Chapter 2

Stem Cell Culture Techniques: Mesenchymal Stem Cells

Jeanne Adiwinata Pawitan

A. Introduction

Cell therapy, especially stem cell therapy, has gained lots of attention worldwide, including in Indonesia. However, most cell therapies are not standardized, except the use of hematopoietic stem cells (HSCs) to repopulate the bone marrow after radiation or chemotherapy in hematological malignancies. For every condition, most centers have their own methods of cell production, preservation, and cell therapy application methods with regard of the source of cells, dose, route of administration, and how many repeats that are needed, as well as the intervals between the repeats (Yoneda et al., 2022).

As a developing country, Indonesia needs to be independent in health services, including in cell therapy services. As Indonesia

J. A. Pawitan

National Research and Innovation Agency, e-mail: ariy007@brin.go.id

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regulation prohibits the use of nonhuman cell sources, as well as embryonic stem cells, only adult stem cells can be used. Adult stem cells can be derived from various adult tissues, such as bone marrow, adipose tissue, dental pulp, hair bulb, and mobilized peripheral blood, as well as partition waste, such as umbilical cord tissue, umbilical cord blood, amnion or placenta (Sipp et al., 2018). Moreover, some studies have succeeded to isolate stem cells from menstrual discharge (Sipp et al., 2018), urine (Fu et al., 2014), or milk (Kersin & Ozek, 2021).

As for cell therapy, usually lots of stem cells are needed. While cell extraction from tissues is usually limited in number, multiple donors are needed when allogeneic cell is intended to be used, or the cells should be expanded in vitro by culturing, which also apply when autologous cells will be used. The type of adult stem cell that is easy to be expanded in vitro is mesenchymal stem cell (MSCs). In order to be independent in cell therapy for various conditions, Pawitan et al. (2013, 2014, 2014-2015) began to isolate MSCs from various tissues, and succeeded to develop methods for easy, and economical MSCs production using an in house developed domestic derived supplement. Pawitan et al. (2013, 2014, 2014-2015) methods to produce MSCs from bone marrow, adipose, and umbilical cord tissue have been applied in a current Good Manufacturing Practices (cGMP) laboratory, and the cells have been used in various animal studies, and clinical trials. In this occasion, Pawitan et al. (2013, 2014, 2014-2015) would like to share their methods of MSCs isolation, propagation, and cryopreservation, which were derived from three kinds of tissues, bone marrow, adipose, and umbilical cord tissue. Therefore, the author will address the MSCs, sample collection and transportation, isolation and culture of MSCs from bone marrow, adipose tissue and umbilical cord tissue, upscale culture of MSCs, preservation of the cells by cryopreservation, and the importance of aseptic technic to prevent culture contamination.

B. Mesenchymal Stem Cell (MSC) from Bone Marrow, Adipose, and Umbilical Cord Tissue

The term mesenchymal stem cell (MSC) was first used in 1991 by Arnold Caplan, who is a US biologist, to describe a cell type, which was derived from bone marrow that could differentiate into various types of tissues of mesenchymal origin. After that, MSC can be isolated from various adult tissues. They are similar but have subtle differences in their potential to differentiate and their other potencies. Moreover, different laboratories might use different surface markers for MSC characterization, and there were ambiguities and inconsistencies in MSC potencies. Therefore, in 2006, International Society for Cell Therapy (ISCT) proposed the term multi-potent 'mesenchymal stromal cell'. According to ISCT, MSCs should fulfill a certain criteria, i.e., they are fibroblastic in morphology, adherent to plastic vessel where they are cultured, have specific surface markers (CD 90, CD73, and CD105 should be ≥95%, and lineage negative CD should be ≤2%), and should be able to differentiate into three lineages namely osteogenic, chondrogenic, and adipogenic lineages. MSCs from bone marrow, adipose, and umbilical cord tissue, all can differentiate into the three lineages. However, the lipid droplets of MSCs from umbilical cord tissue, when they are differentiated into adipocytes, are much smaller compared to those from bone marrow or adipose tissue. Further, in 2017, Arnold Caplan, who at that time no longer believed that MSCs were stem cells, proposed 'medicinal signaling cell' as abbreviation of MSC. However, many studies still refer MSC as 'mesenchymal stem cells' (Sipp et al., 2018).

C. Sample Collection and Transportation

Samples of various tissues that will be used for MSC production should be collected by aseptic technic according to current Good Tissue Practice (cGTP), from individuals who are devoid of infectious

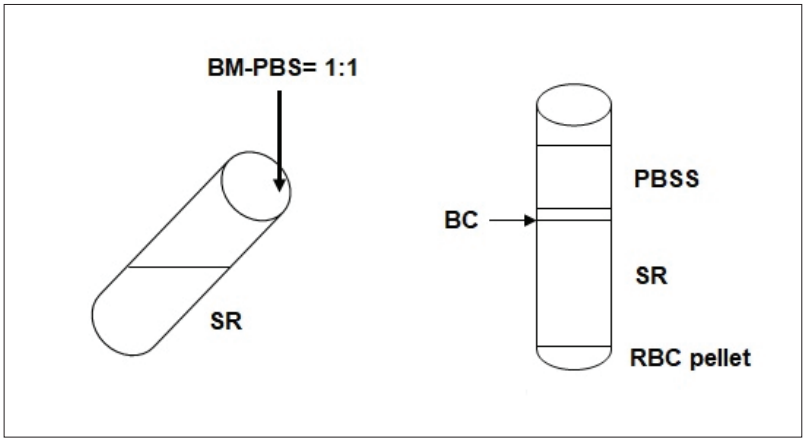
and hereditary diseases and have signed the informed consent form to donate their tissue. Therefore, the donor should be checked for the presence of immunoglobulin G (IgG) and M (IgM) of human immunodeficiency virus 1 and 2 (HIV1-2), hepatitis B virus (HBV), hepatitis C virus (HCV), syphilis, and toxoplasma, rubella, cytomegalo, herpes (TORCH) viruses. In addition, anamnesis of donor pedigree should be carried out to exclude hereditary diseases (Yoneda et al., 2022).

After collection, sample should be placed in a transport medium and kept in a refrigerator at 4°C before and after transportation, and should be process before 24 hours after collection. Transport medium is basal medium such as alpha minimum essential medium (alpha MEM) or Dulbecco's modified Eagle medium (DMEM) that contain antibiotics and antimycotic three times of the usual dose (Pawitan et al., 2014).

D. Isolation and Culture of MSCs from Bone Marrow

The majority of stem cells in bone marrow are HSCs, which should be matching between donor and recipient. Otherwise, the transplanted HSCs will be rejected by the recipient, or when given to repopulate the host' bone marrow, the transplanted cells can regarded recipient's tissue/organ as foreign that can be dangerous for the recipient. Besides HSCs, bone marrow also contains a few MSCs that are found in the mononuclear cell fraction, together with the HSCs (Lucas, 2021).

Isolation of MSCs from bone marrow can be done by either isolating mononuclear cells (MNCs) using a separation medium (Gudleviciene et al., 2014) or directly separating the pellet after centrifugation, which is followed by culture to expand the MSCs (Pawitan et al., 2014-2015). When separation reagent such as Ficoll Hypaque or its equivalent such as Lymphoprep™ is used to isolate the MNCs, bone marrow is mixed with an equal volume of phosphate buffered saline (PBS), and the mixture is dropped carefully onto



Notes: BM: bone marrow;
PBS: phosphate buffered saline;
SR: separation reagent;
PBSS: PBS – serum;
BC: buffy coat;
RBC: red blood cells.

Figure 2.1 Separation of MNCs Using a Separation Reagent

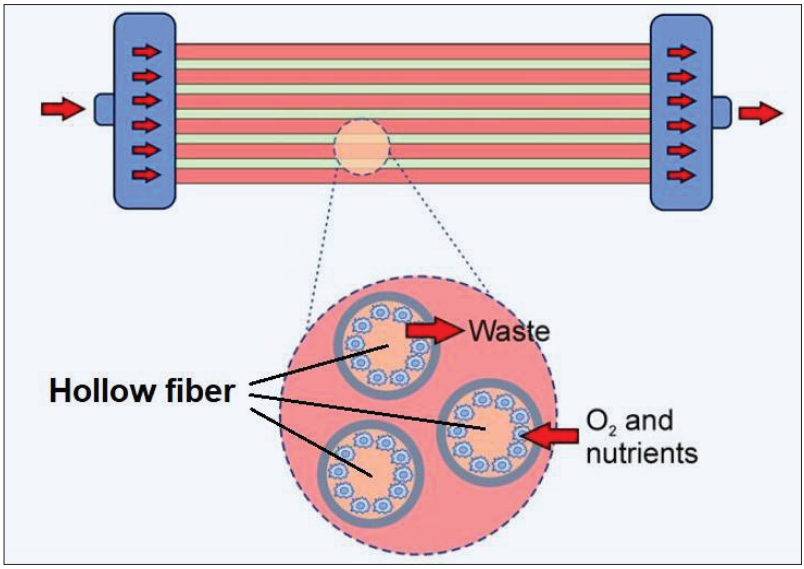
an equal volume of separation reagent, so that the bone marrow mixture does not mix with the separation reagent, but floating. After centrifugation, red blood cells will be pelleted at the bottom, at the upper part of red blood cells is the separation reagent, then the MNCs that form a buffy coat that can be visualized as a white ring, and the uppermost is the PBS and serum (Gudleviciene et al., 2014) (Figure 2.1).

The white ring is carefully taken, and then is washed with PBS, and centrifuged to be pelleted. The pellet is reconstituted with special complete medium for MSCs and then cultured (Gudleviciene et al., 2014).

For direct separation of MNCs, Pawitan et al. (2014-2015) have developed a simple method to culture bone marrow-derived MSCs

(BM-MSCs) without the use of Ficoll Hypaque. In brief, 5–6 ml of bone marrow is mixed with an equal amount of complete MSC medium. The complete medium is alpha MEM that is supplemented with in-house prepared platelet lysate and glutamax. The mixture is centrifuges and pellet that contains the buffy coat together with red blood cells is separated from the supernatant, and transferred into a T25 flask, then a same amount of MSC complete medium is added and cultured. An alternative to culturing whole pellet is to culture only the buffy coat and upper part of erythrocyte pellet. The supernatant is homogenized, and 1 ml is taken and placed in a 12 well plate and cultured. The supernatant is observed beginning day 3 to see cell attachment. Observation of supernatant culture is used to determine the time of washing the pellet culture, as the pellet culture is full of erythrocytes that makes impossible to see MSC attachment, while the supernatant only contains a few MNCs and erythrocytes, so it is readily visible when the MSCs are attached. After the MSCs in the supernatant culture are attached, the pellet culture is washed gently to remove erythrocytes and unattached non-MSCs, then 5–10 ml fresh complete MSC medium is added, and culture is resumed. Further, medium change is done every 3 to 4 days, until enough colonies appear, and the MSCs can be harvested (Pawitan et al., 2014-2015).

Harvesting is done by detaching the cells using an enzyme, which is TrypLE Select, that is milder compared to Trypsin. Before adding TrypLE Select, the cells should be washed gently and thoroughly by PBS, as platelet lysate in the complete medium has anti-enzyme activity. After washing, 1 ml of TrypLE select is added and put in the incubator for five minutes. After five minutes, the flask is observed to see whether detachment occurs, which is visible as rounded shining cells that are floating in the enzyme solution. When the cells are rounded, but still attached to the bottom, the bottom of the flask is tapped gently, or the flask is banged gently to a hard surface. When not all cells are detached, the flask is put in the incubator for another 5 minutes, until all cells are detached. After all cells are detached,



Source: Jankovic et al. (2023)

Figure 2.2 Quantum Bioreactor

cell suspension in TrypLE Select is transferred to a tube, and an equal amount of complete medium is added to stop the enzyme. The mixture can be directly cultured to a new flask or washed by PBS, reconstituted in complete medium and cultured to be passage-1. Before culturing, the amount of cells needs to be counted, and culturing is done by seeding 5000 viable cells/cm^2 of vessel surface area (Pawitan et al., 2014-2015).

Another method for direct separation and culture of MSCs may use a Quantum bioreactor that contains hollow fibers (Figure 2.2). Before culture, the hollow fibers' outer surface is coated overnight at 37°C with human fibronectin (huFN) or other reagents, such as gelatin, collagen, human serum albumin, poly-L-lysine, pooled human cryoprecipitate (PHCP), or human vitronectin (huVN) as a cell attachment factor. After coating, 12.5–25 ml of whole bone marrow that is mixed with heparin and complete medium until the volume reaches 100 ml is loaded in the Quantum bioreactor and

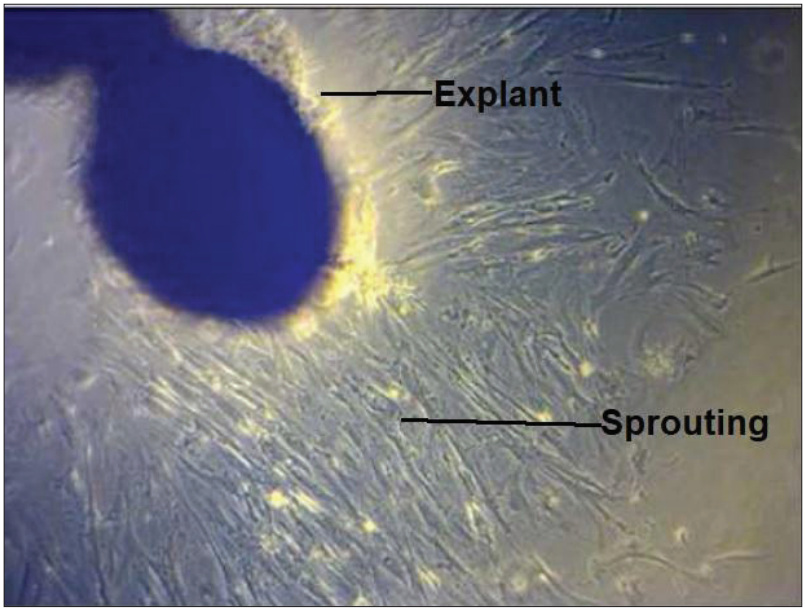
fresh complete MSC medium continuous flow is maintained at the outer and inner space of the hollow fibers, MSCs attached to coated hollow fibers, while other cells are flushed by the continuous medium flow. Comparison between coating reagents showed that coating with huFN, PHPC, or huVN, yielded similar results that were much better than other coating reagents (Frank et al., 2019).

E. Isolation and Culture of MSCs from Adipose Tissue

In adipose tissue, MSCs are found in the vicinity of blood vessels. Isolation of MSCs from adipose tissue can be done by explants' method (Li et al., 2018) or after extraction of cells by either enzymatic (Pawitan et al., 2013) or mechanical method (Gentile et al., 2019).

1. Isolation and Culture of MSCs from Adipose Tissue by Explant Method

In explant method, adipose tissue is minced into small fragments, and put on plastic vessels, such as petri disks, multi-wells, or flasks. As adipose tissue tends to float, explants' method needs a special care to prevent the tissue from floating, either by placing a cover slip on the explants or by putting the explants at the upside of a flask, while giving medium at the bottom to prevent drying until the explants attached. After the explants are attached, the flask is reversed, so that the upside becomes the bottom. The act of reversion should be done very carefully to prevent detachment of the tissues. When using cover slips, care should be taken to avoid air bubble formation. Another method is by putting the explant at the bottom and giving a just enough medium to wet the explants and put the vessel in an incubator until the explants are attached, before carefully giving the usual amount of medium to prevent detachment of the explants. Care should be taken to prevent floating of explants, because cells will only sprout out of the explants if the explants are attached to plastics (Figure 2.3) (Li et al., 2018).



Source: Pawitan et al. (2014)

Figure 2.3 Plastic vessel attached explant culture facilitates "sprouting".

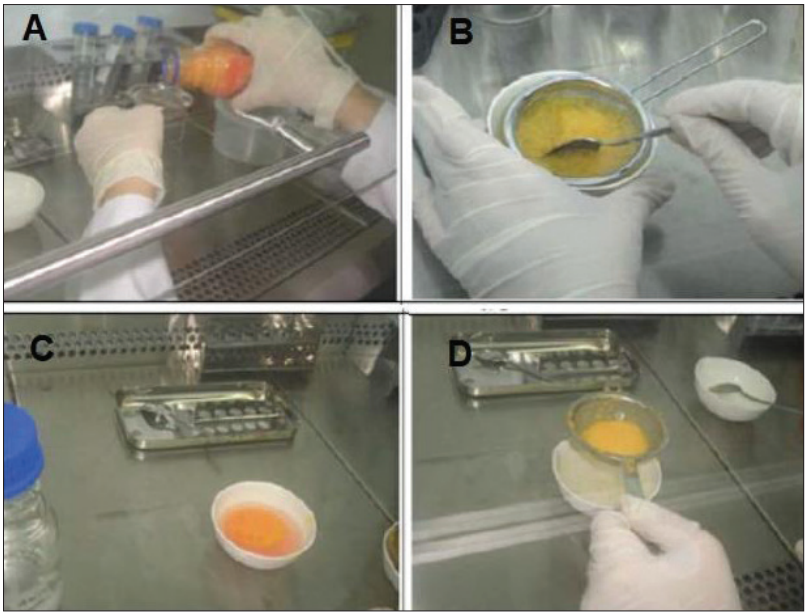
2. Isolation and Culture of MSCs from Adipose Tissue After Extraction of Cells

As isolation by explant method needs special expertise to prevent floating and make the adipose tissue attach to the plastic vessel, cultures after extraction of cells are preferable. Extraction of cells to separate the cells from extracellular matrix can be done by mechanical or enzymatic methods. In mechanical method, the tissue is minced into small pieces, put into erythrocyte lysis solution, followed by agitation by vortex, or other mechanical methods, such as using ultrasonic cavitation device, ultrasound, combination of centrifugation and filtration, or using various mechanical devices. Studies showed that this method yielded enough cells that was comparable to enzymatic method (Gentile et al., 2019). However, another study

showed that enzymatic method gave a lot more cells compared to mechanical method (Aronowitz et al., 2015), which was proven by Pawitan et al. (2013) own experience. Therefore, extraction of cells by enzymatic method is preferable to mechanical method though it is more expensive, as an appropriate enzyme to detach the cells from the tissue matrix is needed. When compared to explant or mixed (enzymatic-explant) method, enzymatic method is preferable, as fat tissue tends to float that leads to explant or mixed culture failure.

In enzymatic method, when the adipose tissue is derived from surgical incision procedure, the tissue should be minced to small pieces, before subjected to enzyme treatment. However, when the tissue is derived from liposuction and already in the form of small pieces, it needs to be washed thoroughly to remove liposuction solution, which is toxic to cells. Moreover, washing step is important to remove red blood cells and free lipids from damaged fat cells that may interfere with cell attachment. Pawitan et al. (2013) found a faster, simpler, and washing solution saver method to wash lipoaspirate by using a coffee filter (Figure 2.4), compared by a previous method, which used extensive washing by repeated centrifugation steps (Zuk et al., 2001), to wash the lipoaspirate until it is really cleaned from contaminants.

To extract cells from adipose tissue, various enzymes and enzyme combinations can be used, such as trypsin, TrypLE Select or collagenase type 1, which can digest adipose tissue matrix. In Pawitan et al. (2013)'s laboratory, they followed a previous method with modification (Zuk et al., 2001), by digesting the lipoaspirate using 0.75% collagenase type 1 in PBS with a lipoaspirate: collagenase solution ratio is 1:2, for one hour at 37^{o}C with manual mixing every five minutes until the tissue become oily and bright yellow in color, which show that the digestion is complete. If the color is still pale yellow, then digestion time needs to be prolonged until the color become bright yellow and oily (Pawitan et al., 2013). After digestion, adipose tissue remnants are discarded, and the infranatant is filtered

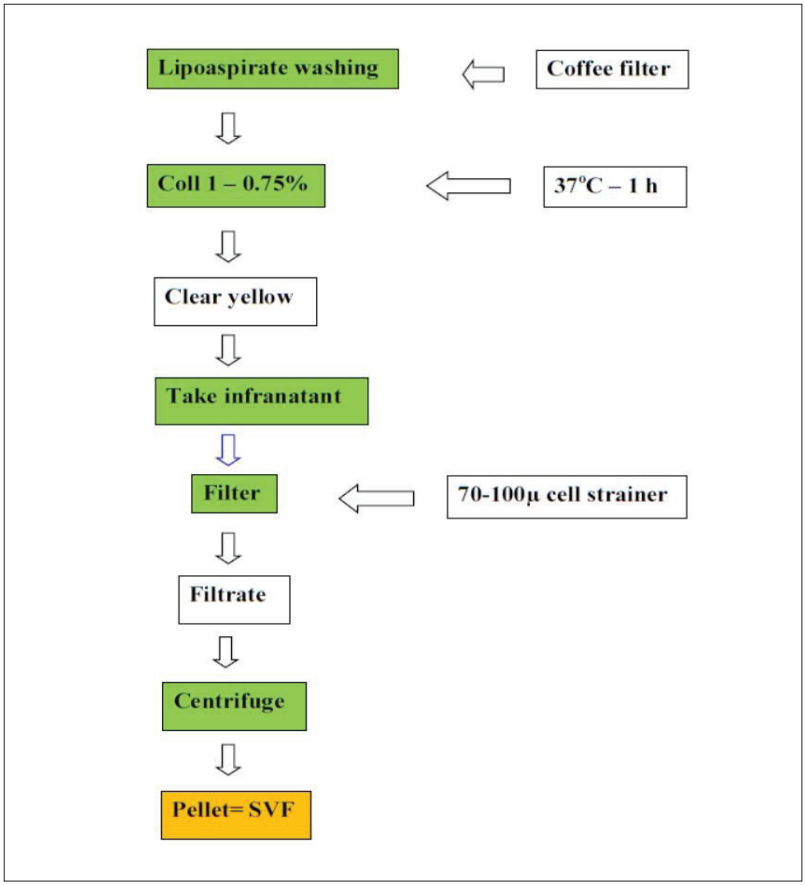


Notes: (A) Lipoaspirate is poured in a sterile stainless steel coffee filter and the fluid was collected in a glass beaker;
(B) Lipoaspirate washing: the lipoaspirate containing coffee filter is soaked in a phosphate buffered saline containing porcelain bowl;
(C) Contaminants and free lipids are left in the bowl;
(D) Clean lipoaspirate.

Source: Pawitan et al. (2013)

Figure 2.4 Simple Lipoaspirate Washing Using a Coffee Filter

using a 70 or 100µ cell strainer, centrifuged, and the pellet is washed using PBS. If the pellet appears red, then it should be subjected to erythrocyte lysis buffer for 10 minutes at room temperature and washed by PBS, followed by centrifugation to get the pellet. The pellet, which contains myriads of cells including MSCs, is called processed lipoaspirate (PLA) or stromal vascular fraction (SVF), as MSCs are found near blood vessels, and some researchers regarded these cells as derivative of pericytes. The SVF pellet is re-suspended in complete medium (Figure 2.5).



Notes: Coll 1: collagenase 1;
1 h: one hour;
µ: micrometer;
SVF: stromal vascular fraction.

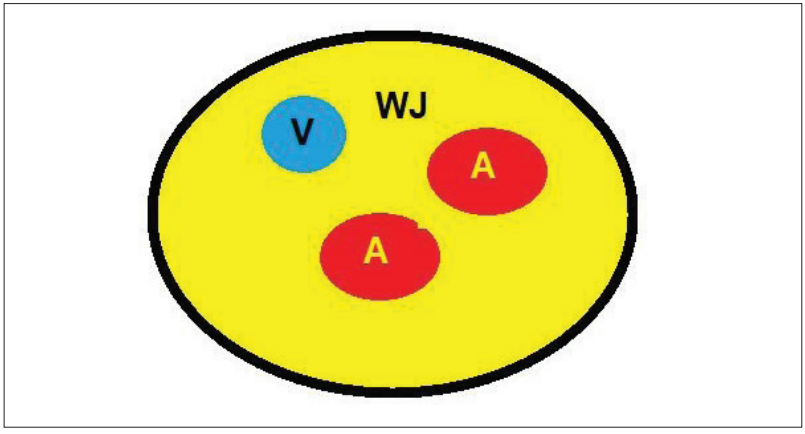
Figure 2.5 Flowchart of the Isolation of Adipose Tissue Derived Stromal Vascular Fraction

Complete medium that is used for culture should be xenofree if the cells are intended for the patient. There are various xenofree commercial media, each sold with their respective supplement, such as MesenCult ™ Basal Medium (human), and Stem Pro culture medium.

However, in Pawitan et al. (2013)'s laboratory, they use Alpha MEM that is supplemented by 10% in house prepared platelet lysate and glutamax. Initial seeding is 170,000 viable nucleated cells or more in a twelve well plate or 42,500 viable nucleated cells/cm^2. Less seeding density may delay the culture to become confluent, while more seeding density may lead to faster confluency. Harvest can be done after 80%–90% confluent. Care should be taken to prevent 100% or more, as harvest after 100% or more may cause senescence of the MSCs. After harvest, the cells can be passaged with a seeding density of 5,000 viable cells/cm^2.

F. Isolation of MSCs from Umbilical Cord Tissue

In umbilical cord tissue MSCs are mainly found in Wharton's jelly (Figure 2.6). Isolation of MSCs from umbilical cord tissue can be done by either explants or enzymatic method. In isolation of MSCs from Wharton's jelly umbilical cord tissue by explant method, care should be taken so that the Wharton's jelly is facing downward and not vice versa. On the other hand, isolation by enzymatic method should



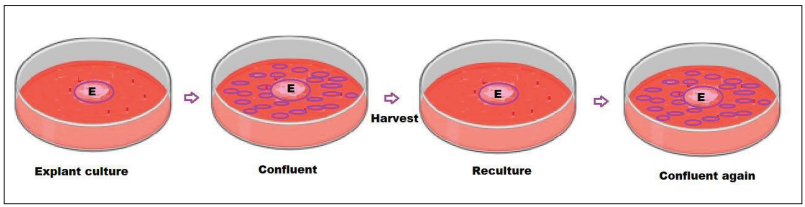
Notes: A: Artery;
V:Vein;
WJ: Wharton's jelly.

Figure 2.6 Schematic Picture of Umbilical Cord

pay attention on the extracellular matrix (ECM) that surrounds the MSCs. Knowledge of the ECM is crucial for selecting the enzymes that are needed to digest the ECM to free the MSCs from the ECM (Varaa et al., 2019).

1. Isolation of MSCs from Umbilical Cord Tissue Using Explant Method

For explant method, Pawitan et al. (2014) have developed multiple harvest explant method (Figure 2.7), as the explant can be re-cultured for several times after the first harvest.



Notes: E: Explant

Figure 2.7 Schematic Picture of Multiple-Harvest Explants Method

Before processing the umbilical cord, the cord tissue should be decontaminated, especially when it is derived from a normal delivery, as the umbilical cord is contaminated with vaginal flora. Around 5–10 cm umbilical cord tissue is washed briefly in 0.5% povidone iodine (betadine) containing PBS pH 7.4, followed by washed in PBS twice. Both ends are discarded and the umbilical cord is cut into several pieces, about 1 cm long. The umbilical veins and artery are pulled out and the umbilical cord is dissected. The tissue can be either directly minced into small pieces around 2–5 mm in with and length, or thinly sliced to remove the outer glistening part (amnion) and then minced into small pieces. Mincing of the umbilical cord tissue is done while wetting the tissue with complete medium to prevent the tissue from drying. Explant culture can be done in 24 or 12 well plates or T25 flasks. For 24 or 12 well plates, one, two, to three pieces are placed in the well, respectively. For T25 flask, many pieces are placed in the flask

and arranged at a distance from each other. The plates and flasks are put in an incubator at 37°C for 1 hour. After the explants are attached, a few complete MSC medium is given cautiously, to prevent floating of the pieces, and the plates and flasks are put back in the incubator. When using direct mincing method, care should be taken that the Wharton's jelly is facing downward, otherwise sprouting of cells will not occur. However, when the amnion is removed before mincing, both surfaces may face downward. The culture needs to be observed daily to detect contamination, the need to add complete MSC medium due to drying or sprouting. After sprouting, usual amount of medium is added and the medium is changed every 3 to 4 days, until 80%–90% confluent. After the culture is confluent, the cells can be harvested and passaged. After harvesting, the explant can be re-cultured followed by several times harvests to yield more cells (Pawitan et al., 2014).

As alternative to Alpha MEM, Budiyanti et al. (2015)'s result in culturing umbilical cord derived MSCs showed that DMEM low glucose or high glucose (DMEM-LG or HG) and a commercial medium MesenCult showed lower performance compared to Alpha MEM.

2. Isolation of MSCs from Umbilical Cord Tissue Using Enzymatic Method

Enzymatic method aims to extract the MSCs from the tissue, which differs from explant method that directly culture tissue pieces without cell extraction. For enzymatic method, after decontamination and mincing of umbilical cord tissue, the tissue fragments are subjected to an enzyme, i.e., 0.1% collagenase for 20 hours at 37°C, followed by filtration using a 100 µm cell strainer to remove tissue remnants. The cell suspension that passes through the strainer is centrifuged; the pellet is washed by PBS twice and the pellet is resuspended in complete MSC medium and cultured (Chen et al., 2016). However, when the author followed Chen et al.'s method, after collagenase digestion, digestion result was a very sticky mucous that could not pass through the strainer (Pawitan et al., 2011). This failure is due to the fact that

Wharton's jelly ECM is composed of collagen and hyaluronic acid, and the hyaluronic acid cannot be digested by collagenase alone. Another method that was more reliable used an enzyme cocktail, which consists of collagenase, hyaluronidase, and trypsin for 45 minutes to two hours at 37°C. After digestion with the enzyme cocktail, the resulting digest was a liquid that could be filtered using a cell strainer. Further, the cells that passed the cell strainer were cultured to yield MSCs (Varaa et al., 2019). When enzymatic is compared to explant method, it is much more expensive as it needs various enzymes to be successful, and initial processing is more laborious so that it takes longer time, but the MSCs grow faster to become confluent, so the MSCs can be harvested faster in primary culture (Varaa et al., 2019). However, explant method is preferable as it is much cheaper, simpler in initial processing, and after primary culture, re-culture takes similar time with enzymatic method (Pawitan et al., 2014).

a. Upscale Culture of MSCs

Initial MSC culture is usually done using T25 flasks. To expand the MSCs, larger vessels can be used, such as T75, T150, T175, and T225 flasks. Since patient may need a lot of cells, to get enough cells for patients, many large flasks can be used that may occupy the whole incubator and be time consuming and labor intensive. To address this issue, hollow fiber system (Frank et al., 2019), multiple flasks (Jankovic et al., 2023), hyperflask (Kearney et al., 2017) and cell factory (Rout-Pitt et al., 2018), both of which have ten layers and micro-carrier-bioreactor system, are developed (Nurhayati et al., 2021).

Two replications of whole bone marrow expansion using Quantum bioreactor, which contained huFN coated hollow fibers, with fresh circulating complete MSC medium in inner and outer space of the hollow fibers showed that the MSC yield were 1.7×10^8 and 5.5×10^7 cells respectively (Frank et al., 2019).

A Beckton-Dickinson (BD) multiple flask with T175 dimension can be found as a Triple flask that has three layers, or a Multi flask that has five layers, with a growth surface area of 525 and 875 cm^2,

respectively or more. These Triple- and Multi flasks are designed to allow easy pipetting to add reagents or to recover detached MSCs, as well as minimal residual liquid retention when pouring technique is preferred over pipetting (Jankovic et al., 2023).

Corning Hyperflask® is a multi-layer flask with a T175 flask dimension, which walls in between layers are in the form of gas permeable membranes. These membranes provide gas and culture media exchange between layers, which harbor the cells. The total area is 1720 cm^2 or around ten times that of a T175 flask area. Each Hyperflask may contain a total of 560–565 ml media without air space. The total media volume is equivalent to eleven T175 flasks. The Hyperflask can be stacked to save place (Kearney et al., 2017).

NUNC™ EasyFill™ Cell Factory™, which is developed by Thermo Fisher Scientific, consists of ten layers. The whole surface area of a Cell Factory is equivalent to 36 pieces of T175 flasks, while the whole dimension is much less than the area that is occupied by 36 pieces of T175 flasks (Rout-Pitt et al., 2018).

For attached cells such as MSCs, culture in a spinner bioreactor needs Collagen-coated micro-carrier beads for cell attachment. Nurhayati et al. (2021) compared flask with spinner bioreactor culture in two experiments using different umbilical cord, and platelet lysate donors. In spinner bioreactor culture, the cells were seeded by mixing 14×10^3 cells/cm^2 with two grams of micro-carrier beads in 50 ml culture media, initially without stirring to facilitate cell attachment. After 16 hours, the medium was added to become 100 ml, and stirring was set at 50 rpm. The spinner flask was put in a fully humidified CO_2 incubator at 37°C. Harvesting of cells from a spinner bioreactor was done using TrypLE select to detach the cells, followed by filtration using cell strainers to separate the cells from the micro-carrier beads. Their result showed that the population doubling time (PDT) of experiment-1 were 12.3 (flask) and 14.8 hours (spinner bioreactor), whereas in experiment-2, they were 17.7 (flask) and 16.9 hours (spinner bioreactor), which were comparable (Nurhayati et al., 2021).

Up-scaling using larger spinner bioreactor may need cell strainer with larger diameter to speed up separation of cells from the micro-carrier.

b. Cryopreservation of Mesenchymal Stem Cells

When MSCs are used for therapy in patient, they should be used within 24 hours, as Krishnanda et al. (2017) and Nofianti et al. (2018) showed that the quality of MSCs was reduced when they were kept for longer time. When harvested cells are not used, they should be stored in a cryopreservation medium, frozen, and kept in a cryo-tank at a temperature of -196^{o}C. In freezing the cells, a decrease in temperature should occur slowly, i.e., 1^{o}C per minute, which can be done in either a controlled rate freezer, or in a device that is called Mr. Frosty, to avoid the formation of ice crystal that is harmful to cells. However, when the cells are to be used, thawing should be done fast, by putting the cryo-vial in a water bath of 37^{o}C. As for cryopreservation medium, there are various cryopreservation media that might differ in type of reagents and their concentrations. Most cryopreservation media use 10% dimethyl sulfoxide (DMSO), but other reagents might differ, such as the use of fetal bovine serum (FBS) that is a xenomaterial, which might contain nonhuman sialic acid Neu5Gc that can be internalized into cryopreserved MSCs (Nasonkin & Koliatsos, 2006), and thus elicit immune response (Villacrés et al., 2021). Therefore, Goei et al. (2015) studied the use of platelet lysate to replace the FBS and found that platelet lysate usage in cryopreservation medium showed better performance after cryopreservation in proliferation rate and cell size, which is a surrogate for senescence. Goei et al. (2015)'s in house cryopreservation medium, which was composed of 10% DMSO and 10% platelet lysate in alpha MEM, was used to cryopreserved Passage-1 umbilical cord derived MSCs, and the results showed that passaging of the cryopreserved cells until Passage-8 with cumulative population doublings (CPD) of more than 34.34 showed less than 5% senescence (Pawitan et al.,2017). Further, the use of Goei et al. (2015)'s cryopreservation medium in adipose derived (AD)- MSCs and BM-MSCs showed that AD-MSCs showed better performance

after cryopreservation, in term of senescence and population doubling time, which is a surrogate of proliferation rate (Ismail et al., 2018).

c. Aseptic Technic to Prevent Culture Contamination

MSCs are easy to be isolated and propagated by culturing from bone marrow, adipose tissue, or umbilical cord, as long as the laboratory officer can keep them in sterile condition. To keep the culture in sterile condition, care should be taken to avoid contamination by bacteria, yeast, fungi, or other microorganism that are difficult to detect, such as mycoplasma, protozoa, viruses, and other cell lines (Biocompare, 2022). Avoiding contamination can be done by using sterile tools, equipments, and all needed culture materials. In addition, every step from isolation, culture, and harvest should be done by aseptic technique (Biocompare, 2022). When operators are not sure of their aseptic technique, antibiotic and antimycotic can be used, but they are only for contamination prevention measures, and cannot replace sterilization and aseptic technique.

1) Sterilization

Before every step, preparation of sterile items should be done one day before. In preparation, tools, vessels, reagents and media need to be prepared and sterilized, or purchased, and large equipment, such as biosafety cabinet and incubator, should be checked whether they work well or not (Pawitan et al., 2012).

Sterilization can be done by heat for heat resistant things, by irradiation using gamma or UV rays for plastics that cannot be autoclaved, or by filtration for non-heat resistant liquids. Sterilization by heat can be done either by dry heat in an oven at 150 to 170°C, for very heat resistant things, such as metal tools, or Pyrex glass wares, or by autoclave at 115 to 121°C and 8 kg or one atmosphere pressure for low heat resistant things, such as blue capped Schott bottles, or heat resistant solutions, such as PBS (Biocompare, 2022).

For things that are purchased in sterile condition, such as large volume of media, regents, and supplements, attention should be paid,

when the volume be only a little left, as they might be contaminated from the frequent taking. Therefore, aliquots need to be done for large volumes, and the aliquot is used for taking. When aliquot has not been done, filtration might be needed for the little left over. In addition, sterile plastic wares, such as flasks, petri dishes, 5 or 15 or 50 ml tubes, are usually not individually packed; flask and petri dishes may contain 10 to 20 per pack, and tubes may contain 25 to 50 per pack. These packs should be opened in the BSC, and directly closed after the needed amount is taken. When a little number is left in the pack, they might not be sterile anymore. Therefore, the left-over need to be sterilized by UV irradiation for a minimal of 15 minutes, together with other things that need to be UV irradiated before the work begin (Pawitan et al., 2012).

Therefore, for every step, knowledge of type and number/amount of tools, vessels, reagents and media that are needed is very important. For a beginner, the number should be doubled or tripled, as everything that has been in contact with non-sterile things should not be used. Unsterile things can be the wall or the floor of incubator, outer surface of reagent or waste bottles, beaker glass, operator hands, etc (Pawitan et al., 2011).

Small metal tools, such as tweezers, scalpels, or scissors, should be placed in a metal box with clips to prevent accidental opening. Before placing them in the box, the end of the tool that will come into contact with specimen or cells to be cultures should be wrapped in aluminum foil and placed regularly in the box. The sterile end of the tool is placed at one side, while the other end that will be held by the hand is placed at the opposite side of the box. Small things, such as Eppendorf tube or cryovials should be placed in a heat resistant pouch and sealed by a special tape. As an alternative of heat resistant pouch, a laboratory officer can use autoclave resistant bottles with metal cap, such as used bottles of jam, spaghetti, or barbeque sauce. The metal box, small thing containing pouch or bottles may be opened only inside a BSC (Pawitan et al., 2012).

2) Aseptic Technique

Every isolation and culture related step should be done by aseptic technique, i.e., all works should be done in a BSC and before usage the UV lamp should be on for a minimum of 15 minutes, with all things that need to be UV irradiated inside. Operators should use culture attributes, which are laboratory coat, hair cap, mask, and gloves (Biocompare, 2022). Before working, the BSC table should be wiped with disinfectant, disinfectant spray should be sprayed on all things that will be put inside the BSC, and during work disinfectant spray should often be used to disinfect the gloves (Pawitan et al., 2012).

When working, the BSC should be on, so that sterile air wall from the BSC can protect the culture, as air from outside BSC cannot come into the BSC, and air from inside BSC cannot go outside, which protect the operator, in case the sample that is handled is infectious. Things should be placed in the BSC according to operator convenience; there is no strict rule on how to place things in the BSC, as every person has their own convenience that might be different between persons. To make the culture work easy, usually things that are not readily used are put far from the operator, at the back of the BSC, and things that are in use are put in front in the BSC, near the operator. However, one thing that should be kept in mind is that the row of holes at the front and back of BSC should be free of things, otherwise the air wall will be disturbed and there will be holes in the air wall, so that air from outside can move inside and vice versa, which may cause contamination of the culture (Pawitan et al., 2012).

The most important things that should be kept in mind are that operators must always be aware that they should never touch unsterile things to specimen, medium, or whatever that is related to the culture, and never pass unsterile things on specimen, medium, or whatever that is related to the culture that is in open condition. Unsterile things include tools that have been fallen to BSC table, have touched BSC wall, or the outside of a medium or reagent bottle or tube

or waste vessel or gloves or whatever that is unsterile, such as pipettor, micropipette, racks, outside of boxes, etc (Pawitan et al., 2012).

3) Antibiotic and Antimycotic

Antibiotic and antimycotic that are used should be those that are special for tissue culture, the final concentration in culture media can be seen in Table 2.1. Concentration of antibiotic and antimycotic for tissue/cell culture is different from for drugs and can differ from brand to brand. Therefore, care should be taken when counting the amount that is needed to make a final concentration. For instance, penicillin is usually sold as combination penicillin 10,000U/ml, and streptomycin 10,000µg/ml, so dilution to get a final concentration is 1:100 (Pawitan, et al., 2012).

Table 2.1 Final Concentration of Various Antibiotics and Antimycotics

Reagent	Function	Final Concentration
Penicillin	Antibiotic	100U/ml
Streptomycin	Antibiotic	100µg/ml
Kanamycin	Antibiotic	100µg/ml
Gentamycin	Antibiotic	50µg/ml
Polymyxin B sulphate	Antibiotic	100U/ml
Nystatin (mycostatin)	Antimycotic	50µg/ml
Amphotericin B (fungizon)	Antimycotic	0.25 – 2.5µg/ml

Notes: U: unit;
µg: microgram;
ml: milliliter.

G. Concluding Remark

Pawitan et al. (2013, 2014, 2014-2015) have developed simple methods for isolation and culture of BM-MSCs, AD-MSCs, and umbilical cord derived MSCs using direct MNC separation after centrifugation, simple washing step using a coffee filter, and multiple harvest explant method, respectively. In addition, a xeno-free cryopreservation medium used in house processed platelet lysate was developed. For

the success of isolation and culture of MSCs from various tissues, mastering an aseptic technique is very important.

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