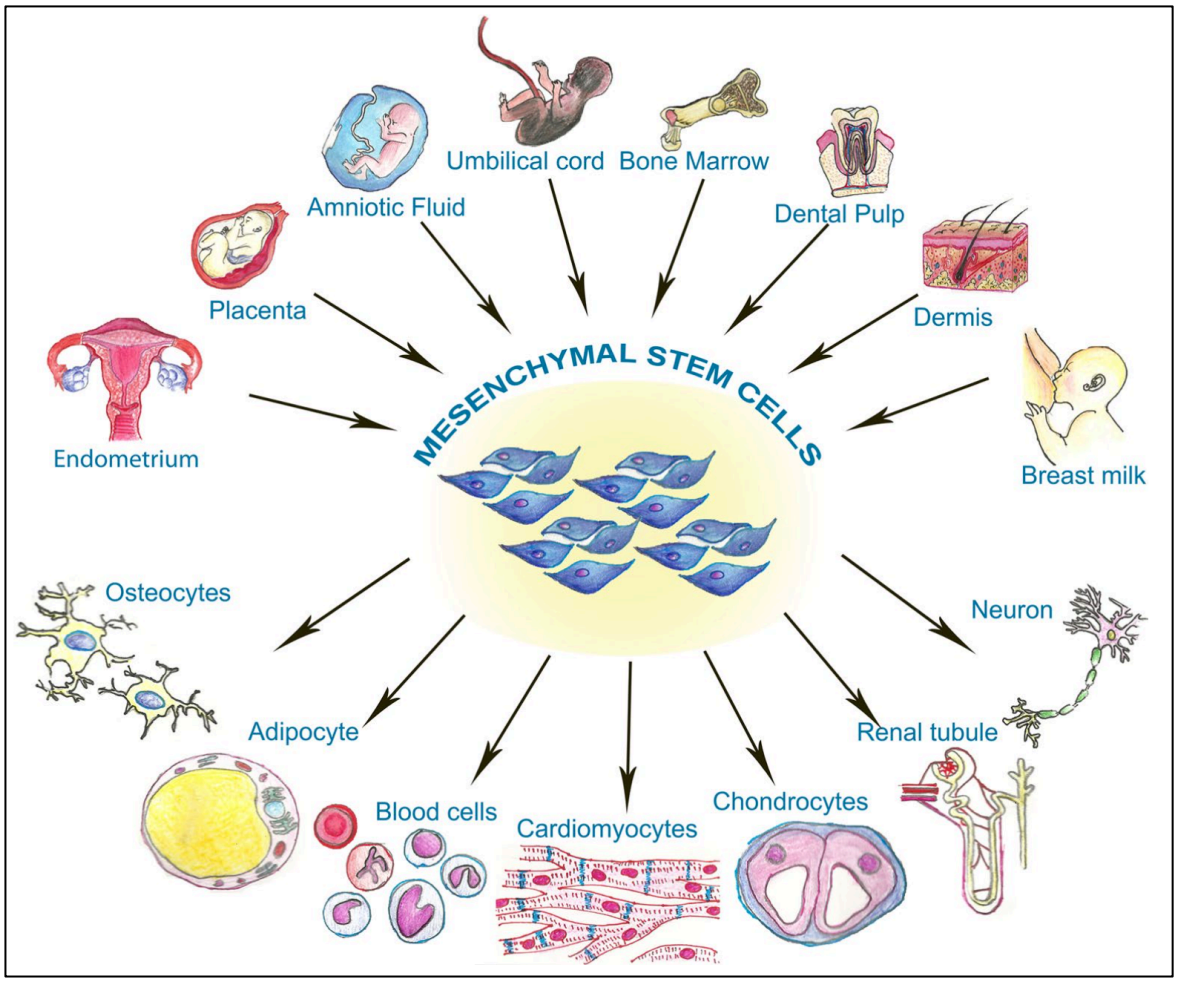


Figure 1.10. Various MSCs sources [56]

MSCs are critical for tissue regeneration, cell therapy and use in regenerative medicine. For these cells to be used in these areas, various isolation methods are applied. Stem cells are found in small amounts in tissues and organs, so effective isolation methods should be used. Cell isolation methods differ from tissue to tissue. Since cells are variable and viable, the isolation process must be rapid, and the viability of the cells must be preserved [57].

Human MSCs are easily isolated from bone marrow (BM) by their adhesion to tissue culture plastic in medium containing high concentration of fetal bovine serum (FBS) [58]. As Friedenstein stated, the most basic feature of BM cells is that they can form colonies even at low density [59]. Critical steps of MSC isolation from bone marrow are i) mononucleated cells isolation from bone marrow aspiration, ii) cells culturing and proliferation and iii) recovery of cells under standard serum conditions to obtain passage zero [60]. Various techniques have been developed for stem cell isolation from bone marrow. These techniques

are the use of density gradient centrifuge, blood cell separation [61] and Hydroxyethyl starch (HES) [62]. Initially, manual methods were used for MSC isolation from BM. Although the manual isolation method is effective, it is open more to bacterial contamination due to exposure to the environment during processing. In addition, undesirable components such as platelets and neutrophils can be found in the culture medium after isolation [63]. As Wells *et al*. stated, BM-MSC can also be isolated with Ficoll-Hypaque, and with this method, red blood cell (RBC) is almost absent in the culture medium after isolation [64]. The isolation efficiency with the Ficoll-Hypaque method is generally 50 percent higher than other manual methods [65]. In addition to manual methods, automatic methods are used for MSC isolation For example, MSC isolation is done quickly and simply in a closed and sterile environment. In 2006, the Sepax system was adopted as a routine procedure for MSC isolation from BM for use in cellular therapeutics by maintaining high standards. The Sepax system's working principle is based on the Ficoll density gradient and can be performed both manually and automatically [57].

In 2001, Zuk *et al.* stated that MSC isolation was made from adipose tissue, and thereupon, interest in MSC isolation from adipose tissue increased [66]. It has been stated that stem cells in adipose tissue are associated with the vascular bed [67] and named as Adipose Derived Mesenchymal Stem Cells (ADMSCs) [60]. ADMSCs are extremely attractive somatic stem cells because of the availability, accessibility, and abundancy. The possibility of obtaining ADMSCs therapeutically raises the interest for their use in tissue engineering applications and stem cell therapies. While ADMSCs are used in treatments such as wound healing and osteochondral repair [68], they are also used for regenerative applications due to their liver [69] or neuronal [70] differentiation capacity. There are important variables for the isolation of ADMSCs and cell proliferation [71]. For this reason, isolation should be done by following the standard procedures. As with bone marrow, mesenchymal cells found in adipose tissue contain a supporting stroma that allows for the easy isolation [72]. Based on this, adipose tissue is said to be a resource that can have far-reaching effects. Adipose tissue is easier to obtain than other sources and is associated with minimal discomfort and risk with local anaesthesia [73]. There are five macroscopic sources of ADMSCs, which are breast adipose tissue, bone marrow adipose tissue, white adipose tissue, brown adipose tissue, and mechanical adipose tissue [74]. Although ADMSCs are morphologically and immunophenotypically alike BM-MSCs, they generate more CFU-F in cell culture [75]. For

ADMSC isolation from adipose tissue, firstly, subcutaneous adipose tissue must be broken down, followed by enzymatic digestion. After these steps, cell proliferation is performed in cell culture [47]. Similar to BM-MSCs, Zuk *et al*. defined stem cell population from human lipoaspirates (PLA), showed stable growth and proliferation in cell culture, and could differentiate into adipogenic, osteogenic, chondrogenic and myogenic lineages *in vitro* [66,72]. Apart from these differentiations, ADMSCs can also differentiate into macrophages and endothelial cells [76]. ADMSCs isolation methods from PLA and adipose tissue biopsy are different. In PLA isolation method, lipoaspirate samples are mixed with sterile phosphate buffered saline (PBS) to separate adipose tissue. The sample is washed with PBS until completely clear and cells are cultured. Two methods, enzymatic and non-enzymatic, are used for cell isolation from adipose tissue biopsy samples. In the non-enzymatic method, the tissue is divided into small pieces with a scalpel in a sterile environment [60]. In the enzymatic method, the cells are treated with 0.075 percent collagenase type 1 enzyme [72]. ADMSCs cells can be isolated by all methods described.

In 1993, progenitor cells were isolated from amniotic fluid for the first time at the 12th week of pregnancy. These isolated cells were defined as hematopoietic progenitor cells. In 1996, the presence of non-hematopoietic stem cells was also established in amniotic fluid by demonstrating a sub-population of cell isolates had myogenic differentiation potential. However, the characterization, differentiation, and therapeutic applications of amniotic mesenchymal stem cells (aMSCs) have recently begun to be investigated [77]. The number of aMSCs can vary with cellular and chemical composition in amniotic fluid, fetal health, and gestational age. Therefore, as the isolated cellular density changes between samples, the aMSC yield may also change. Isolation of aMSCs from amniotic fluid is through a non-enzymatic and easy-to-use method. After collecting the amniotic fluid with a 30-gauge needle, amniotic fluid is centrifuged. After centrifugation, aMSCs are cultured with appropriate culture media [57,60].

The human placenta is referred to as a postnatal waste product [60]. While placenta plays an important role for fetal nutrition and development, it also contains stem cell reserves. Progenitor cells isolated from placental tissue are important source of cells for regenerative medicine due to their longevity and plasticity. In addition, these cells also have immunomodulatory properties. Placenta tissue can be obtained without any need for an invasive method and does not pose ethical problems [78]. For the isolation of placenta

derived MSCs (PDSCs), the maternal decidua and chorionic villus fragments are first removed. The remaining pieces are cut into small pieces with a scalpel. After this step, PDSCs isolation is applied with different methods. Klein and Fauza isolated PDSCs using the enzymatic method with 10 percent (w/v) collagenase type 2 from the fragmented placenta tissue. Jiang *et al*. isolated PDSC by an enzymatic method which involved the incubation of placenta tissue with 0.075 percent collagenase for 30 minutes at 37°C followed by and incubation with 0.125 percent trypsin for 30 minutes at 37°C. In this method cells were plated onto tissue culture plastic by passing the tissue homogenate through 100 nm filter [79]. Brooke *et al*. incubated the placenta tissue with 100 U/ml collagenase type1 together with 5 µg/mL DNase 1 for 2 hours at 37°C and then isolated the PDSCs using a 70 µm filter [80]. As a result, PDSCs are isolated by different enzymes and enzymatic methods under different conditions.

The presence of MSC in Umbilical Cord Blood (UCB) was evidenced by the presence of fibroblast cells and expression of mesenchymal progenitor antigen [77]. MSCs isolated from UCB are called Umbilical Cord Derived Stem Cells (UCSCs) and can differentiate into different lineages like other stem cells [50]. UCSCs differentiate into adipogenic, osteogenic and chondrogenic as well as neural and cardiogenic tissues. In addition to the Umbilical Cord, UCSCs can also be isolated from the cord lining, the connective tissue protecting the umbilical vessels, and the cord perivascular tissue [81]. When the morphological, immunophenotic and differentiation characteristics of UCSCs were examined, it was observed that these cells were not different from BM-MSCs [56]. For stem cell isolation from umbilical cord, umbilical cord is obtained after full-term birth [82]. Usually, UCSCs are isolated with enzymatic digestion method, however, if the aim is explant culture, then enzymatic digestion can be skipped. For UCSCs isolation, firstly, blood vessels are removed from the tissue and then tissue is treated with enzymes mostly collagenase type 1, although in some studies, collagenase type 2 and collagenase type B are used due to their efficiency to solubilize the Umbilical Cord microfibrils [83].

In tooth growth and development, ameloblasts form the tooth enamel and odontoblasts form the primary dentin [84]. After tooth eruption enamel formation stops and ameloblasts form. However, odontoblasts continue to form the dentin matrix. All of this has led to speculations that it may create a progenitor or root source in the dental pulp [85]. Isolation of stem cells from wisdom teeth was performed for the first time in 2000 by Gronthos *et al* [86], which

was followed by another study where exfoliated deciduous tooth was investigated for the existence of stem cells [87]. The Dental Stem Cell population is subdivided into Dental Pulp Stem Cells (DPSCs), Dental Follicle Stem Cells (DFSCs), Periodontal Ligament Stem Cells (PDLSCs), and Apical Papilla Stem Cells (SCAPs). Like other MSCs, DPSCs can be differentiated into adipocytes, osteocytes, and chondrocytes, in addition to their potential to differentiate neurons, odontoblasts, myocytes, and endotheliocytes. In addition, it has been reported that DPSCs have higher proliferation ability than BM-MSCs [88]. For stem cell isolation from tooth, firstly the tooth enamel is removed to expose dental pulp tissue. which is then harvested by collagenase treatment [86] or mechanical dissociation [89]. In most cases, in enzymatic isolation, collagenase type 1 and protease dispase are used [60].

Breast milk is a bioactive fluid containing countless cells and biological molecules. Breast milk does not only provide nutrition for newborn babies, but also helps to regulate the immune system and tissue development. In the literature, there has not been much research on breast milk and the cells it contains [90]. Most of the cells in breast milk are epithelial cells [91]. In 2007, Cregan *et al*. showed that stem and progenitor cells are also present in breast milk [92]. In 2012, Hassiotou *et al*. published the first paper that showed the presence of cells called human breast milk stem cells (hBSCs) in breast milk, which exhibited stem cell properties and expressed pluripotent markers. Later, Kaingade *et al*. reported that hBSCs secrete some growth factors including vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) [93]. hBSCs isolation is obtained by a non-invasive method, has no ethical and sustainability problems, and these cells do not form tumours. For isolation, firstly breast milk collected in sterile environment is diluted with the same amount of sterile PBS and then centrifuged. After the first centrifugation, the fat layer and skim milk layer are separated, and the pellet (fat layer) is resuspended in sterile PBS and centrifuged again. After three consecutive centrifuges, pelleted cells are cultured in optimal cell culture media [94].

De Bari *et al.* isolated MSC from the synovium (SM) of the knee joints for the first time in 2001 [95]. Studies in isolated cells have found that these cells have stem cell characteristics, and their differentiation potential is versatile, which led them to be named as synovial-derived mesenchymal stem cells (SDMSCs). Compared to other MSCs and their sources, SDMSCs have a more accessible source, low in immunogenicity, high proliferation rate, and greater chondrogenic potential, resulting in increased interest in these cells [96]. Isolation of SDMSCs from synovial fluid can be performed by non-enzymatic methods, where the

synovial fluid diluted with dPBS is first passed through a 70 μm nylon filter to disperse the cells, which are then seeded [97]. For MSC isolation from synovial membrane tissue, SM tissue is cut into small pieces, which is then digested with 440 u/mL collagenase A [98] or 3 mg/mL collagenase type V [97] to remove connective tissue and associated cells. The un-digested tissues pieces are then transferred into cell culture for cell growth after passing through a 70 μm nylon filter.

The endometrium has recently been discovered as a source of stem cells. Stem cells derived from the endometrium are defined as endometrial mesenchymal stem cells (eMSCs) and have the potential to differentiate into various cell types [99]. To obtain eMSCs, endometrial samples can be collected by hysterectomy method or obtained from menstrual blood [100]. It has been reported that eMSCs can differentiate into ectodermal and mesodermal cell lines. In addition, eMSCs can differentiate into neural cells from the ectodermal cell lineage [101]. It is also hypothesized that eMSCs can also differentiate into cardiac muscle cells. Also, it is stated that eMSCs express pluripotent markers, making these cells suitable for programming into iPSCs [102]. Non-enzymatic and enzymatic methods can be used for the isolation of eMSCs from the endometrium. For the non-enzymatic method, the resulting tissue is cut into small pieces using sterile scalpel blades in sterile PBS and grown in optimal cell culture media. For the enzymatic method, 0.05 percent trypsin and 5 mg/mL collagenase type 1 solution are used and grown in optimum cell culture media [103].

1.3.3. Cellular Therapies Using Mesenchymal Stem Cells

There are hundreds of clinical studies for the use of MSCs in the therapeutic interventions of serious diseases, either alone or in combination with drugs. These clinical trials are generally in orthopaedics [104]; degenerative diseases such as eye, heart, liver, kidney, lung [105]; autoimmunity diseases affecting the brain and spinal cord, pancreas, or gastrointestinal tract [106]; and immune rejection in transplantation [107]. Clinical testing is performed worldwide, especially in biomedical institutions and universities in China, the United States, and the European Union [108]. In MSC treatments, actual data are generally at the initial level [109]. The first clinical trial involved the injection of Bone Marrow cells into patients with hematologic malignancies by Lazarus in 1995, after which a significant increase in clinical trials was observed and the studies were registered on clinicaltrials.gov.

19 studies were started in 1995-2005, 159 more studies were started in 2006-2010, 420 studies were started between 2011-2015 and 27 studies were started between 2016-2020. As of June 2020, there were a total of 1138 studies registered worldwide. All these studies, only 18 studies have been published [109]. In September 2021, there were a total of 1045 studies worldwide, of which 61 were about COVID-19 (clinicaltrials.gov). As of September 2021, the most studies worldwide were conducted in East Asia with 339 studies. It is followed by the United States with 197 studies, Europe with 171 studies, the Middle East with 76 studies which 14 studies being conducted in Turkey, Russia with 30 studies, Central and South America with 46 studies, South and Southeast Asia with 67 studies, Canada with 9 studies, Japan with 4 studies, and Pacifica with 4 studies (clinicaltrials.gov). Although the use of MSCs in clinical trials is increasing day by day, the age of the donor at which cells are isolated remains an issue to be considered. Besides the importance of donor age, the density of the cell isolated from the donor obtained is low [110]. New approaches should be tried in clinical studies due to the differentiation capacity of MSCs, their growth rate and the ability of cells to migrate to damaged areas [107].

1.4. MENSTRUAL BLOOD MESENCHYMAL STEM CELLS

A woman experiences an average of 400 menstrual cycles during her reproductive years, and menstrual blood is discarded. Since 2007, the discovery of MSCs in menstrual blood, they were seen as a new, powerful source of MSCs for regenerative medicine [107]. Cells isolated from menstrual blood are called by various names. These are endometrial menstrual MSCs, menstrual blood MSCs (MenSCs), endometrial regenerative cells (ERCs), menstrual blood derived MSCs (MMSCs), endometrial decidual tissue MSCs (EDT-MSCs) and menstrual blood progenitor cells (MBPCs) [54]. Patel *et al*. in 2008, demonstrated the clonogenic properties of stem cells isolated from menstrual blood. In addition, it has been shown that MenSCs can differentiate into tissues derived from ectoderm and mesoderm. It has been reported that MenSCs express pluripotent markers such as Nanog, Oct-4 and SSEA-4 expressed in embryonic stem cells [111], and possess human telomerase reverse transcriptase (hTERT) and human telomerase activity [112]. MenSCs are suggested to be a better alternative to other stem cell sources such as bone marrow and adipose tissue. The reason is because MenSCs have a higher proliferation rate than other stem cells and can be accessed without hospitalization or surgery. In addition, MenSCs are far from ethical

dilemmas, which is shown as a new feature compared to other stem cells known so far [113]. Meng *et al*. showed that the doubling rate of MenSCs was every 19.4 hours. This rate is twice as fast as in BM-MSCs with a doubling rate of 40-45 hours [16]. While the expression of MSC markers such as CD29, CD73, CD105 and CD90 is positive, the expression of markers such as CD34 and CD45, which are hematopoietic markers, is negative for MenSCs [114]. Therefore, it is questioned whether MenSCs represent a more primitive progenitor cell than other MSCs [113].

MenSCs can differentiate into bone, fat, cartilage, pancreatic, nerve and liver cells. In addition to these cells, MenSCs can also differentiate into neurocytes, myocytes, and respiratory epithelial cells [16]. Liet *et al.* showed that MenSCs can be a new source for induced pluripotent stem cells (iPSCs) [115]. However, it was stated that these experiments were performed only *in vitro,* and the differentiation was only shown phenotypically. Although various studies have been conducted to learn the immune properties of MSCs, studies for MenSCs are still in their infancy [113].

It is known that the endometrium, as an integral part of the mucosal immune system, protects the integrity of the fetus and is protective against infectious pathogens [116], therefore, MenSCs were expected to show strong immune effects. In animal studies to determine whether MenSCs are teratoma-forming, no teratoma development was found in NOD/SCID mice at screening (after 16 weeks) after subcutaneous injection [113]. To test the anti-tumour effect of rat glioma model was injected with MenSCs and a significant inhibition of tumour growth was observed [117].

As of September 2021, there are three studies in which MenSCs have been used therapeutically in clinical trials. Two of these studies are carried out in China and one in Iran. A clinical trial titled "Phase 1/2 Study of Human Menstrual Blood-derived Mesenchymal Stem Cells Transplantation for the Evaluation of the Efficacy and Safety in Patients With Liver Cirrhosis" was initiated in 2010 in China. Orthotopic liver transplantation (OLT) method, which is effectively used for end-stage liver disease, was being evaluated for the efficacy and safety of stem cell transplantation from menstrual blood for liver cirrhosis because of the scarcity of donor organs, immune rejection, and high cost. However, the status of the study is yet to be announced. The second study, titled "Phase 1/2 Clinical Study of MenSCs Infusion in Type 1 Diabetes" for type 1 diabetes patients, was initiated in 2012 in China. In the study, the safety and effectivity of MenSCs treatment for

Type I diabetes patients was investigated. No information about the status of this study was released. Finally, in April 2021, a clinical trial titled "Safety and Efficacy Study of Allogeneic Human Menstrual Blood Stem Cells Secretome to Treat Severe Covid-19 Patients, Clinical Trial Phase I&II" was started in Iran for COVID-19. The study is a randomized controlled trial, and the effectivity of MenSCs on cytokine storm severe cases of COVID-19 was investigated (clinicaltrials.gov).

1.5. AIM OF THE THESIS

With the discovery of MSCs in menstrual blood in 2007, menstrual blood was seen as a new source of stem cells, and MenSCs became a source of interest in stem cell studies. Over the past 13 years, several studies have been conducted on MenSCs because cell isolation is easy, consistently available, does not require surgery, and does not cause ethical problems. Various studies have been conducted, such as the stem cell properties of MenSCs isolated from menstrual blood, and their use in the treatment of various diseases such as COVID-19, diabetes, and liver diseases. However, different isolation methods of MenSCs from menstrual blood was carried out in all these studies. These methods have not been compared in terms of their potential to maintain stemness of the isolated cell population. In this context, the purpose of this thesis is to compare the three different isolation methods including non-enzymatic, collagenase type 1 and dual digestion method and investigate the potential of MSCs isolated from menstrual blood to maintain their stemness until late passages. Menstrual blood of healthy volunteers will be collected under ethical approval from local ethical committee using menstrual cups in first 3 days of menstrual cycle.

2. MATERIALS

2.1. INSTRUMENTS

Laminar flow cabinet (ESCO Lab culture Class II Biohazard Safety Cabinet 2A, Singapore), CO_2 incubator (In-Vitro Cell ES NU-5800, NuAire, USA), Centrifuge (Gyrozen M416, USA), Microcentrifuge (Beckman Coulter, A46473, Germany), Rotator (BioSan, RS-24, Latvia), Carl Zeiss PrimoVert Microscopy with Axio Cam 105 colour (GmbH 37081, Germany), Zeiss SteReo Discovery V20 (Germany), -80°C freezer (Thermo Forma -86 C ULT Freezer, USA), Waterbath (Stuart, SBS40, UK), Vortex (Stuart SA8, UK), FACS Calibur (BD Biosciences, USA), Mp Minipure Dest Up Distilled Water (SentezLab, Turkey), Weighing Scale (Shimaozu, Tw423L, Japan), SpectraMax Paradigm Spectrometry Multi-Mode Plate Reader

2.2. EQUIPMENT

Menstrual Cup (Model1, Lunette, Finland), Electronic pipette (CAPP aid, Denmark), Serological pipettes (2 ml, 5 mL, 10 mL, 25 mL, SPL Life Sciences, USA), Micropipettes (10 µL, 20 µL, 100 µL, 200 µL, 1000 µL, Thermo Scientific, USA), Pipette Tips (10 µL, 200 µL, 1000 µL, CAPP Expell Plus, Denmark), Falcon tubes (15 mL, 50 mL, Isolab, Germany), Polypropylene eppendorf tubes (2 mL, 1.5 mL, Isolab, Germany), Cell culture flasks (T25, T75, TPP, Switzerland), cryovial (2 mL, NEST, China), Bright-Line Hemacytometer (Sigma Aldrich, USA), Cell Strainer (0.70 µm Nylon) (Corning, 352350, USA), 0.22 µm filter (TPP, Switzerland), Cover slip (Sigma Aldrich, USA), Hemocytometer (Marienfelt, Germany), Well Plates (Sigma, USA)

2.3. CHEMICALS

Dulbecco's Modified Eagle Medium low glucose (1x DMEM, 1 g/L Glucose, L- glutamine, sodium (Gibco 31885-023, Thermo Fisher), Heat-inactivated Fetal Bovine Serum (FBS) tested (Gibco, A3840401, UK), MEM Non-essential amino acid 100x (INVITROGEN / 11140-035), Antibiotic-Antimycotic (Gibco, USA), Dulbecco's Phosphate Buffered Saline

(dPBS) (10X, Gibco 14080055, USA), Trypsin-EDTA (0.05 percent, Lonza, BE02-007E, Belgium), Collagenase (powder) (Gibco, 17018029, USA), Paraformaldehyde (Merck, 1,04005,1000, Germany), EDTA (Merck, 1,08418,0100, Germany), Crystal Violet (Sigma, P4333, USA), Ammonium Chloride (Sigma, A9434, USA), Histo-Paque (Sigma, RNBK3107, USA), Cell Proliferation Reagent WST-1 (Merck, 11644807001, Germany)

3. METHODS

3.1. MENSTRUAL BLOOD MESENCHYMAL STEM CELL ISOLATION METHODS

Menstrual Blood Mesenchymal Stem Cells (MenSCs) were obtained using three different isolation protocols namely non-enzymatic, collagenase type 1 digestion and dual enzyme (collagenase type 1 and trypsin) digestion methods.

3.1.1. Non-Enzymatic Method for MenSCs Isolation

Approximately, 10 mL Menstrual Blood (MB) collected in sterile menstrual cup was slowly placed into the isolation buffer (1:1) that contains 1 mM (w/v) EDTA, 2 percent (v/v) Penicillin/ Streptomycin/ Amphotericin (PSA) in 20 mL 1X Phosphate Buffered Saline at pH 7.4 (PBS). Diluted blood sample was slowly placed onto the Histo-Paque with 2:1 ratio and centrifuged at 400 *xg* for 30 minutes. After centrifugation, buffy coat (middle layer) was transferred into a new centrifuge tube and 5 mL 1X PBS was added. Following another centrifugation step at 100 *xg* for 10 minutes, pellet was resuspended in MenSCs media which is composed of Low Glucose Dulbecco's modified medium (lgDMEM) supplied with 20 percent (v/v) Fetal Bovine Serum (FBS), one percent (v/v) Penicillin/ Streptomycin/ Amphotericin (PSA) and one percent (v/v) Non-Essential Amino Acid (NEAA) and seeded into 6-well plate. Cells were transferred into a mammalian incubator with 37^{o}C temperature, 80-95 percent humidified air and 5 percent CO_2 conditions, to provide optimal growth conditions. Medium was changed at every other day with MenSCs media.

3.1.2. Collagenase Type I Digestion Method for MenSCs Isolation

As described above (Section 3.1.1.) menstrual blood was collected and mixed with the isolation buffer with 1:1 ratio. Blood sample was decanted gently onto Histo-Paque with 2:1 ratio and centrifuged at 400 *xg* for 30 minutes. Buffy coat (middle layer) was collected after centrifuge and washed with 5 mL 1X PBS and centrifuged at 250 *xg* for 10 minutes. After centrifugation, 160 mM (w/v) Ammonium Chloride (NH_4Cl) was added onto pellet to lyse

erythrocytes for 10 minutes at room temperature with gentle agitation. After inoculation, cell fragments were washed with 1X PBS and centrifuged at 200 *xg* for 10 minutes. After centrifugation, 0.1 percent (w/v) collagenase type I was added onto the slurry. Following 30 minutes incubation at room temperature with gentle agitation, collagenase type I was inactivated with the addition of MenSCs media (twice the total volume). After centrifugation at 250 *xg* for 10 minutes, the cell pellet was washed with 1X PBS and centrifuged 250 *xg* for 5 minutes. Obtained cell pellet was then dissolved in MenSCs media and filtered thorough cell strainer (70 µ Nylon) to get rid of tissue particles and cell debris. Finally, cells were seeded into 6-well plate and transferred into a mammalian incubator set to provide the optimal growth conditions. Medium was change was performed every other day as indicated above (Section 3.1.1.).

3.1.3. Dual Enzyme Digestion Method for MenSCs Isolation

MenSCs isolation via collagenase type I and trypsin dual digestion was similar to collagenase type I digestion method. Only difference was using combination of 0.05 percent (w/v) collagenase type I and 0.05 percent (w/v) trypsin instead of using 0.1 percent (w/v) collagenase type I described above (Section 3.1.2.).

3.2. MAINTENANCE of MENSTRUAL BLOOD MESENCHYMAL STEM CELLS

3.2.1. Cell Passaging of MenSCs

When cell confluency reached to 70-80 percent, cell passaging was performed. Firstly, growth medium was discarded, cell monolayer was washed with 3 mL 1X PBS and 3 mL 0.05 percent (w/v) trypsin was placed onto the cell monolayer. After a five-minute incubation in the mammalian incubator, flasks were removed and checked for cell dissociation under an inverted microscope. Next, 6 mL of MenSCs media was added for the inactivation of trypsin, and dislodged cells were collected into a 15 mL centrifuge tube. Cell suspension was centrifuged. After 300 *xg* / 5 minute-centrifugation step, the cell pellet was dissolved in MenSCs media and cells were seeded into a fresh culture flask or prepared for cryopreservation.

3.2.2. Determination of Cell Number

After trypsinization, cells were centrifuged at 300 *xg* for 5 minutes as described above (Section 3.2.1.) and pellet was resuspended in MenSCs media. 10 µL from cell suspension was taken and loaded into each grid of the hemocytometry. The number of cells was counted at 10x objective using an inverted light microscope. Number of cell per 1 mL of cell suspension was calculated using the following equation:

$$Cell\ Number/mL = \frac{The\ average\ number\ of\ counted\ cells\ x\ Dilution\ factor}{10^{-4}}$$

3.2.3. Cell Thawing

Formerly frozen cells in cryovials were taken from either liquid nitrogen container or -80ºC were heated to 37ºC and transferred into new falcon tube. 5 mL MenSCs media was added slowly to avoid building of the osmotic pressure. Tubes were then centrifuged at 300 *xg* for 5 minutes, and pellet was resuspended in MenSCs media. Cells placed into T-25 flask and placed in a mammalian incubator with optimal growth conditions.

3.2.4. Cryopreservation of Menstrual Blood Mesenchymal Stem Cells

Following cell passaging (Section 3.2.1.), $1x10^6$ cells were centrifuged and cell pellet was resuspended in freezing mix composed of 90 percent FBS and 10 percent dimethyl sulfoxide (DMSO). Cells were then transferred into sterile cryotubes and stored at -80ºC for short term (up to six months) or at liquid nitrogen at -196ºC for long term preservation.

3.3. CHARACTERIZATION of MENSTRUAL BLOOD MESENCHYMAL STEM CELLS with FLOW CYTOMETRY

Characterization of MenSCs were done with flow cytometry. Trypsinization of cells were performed as described in Section 3.2.1. and fixed in 1 mL of 4 percent (v/v) paraformaldehyde (PFA) for overnight at +4ºC. Next day, cells were washed with 8 mL 1x dPBS, pH 7.4 and centrifuged at 400 *xg* for 10 minutes. After centrifugation, cell pellet was

washed with 1x PBS and cells were counted as indicated above. $1.9x10^5$ cells per 100 µL of PBS was incubated with 1 µL of antibodies against surface markers conjugated with fluorescent dye (Table 3.1) for 1 hour at room temperature in dark. After 1 hour incubation, cells were washed with 1 mL 1x dPBS pH 7.4 and centrifuged at 400 *xg* for 10 minutes. Cells resuspended in 300 µL 1x dPBS pH 7.4 were transferred into flow cytometry tubes for analysis using FACS Calibur. At least, 10000 events for each sample were recorded for eleven CD markers and normal mouse IgG-FITC was used as negative control.

Table 3.1. Mesenchymal Stem Cell Markers with their conjugated dye

CD Marker	Labelling Dye	Company	Catalog Number
CD 29	PE (positive)	Biolegend	303004
CD 44	FITC (positive)	Biolegend	103022
CD 73	PE (positive)	Biolegend	344004
CD 90	FITC (positive)	Biolegend	328108
CD 105	PE (positive)	Abcam	Ab53321
PDGFR	PE (positive)	Biolegend	323606
W5C5	PE (positive)	Biolegend	327406
Integrin β1	PE (positive)	Abcam	Ab27314
CD 14	FITC (negative)	Abcam	Ab82434
CD 34	FITC (negative)	Biolegend	343504
CD 45	PE (negative)	Biolegend	304008
Negative Control (NC)	FITC	Santa Cruz	Sc-2855

3.4. COLONY FORMING UNIT ASSAY

Colony forming unit assay was done to test colony forming characteristics of MenSCs. For this purpose, MenSCs were seeded into 6-well plate at a clonal density of 236 cells/cm^2 in MenSCs media for 14 days with one media change per week. At 14^{th} day, cell colonies were rinsed with 1x dPBS, pH 7.4. and were stained with Crystal Violet in 20 (w/v) percent methanol for 30 minutes at room temperature. After the cell-wash with 1x dPBS, pH 7.4., wells were washed with distilled water twice and air dried. Images were captured using Zeiss SteReo Discovery V20 microscopy at 7.5x objective.

3.5. CELL PROLIFERATION ASSAY with WST-1

MenSCs proliferation was determined using WST-1. For experimental set up, standard plate with 2000, 5000, 10000, 15000, and 20000 MenSCs per well of a 96-well plate was prepared and incubated overnight in the mammalian incubator. Next day, 5 µL WST-1 reagent was mixed with 45 µL MenSCs media and placed into each well after removal of the old media. Standard curve plate was then incubated for 1 hour in the mammalian incubator and absorbance values were measured at 450/630 nm by Spectra Max Paradigm Multi-Mode Microplate Reader. For experimental set up, 2000 MenSCs per well were seeded into 96-well plate and incubated for 24, 48, 72, and 96 hours. After the completion of each incubation time point, 45 µL complete media and 5 µL WST-1 reagent was mixed and placed into each well as indicated above. Following the analysis of absorbance values (450/630 nm), viable cell number was calculated by extrapolating from the standard curve that is plotted by the mean absorbance (y axis) against the cell number (x axis) for standard curve plate.

3.6. STATISTICAL ANALYSIS

GraphPad Prism 9 program was used for all data analysis. Standard deviations were calculated with three independent replicates (means ± SD). In addition, data showing parametric distribution were analysed with t-test, while one-way ANOVA test was used for non-parametric data.

4. RESULTS

4.1. ISOLATION OF MENSTRUAL BLOOD MESENCHYMAL STEM CELLS WITH ISOLATION METHODS

With the approval of the local ethics committee, MenSCs were isolated from the menstrual blood collected from subjects with three different isolation methods as detailed in Section 3.1. The images of the isolated cells at the end of the Day2 were shown in Figure 4.1.

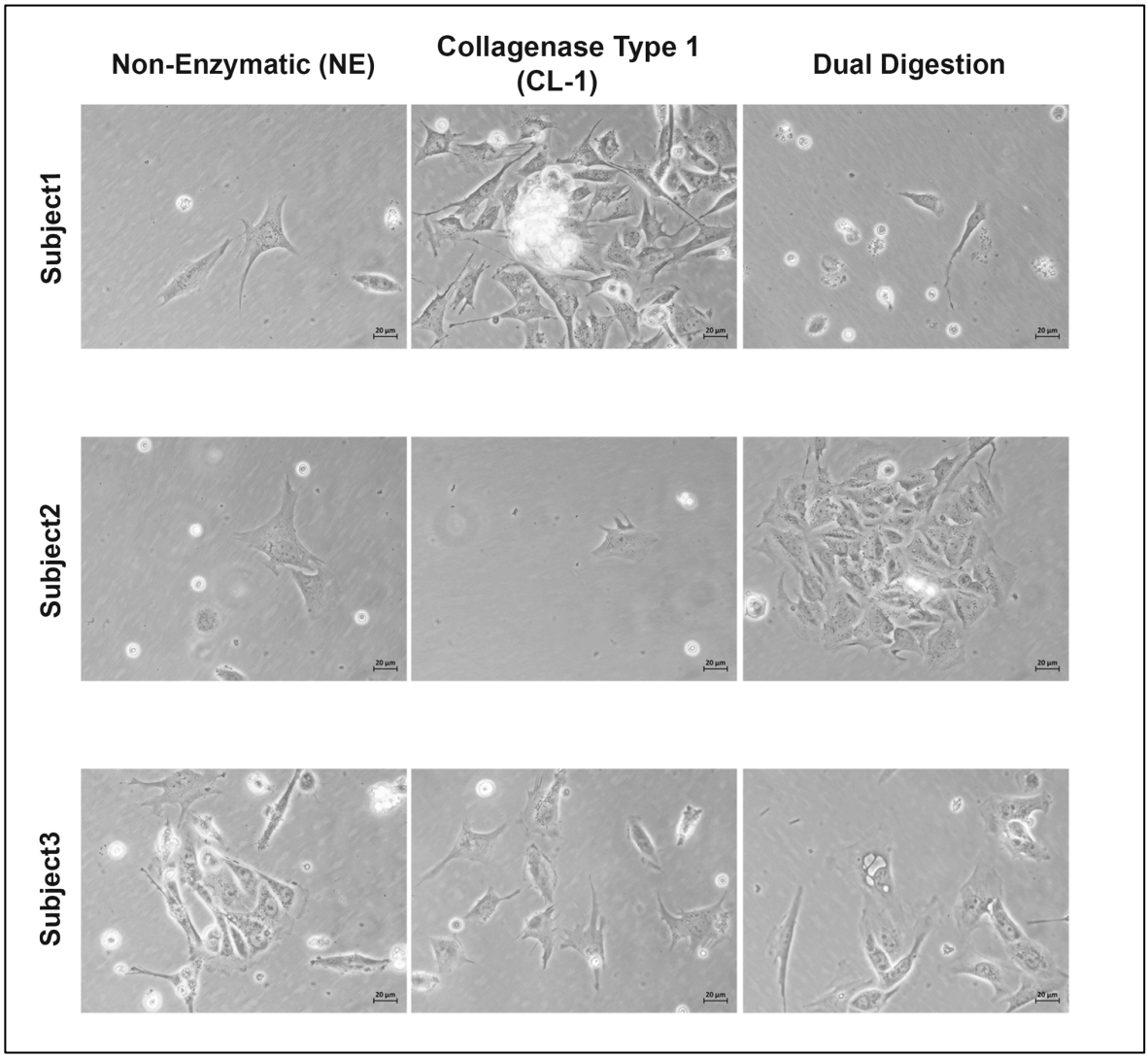


Figure 4.1. Representative images of MenSCs from three subjects with three isolation methods. Images were taken with a ZEISS phase-contrast microscope with a 40x objective. NE represents non-enzymatic, CL-1 represents collagenase type 1, and scale bar represents 20µm

At Day2 isolated MenSCs were attached to the plastic surface. MenSCs from Subject1, most cells were isolated with collagenase type 1 (CL-1) isolation method, MenSCs isolated with non-enzymatic (NE) isolation method had fewer erythrocytes in the cell culture than cells isolated with dual digestion method. MenSCs from Subject2, most cells isolated with dual digestion method. Although there were more erythrocytes in cell culture isolated with NE method than dual digestion method, more MenSCs were also isolated with NE isolation method. In Subject3, most MenSCs were isolated with NE and there were more erythrocytes in the cell medium compared to other isolation methods. In addition, when the CL-1 isolation method was compared with dual digestion method, it is observed that there were fewer erythrocytes in the cell medium isolated with dual digestion method. In addition to all these, all isolated cells show stem cell characteristics morphologically (Figure 4.1.).

4.2. COMPARISON OF STEMNESS CHARACTER MAINTENANCE AMONG DIFFERENT MenSCs ISOLATION METHODS

4.2.1. Analysis of Cell Surface Markers at Early and Late Passage MenSCs Isolated by Different Methods

In order to analyse the stem cell characterization of MenSCs which were morphologically display MSC characteristics (Figure 4.1.), flow cytometry analysis was performed (Section 3.3.). For flow cytometry analysis, MenSCs were subjected for the expression of antibodies. Passage 3 was chosen for early and passage 8 for late passage [103]. For this purpose, MenSCs at early passage were harvested and probed for CD29, CD44, CD73, CD90, CD105, PDGFR, W5C5, ITGβ1, CD14, CD34, and CD45 antibodies. The characterization of MenSCs isolated by NE, CL-1 and dual digestion methods were shown in Figure 4.2, 4.3 and 4.4 respectively.

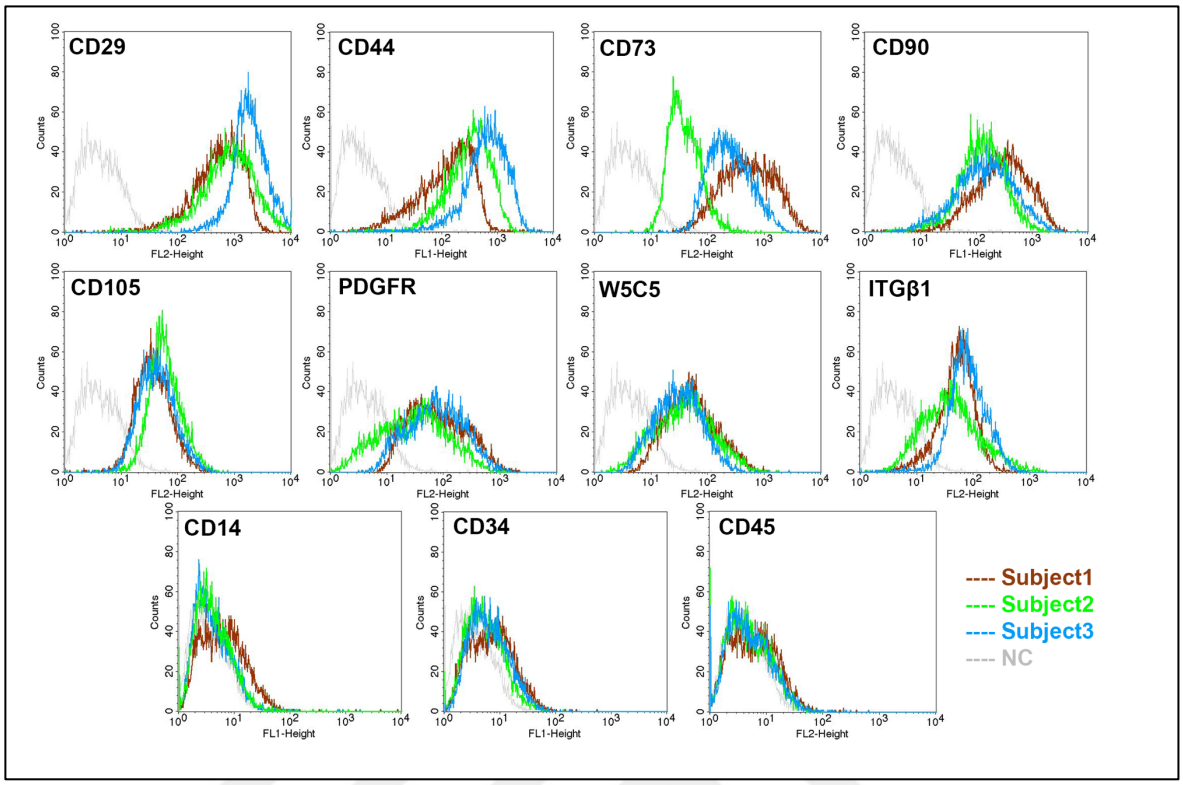


Figure 4.2. Characterization of MenSCs isolated via non-enzymatic isolation method at Ep_MenSCs by flow cytometry. $1x10^4$ cells were fed into the flow cytometry for each marker and cells were incubated with isotype IgG antibody as a negative control (NC) as presented with grey line. Ep_MenSCs from Subject1 was represented with red line, Subject2 was represented with green line and Subject3 was represented with blue line

Early passage MenSCs (Ep_MenSCs) for all three subjects isolated by NE isolation were tested positive for MSC markers CD29, CD44, CD73, CD90, CD105, PDGFR, W5C5, and ITGβ1 and negative for hematopoietic markers (Figure 4.2.) CD14, CD34, and CD45. Ep_MenSCs isolated from Subject1 with NE isolation method expressed 98.75 percent CD29, 85.01 percent CD44, 99.75 percent CD73, 97.52 percent CD90, 63.06 percent CD105, 77.57 percent PDGFR, 59.65 percent W5C5, 71.38 percent ITGβ1, 1.97 percent CD14, 2.38 percent CD34 and 1.59 percent CD45. Similarly, Ep_MenSCs isolated from Subject2 with NE isolation method expressed 99.61 percent CD29, 99.27 percent CD44, 72.13 percent CD73, 96.93 percent CD90, 93.44 percent CD105, 56.83 percent PDGFR, 64.96 percent W5C5, 65.63 percent ITGβ1, 1.28 percent CD14, 3.38 percent CD34 and 2.87 percent CD45. Likewise, Ep_MenSCs isolated from Subject3 with NE isolation method expressed 99.94 percent CD29, 99.73 percent CD44, 100.00 percent CD73, 96.95 percent

CD90, 95.37 percent CD105, 83.02 percent PDGFR, 78.88 percent W5C5, 99.74 percent ITGβ1, 3.35 percent CD14, 13.19 percent CD34 and 11.87 percent CD45 (Table A.1.).

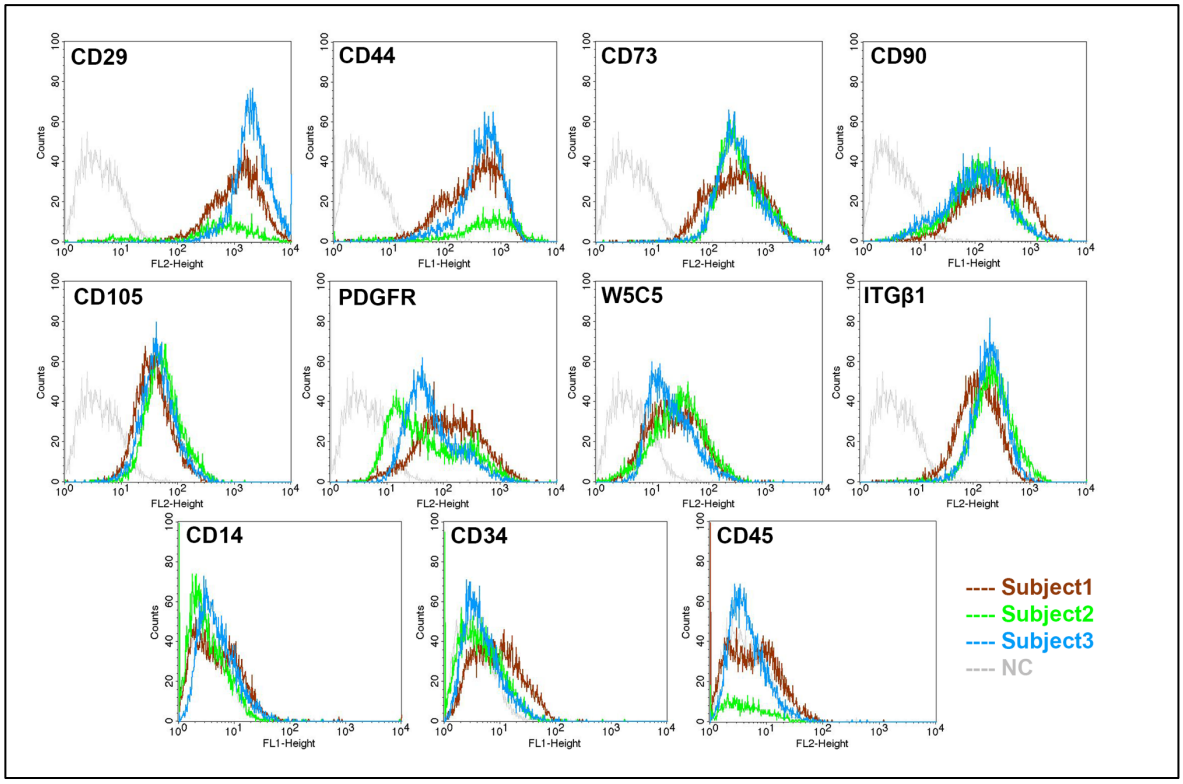


Figure 4.3. Characterization of MenSCs isolated via CL-1 isolation method at Ep_MenSCs by flow cytometry. $1x10^4$ cells were fed into the flow cytometry for each marker and cells were incubated with isotype IgG antibody as a negative control (NC) as presented with grey line. Ep_MenSCs from Subject1 was represented with red line, Subject2 was represented with green line and Subject3 was represented with blue line

Early passage MenSCs (Ep_MenSCs) for all three subjects isolated by CL-1 isolation were tested positive for MSC markers CD29, CD44, CD73, CD90, CD105, PDGFR, W5C5, and ITGβ1 and negative for hematopoietic markers (Figure 4.3.) CD14, CD34, and CD45. Ep_MenSCs isolated from Subject1 with CL-1 isolation method expressed 99.73 percent CD29, 98.00 percent CD44, 99.17 percent CD73, 96.29 percent CD90, 70.90 percent CD105, 90.64 percent PDGFR, 47.61 percent W5C5, 97.07 percent ITGβ1, 2.26 percent CD14, 13.25 percent CD34 and 5.54 percent CD45. Similarly, Ep_MenSCs isolated from Subject2 with CL-1 isolation method expressed 90.11 percent CD29, 95.54 percent CD44, 99.89 percent CD73, 93.83 percent CD90, 97.01 percent CD105, 68.31 percent PDGFR,

56.61 percent W5C5, 99.45 percent ITGβ1, 1.91 percent CD14, 6.12 percent CD34 and 1.32 percent CD45. Likewise, Ep_MenSCs isolated from Subject3 with CL-1 isolation method expressed 99.94 percent CD29, 99.37 percent CD44, 99.55 percent CD73, 85.41 percent CD90, 75.03 percent CD105, 74.24 percent PDGFR, 30.30 percent W5C5, 99.61 percent ITGβ1, 2.13 percent CD14, 2.54 percent CD34 and 1.81 percent CD45 (Table A.2.).

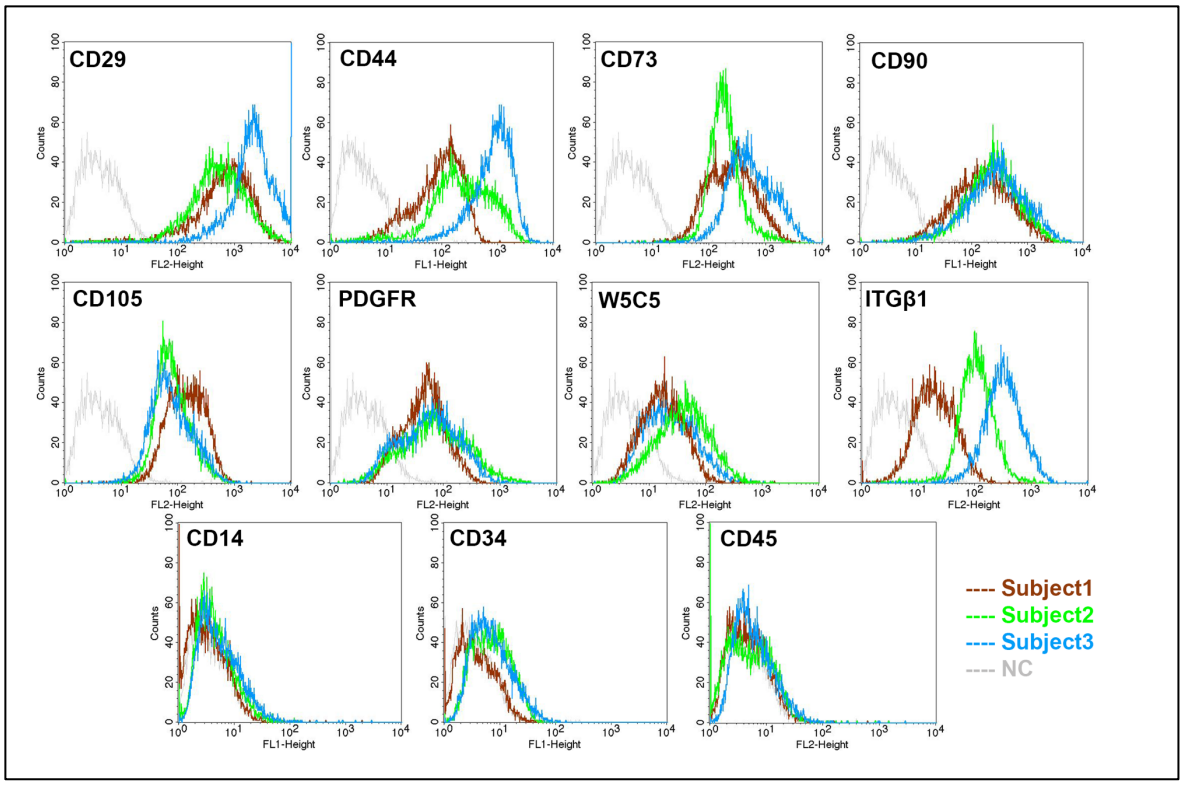


Figure 4.4. Characterization of MenSCs isolated via dual digestion isolation method at Ep_MenSCs by flow cytometry. $1x10^4$ cells were fed into the flow cytometry for each marker and cells were incubated with isotype IgG antibody as a negative control (NC) as presented with grey line. Ep_MenSCs from Subject1 was represented with red line, Subject2 was represented with green line and Subject3 was represented with blue line

Early passage MenSCs (Ep_MenSCs) for all three subjects isolated by dual digestion method were tested positive for MSC markers CD29, CD44, CD73, CD90, CD105, PDGFR, W5C5, and ITGβ1and negative for hematopoietic markers (Figure 4.4.) CD14, CD34, and CD45. Ep_MenSCs isolated from Subject1 with dual digestion method expressed 98.48 percent CD29, 92.56 percent CD44, 99.66 percent CD73, 89.84 percent CD90, 99.50 percent CD105, 79.74 percent PDGFR, 30.70 percent W5C5, 98.64 percent ITGβ1, 1.75 percent

CD14, 2.34 percent CD34 and 2.39 percent CD45. Similarly, Ep_MenSCs isolated from Subject2 with dual digestion method expressed 98.90 percent CD29, 95.26 percent CD44, 99.83 percent CD73, 97.80 percent CD90, 97.33 percent CD105, 73.80 percent PDGFR, 60.32 percent W5C5, 99.04 percent ITGβ1, 1.95 percent CD14, 1.16 percent CD34 and 1.44 percent CD45. Likewise, Ep_MenSCs isolated from Subject3 with dual digestion method expressed 99.65 percent CD29, 99.52 percent CD44, 99.83 percent CD73, 99.64 percent CD90, 89.70 percent CD105, 78.08 percent PDGFR, 39.17 percent W5C5, 99.74 percent ITGβ1, 2.66 percent CD14, 5.06 percent CD34 and 2.84 percent CD45 (Table A.3.).

For the characterization of MenSCs isolated by NE, CL- 1 and dual digestion methods at late passage MenSCs (Lp_MenSCs) flow cytometry results were shown in Figure 4.5, 4.6 and 4.7 respectively.

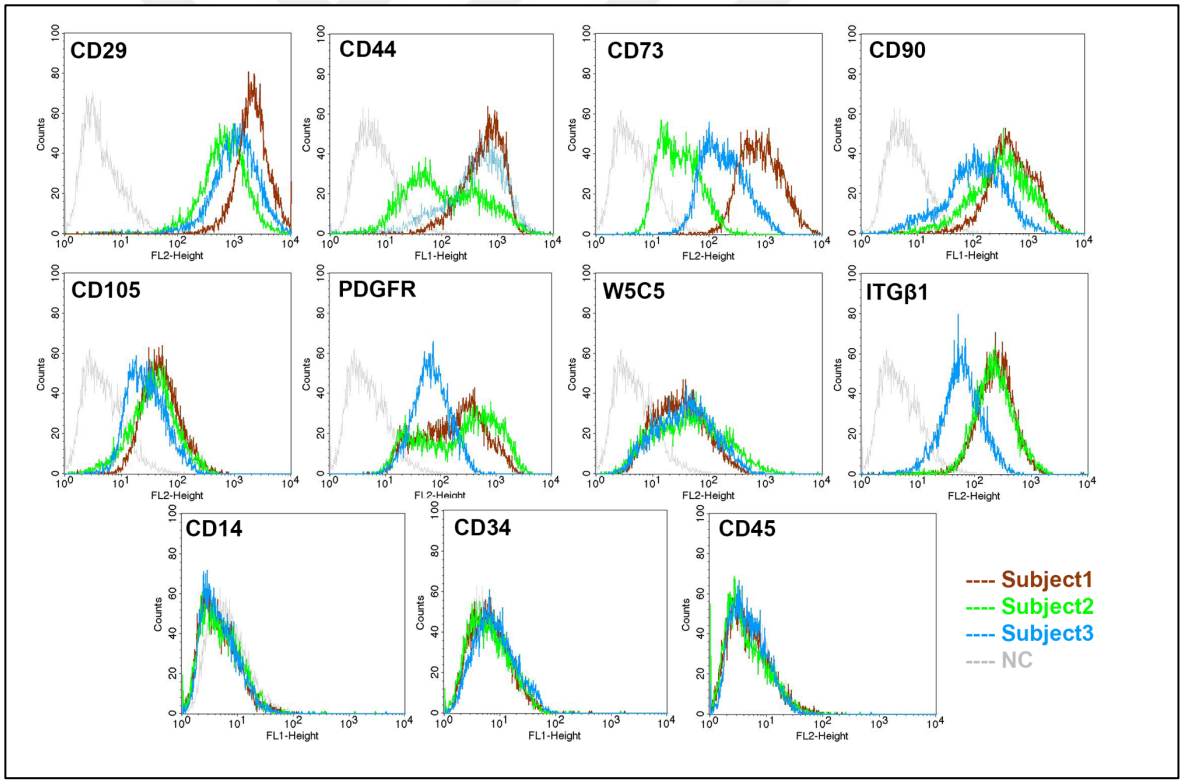


Figure 4.5. Characterization of MenSCs isolated via NE isolation method at Lp_MenSCs by flow cytometry. 1x10^4 cells were fed into the flow cytometry for each marker and cells were incubated with isotype IgG antibody as a negative control (NC) as presented with grey line. Ep_MenSCs from Subject1 was represented with red line, Subject2 was represented with green line and Subject3 was represented with blue line

Late passage MenSCs (Lp_MenSCs) for all three subjects isolated by NE isolation method were tested positive for MSC markers CD29, CD44, CD73, CD90, CD105, PDGFR, W5C5, and ITGβ1 and negative for hematopoietic markers (Figure 4.5.) CD14, CD34, and CD45. Lp_MenSCs isolated from Subject1 with NE isolation method expressed 99.90 percent CD29, 99.83 percent CD44, 99.96 percent CD73, 99.96 percent CD90, 88.98 percent CD105, 93.37 percent PDGFR, 63.96 percent W5C5, 99.71 percent ITGβ1, 2.82 percent CD14, 7.22 percent CD34 and 4.53 percent CD45. Similarly, Lp_MenSCs isolated from Subject2 with NE isolation method expressed 99.54 percent CD29, 85.58 percent CD44, 44.72 percent CD73, 99.71 percent CD90, 69.23 percent CD105, 81.72 percent PDGFR, 64.34 percent W5C5, 86.60 percent ITGβ1, 1.97 percent CD14, 7.22 percent CD34 and 3.71percent CD45. Likewise, Lp_MenSCs isolated from Subject3 with NE isolation method expressed 99.67 percent CD29, 97.72 percent CD44, 99.73 percent CD73, 89.78 percent CD90, 54.68 percent CD105, 93.15 percent PDGFR, 65.35 percent W5C5, 86.60 percent ITGβ1, 1.55 percent CD14, 3.56 percent CD34 and 1.97 percent CD45 (Table A.4.).

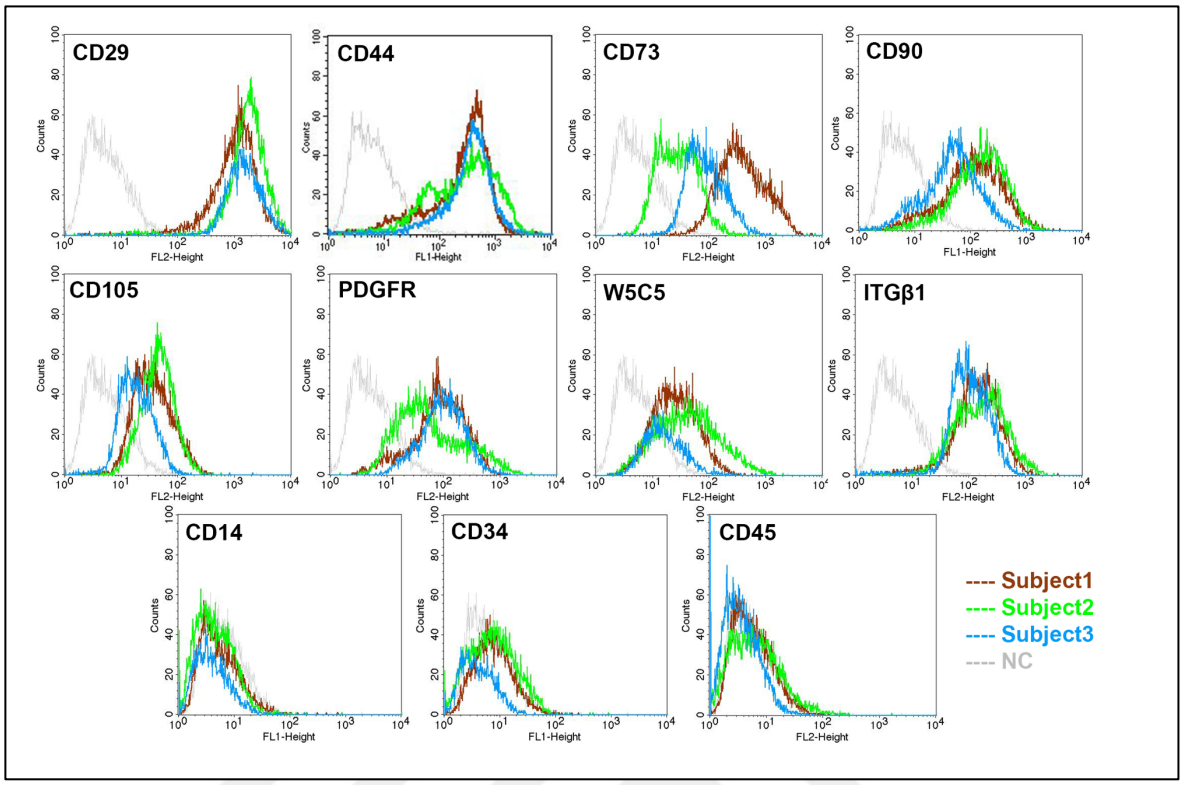


Figure 4.6. Characterization of MenSCs isolated via CL-1 isolation method at Lp_MenSCs by flow cytometry. $1x10^4$ cells were fed into the flow cytometry for each marker and cells were incubated with isotype IgG antibody as a negative control (NC) as presented with grey line. Ep_MenSCs from Subject1 was represented with red line, Subject2 was represented with green line and Subject3 was represented with blue line

Late passage MenSCs (Lp_MenSCs) for all three subjects isolated by CL-1 isolation method were tested positive for MSC markers CD29, CD44, CD73, CD90, CD105, PDGFR, W5C5, and ITGβ1 and negative for hematopoietic markers (Figure 4.6.) CD14, CD34, and CD45. Lp_MenSCs isolated from Subject1 with CL-1 isolation method expressed 99.48 percent CD29, 93.34 percent CD44, 99.71 percent CD73, 99.94 percent CD90, 61.20 percent CD105, 85.79 percent PDGFR, 45.49 percent W5C5, 95,89 percent ITGβ1, 3.32 percent CD14, 6.08 percent CD34 and 3.70 percent CD45. Similarly, Lp_MenSCs isolated from Subject2 with Cl-1 isolation method expressed 99.31 percent CD29, 97.63 percent CD44, 54.47 percent CD73, 95.39 percent CD90, 87.22 percent CD105, 74.62 percent PDGFR, 68.80 percent W5C5, 98.19 percent ITGβ1, 1.20 percent CD14, 11.46 percent CD34 and 8.02 percent CD45. Likewise, Lp_MenSCs isolated from Subject3 with CL-1 isolation method expressed 99.63 percent CD29, 99.31 percent CD44, 99.35 percent CD73, 83.90

percent CD90, 49.27 percent CD105, 96.80 percent PDGFR, 39.42 percent W5C5, 98.21 percent ITGβ1, 2.13 percent CD14, 1.31 percent CD34 and 1.31 percent CD45 (Table A.5.).

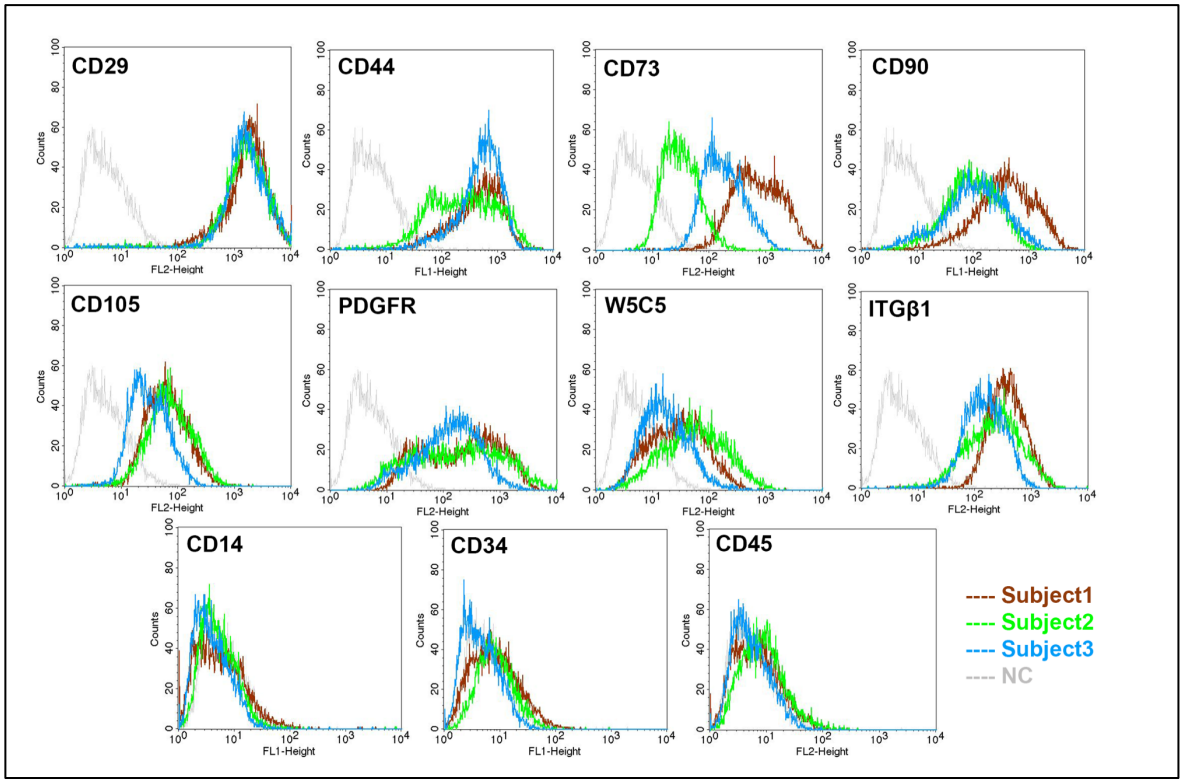


Figure 4.7. Characterization of MenSCs isolated via dual digestion isolation method at Lp_MenSCs by flow cytometry. $1x10^4$ cells were fed into the flow cytometry for each marker and cells were incubated with isotype IgG antibody as a negative control (NC) as presented with grey line. Ep_MenSCs from Subject1 was represented with red line, Subject2 was represented with green line and Subject3 was represented with blue line

Late passage MenSCs (Lp_MenSCs) for all three subjects isolated by dual digestion method were tested positive for MSC markers CD29, CD44, CD73, CD90, CD105, PDGFR, W5C5, and ITGβ1 and negative for hematopoietic markers (Figure 4.6.) CD14, CD34, and CD45. Lp_MenSCs isolated from Subject1 with dual digestion method expressed 99.58 percent CD29, 97.59 percent CD44, 99.77 percent CD73, 96.03 percent CD90, 63.43 percent CD105, 78.97 percent PDGFR, 34.27 percent W5C5, 99.60 percent ITGβ1, 1.84 percent CD14, 3.62 percent CD34 and 2.24 percent CD45. Similarly, Lp_MenSCs isolated from Subject2 with dual digestion method expressed 99.23 percent CD29, 96.67 percent CD44, 44.58 percent CD73, 92.05 percent CD90, 89.26 percent CD105, 83.76 percent PDGFR,

72.10 percent W5C5, 98.06 percent ITGβ1, 1.57 percent CD14, 6.01 percent CD34 and 8.97 percent CD45 Likewise, Lp_MenSCs isolated from Subject3 with dual digestion method expressed 99.10 percent CD29, 99.58 percent CD44, 99.75 percent CD73, 90.77 percent CD90, 56.32 percent CD105, 88.99 percent PDGFR, 30.57 percent W5C5, 98.96 percent ITGβ1, 1.89 percent CD14, 3.36 percent CD34 and 2.42 percent CD45 (Table A.6.).

Table 4.1. Average percentage of stem cell markers of Ep_MenSCs isolated from three different subjects with three isolation methods. NE represents non-enzymatic isolation method and CL-1 represents collagenase type 1 isolation method

Ep_MenSCs	NE	CL-1	Dual Digestion	p Value
CD29	99.43 ± 0.01	96.59 ± 0.06	99.01 ± 0.01	ns
CD44	94.67 ± 0.08	97.64 ± 0.02	95.78 ± 0.04	ns
CD73	90.63 ± 0.16	99.54 ± 0.00	99.77 ± 0.00	ns
CD90	97.13 ± 0.00	91.84 ± 0.06	95.76 ± 0.05	ns
CD105	83.96 ± 0.18	80.98 ± 0.14	95.51 ± 0.05	ns
PDGFR	72.47 ± 0.14	77.73 ± 0.12	77.21 ± 0.03	ns
W5C5	67.83 ± 0.10	44.84 ± 0.13	46.40 ± 0.12	ns
ITGβ1	78.92 ± 0.18	98.71 ± 0.01	99.14 ± 0.01	ns
CD14	2.20 ± 0.01	2.10 ± 0.00	2.12 ± 0.00	ns
CD34	6.32 ± 0.06	7.30 ± 0.05	2.88 ± 0.02	ns
CD45	5.44 ± 0.06	2.89 ± 0.02	2.22 ± 0.01	ns

The mean percentage values for stem cells markers of MenSCs isolated from three subjects by three isolation methods at Ep_MenSCs were represented in Table 4.1. and standard deviations of three different subjects were shown with ±. Results were analysed in GraphPad Prism9 software program by one-way ANOVA multiple comparisons. According to Table 4.1., it was observed that Ep_MenSCs preserved their stem cell characteristics in all three isolation methods. In addition, when each surface marker was compared in terms of isolation methods, no difference was observed between isolation methods.

Table 4.2 Average percentage of stem cell markers of MenSCs isolated from three subjects with three isolation methods at Lp_MenSCs. NE represents non-enzymatic isolation method and CL-1 represents collagenase type 1 isolation method

Lp_MenSCs	NE	CL-1	Dual Digestion	p Value
CD29	99.70 ± 0.00	99.47 ± 0.00	99.30 ± 0.00	ns
CD44	94.38 ± 0.08	96.76 ± 0.03	97.95 ± 0.01	ns
CD73	81.47 ± 0.32	84.51 ± 0.26	81.37 ± 0.32	ns
CD90	95.76 ± 0.05	92.74 ± 0.08	92.95 ± 0.03	ns
CD105	70.96 ± 0.17	65.90 ± 0.19	69.67 ± 0.17	ns
PDGFR	89.41 ± 0.07	85.74 ± 0.11	83.91 ± 0.05	ns
W5C5	64.55 ± 0.01	51.24 ± 0.16	45.65 ± 0.23	ns
ITGβ1	91.82 ± 0.07	97.40 ± 0.01	98.87 ± 0.01	ns
CD14	2.11 ± 0.01	2.22 ± 0.01	1.77 ± 0.00	ns
CD34	5.68 ± 0.02	6.28 ± 0.05	4.33 ± 0.01	ns
CD45	3.4 ± 0.01	4.34 ± 0.03	4.54 ± 0.04	ns

The mean percentage values for stem cells markers of MenSCs isolated from three subjects by three isolation methods at Lp_MenSCs were represented in Table 4.2 and standard deviations of three different cells samples with isolation methods were shown with ±. Results were analysed in GraphPad Prism9 software program by one-way ANOVA multiple comparisons. According to Table 4.2, it was observed that Lp_MenSCs, the percentage values for Lp_MenSCs decreased relative to Ep_MenSCs as expected, preserved their stem cell characteristics in all three isolation methods. In addition, when each surface marker was compared in terms of isolation methods, no difference was observed between isolation methods at Lp_MenSCs.

4.2.2. Determination of Colony Forming Unit for Menstrual Blood Mesenchymal Stem Cells

Colony forming unit (CFU) of MenSCs was tested as described in Section 3.4. Since the potential of CFU is one of the characteristic features of mesenchymal stem cells, the colony-forming unit properties of MenSCs isolated from the subjects by three different methods were examined at early and late passages. For this purpose, MenSCs seeded in 6-well plates at 118 cells/mL were allowed to colonize for 14 days in their optimum growth medium. At

the end of 14 days, colonies were captured and cell number in each colony were counted, and colony size were determined using the open-source software Fiji program (Figure 4.8 and 4.9). Colony formation efficiency (CFU-E) was calculated by dividing the total number of colonies by the total number of seeded cells.

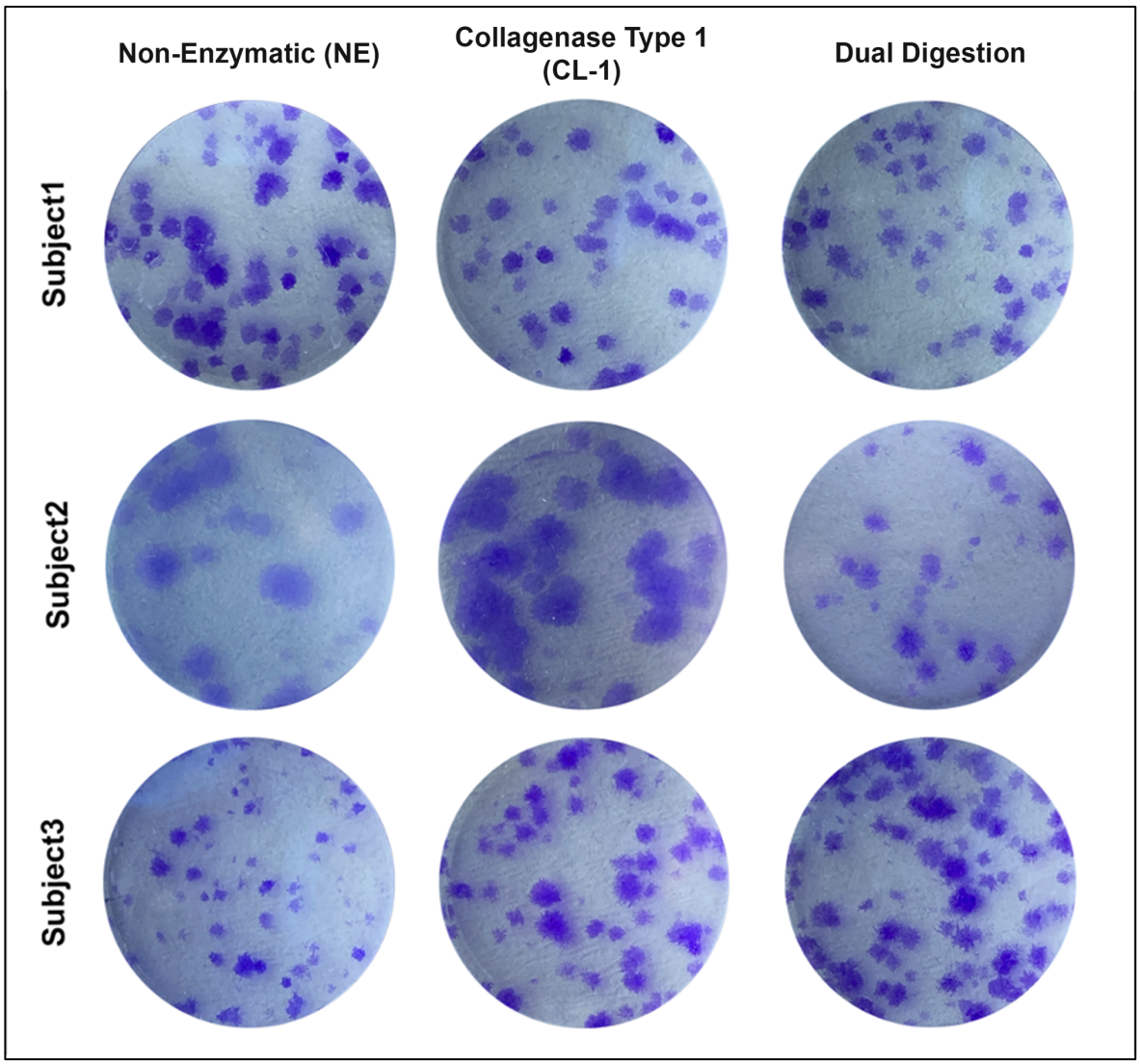


Figure 4.8. Colony formation capacity of MenSCs. Photographs of MenSCs' from three subjects with three isolation methods at Ep_MenSCs were taken at the end of day 14

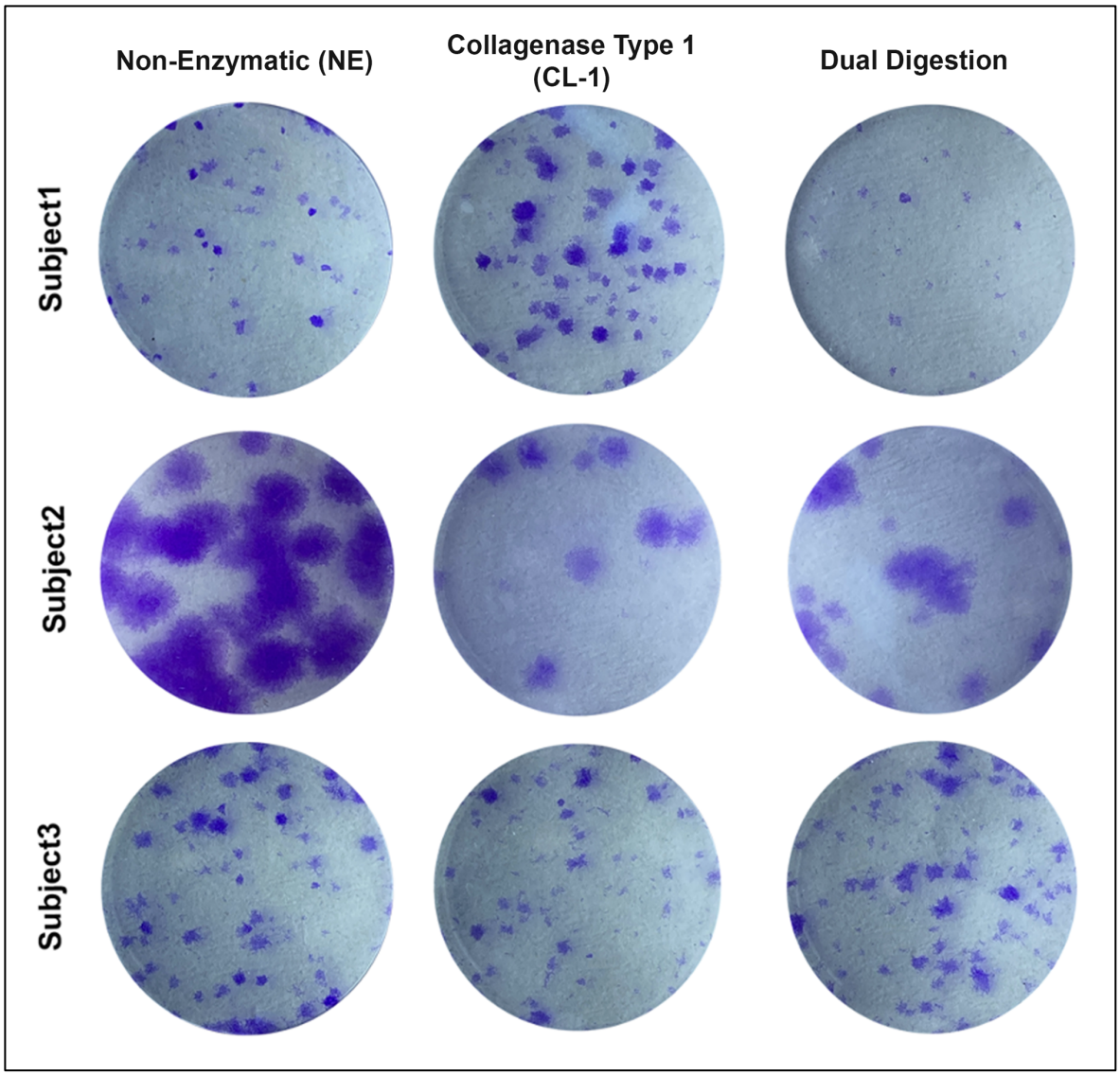


Figure 4.9. Colony formation capacity of MenSCs. Photographs of MenSCs' from three subjects with three isolation methods at Lp_MenSCs were taken at the end of day 14

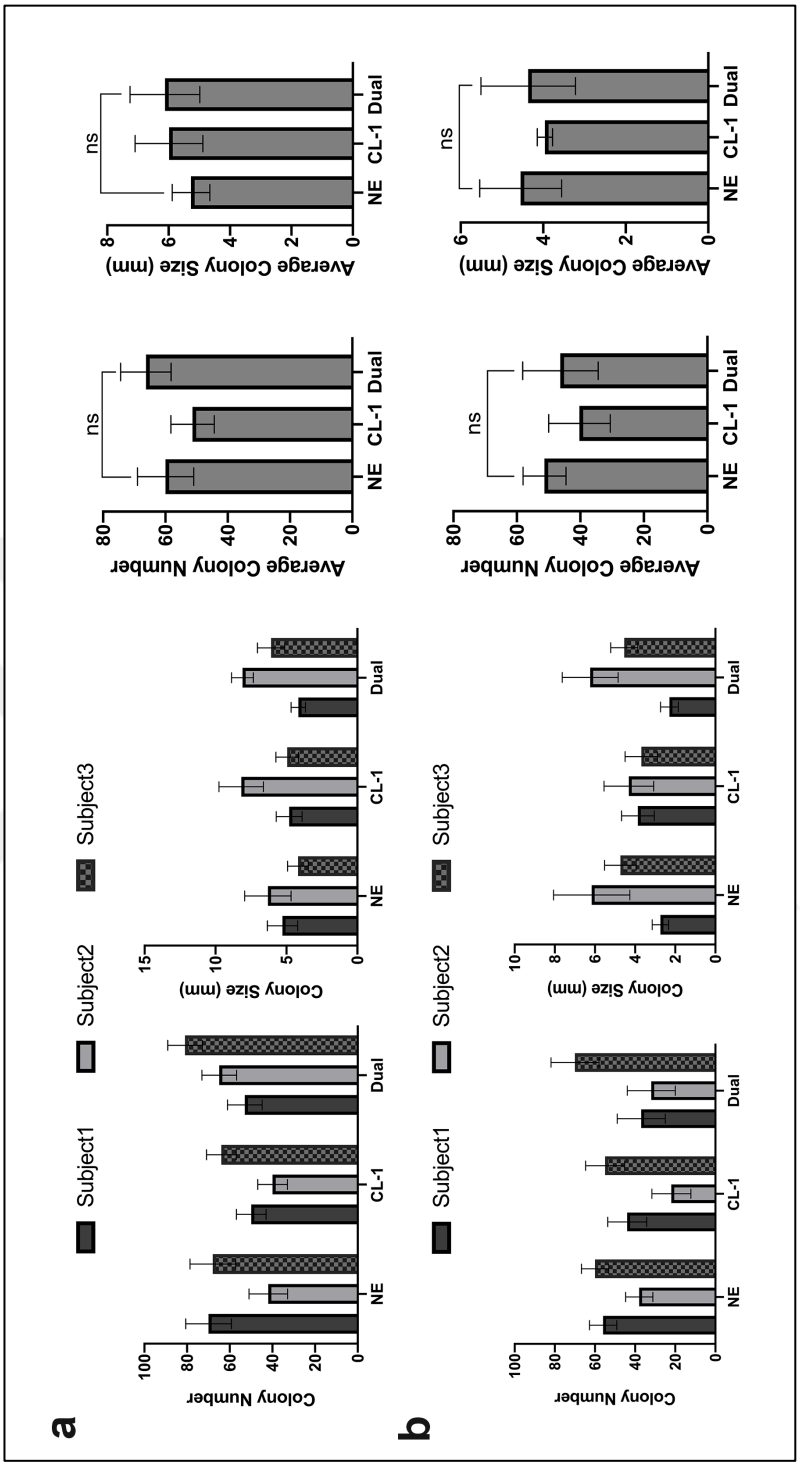


Figure 4.10. Determination of colony number and size in early and late passage MenSCs isolated by NE, CL-1, and dual digestion. (a) Analysis of colony number ability of Ep_MenSCs, analysis of colony size (mm), and average MenSCs obtained by NE, CL-1 and dual digestion isolation methods. (b) Analysis of colony number ability of Lp_MenSCs, analysis of colony size (mm), and average MenSCs according to isolation methods. Prism 9 one-way ANOVA multiple comparison program was used in statistical analysis

Comparison of colony number recorded for Ep_MenSCs of Subject1 among different isolation methods showed that 70 colonies for NE method, 50 colonies for CL-1 method and 53 colonies for dual digestion method was formed. Average sizes of these colonies were analysed respectively as 5.3 mm, 4.8 mm, and 4.2 mm for NE, CL-1, and dual digestion. Evaluation of colony formation efficiency (CFU-E) of Ep_MenSCs (Table 4.3.), demonstrated that CFU-E for NE was the highest with 29.7, while CL-1 and dual digestion methods CFU-E was found to be 21.2 and 22.5 respectively. For Lp_MenSCs of Subject 1, while colony numbers were calculated as 56, 44, and 37, CFU-E were evaluated as 23.7, 18.6 and 15.7 and sizes of these colonies were analysed as 2.7 mm, 3.9 mm and 2.3 mm for NE, CL-1, and dual digestion, respectively. These results suggested that use of NE method for isolation of MenSCs provided significantly higher colony number, CFU-E, and size for Subject1. While comparison between early and late passages showed that both colony number and size decreased by passaging regardless of the isolation type.

Table 4.3. Efficiency of colony forming unit according to isolation methods at early and late passages

		Subject 1	**Subject 2**	**Subject 3**	**Average**	**p Value**
Ep_MenSCs	**NE**	29.7	17.8	28.8	25.4	ns
	CL-1	21.2	16.9	27.1	21.8	
	Dual Digestion	22.5	27.5	34.3	28.1	
Lp_MenSCs	**NE**	23.7	16.1	25.4	21.8	ns
	CL-1	18.6	9.3	23.3	17.1	
	Dual Digestion	15.7	13.6	29.7	19.6	

Comparison of colony number recorded for Ep_MenSCs of Subject2 among different isolation methods showed that 42 colonies for NE method, 40 colonies for CL-1 method and 65 colonies for dual digestion method was formed. Average sizes of these colonies were analysed respectively as 6.3 mm, 8.2 mm, and 8.1 mm for NE, CL-1, and dual digestion. CFU-E of Ep_MenSCs demonstrated that CFU-E for dual digestion was the highest with 27.5, while NE and CL-1 methods CFU-E was found to be 17.8 and 16.9 respectively (Table 4.3.). For Lp_MenSCs of Subject2, while colony numbers were calculated as 38, 22, and 32, CFU-E were evaluated as 16.1, 9.3 and 13.6 and sizes of these colonies were analysed as 6.2 mm, 4.3 mm and 6.3 mm for NE, CL-1, and dual digestion, respectively. These results

suggested that use of dual digestion method for isolation of MenSCs provided significantly higher colony number, CFU-E, and size for Subject 2. While comparison between early and late passages showed that both colony number and size decreased by passaging regardless of the isolation type.

Comparison of colony number recorded for Ep_MenSCs for Subject3 among different isolation methods showed that 68 colonies for NE method, 64 colonies for CL-1 method and 81 colonies for dual digestion method was formed. Average sizes of these colonies were analysed respectively as 4.2 mm, 5.0 mm, and 6.1 mm for NE, CL-1, and dual digestion methods. CFU-E of Ep_MenSCs demonstrated that CFU-E for dual digestion was the highest with 34.3, while NE and CL-1 methods CFU-E was found to be 28.8 and 27.1 respectively. For Lp_MenSCs of Subject3, while colony numbers were calculated as 60, 55, 70, CFU-E were evaluated as 25.4, 23.3, and 29.7 and sized of these colonies were analysed as 4.7 mm, 3.7 mm, and 4.5 mm for NE, CL-1, dual digestion, respectively (Table 4.3.). These results suggested that use of dual digestion method for isolation of MenSCs provided significantly higher colony number, CFU-E, and size for Subject3. While comparison between early and late passages showed that both colony number and size by passaging regardless of the isolation type.

Table 4.4. Colony size (mm) according to isolation methods at early and late passages

		Subject 1	**Subject 2**	**Subject 3**	**Average**	**p Value**
Ep_MenSCs	**NE**	5.3	6.3	4.2	5.3	ns
	CL-1	4.8	8.2	5.0	6.0	
	Dual Digestion	4.2	8.1	6.1	6.1	
Lp_MenSCs	**NE**	2.7	6.2	4.7	4.5	ns
	CL-1	3.9	4.3	3.7	4.0	
	Dual Digestion	2.3	6.3	4.5	4.3	

When the average colony number and colony size (Table 4.4.) at early passage were compared in terms of isolation methods, it was seen that 60 colonies for NE method, 51 colonies for CL-1 method and 66 colonies for dual digestion method was formed (Figure 4.10.). When average sizes of these colonies were analysed 5.3 mm, 6.0 mm, and 6.1 mm for NE, CL-1, and dual digestion was respectively determined. While colony number of Lp_MenSCs were determined as 51, 40, and 46, their colony sizes were found as 4.5 mm,

4.0 mm, and 4.3 mm for NE, CL-1, and dual digestion, respectively. Average CFU-E of Ep_MenSCs demonstrated that CFU-E for dual digestion was the highest with 28.1, while NE and CL-1 methods CFU-E was found to be 25.4 and 21.8, respectively. Likewise, average CFU-E of Lp_MenSCs demonstrated that CFU-E for NE method was the highest with 21.8, while CL-1 and dual digestion methods CFU-E was found to be 17.1 and 19.6, respectively (Table 4.3.). When comparison was made about CFU-E and colony size for the maintenance of stemness, it was found that there were no significant changes in neither isolation methods nor Ep_MenSCs and Lp_MenSCs when one-way ANOVA multiple comparison was done. Likewise, when Ep_MenSCs and Lp_MenSCs were compared with each other in terms of colony number, no significant difference was found in isolation methods, when t-test was applied. Lastly, according to colony number for each isolation method, fold change was determined, and it was found that there were as 14.4, 21.4, and 30.2-fold increase for NE, CL-1, and dual digestion isolation methods, respectively.

4.2.3. Characterization of Cell Proliferation in MenSCs via WST-1

In order to determine the cell proliferation potential of MenSCs isolated from three subjects by NE, CL-1 and dual digestion methods, WST-1 method was performed as mentioned in Section 3.5 for 24, 48, 72 and 96 hours (Figure 4.11.). To convert the absorbance values to be cell number; 2000, 5000, 10000, 15000 and 20000 cells were seeded per well to construct standard curve. The cell proliferation rate for Ep_MenSCs and Lp_MenSCs was measured according to the isolation methods from the linear equation obtained from the absorbance values of the cells seeded in standard plate.

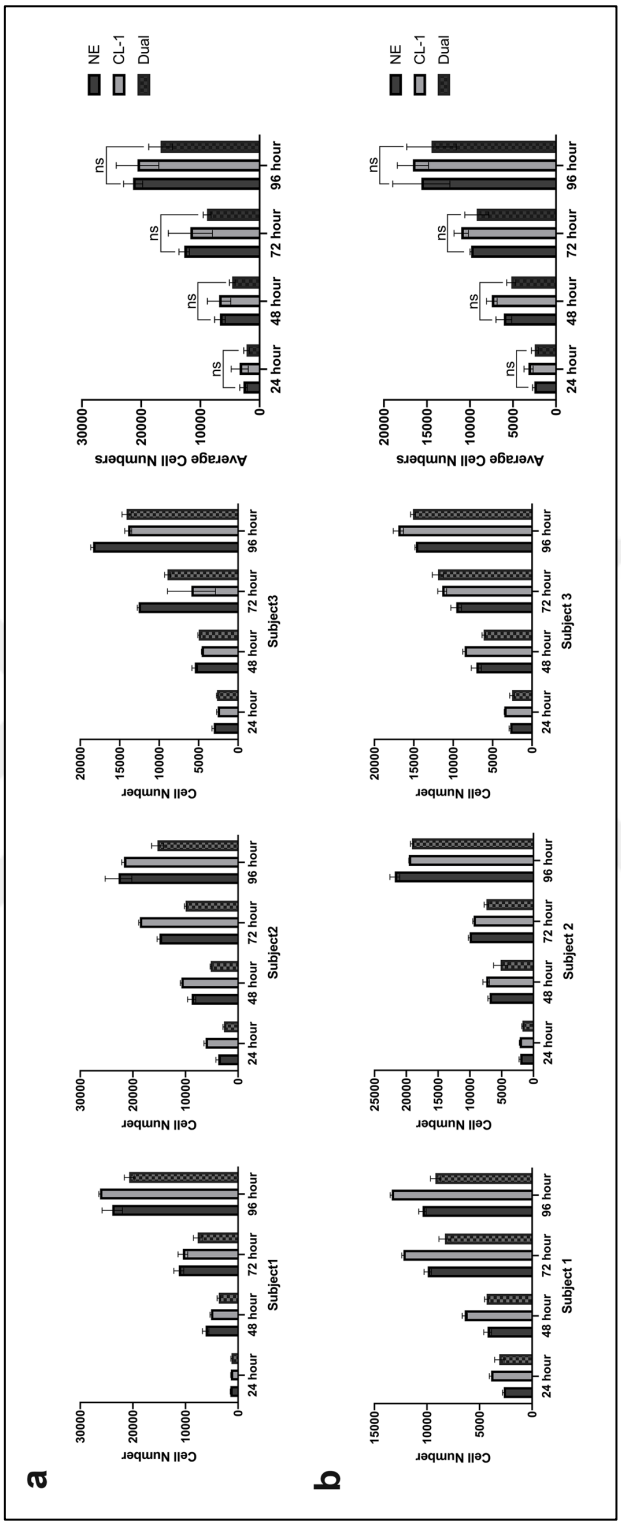


Figure 4.11. Evaluation of proliferation rate for Ep_MenSCs and Lp_MenSCs. (a) Analysis of cell numbers for each subject's Ep_MenSCs (a) and Lp_MenSCs (b) isolated using NE, CL-1, and dual digestion isolation methods. Measurements were taken at 24, 48, 72 and 96 hours. Average cell numbers for both passages were presented on the far-right graphs. Prism 9 one-way ANOVA multiple comparison program was used in statistical analysis

Cell proliferation rate for Ep_MenSCs from Subject1 was determined via WST-1 reagent at 24, 48, 72, and 96 hours (Figure 4.11.a.). When cell growth kinetics obtained for Ep_MenSCs isolated by three different methods were compared with each other, it was seen that although in all isolation methods the number of cells increased as time progressed, the isolation method that reached the highest number of cells after 96 hours was the CL-1 isolation method. When cell proliferations of Lp_MenSCs from Subject1 with three isolation methods were determined (Figure 4.11.b.), an increase in cell number was observed as time passed, and the highest number of MenSCs was observed in cells isolated by CL-1 isolation method.

As with cell proliferation rate determination for Subject1 was applied for Subject2, too. When, isolation methods at Ep_MenSCs were compared with each other, it was observed that as time progressed, cell numbers increased, and the highest cell number was observed at NE isolation method (Figure 4.11.a). As with Ep_MenSCs, cell proliferations rate of Lp_MenSCs from Subject2 was determined (Figure 4.11.b). Like Ep_MenSCs from Subject2, cell number was increased as time progressed at Lp_MenSCs from Subject2, and the highest number of MenSCs was observed in NE isolation method.

Lastly, cell proliferation rate was determined for Subject3 with WST-1 reagent at certain time points. When, isolation methods at Ep_MenSCs were compared with each other, it was observed that as time progressed, cell numbers increased, and the highest cell number was observed at NE isolation method for Ep_MenSCs (Figure 4.1.a.). Cell proliferation rate of Lp_MenSCs from Subject3 was determined as with Ep_MenSCs (Figure 4.11.b.). It was observed that cell number was increased as time progress like Ep_MenSCs. However, the highest cell number was observed at CL-1 isolation method for Lp_MenSCs, unlike Ep_MenSCs.

When the average cell proliferation rates of MenSCs were compared in terms of isolation methods at Ep_MenSCs, it was observed that cell numbers were increased as time progressed, MenSCs populations isolated via three isolation methods have similar proliferation rates at 24 hours as shown in Figure 4.11.a., and the highest cell proliferation rate was found in NE isolation method. Moreover, at the end of 48 hours, it was observed that there were 2.5-, 2.0-, and 2.1-fold increase in doubling time for NE, CL-1, and dual digestion isolation methods, respectively. Likewise, while there were 1.2-, 1.7-, and 1.9-fold increase at 72 hours, 1.7-, 1.8-, and 1.9-fold increase were observed at 96 hours for NE, CL-

1, and dual digestion isolation methods, respectively. On the other hand, when cell proliferation rate of Lp_MenSCs was analysed in terms of isolation methods, it was observed that there were similar proliferation rates between isolation methods at 24 hours (Figure 4.11.b.), and the highest cell proliferation rate was found in CL-1 isolation method. Likewise, while there were 2.4-, 2.3-, 2.1-fold increase at 48 hours,1.6-, 1.5-, and 1.8-fold increase at 72 hours, 1.6-, 1.5-, and 1.5-fold increase were observed at 96 hours for NE, CL-1, and dual digestion isolation methods, respectively. When Ep_MenSCs and Lp_MenSCs were compared in terms of proliferation rate, there were 1.1, 1.1, 1.3, and 1.4-fold increase at 24, 48, 72, and 96 hours for NE isolation method, respectively. Similarly, when proliferation rates of Ep_MenSCs and Lp_MenSCs were compared with each other, 1.1, 1.1, 1.1, and 1.2-fold increase was observed at 24, 48, 72, and 96 hours for CL-1 isolation method, respectively. Lastly, 1.1, 1.1, 1.0, and 1.2-fold increase were observed for dual digestion isolation method at 24, 48, 72, and 96 hours, respectively, when Ep_MenSCs, and Lp_MenSCs were compared in terms of proliferation rate. However, when isolation methods were compared with each other at early and late passage, a significant increasement ($p<0.05$) was found only at 72 hours for MenSCs isolated via NE isolation method. Likewise, no significant difference was detected between the isolation methods in the early and late transitions at other isolation methods and time points. When doubling time of Ep_MenSCs was calculated, 21.6 hour for NE isolation method, 22.2 hour for CL-1 isolation method and 31.2 hour for dual digestion isolation method was determined. Similarly, doubling time of Lp_MenSCs were found as 26.1 hour for NE isolation method, 21.5 hour for CL-1 isolation method, and 27.3 hour for dual digestion isolation method respectively. No significant change in neither isolation methods nor Ep_MenSCs and Lp_MenSCs was seen when one-way ANOVA multiple comparison was done.

5. DISCUSSION

With the presence of mesenchymal stem cells in the bone marrow, studies on MSCs have increased and its properties have been revealed [39]. With the properties of MSCs, interest in these cells has increased and different stem cell sources have begun to be investigated. Although stem cells were isolated from different sources and named differently, all of them categorized as MSCs. These features are i) adherence to plastic and able to form colonies when maintained under standard cell conditions, ii) expression of CD73, CD90, and CD105 surface markers, without CD11b, CD14, CD19, CD34, CD45, and CD79a surface marker expression, and iii) *in vitro* differentiation into adipocytes, chondrocytes, and osteoblasts [41]. Research on different sources of MSCs revealed a heterogeneous cell population with the differentiation ability into tissues such as epithelial, endothelial, and neural. Furthermore, MSCs have been crucial for clinical studies after it was noted that MSCs had the ability to inhibit immune responses [47]. Since high number of MSCs is required for clinical studies, new sources have been sought.

After the discovery of MSCs in the endometrium, it has been stated that eMSCs have a role in the regeneration of the endometrial layer during the menstrual cycle [112]. Chan *et al*. reported that the endometrium and menstrual blood are rich sources of stem cells. Patel *et al.* in 2008 stated that MenSCs have clonogenic properties and that MenSCs can differentiate from ectoderm and mesoderm origin [116]. MenSCs are accepted as a new source of MSC because they do not require surgery for isolation, do not create an ethical dilemma and have high proliferation characteristics. It is indicated that MenSCs have greater proliferation and differentiation properties compared to BM-MSCs [16]. In addition to these features, MenSCs from menstrual blood are seen as a promising source for use in regenerative medicine applications due to the ease of obtaining from the same donor by repeated non-invasive methods [118]. Compared with MSCs isolated from other tissues, although MenSCs are more useful cells than other MSCs, further research is still needed for clinical applications and basic research [119].

Despite the isolation of MenSCs using different methods after the discovery of MSCs in menstrual blood, these isolation methods were not compared in terms of maintenance of the stem cell potential of MenSCs. Therefore, in this thesis, MenSCs were isolated by non-enzymatic, collagenase type 1 and dual enzyme digestion methods. Although there is no

resource stating the pros and cons of the isolation methods about MenSCs, when comparing non-enzymatic and enzymatic isolation methods for adipose MSCs, the non-enzymatic isolation method was suggested as a better choice than other isolation methods in terms of maintaining ECM-cell surface interaction. In addition, it has been stated that ECM integrity may be disrupted by proteolytic degradation in primary cell isolation by enzymatic methods [120]. In line with this finding, as shown in phase-contrast microscope images (Figure 4.1.), MenSCs were spindle-shaped, with a large nucleus and multiple nucleoli, and isolated cells were close to each other in the TCP environment, in a clustered form. The number of cells isolated through all three methods differed among volunteers, due to the difference in the amount of blood collected. In Subject1 the best isolation method was found as CL-1 isolation method with respect to isolated cell number, in Subject2 the dual isolation method and in Subject3 the NE isolation method conferred an advantage.

Although cells isolated by the three isolation methods can be morphologically identified as MSC, molecular characterization of cells was analysed by flow cytometry analysis. The surface markers CD29, CD44, CD73, CD90, CD105 and ITGβ1, known to be positively expressed by all MSCs, were used for flow cytometry analysis, and MenSCs were also noted to be negative for CD14, CD34, and CD45 (hematopoietic cell markers) [36,103]. Our results suggested that cells isolated by three isolation methods possessed MSC surface markers. In addition, PDGFR and W5C5 markers [36], which were previously stated to be specific markers for MenSCs [118], were used for flow cytometry, and MenSCs were found to be positive in these markers regardless of the isolation method. When the isolation methods were compared with each other in terms of expression level of surface markers in the Ep_MenSCs; no significant difference in the expression levels of surface markers was determined. Similarly, when the NE isolation methods was compared to CL-1 or dual digestion, no significant difference in the expression level of surface markers was seen at early passages. At Lp_MenSCs regardless of isolation methods, cells retained their stem cell properties, but percentages values of PDGFR and ITGβ1 were slightly increased, although there was a slight decrease in CD73, CD90, CD105 and W5C5 percentage values in all MenSCs. PDGFR is responsible for cell proliferation, growth and development and cellular differentiation, and these features are expected to decrease as the cell ages. ITGβ1 responsible for cell adhesion, embryogenesis, haemostasis, tissue repair, and immune response. Expression level of ITGβ1 was expected to be decrease, however, like PDGFR

expression level, our results showed that, ITGβ1 expression level was also increased at late passages. Therefore, it can be said that these features may still be conserved in MenSCs or due to PDGFR were found located in the perivascular region, MenSCs may enter vascularization state spontaneously at late passages with increased cell adhesion ability gained with ITGβ1 upregulation [121]. As expected, the expression levels of CD73, CD90, and CD105 expression levels were slightly decreased at late passages suggesting initiation of differentiation process that is accompanied by a decline in stemness markers. Unlike reduction in the expression level of CD73, CD90 and CD105, a slight incline in the expression level of PDGFR and W5C5 was also evident for the late passage MenSCs suggesting that as differentiation towards epithelial lineage [122]. According to our results, although MenSCs obtained by the isolation methods were seen to positively express surface markers, none of the isolation methods confers an advantage for the maintenance of CD markers at late passages.

After verification of MSC characteristics of MenSCs via flow cytometry analysis, colony formation ability, which is one of the characteristics of stem cells, was also examined for these cells. For CFU analysis at early and late passage, 236 cells were seeded onto 6-well plate and cells were stained with crystal violet after 14 days incubation. According to our results, at the early passage the highest number of colonies was formed by MenSCs when isolated by dual digestion method, then MenSCs isolated by NE and CL-1 isolation methods respectively provide the highest number of colonies. Unlike Ep_MenSCs, highest colony number was achieved by MenSCs obtained through NE isolation method, then the dual digestion, and lastly CL-1 isolation method at late passages. However, no significant difference among isolation methods was found both for Ep_MenSCs, and Lp_MenSCs in terms of colony number. Similarly, when isolation methods were compared with each other in terms of colony size (mm), it was detected that MenSCs isolated via dual digestion isolation method formed bigger colonies than CL-1 and NE isolation methods at early passage. However, MenSCs isolated by NE isolation method were formed bigger colonies than CL-1 and dual digestion isolation method at late passages. As expected, both colony number and size were decreased in all isolation methods towards to the late passages. While the highest CFU-E at Ep_MenSCs was found in the dual digestion isolation methods, for Lp_MenSCs, the highest CFU-E was found for the NE isolation method. When isolation methods compared to each other at Ep_MenSCs and Lp_MenSCs in terms of CFU-E, no

significant difference was found. Similarly, when CFU-E was compared between early and late passages for every isolation method, no significant difference was found for any isolation methods. Comparison of colony numbers for MenSCs obtained through different isolation methods showed non-significant fold change for both for early and late passages, although the decrease in colony number was more prominent in the dual digestion Lp_MenSCs. Similarly paired comparison between the NE isolation methods to CL-1 or dual digestion, showed no significant difference in the colony number. Due to the importance of cell number and stemness maintenance in cell-based therapy and stem-cell therapy [121], our results showed that the best isolation methods for CFU was dual digestion isolation method.

Following flow cytometry and CFU analysis for maintenance of stemness, cell proliferation rates of MenSCs isolated by three isolation methods at early and late passages was examined. When isolation methods were compared at early passage, cell proliferation was higher in NE isolation method, then CL-1 and dual digestion isolation methods. For Lp_MenSCs, the highest cell proliferation rate was found for CL-1 isolation method, then NE, and dual digestion isolation methods. Chen *et. al* showed that, doubling time of MenSCs initially slower, and it reached a peak at passage8, after passage8 doubling time of MenSCs were decreased gradually [123]. According to our results, while there was no significant difference between isolation methods at early passages, NE isolation method may be a better choice because doubling time of Ep_MenSCs as NE_MenSCs duplicated faster than MenSCs isolated with CL-1 and dual digestion isolation methods. Proliferation rate was decreased thorough late passages in all isolation methods as expected, according to our results. When isolation methods were compared with each other for the proliferation rate at Ep_MenSCs, NE_MenSCs showed higher rate than MenSCs isolated CL-1 and dual digestion isolation methods. For Lp_MenSCs, the highest cell proliferation rate was found for CL-1 isolation method, then NE, and dual digestion isolation methods. However, statistical analysis showed no significant difference among the isolation methods at both early and late passages MenSCs in terms of proliferation rate. On the other hand, when proliferation rates of Ep_MenSCs and Lp_MenSCs were compared with each other for each isolation methods at each time point, and a significant decrease ($p<0.05$) was found only at 72 hours for NE isolation method. Albeit no significance was found, our results suggest NE

isolation method as the best isolation method for the maintenance of self-renewal potential for MenSCs at early passages.

6. CONCLUSION AND FUTURE PERSPECTIVE

After the discovery of mesenchymal stem cells, their properties over time and their importance in clinical studies, other stem cell sources began to be sought. On the discovery of stem cells in the endometrium, including our lab, research has begun on eMSCs. With the discovery of eMSCs, studies have been carried out on the hypothesis that there may be stem cells in menstrual blood, and the presence of MenSCs has been found. While MenSCs were obtained with different isolation methods, the importance of isolation methods in term of stemness maintenance was not determined. With our current study, non-enzymatic and enzymatic methods which were collagenase type 1 and dual digestion, were compared in terms of maintenance of stemness at early and late passages. No difference in cell morphology was observed when the isolation methods were compared, and MenSCs isolated by NE, CL-1 and dual digestion methods were found to preserve stem cell characteristics in late passages. Dual digestion isolation method was found to be the best isolation method for CFU properties in terms of maintenance of stemness. According to cell proliferation rates, best isolation method was found as NE isolation method for early passages, and CL-1 isolation method for late passages. Since early passage MSCs are used in cell-based studies and tissue differentiation because they do not lose their MSC properties [121], it can be said that MenSCs isolated by NE isolation method should be used in the early passage if they are to be used in this field. Although late passage MSCs are not desired to be used in cell-based studies and tissue differentiation as they begin to lose their MSC properties, in application that require long term culturing such as use of eMSCs as feeder cells for embryonic stem cell [124] MenSCs isolated by CL-1 isolation method can be used.

As our results show that there is no difference in maintenance of stemness in MenSCs with enzymatic and non-enzymatic isolation methods, in future additional studies will be needed to compare isolation methods with larger sample numbers ($n>6$). In addition, future studies can focus on the effect of isolation methods in the differentiation of MenSCs into adipogenic, osteogenic, chondrogenic lineage. In this context, levels of lineage specific protein can be analysed by Immunocytochemistry and Western Blot expression of lineage specific genes can be analysed by quantitative real time PCR, in order to determine which isolation method is the best for differentiation.

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APPENDIX A: ETHICAL COMMITTEE REPORT

ACIBADEM MEHMET ALİ AYDINLAR ÜNİVERSİTESİ
TIBBİ ARAŞTIRMALAR DEĞERLENDİRME KURULU (ATADEK)

Etik onay istenen tıbbi araştırmanın başlığı:

Menstrüel kandan endometriyal kök hücre izolasyonu için farklı yöntemlerin bu hücrelerin başkalaşma potansiyeli üzerine etkisi

Etik onay istenen tıbbi araştırmanın yürütücüsü (sorumlusu):

Prof. Dr. Dilek TELCİ TEMELTAŞ, Uzm. Dr. Melike BATUKAN, Prof. Dr. Ahmet Cem BATUKAN

Karar:

Kabul (Etik olarak uygun) (✓) **Revizyon** ( )* **Etik olarak uygun değil** ( )**

Toplantı Tarihi: 03.12.2021 **Karar Numarası: 2021-23/13**

		Karara	Karara
Kurul Üyesi-Unvan Ad-Soyad	**İmza**	**Katılıyorum**	**Katılmıyorum*** **
Prof. Dr. Murat BAŞ (Başkan)		(✓)	( )
Prof. Dr. Ükke KARABACAK (Başkan Yrd.)		(✓)	( )
Prof. Dr. Yasemin ALANAY		(✓)	( )
Prof. Dr. Şeyda TÜRK		(✓)	( )
Prof. Dr. A. Yiğit ÇAKIROĞLU		(✓)	( )
Doç. Dr. Figen DEMİR		( )	( )
Doç. Dr. Erman AYTAÇ		( )	( )
Doç. Dr. Fatih ARTVİNLİ		( )	( )
Dr. Öğr. Üyesi Sinem Burcu ERDOĞAN		(✓)	( )
Dr. Öğr. Üyesi Zekiye Kübra YILMAZ		( )	( )
Dr. Öğr. Üyesi Ceren Gülser İLİKAN RASİMOĞLU		(✓)	( )
Dr. Öğr. Üyesi Nazlı KESKİN		(✓)	( )
Melisnaz ÇOLAK		( )	( )

APPENDIX B: RESULTS OF FLOW CYTOMETRY PERCENTAGE VALUES OF THREE SUBJECTS WITH ISOLATION METHODS AT EARLY AND LATE PASSAGES

Table A.1, Table A.2, and Table A.3 represents the percentage values of flow cytometry for stem cells markers of Ep_MenSCs isolated from three subjects by three isolation methods which were NE, CL-1, and dual digestion, respectively.

Table A.1. Flow cytometric analysis for percentage values of Ep_MenSCs from three subjects with non-enzymatic isolation method

Ep_MenSCs	Subject1	Subject2	Subject3
CD29	98.75	99.61	99.94
CD44	85.01	99.27	99.73
CD73	99.75	72.13	100.00
CD90	97.52	96.93	96.95
CD105	63.06	93.44	95.37
PDGFR	77.57	56.83	83.02
W5C5	59.65	64.96	78.88
ITGβ1	71.38	65.63	99.74
CD14	1.97	1.28	3.35
CD34	2.38	3.38	13.19
CD45	1.59	2.87	11.87

Table A.2. Flow cytometric analysis for percentage values of Ep_MenSCs from three subjects with collagenase type 1 isolation method

Ep_MenSCs	Subject1	Subject2	Subject3
CD29	99.73	90.11	99.94
CD44	98.00	95.54	99.37
CD73	99.17	99.89	99.55
CD90	96.29	93.83	85.41
CD105	70.90	97.01	75.03
PDGFR	90.64	68.31	74.24
W5C5	47.61	56.61	30.30
ITGβ1	97.07	99.45	99.61
CD14	2.26	1.91	2.13
CD34	13.25	6.12	2.54
CD45	5.54	1.32	1.81

Table A.3. Flow cytometric analysis for Ep_MenSCs from three subjects with dual digestion isolation method

Ep_MenSCs	Subject1	Subject2	Subject3
CD29	98.48	98.90	99.65
CD44	92.56	95.26	99.52
CD73	99.66	99.83	99.83
CD90	89.84	97.80	99.64
CD105	99.50	97.33	89.70
PDGFR	79.74	73.80	78.08
W5C5	39.70	60.32	39.17
ITGβ1	98.64	99.04	99.74
CD14	1.75	1.95	2.66
CD34	2.43	1.16	5.06
CD45	2.39	1.44	2.84

Table A.4, Table A.5, and Table A.6 represents the percentage values obtained from flow cytometry analysis of stem cells markers of Lp_MenSCs isolated from three subjects by three isolation methods which were NE, CL-1, and dual digestion, respectively.

Table A.4. Flow cytometric analysis for Lp_MenSCs from three subjects with non-enzymatic isolation method

Lp_MenSCs	Subject1	Subject2	Subject3
CD29	99.90	99.54	99.67
CD44	99.83	85.58	97.72
CD73	99.96	44.72	99.73
CD90	99.79	97.71	89.78
CD105	88.98	69.23	54.68
PDGFR	93.37	81.72	93.15
W5C5	63.96	64.34	65.35
ITGβ1	99.71	86.60	89.14
CD14	2.82	1.97	1.55
CD34	7.22	6.25	3.56
CD45	4.53	3.71	1.97

Table A.5. Flow cytometric analysis for Lp_MenSCs from three subjects with collagenase type 1 isolation method

Lp_MenSCs	Subject1	Subject2	Subject3
CD29	99.48	99.31	99.63
CD44	93.34	97.63	99.31
CD73	99.71	54.47	99.35
CD90	98.94	95.39	83.90
CD105	61.20	87.22	49.27
PDGFR	85.79	74.62	96.80
W5C5	45.49	68.80	39.42
ITGβ1	95.89	98.19	98.12
CD14	3.32	1.20	2.13
CD34	6.08	11.46	1.31
CD45	3.70	8.02	1.31

Table A.6. Flow cytometric analysis for Lp_MenSCs from three subjects with dual digestion isolation method

Lp_MenSCs	Subject1	Subject2	Subject3
CD29	99.58	99.23	99.10
CD44	97.59	96.67	99.58
CD73	99.77	44.58	99.75
CD90	96.03	92.05	90.77
CD105	63.43	89.26	56.32
PDGFR	78.97	83.76	88.99
W5C5	34.27	72.10	30.57
ITGβ1	99.60	98.06	98.96
CD14	1.84	1.57	1.89
CD34	3.62	6.01	3.36
CD45	2.24	8.97	2.42