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Isolation of Mesenchymal Stem Cells (MSCs) from Wharton's Jelly (WJ) Tissue of Human Umbilical Cord (hUC); a Protocol

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ABSTRACT

Mesenchymal stem cells (MSCs) with their spindle like shapes are a lineage of stem cells with the capacity to self-renew and differentiate into osteoblasts, adipocytes, and chondrocytes and with CD105, CD73, and CD90 expression and the lack of CD34, CD14, CD45, and HLA - DR expression. The immunomodulatory, angiogenic, antiapoptotic, antimicrobial, and antioxidative characteristics of these cells made them more attractive in the field of cell - therapy for several autoimmune and inflammatory diseases, including diabetes, neurological disorders, sepsis, cardiac ischemia, and GvHD. For this reason, various protocols have been proposed to isolate mesenchymal stem cells from different tissue sources, such as adipose tissue (AT), umbilical cord (UC), Wharton's jelly (WJ), bone marrow (BM), dental pulp, and even menstrual fluid. Considering the ease of access to the umbilical cord tissue and the fact that this tissue is rich in MSCs with embryonic origin and higher proliferation rate and lower senescence of the cells, the umbilical cord became a suitable source for explant MSC culture. In this study, we decided to introduce an explant culture protocol of MSCs that is less expensive and cost - effective achieving a high yield of MSCs.

INTRODUCTION

Mesenchymal stem cells (MSCs) are multilineage stem cells associated with CD105, CD73, and CD90 expression and the lack of CD34, CD14, CD45, and HLA - DR expression (1). MSCs are known for their ability to attach to plastic surfaces and differentiate into osteogenic, adipogenic, and chondrogenic lineages (1, 2). Due to various attractive immunological features, including immune system modulation and wound healing, mesenchymal stem cells have been widely used in regenerative medicine (3). Recently, special attention has been paid to MSC secretions containing extracellular vesicles (EVs), cytokines, growth factors, etc. which has made them more intriguing to scientists (4-6). Therefore, efforts to find a reliable source of MSCs that has the highest yield and lowest cost are inevitable.

Mesenchymal stem cells can be obtained from various sources with different biological characteristics, such as adipose tissue (AT), umbilical cord (UC), Wharton's jelly (WJ), bone marrow (BM), dental pulp, and even menstrual fluid (7-10). Wharton's jelly (WJ) is a gelatinous perivascular portion of the umbilical cord that is rich in MSCs and is easily and non-invasively accessible since UC is usually discarded after birth. Studies reported that WJ - MSCs strongly modulate the immune system and show higher proliferation rates in comparison with MSCs isolated from other tissues (11, 12). In a study conducted by Wang et al., it was reported that only WJ - MSCs can improve survival and increase bacterial clearance in septic mice. Additionally, in a study comparing human fetal BM- MSCs (F - BM - MSCs), WJ - MSCs, and AT - MSCs, it was showed that F- BM- MSCs and WJ - MSCs have higher proliferation

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rates than AT - MSCs, while the gene expression pattern of WJ - MSCs is similar to AT - MSCs (13).

In terms of MSC isolation methods, there are two main methods to culture mesenchymal stem cells from different sources: the enzymatic method and the explant culture method. In the first method, one or more proteolytic enzymes (including trypsin, collagenase, and hyaluronidase) are used to degrade the extracellular matrix (ECM) of tissue, and MSCs are released and cultured with appropriate media in cell culture flasks. Unlike the aforementioned method, in the second procedure, by considering the migratory potential of MSCs, the target tissue is cut into small pieces and placed into a cell culture flask or plate. Appropriate media is added to the flask and wait until MSCs migrate out of the target tissue and cover the surface of the flask. This method is easy and less - expensive than the enzymatic method (11, 14).

In this study, we try to provide an easy and efficient protocol to obtain the maximum number of WJ - MSCs with the minimum facilities and the lowest expenditures. In the next step, we focused on the collection of serum - free media for MSC - EVs extraction.

Table 1. Comparison between the explant cell culture method and the enzymatic cell culture method

Explant culture method	Enzymatic method
Higher yield	Low yield
Intact ECM	Dissociated ECM
Higher cell viability	Proteolytic stress
Higher proliferation rates in a shorter time	Longer population doubling time
Less heterogeneous cell populations	Heterogenous cell population
Shorter time for cell adherence	Longer time for cell adherence
Release of cytokines and growth factors into the medium	

The advantages and disadvantages of each method are discussed in the table.

MATERIALS

***All materials mentioned below should be sterile**

1. 50 ml conical tube (SPL, Cat: 50050)
2. 15 ml conical tube (SPL, Cat: 50015)
3. Phosphate buffered solution (PBS), pH= 7.2-7.4
4. Plastic petri dish or autoclaved glass petri dish (SPL, Cat: 20100)
5. 3 ml sterile pipette (Biologix, Cat: 30-0138A1)
6. 10 cm cell culture plate (SPL, Cat: 20150)
7. T25 cell culture flask (SPL, Cat: 70025)
8. T75 cell culture flask (SPL, Cat: 70175)
9. Syringe needle
10. Dulbecco's Modified Eagle's Medium / Nutrient Mixture F12 (DMEM/F12), pH= 7.2-7.4 (Gibco, Cat: 11320033)
11. Fetal bovine serum (FBS) (Gibco, Cat: 16000044)
12. Penicillin / streptomycin (Gibco, Cat: 15140122)
13. Trypsin - EDTA 0.5% (Gibco, Cat: 15400054)
14. Mouse Anti - Human CD45 - FITC (BioLegend, Cat: 304005)
15. Mouse Anti - Human CD34 - PE (IQ Products, Cat: IQP - 144R)
16. Mouse Anti - Human CD73 - PE - cy7 (BioLegend, Cat: 344009)
17. Mouse Anti - Human CD90 - APC (BioLegend, Cat: 328113)
18. Mouse Anti - Human CD105 - PE (BioLegend, Cat: 323205)
19. Mouse Anti - Human CD14 - FITC (BioLegend, Cat: 325603)
20. EXOCIB kit (Cibbiotech, Cat: 3603-450)
21. CD9 (C-4) (Santa Cruz Biotechnology, Cat: sc - 13118)
22. CD63 (MX - 49.129.5) (Santa Cruz Biotechnology, Cat: sc - 5275)
23. CD81 (B-11) (Santa Cruz Biotechnology, Cat: sc - 166029)
24. Mouse anti - rabbit IgG - HRP (Santa Cruz Biotechnology, Cat: sc - 2357)

Equipment:

1. Inverted microscope (OPTIKA, model: IM - 3 series)
2. Laminar flow cabinet (JAL TAJHIZ, model: JTLVC2S)
3. CO2 incubator (Memmert, model: ICO50)
4. Sterile or autoclaved scissors, forceps, and scalpel (KLS martin, Cat: 11-100-11-07, Cat: 12-100-10-07, and Cat: 10-155-10-04, respectively)
5. BD FACS Calibur Flow Cytometer (BD Biosciences, San Jose, CA)

6. Dynamic light scattering (DLS) (Zetasizer ver.7.11)
7. Transmission electron microscope (TEM) (Zeiss LEO 906)
8. Field emission scanning electron microscope (FESEM) (MIRA3 - TESCAN)

Procedure:

A. Transferring an umbilical cord tissue to the laboratory

1. The umbilical cord was received from the Royan Institute, Tehran, Iran. After obtaining informed consent from a patient.
2. After receiving the umbilical cord, it was sent to the laboratory under cold and sterile conditions in a transferring buffer containing PBS. All the below experiments were carried out within 24 hours after the delivery and under the sterile conditions of a laminar flow cabinet. We do not recommend using any disinfectant in this step to wash the umbilical cord since it can damage the tissue structure and cells inside.

B. Removing the vessels and cutting the tissue

1. The umbilical cord tissue was cut into 3-4 cm pieces with autoclaved scissors on a sterile plate.
2. Each section was placed into a 50 ml conical tube containing PBS, to prevent each part from dryness.
3. To eliminate the red blood cells (RBCs) from the umbilical cord tissue, conical tubes containing umbilical cord pieces were shaken vigorously for 30 seconds.
4. After 30 seconds, a piece of umbilical cord was placed into a sterile plate with sterile forceps to resect the arteries and a vein. The umbilical cord contains two arteries with a small diameter and one vein with a large diameter (Fig. B.1.H).
5. To remove the vessels, first, the outer surface of the cylindrical umbilical cord tissue was cut just above the space between the two arteries with a scalpel.
6. Then, the first and the second arteries were removed with the help of a scalpel that cuts the tissue below these vessels.
7. All parts of the umbilical cord tissue that certainly do not contain any arteries were cut into smaller pieces (0.5-1 cm) and they were set aside.
8. The last part that remains in the umbilical cord tissue and is close to the vein was discarded.

C. WJ - MSC explant culture process

9. The parts left aside were cut into pieces of 0.5-1 cm. The jelly - like surface (the inner part of the umbilical cord, which is called Wharton's jelly) was scratched with a sterile scalpel or a sterile needle to allow easier migration of mesenchymal stem cells from the tissue.
10. Using a sterile pipette or a needle connected to a syringe, the tissues were placed into a sterile cell culture plate or T25 cell culture flask so that the inner, jelly - like surface of the tissue is placed towards the bottom of the flask or plate.
11. For the tissues to stick to the bottom of the plate or flask, leave the plate or flask aside for a short time (about 3 minutes). At this stage, if the tissue is not properly attached to the bottom of the plate, after adding the culture medium, the tissues will float.
12. Next, DMEM / F12 medium enriched with 15-20% FBS and containing 1% penicillin/streptomycin was slowly added to the tissues. The added medium should be enough to just cover the tissues and the bottom of the flask or plate and the tissues should not float in the liquid. The initial recommended volume to be added to the T25 flask is 3 ml and about 6 ml to a 10 cm cell culture plate. If the volume of the medium is very low, FBS - enriched medium can be added to them again the next day.
13. The flasks and plates were placed gently and carefully in a CO2 incubator with 5% CO2, 95% humidity, and 37 ºC temperature.
14. After 5-7 days, the medium of flasks or plates was changed. Changing the medium was done with medium enriched with 15-20% FBS containing 1% penicillin / streptomycin. The first time the medium was changed, the medium inside the flask was thick and viscous.
15. After 10-15 days, the cells migrated out of the tissues to such an extent that the tissues needed to be moved from their current location to a different place in the same flask or plate. At this stage, if the number of migrated cells was very high and they became condensed, subculture was required.

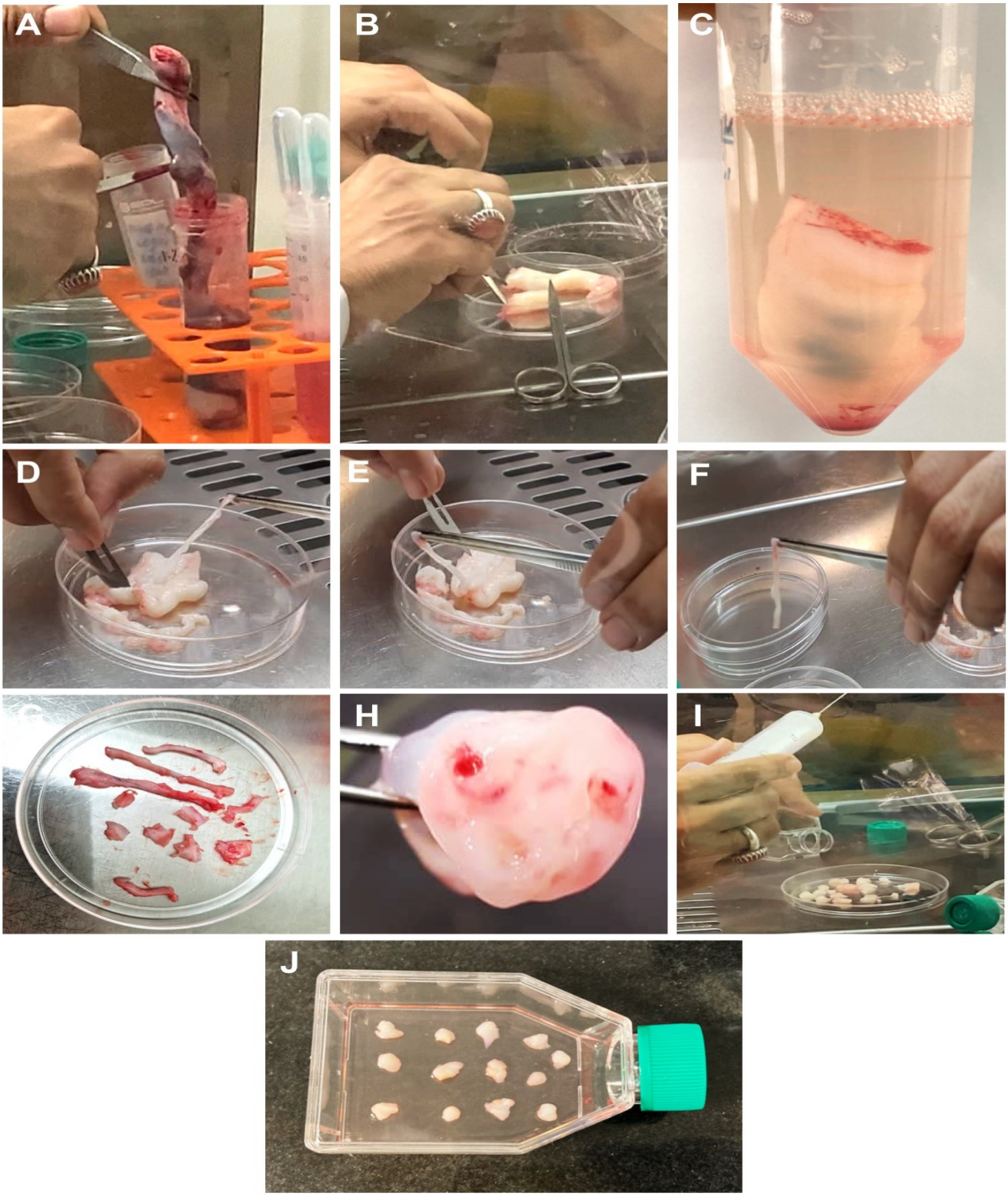

FIGURE C.1. Stages of MSC explant culture in a T25 flask

(A) The UC tissue moved out of the sterile conical tube containing transferring medium. (B) The UC tissue was cut into 3-4 cm pieces. (C) Each section was put into a 50 ml conical tube containing PBS and was shaken vigorously for 30 seconds. (D) & (E) A piece of umbilical cord was placed into a sterile plate with sterile forceps to resect the arteries and a vein. (F) The resection of one of the arteries was shown. (G) Two arteries and one vein were resected from tissue and the other parts were cut into small pieces for further steps. (H) A cross - section of the UC shows two arteries with a small diameter and one vein with a large diameter. (I) Using a needle connected to a syringe, the tissues were placed in a T25 cell culture flask. (J) The FBS - enriched medium was slowly added to the tissues. The flask was gently and carefully placed in a CO2 incubator.

Tips:

1. All materials used in this process should be sterile.
2. It is recommended that the process of explant MSC culture should begin as soon as possible after delivery.
3. When 1 cm pieces of tissue are left aside in flasks or plates, the time should not exceed more than 10 mins since the tissue will be dried.
4. Handling and transferring flasks and plates containing tissue pieces should be done carefully and gently.
5. Each time, changing the medium should be done according to the point that 2/3 of the previous medium was removed and 1/3 of it containing growth factors and secretions from the cells remained inside the flasks or plates. Then, 2/3 of the fresh medium enriched with FBS was added to the flasks or plates.
6. Due to the high proliferation rate of WJ - MSCs, flasks and plates needed to be checked daily.

Troubleshooting:

1. If the MSCs do not migrate out of the tissue pieces within the stated time above, the tissues should not be discarded. In some cases, MSCs migrate with a delay of 2 weeks (or more) and patience is required.
2. If the MSCs do not migrate out of the tissue pieces even after 4 weeks, it might be due to the large size of the tissue. You should cut them into smaller pieces and scratch the inner side of them with the scalpel.
3. The preferred medium is DMEM / F12 medium since it supports cells to grow and proliferate faster. However, DMEM medium can use interchangeably by considering the longer proliferation rate it takes.
4. If the tissue pieces float in the flask or the plate, decant the medium and let the tissue pieces attach to the flask or the plate. Then, add fresh medium to them.

D. Subculturing process of MSCs

1. After the cells reached 80% confluency, subculturing is required. In this step, first, the supernatant of the cells should be removed.
2. Then, the flask was washed 2 times with sterile PBS (each time about 2 ml to the T25 flask and about 3 ml to the 10 cm plate and T75 flask). This way, the flask and the plate were washed with PBS thoroughly. The purpose of this step is to remove the excess FBS remaining from the FBS - enriched medium.
3. In this step, the cells detached from the surface of the flask or the plate and from each other. 1X Trypsin - EDTA was added to the flask or the plate (2 ml to the T25 flask and 3 ml to the 10 cm plate and T75 flask) and the flask or the plate was placed in the cell incubator for 2 minutes.
4. After 2 minutes of incubation, the flask or the plate was taken out. By pipetting, the cells became single cells and did not have cell clamps. Then, the separation of cells was checked under an inverted microscope.
5. At this stage, neutralization of trypsin by FBS should be done. For this purpose, the FBS - enriched medium (15-20% FBS) should be used. FBS - enriched medium (the same volume as trypsin) was added to the flask or the plate. The pipetting should be done slowly so that foam or bubbles does not form. The formation of foam or bubble is due to the presence of proteins in FBS.
6. The cell suspension containing 1X trypsin - EDTA inside the flask or the plate was transferred to the 15 ml conical tube and the tube was centrifuged at 1,500 RPM for 5 minutes until the cells accumulated at the bottom of the tube. The liquid inside the tube was decanted and the cell pellet at the bottom of the tube was rigorously vortexed until it was completely mixed.

Then, depending on the size of the flask or the plate, the medium enriched with 15-20% FBS was added to the cell pellet inside the conical tube. The surface of the flask or plate was getting wet by adding 1-2 ml of enriched medium and then the cell suspension was added to the flask or the plate.

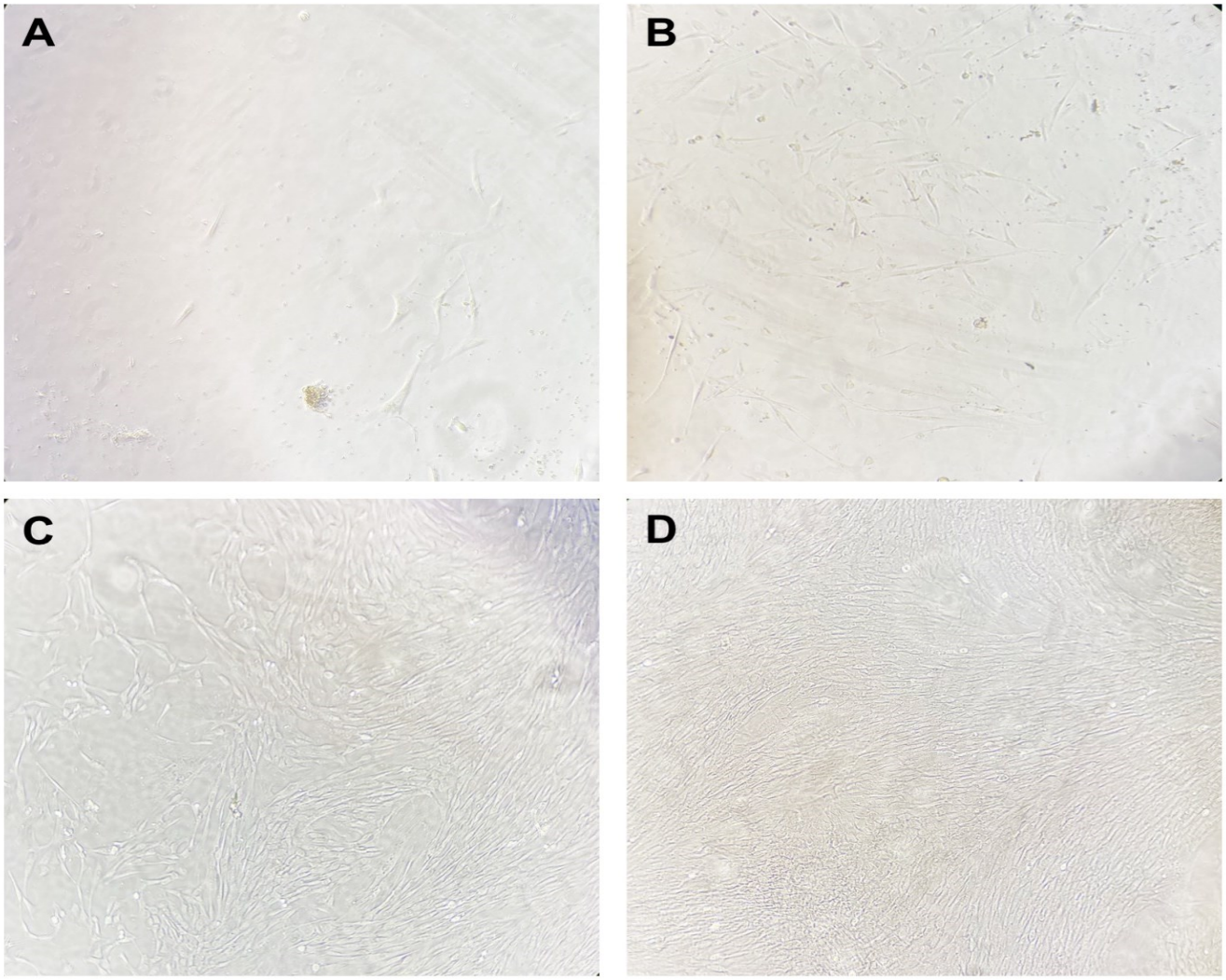

FIGURE D.1. Stages of MSCs growth and proliferation
(A) < 10% confluency, after 10 days. (B) 40% confluency, day 14. (C) 60% confluency, day 16. (D) 90% confluency, day 18.

Tips:

1. In step 3, the time required to incubate cells with trypsin can be varied depending on the cell type, the type and the brand of trypsin, and the concentration of trypsin.
2. Pipetting after trypsinization is very crucial to single the cells. However, excessive pipetting can cause cell damage and death.
3. The foam or bubble formation can be harmful to the cells and can cause cell death.

Troubleshooting:

1. A low population of cells seeding into the flask can cause shape transformation from a spindle-shape to a circular shape. It is highly recommended that the high population of cells will be seeded into the flask because these cells needed to interact and transfer signals with each other.
2. When cells become loose and the circular rather than spindle - shape or granule formation observed in the cytoplasm of them, the flask should be discarded.
3. If the cells do not detach after 2 mins and pipetting, the flask can stay in the incubator for 5 mins. However, longer periods can increase the rate of cell death.

Pipetting is very important in steps 4 and 5 to detach cells to become single cells. Cell clamps can cause disintegrity in cell growth at the flask surface and cause spherical growth of MSCs.

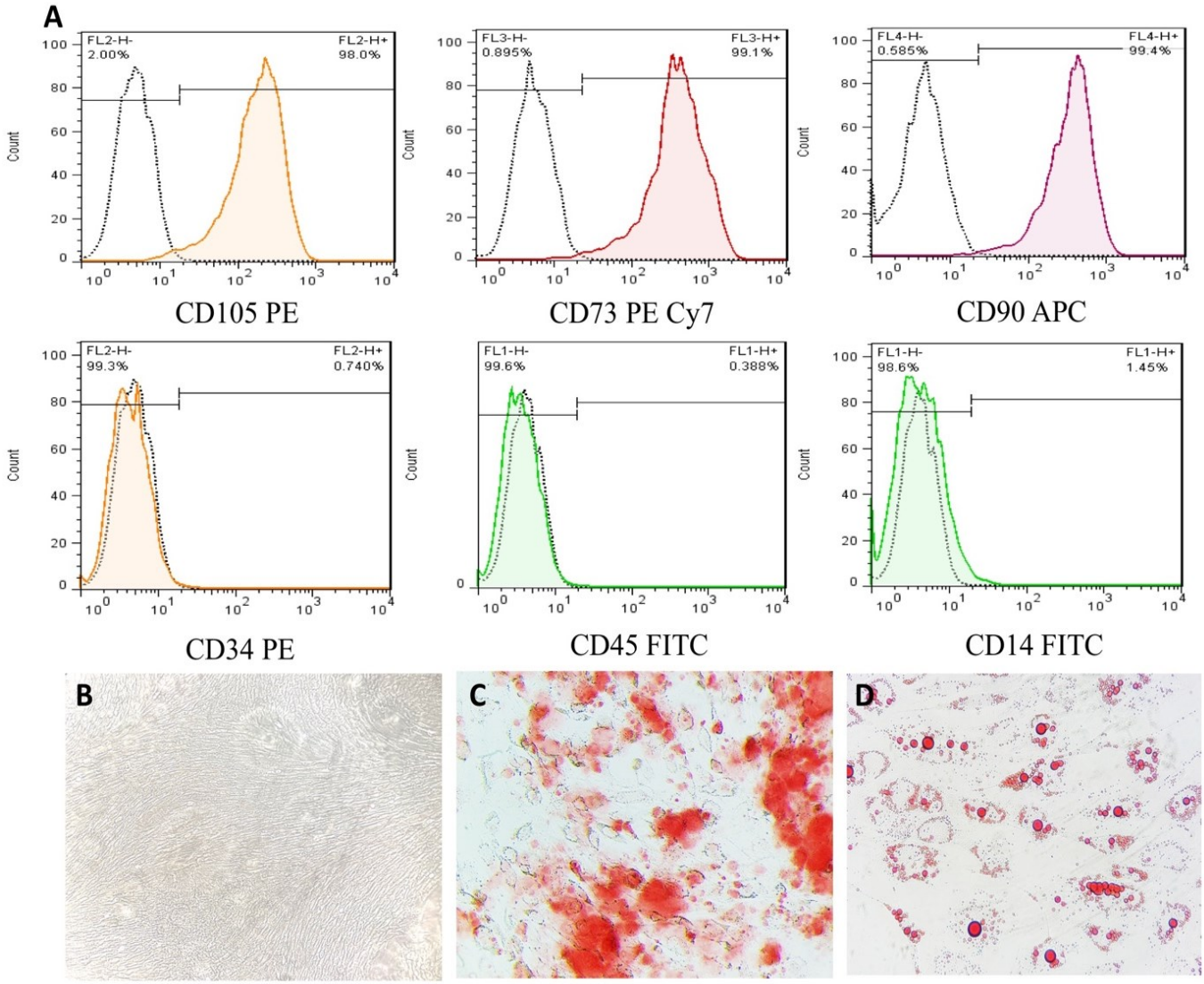

FIGURE D.2. Characterization of MSCs by surface marker expression and differentiation to osteoblastic and adipocytic lineages (A) The expression of CD105, CD73, and CD90 markers were positive and the expression of CD34, CD45, and CD14 markers were negative on the surface of the MSCs. (B) MSCs were spindle - like shape under inverted microscope (X40). (C) MSCs differentiated to osteoblasts followed by Alizarin Red staining process (X100). (D) MSCs differentiated to adipocytes followed by Oil Red O staining process (X400).

E. Supernatant collection for MSC-EVs extraction

1. After the MSCs reached 90% confluency in a T75 flask, DMEM/F12 medium with 15% FBS was replaced with medium without FBS. The supernatant of the cell (containing 15% FBS) was removed from the flask by a sterile pipette and then add the fresh medium with 0% FBS.
2. After 3-4 days, the yellowish supernatant was collected from the flasks by a sterile pipette and poured into a sterile conical tube or sterile bottle. The supernatants were stored at -20 ºC temperature (or -80ºC for longer periods).
3. After supernatant collection, the cells were checked under an inverted microscope to investigate their condition.

The MSC - EVs were extracted from the collected supernatant by the EXOCIB kit instruction.

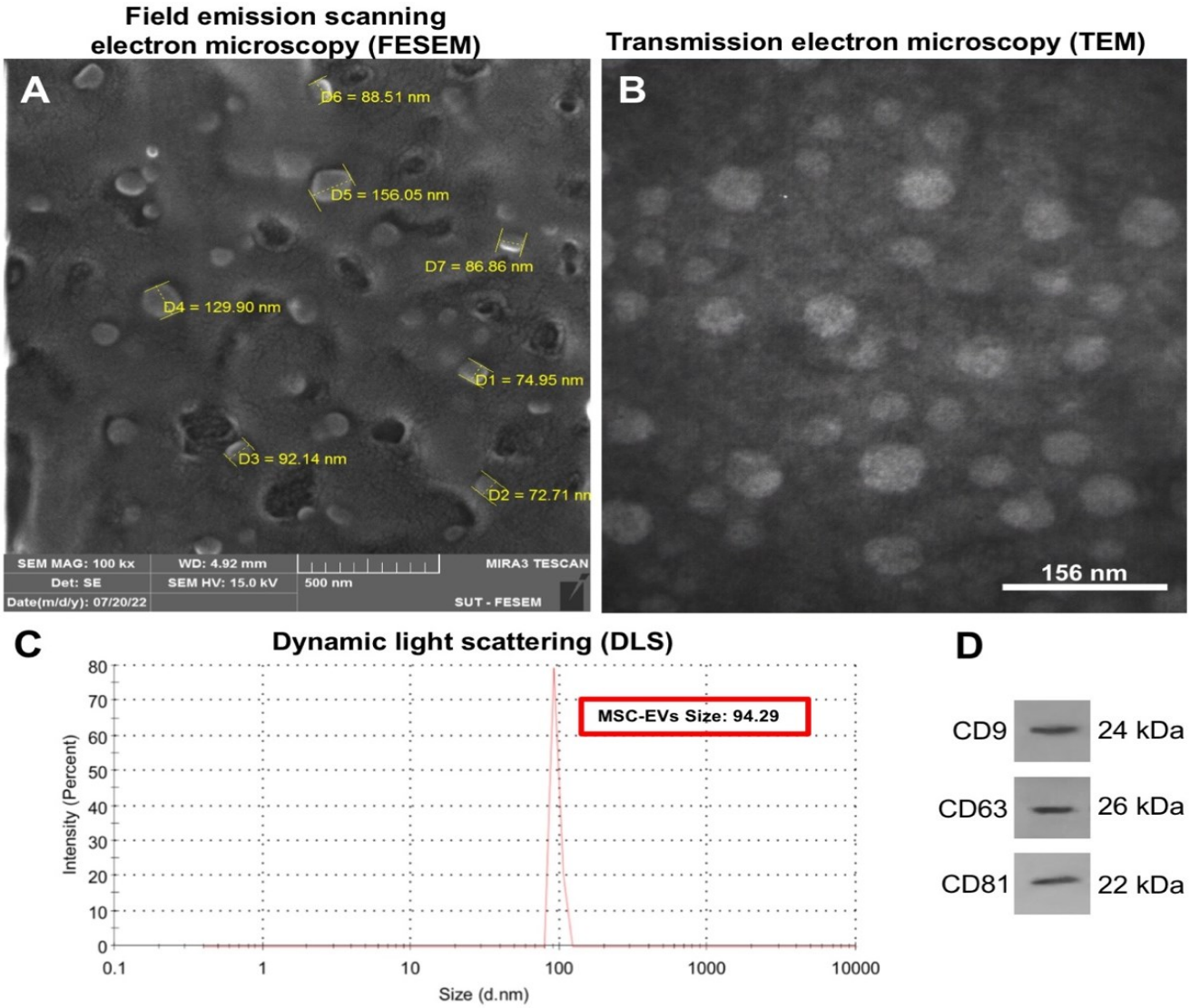

FIGURE E.1. Characterization of MSC-EVs by size, shape, and surface marker expression
(A) & (B) The shape of MSC - EVs was characterized by the field emission scanning electron microscopy (FESEM) and transmission electron microscopy (TEM). (C) The size of MSC - EVs was characterized by the dynamic light scattering (DLS). The mean size of MSC - EVs extracted from the collected supernatant was 94.29 nm. (D) The expression of CD9, CD63, and CD81 on the MSC - EVs was determined by the western blot technique.

Tips:

1. Always follow the rule of replacing 2/3 of the supernatant with fresh medium.
2. It should be noted that in the first step, the minimum volume of medium that can be poured into a flask should be added because the goal is to obtain a concentrated supernatant containing MSC - EVs.
3. It took about 3-4 days (or even longer) for the color of the medium to change from pink to yellow, indicating the activity and the proliferation of MSCs and the release of MSC - EVs into the supernatant.

In the case of using DMEM medium instead of DMEM / F12, it should be considered that it takes longer time to turn yellow, therefore; it is recommended to change the medium after 4 days.

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CONFLICT OF INTEREST
All authors declare no conflicts of interest.
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