

Simple Procedure for the Isolation of Mesenchymal Stem Cells from Different Parts of the Human Umbilical Cord

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Abstract

The umbilical cord and placenta are both sources of mesenchymal stem cells (MSCs) that are promising for cell-based therapy. Furthermore, compared to other MSCs sources, they are easy to obtain with no invasive procedures. This study presents an adapted method for stem cell isolation from three different parts of the human umbilical cord, including Wharton's jelly (WJ), cord lining (CL), and cord-placenta junction (CPJ). The isolation consists of sample preparation, tissue dissection into distinct anatomical regions, mincing and enzyme digestion, and explant culturing. In addition, we monitored when the cells migrated from the explant to the bottom of the cell culture dish and passed the cells after they became confluent. This study found that WJ cells were the first to reach confluence at Passage 0 (P0). In contrast, CL cells needed the longest time to get confluence at P0 but displayed faster cell growth after subsequent passages (P1-P2). In addition, CPJ cells showed growth retardation after P1 and P2. Altogether, we could extract the MSCs from umbilical cord tissue explants by using DMEM supplemented with 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin as general cell culture medium and omitting the use of gentamicin. However, the MSCs may need a more complex specified medium for optimum cell regeneration for further cell expansion.

Keywords: mesenchymal stem cells, umbilical cord, Wharton's jelly, cord lining, cord-placenta junction.

INTRODUCTION

Stem cells are a population of cells having the properties to renew themself and differentiate into other cell types. Those properties make stem cells an exciting topic in regenerative medicine. Stem cells may come from embryos (embryonic stem cells/ESCs), induced from adult cells (induced pluripotent stem cells), or fetal or adult tissues (Bacakova, *et al.*, 2018). Embryonic and induced pluripotent stem cells have a barrier to applications in humans. Despite ethical issues, especially ESCs, they can potentially cause teratomas after

transplantation (Lee, *et al.*, 2013, Tao, *et al.*, 2018). Mesenchymal stem cells or mesenchymal stromal cells (MSCs) are any cell in the stroma (connective tissue) that possesses stemness (Short, *et al.*, 2003). They are considered the most effective therapy tool (Saeedi, *et al.*, 2019). Multiple mechanisms

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(paracrine, trophic, immunomodulatory, and differentiation) are simultaneously activated in MSCs during the regeneration of damaged tissues (Arutyunyan, et al., 2016). Mesenchymal stem cells have been isolated from adult tissues (bone marrow, adipose tissue, inner organs, blood vessels) and birthderived tissues (amniotic fluid, amniotic membrane, umbilical cord, placenta) (Elahi, et al., 2016). Although bone marrow-derived mesenchymal stem cells (BM-MSCs) are considered the gold standard for clinical research, obtaining them can result in pain, bleeding, or infection (Berebichez-Fridman and Montero-Olvera, 2018). This leads to efforts in harvesting MSCs with less or non-invasive procedures.

Umbilical cords, a source of MSCs can be obtained from less or non-invasive procedures (by Caesarian section or vaginal delivery) (Pereira, *et al.*, 2008). Stem cells derived from the umbilical cord (UC-MSCs) have superior properties to BM-MSCs. They have a higher cell proliferation rate, a higher frequency of CFU-fibroblasts, a higher overall immunomodulatory effect with increased expression of potent immunosuppressive factors (CD200, leukemia inhibitory factor, TGF- β 2), greater anti-inflammatory effects, lower immunogenicity, and undergoing slower senescence (Chia, *et al.*, 2021).

Isolation of mesenchymal stem cells has been perceived as a time-consuming, labor-intensive, and cost-ineffective procedure (Sibov, et al., 2012; Yoon, et al., 2013; di Scipio, et al., 2017). The procedure often involves using different combinations of enzymes (collagenase, hyaluronidase, DNase, trypsin) and specified medium (Tsagias, et al., 2011). Beeravolu, et al. (2017) have successfully isolated MSCs from the human placenta and umbilical cord by preparing minced explant pretreated with recombinant enzyme TrypLE to avoid zoonosis disease transmission and halal issues in the future application, and also TrypLE is commonly used in general cell culture lab for cell detachment. In this study, we adapted

this protocol to isolate MSCs from the umbilical cord in our mammalian cell culture lab. We found that this procedure is readily adapted in the common cell culture laboratory that previously had not been experienced in MSCs isolation.

MATERIALS AND METHODS

We adapted the procedure Beeravolu, *et al.* (2017) reported. In addition, we modified some steps based on the availability of reagents in our lab, which include the omission of gentamicin (0.1%, $50 \mu g/mL$) in the collection and growth media. On the other hand, we performed surface sterilization of the umbilical cord by briefly dipping the tissue in 70% ethanol after washing and before the dissecting steps. While dissecting the samples, we used icecold PBS (1x, pH 7.4) to keep them moist and avoid keeping them on ice, as the melting ice increases the chance of contamination. Our detailed modified protocol is as follows.

Preparation of Human Umbilical Cord

Prior to processing the umbilical cord, space for washing and cutting the umbilical cord was allocated and cleaned thoroughly. All surfaces were sterilized with 70% alcohol and covered with aluminum foil. A burner was prepared to sterilize the surgical tools while cutting the larger umbilical cord into smaller pieces.

The umbilical cord and placenta were obtained from one of the authors, who had undergone a cesarean surgery by a gynecologist and immediately transferred to the lab by placing it on ice in a cooler box. A sample of about 5 cm long of the cord-placenta junction (CPJ) and about 5 cm long of the umbilical cord (UC) were taken from the umbilical cord using sterilized scissors and forceps. First, the sample was washed thoroughly with cold PBS (Merck, Darmstadt, Germany) until all blood cells and clots were removed. Then, the excised tissue was dipped briefly in 70% ethanol (Merck) and subsequently sank in the transfer medium



consisting of 1% penicillin-streptomycin (GibcoTM, New York, USA) in high glucose DMEM (Sigma, St. Louis, USA). All further work was done inside of a Class II BSC.

Dissection of Human Umbilical Cord

The tissue sample was dipped briefly in 70% ethanol and washed with PBS 2x10 mL. It was kept in the transfer medium before further processing. The sample was cut into two distinct anatomical regions (UC and CPJ). Each part was placed into separate dishes. The UC was held using forceps, and any twists were released. It was cut using a scalpel along the length to completely expose the blood vessels (vein and arteries). The vessels were removed. WJ was scraped away from opened UC and around BVs using a scalpel and collected into a separate dish. The resulting cord lining (CL) was then collected into a different dish.

Mincing and Partial Enzymatic Digestion

Each tissue was chopped into 1-2 mm pieces. All liquid was removed from the tissues. Several undigested tissue pieces of each region (CL, WJ, CPJ) were cryopreserved in 1 mL of freezing medium consisting of 10% DMSO (Sigma) in the growth medium. The remaining tissue pieces of each region were digested with 5 mL of TrypLE (GibcoTM) at 37° C, 5% CO₂, 30 min. Partially digested tissue pieces were neutralized with 5 mL of growth medium consisting of 10% FBS (GibcoTM), 2 mM L-Glutamine (GibcoTM), 1% penicillin-streptomycin (GibcoTM) in high glucose DMEM (Sigma). They were transferred into 50-mL tubes and left to settle for 3 min. Any liquid was removed from the tissue pieces.

Explant Culturing

Five to seven partially digested tissue pieces of each region were plated onto a T-25 flask. Three mL of growth medium were carefully added to the flask. The explants were incubated at 37°C, 5% CO₂, for three days. Any disturbance to the

flask was avoided during incubation. The remaining tissue pieces should be cryopreserved in 1 mL of a freezing medium.

Cell growth from the explants was monitored daily. Any contaminated culture vessels were discarded accordingly. The medium was replaced every 3 days. Subculture was done when cell growth reached 70% confluence. Cultures were cryopreserved from a larger vessel (a 10-cm dish) at P2.

Observation of Cell Growth and Morphology

We observed the growth and morphology of the extracted cells (spindle-shaped, fibroblast-like/fibroblastoid) by performing a microscopic investigation, then evaluated the cell morphology compared with the reference.

RESULTS

Steps in Isolating Mesenchymal Stem Cells from Human Umbilical Cord

Using the adapted version of Beeravolu, *et al.* (2017), MSCs were successfully generated from cord lining and Wharton's jelly of the umbilical cord and cord-placenta junction. The steps in isolating MSCs from the human umbilical cord-placenta were 1) preparation of umbilical cord-fetal placenta junction, 2) dissection of the umbilical cord and cord-placenta junction, 3) mincing and partial enzymatic digestion, and 4) explant incubation and culture monitoring (Figure 1).

In our adapted version, the cord-placenta was dipped briefly in 70% ethanol and kept in transfer medium in Step 1 and Step 2. We also omitted the use of gentamicin from the beginning of culture establishment to expansion. The omission of gentamicin does not significantly affect the culture. We obtained cells morphologically similar to those of Beeravolu, *et al.* (2017). Those cells are fibroblast-like (fibroblastoid), a characteristic morphology of mesenchymal stem cells (Hematti, 2012; Denu, *et al.*, 2016; Tanaka, *et al.*, 2022).



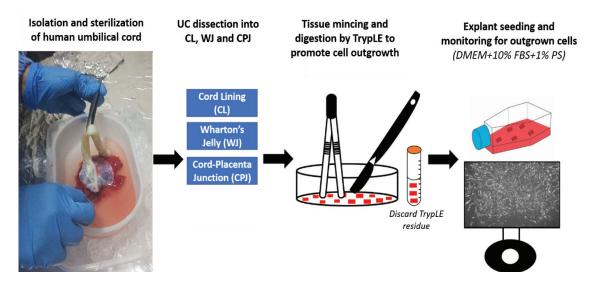


Figure 1. Schematic representation of the isolation of stem cells from the human umbilical cord/placenta. Step 1. Quick sterilization with 70% ethanol to remove blood traces and blood clots from the sample by washing extensively in PBS. Step 2. Dissection and isolation of umbilical cord (UC) and cord-placenta junction (CPJ), followed with separation of the UC into cord lining (CL) and Wharton's jelly (WJ) using forceps and a scalpel. Step 3. Tissue mincing into 1- to 2-mm pieces and partial digestion with recombinant TrypLE. Step 4. Explant incubation and culture monitoring.

Culture Condition from Initial Outgrowth to Several Passages

Initial outgrowths were observed on different days for different tissues. Cord-placenta junction was the first to have the initial outgrowth (Day 6), whereas the initial outgrowth appeared at the same time for cord lining and Wharton's jelly (Day 7) (Figure 2A). Explants of each tissue have different numbers of cells at initial outgrowth. WJ had the most cells, followed by CPJ and CL at initial outgrowth.

Explants from different tissues have a distinct ability to reach confluence (70-80%). Those cells at such confluence were obtained at Passage 0 (P0). WJ was the first to reach confluence (D9), followed by CPJ (D10), and CL (D15) (Figure 2B). Cells from different tissues had different abilities to multiply. CL stayed the shortest in P1 (5 days), followed by CPJ (10 days), and WJ (11 days) (Figure 3A). CPJ was decided to be subcultured to P2 because there was no apparent growth on that

day compared to previous days. After two days at P2, CL and WJ had similar confluence, whereas CPJ had the least (Figure 3B).

Cell Morphology

Cells obtained in this study can be differentiated into type 1 and type 2 cells (Figure 4). Type 1 cells were wide and flattened, while type 2 cells were long, slender, and spindle-shaped. In addition, the number of type 1 cells decreased along the passage (P0-P2).

DISCUSSION

Mesenchymal stem cells have been isolated from various body parts including extraembryonic tissues such as the umbilical cord and placenta. The use of extraembryonic tissues possesses no ethical concerns considering that they are generally discarded after delivery and involve a more comfortable collection method (Marcus and



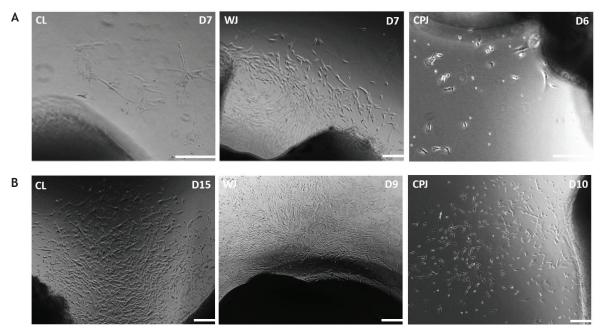


Figure 2. A. Representative explants of each tissue for initial outgrowth. B. Representative cells outgrew from explants of each tissue that reached confluence (70-80%). Scale bars: 100 µm.

Woodbury, 2008). Generally, MSCs can be obtained through 3 different methods: enzymatic digestion (Tsagias, *et al.*, 2011), explant method (Hasan, *et al.*, 2017), and a combination of explant-enzymatic method (Beeravolu, *et al.*, 2017). Additionally, Zheng, *et al.* (2022) modified the explant-enzymatic method by doing the enzymatic digestion of the explant after 7 days of culture in a CO₂ incubator (Zheng, *et al.*, 2022).

We performed MSCs isolation according to Beeravolu, *et al* (2017) protocols to isolate stem cells from the umbilical cord by using a common culture medium; high glucose DMEM supplemented with 10% FBS, 100 IU/mL penicillin, and 100 μ g/mL streptomycin, and obtained cells derived from umbilical cord lining. We added the sterilization step by quickly dipping the isolated tissue in 70% ethanol to disinfect the tissues. Additional antibacterial protection was achieved by dipping in an antibiotics-containing transport medium.

Although we omitted gentamicin in our culture, we could still obtain cultures free of bacterial contamination. Gentamicin is a xenobiotic, and it may affect MSCs. Gentamicin use at the recommended concentration (50 μg/mL) does not affect cell viability and proliferation of bone marrow-derived human MSCs (Chang, *et al.*, 2006). But, the activity of alkaline phosphatase (a marker for osteogenic differentiation) decreased during the early stage of osteogenesis (Days 4 and 8) in cells pre-exposed to gentamicin at 50 μg/mL or greater concentrations. This finding is similar to that of Kagiwada, *et al.* (2008) in which gentamicin at 50 μg/mL or higher inhibits osteoblastic differentiation.

Several aspects should be considered when establishing the culture of MSCs. They are initial culture condition (initial outgrowth appearance, cell number at initial outgrowth, duration of explants in primary culture), cell number at harvest/seeding for next passage, and cell morphology. Here, we obtained MSCs from CPJ, CL, and WJ. We found that harvesting the cells and reseeding may promote cell growth in the case of WJ and CL, but not CPJ. In addition, the cell growth and proliferation decreased when we kept maintaining the cells in



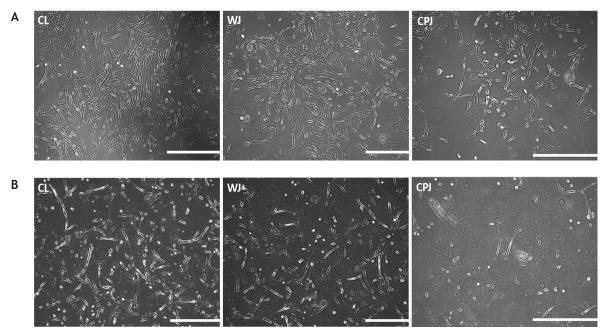


Figure 3. A. hUC stem cells at P1 (D20). B. hUC stem cells at P2 (D22). Scale bars: 100 μm.

DMEM medium, especially for CPJ. In contrast to Beeravolu, *et al* (2017) report, we did not obtain highly proliferating CPJ. This can be interpolated from the low cell numbers of any passage (P0-P2) (Figures 2 and 3).

The difference in cell number at initial outgrowth affects the time needed to reach confluence. We found that WJ has the most cells at initial outgrowth and reaches confluence the fastest. This is similar to the one observed by Subramanian, *et al.* (2015). They found that WJ had the most

number of surviving cells and produced monolayers faster than PV (perivascular area), SA (subamnion), AM (amnion), and MC (mixed cord). Nevertheless, even though CL has the least cells at initial outgrowth, it showed a higher proliferation rate than WJ. The number of cells at initial outgrowth is affected by the ability to migrate out of the tissue piece (explant).

Explants should be incubated appropriately in the incubator to ensure adequate cell number is reached before subculturing. Some researchers left

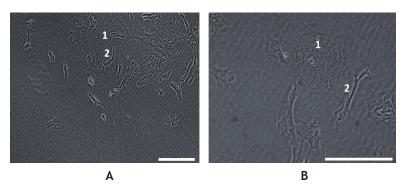


Figure 4. Two types of cells were found in hUC-MSC culture. A. At a magnification of 4x objective. B. At a magnification of 20x objective. Scale bars: 50 μm.



the explants for two weeks before removing them (Yoon, et al., 2013; Hassan, et al., 2017; Semenova, et al., 2021). Another study even showed the explants incubation for three weeks (Mennan, et al., 2013). Earlier explant removal may cause fewer cells for the next passage.

Low seeding density increases the cumulative population doubling level and speeds up aging. In another circumstance, later explant removal will cause over-confluence at explant-toexplant and entire vessel level. Over-confluence will cease cell division (due to contact inhibition) and trigger cells to differentiate. Hence, explant removal should be carefully considered to avoid leaving the cells at a low number or in a state of over-confluence. However, despite discarding the explants, Zheng, et al. (2022) have successfully extracted cells from the explants after the incubation with the medium inside the incubator.

Cell numbers of CPJ seem to be the lowest in either passage (P0-P2). Therefore, subculturing for all cultures was decided to be done after some days (5-11 days from P1 to P2) since there's no apparent growth (no increase in confluence). However, this action resulted in a lower number of cells (especially in the case of CPJ) after the subsequent passage and decreased the ability to expand due to senescence.

CL and WJ have similar confluence from P0 (around the explant) to P2. In addition, many studies have demonstrated the more significant potential of WJ over other extraembryonic tissues to generate hUC-MSCs (Manochantr, *et al.*, 2013; Li, *et al.*, 2014; Chen, *et al.*, 2015; El-Demerdash, *et al.*, 2015; Pu, *et al.*, 2017). But, the potency of CL cannot be ignored. Although it seems to conflict with other results, Stubbendorff, *et al.* (2013) found that MSCs from cord lining have higher proliferation and migration rates than those from cord blood, placenta, and Wharton's jelly.

Like other tissues, extraembryonic tissues consist of different cells. Although not as extensive as the enzymatic method in producing heterogeneous cell mixtures, MSCs generated from

this method should be closely monitored. Two morphologically distinct cells are observed from P0 to P2. Type 1 cells are wide and flattened and type 2 cells are long, slender, and spindle-shaped. The number of type 1 cells decreased along the passage. This phenomenon is similar to the finding of Singh, et al. (2019). They observed that migrated cells presented as a heterogenous population and became more uniformly spindle-shaped with a homogeneous population by the third passage. According to Karahuseyinoglu, et al. (2007), type 1 cells express vimentin (a mesenchymal marker) and pan-cytokeratin (an ecto/endodermal marker). Type 2 cells only express vimentin. This finding may explain why the number of type 1 cells decreased from P0-P2. They may become more committed and less proliferative than type 2 cells. Additionally, media modification or other supplementation needs to be considered for better cell growth and recovery instead of maintaining the cells in the DMEM medium for longer.

CONCLUSION

We isolated MSCs from the umbilical cord and cord-placenta junction by an explant-enzymatic method previously reported by Beeravolu, et al. (2017). We adapted the procedure in our lab by utilizing the available common reagents and supplies for cell culture works with omitting gentamicin usage. Additionally, a typical cell culture medium, DMEM, supplemented with 10% FBS, 100 IU/ mL penicillin, and 100 μg/mL streptomycin, can be used to isolate and induce cell growth from explants without adding specific growth factors. We found that the process is simple and adaptable to the general mammalian cell culture lab that previously did not work on stem cells, and the use of gentamicin is optional. However, by this procedure, it is best to focus on isolating cells from WJ and CL of the umbilical cord with the highest survival and growth rate. Furthermore, the MSCs may need a more complex specified medium for optimum cell regeneration for further cell expansion.



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