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# Isolation of Mesenchymal Stem Cell from Wharton's Jelly of Human Umbilical Cord for Application in Wound Healing

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**Abstract:** Mesenchymal stem cell (MSC) is a promising source of novel cell-based therapies, driven by the hope of finding cures for numerous diseases including skin wound healing. In this study, we isolated MSCs from Wharton's jelly of human umbilical cord by enzymatic method. To determine the effect of MSC conditioned medium on wound healing ability, we examined two MSC conditioned mediums (MSC-CM), which differ in concentration of serum and harvest time. The results showed that in serum starvation condition, MSC-CM showed significantly enhanced keratinocyte migration speed and prolonged culture of MSC in this condition also improve the efficiency of MSC-CM.

*Keywords:* Wharton's jelly, Mesenchymal stem cell-conditioned medium, serum starvation, wound healing.

## 1. Introduction

MSCs, as defined by the International Society for Cellular Therapy, are plastic-adherent cells with a specific surface phenotype that have the capacity to self-renew and under appropriate *in vitro* conditions have the capacity to differentiate into all cells of mesodermal origin, such as adipocytes, osteoblasts, chondrocytes, skeletal myocytes, and visceral stromal cells [1-3].

MSCs are commonly sourced from bone marrow (BM-MSCs) [4]. However, due to the limited number of BM-MSCs available for autologous transplantation, the invasive nature of the procedure, decreased proliferation and differentiation potential with age, an alternative source of MSCs should be selected and applied in regenerative medicine to replace BM-MSCs [3]. Recently, It is reported that MSCs could also be harvested from other sources such as adipose tissue [5], umbilical cord (Wharton's jelly) [5], amniotic fluid [6], and synovial membrane [7]. MSCs derived from Wharton's jelly (WJ-hMSCs) have greater proliferation viability and differentiation ability compared to

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MSCs derived from white adipose tissue (Ad-MSCs) and BM-hMSCs because of their primitive nature [8]. Thus, WJ-hMSC is a promising alternative source to traditional sources of MSCs such as bone marrow for future autologous and therapeutic use [2].

Since the discovery of MSCs and the establishment of stable cell lines, investigations into their applications have increased significantly [9], with a view to find treatments such as skin wound healing [10]. Previous work has demonstrated that MSCs play a central role in the wound healing process [11]. The first popular approach is the injection of MSCs directly into the wounded site or host. Initially upon transplantation, these cells attach and differentiate within the injured tissue into specialized cells [10]. However, only a small percentage of the transplanted cells integrate and survive in host tissues. Thus, the foremost mechanism by which stem cells participate in tissue repair seems to be related to their trophic factors [10]. MSCs have the ability to secrete a multitude of trophic and survival signals including growth factors, chemokines and cytokines [12]. In *in vitro* condition, these molecules can be traced in the conditioned medium (CM) or spent medium harvested from culturing cells [13]. Conditioned medium now serves as a new treatment modality in regenerative medicine and has shown a successful outcome in some diseases [10]. This has encouraged scientists to use of CM in wound healing by modulating wound repair without stem cells being present in the wound. With the emergence of this approach, the aims of our study are isolation of MSCs in Wharton's Jelly of human umbilical cord and the application of CM from these MSCs culture in wound healing model *in vitro*.

## 2. Materials and Methods

### *2.1. Isolation and culture of WJ-MSCs*

Fresh umbilical cord was collected from Vinmec International Hospital Times City with the consent of the infants'parents. After being cut off from the placeta, umbilical cord was transferred immediately to sterile Phosphate Buffered Saline (PBS – Invitrogen, USA) supplemented with 100 units/ml of penicillin,100 µg/mL streptomycin, and 150 µg/mL Gentamycin (Invitrogen, USA) until processing. Typically, the cord was processed within 0 – 6 h of birth. Whole cord was rinsed in sterile PBS three times to remove blood, immersed in 70% ethanol for 30 s, and then immediately washed in PBS before further processing. The cord was cut into 3–5 cm long pieces using a sterile blade and blood vessels are removed from each piece. Remaining tissue was rinsed.

Extracted WJ was cut into approximately 1 $cm^3$ pieces and washed with PBS. Cord tissue were then placed into a sterile 50 ml centrifuge tube and incubated in 25 ml of 1 mg/mL collagenase type I for 16 h at 37° C. After 16h incubation with enzyme, the residual cord pieces were crushed to release as many cells as possible into the solution. Then the digested suspension was centrifuged at 1000g for 5 min. The supernatant was discarded and 3 ml of medium was added to the cell pellet and transferred to 25-$cm^2$ T-flask. The medium was added and the culture flask was incubated at 37°C in 5% $CO_2$ in a humidified incubator.

### *2.2. Flow cytometry analysis*

To examine the mesenchymal phenotype, cells were subjected to flow cytometry analysis, using the standard marker panel for MSC described by the position paper of the International Society for Cellular Therapy (ISCT) [14]. Human MSC analysis kit (BD Biosciences, USA) were used to characterize the isolated MSCs. Cells (second passage e) were harvested and divided into 6 tubes. Cells from tube 1 to 3 were stained with CD90-FITC, CD105-PerCP-Cy5.5, CD73-APC respectively. Cells in tube 4 stayed unstained; In tube 5, cells were stained with hMSC Positive Isotype Control Cocktail (mIgG1κ FITC, mIgG1 κ PerCP-Cy5.5, mIgG1 κ APC) and PE hMSC

Negative Isotype Control Cocktail (mIgG1κPE, mIgG2a, κPE); tube 6: stained with hMSC Positive Cocktail (CD90-FITC, CD105-PerCP-Cy5.5, CD73- APC) and PE hMSC Negative Cocktail (CD11b-PE, CD19-PE, CD45-PE, HLA-DR-PE). Flow cytometry was performed using a Navios Flow Cytometer (Beckman Coulter, USA).

*2.3. Mycoplasma testing*

Before proceeding to the next experiments, a sample of the primary culture was tested for Mycoplasma contamination using the MycoAlertTM Mycoplasma Detection Kit (Lonza, USA) and The Luminometer (Lucetta™, Lonza, USA). 100 µl of sample (cell supernatant) was transfered into a luminometer tube, then 100 µl of MycoAlert™ Reagent was added to each sample followed by 5 minutes wait, tube was placed in the luminometer reader and initiated the program (reading A). Subsequently, 100 µl of MycoAlert™ Substrate was added to each sample for 10 minutes. Similar procedures were conducted to obtain luminometer result (reading B). Calculate ratio = reading B/reading A.

*2.4. Collection of WJ-MSCs conditioned medium*

When cells (second passage) reached roughly 70 – 80% confluence, the culture medium was removed, then washed extensively with PBS and replenished with culture medium RPMI 1640 (Gibco) supplemented with 10% or 0.1% FBS, 100 µg/mL streptomycin and 100 units/mL of penicillin. The medium was collected after 24 h or 72 h cultured in a humidified incubator with 5% $CO_2$ at 37°C. Collected media samples were centrifuged at 350 *x g* for 5 min, and then stored at −20°C until further use.

*2.5. HacaT cell culture*

The human transfomed normal skin keratinocyte (HaCaT) cell line wasa gift from Prof. Dr. Masashi Kato, School of Medicine, Nagoya University. This cell line was cultured in RPMI 1640 medium (Gibco) supplemented with 10% FBS, 100 µg/mL streptomycin and 100 units/mL of penicillin at 37°C and 5% $CO_2$.

*2.6. Scratch wound assay*

The wound healing assay was performed with HacaT cell line. Cells were cultured as confluent monolayer in 6-well plates and a 200-µl pipette tip was used to scratch the monolayer. After wounding, the cell debris was removed by washing with PBS. Wounded monolayers were then replenished with 10% or 0.1% serum WJ-MSC-CM at 24 h and 72 h collected previously. The flasks were incubated in a humidified incubator with 5% $CO_2$ at 37°C. Wound images were recorded with a Canon digital camera attached to an inverted light microscope (Carl Zeiss, Germany) at 0, 8 and 20 h. The average rates of wound closure were calculated from 3 independent experiments.

*2.7. Statistical analysis*

Experimental data were presented as mean ± SEM (standard errors of the mean) calculated from 3 independent experiments. Statistical significance was evaluated using one-way ANOVA followed by individual t-test between each treated group and the control group, otherwise non-parametric tests were used. P values of $<0.05$ were considered as statistically significant.

## 3. Results

*3.1. Isolation and culture WJ-MSCs*

After 2-day culture of the enzyme digested cells, the primary cells had a heterogeneous shape, including fibroblast-like cells and small round cells with a relatively high nuclear to cytoplasm ratio, as well as flat cells (Fig. 1a). In the further passages, we observed that small round cells and flat cells gradually disappeared while the number of fibroblast-like cells became increasingly overwhelming (Fig. 1b-c).

The interval between the primary culture and the first passage was approximately 13 days.

### *3.2. Mycoplasma detection*

We examined Mycoplasma infection in two samples from the second passage, the results showed that in the both samples, the ratios of emitted light intensity (linearly related to the ATP concentration) before and after adding the appropriate substrate were 0.567857 and 0.682353 respectively. These two values were less than 0.9, indicating that there was no presence of Mycoplasma in cell culture.

### *3.3. Characterization of isolated cells derived from human umbilical cord Wharton's jelly*

Flow cytometry analysis showed that the cells expressed high levels of CD90 (99.3%), CD73 (96%), CD105 (63.7%) (Fig. 2(a)), which was known to be expressed on MSCs but not on fibroblasts. Simultaneously, the isolated cells lacked expression of the hematopoietic, macrophage, and endothelial markers, such as: CD45, CD11b, CD19 and HLA-DR (Fig. 2(b)). When examining the combination of markers, the proportion of cells expressing simultaneously 2 markers (CD90, CD73) or 3 markers (CD90, CD73, CD105) were 92.9% and 67.6% respectively. Therefore these data suggested that the isolated cells derived from human umbilical cord Wharton's jelly could potentially be MSCs for usage in the following experiments.

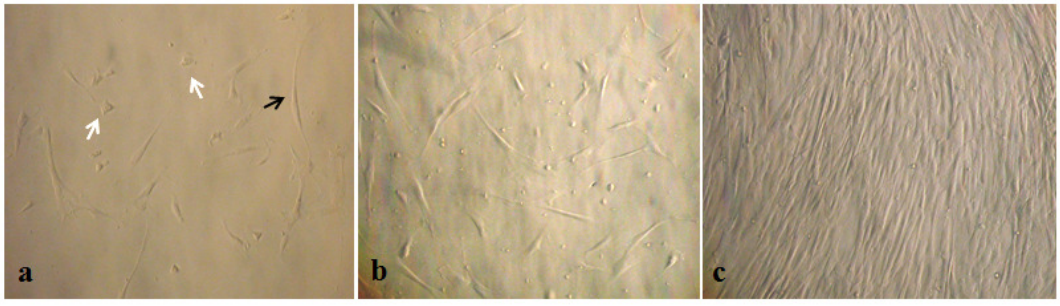


Figure 1. Morphological features of the isolated cells derived from human umbilical cord Wharton's jelly by enzymatic method. Cells on the second day of isolation (a), on day 3 of the 2nd passage (b) and on day 7 of the 3rd passage (c). Three types of isolated cells (fibroblast-like cells which were overwhelming on day 7 of the 3rd passage and characterized as MSC, *black arrow*; and small round cells, *white arrow*; as well as flat cells, *arrowhead*).

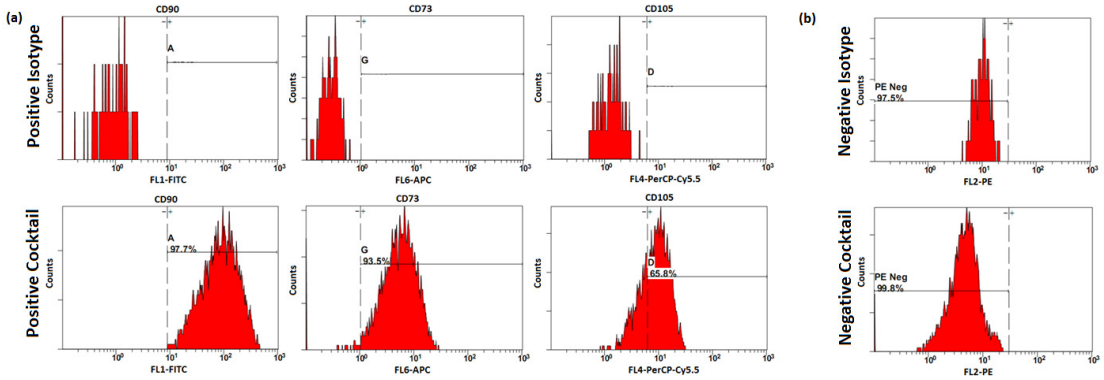


Figure 2. Flow cytometry analysis of surface markers on the second-passage WJ-MSCs. (a) WJ-MSCs expressed CD90, CD73 and CD105. *A, G, D: positive staining of FITC, PE and PerCP-Cy5.5 fluorescence, respectively*. (b) WJ-MSCs did not express CD11b, CD19, CD45 and HLA-DR. *PE Neg: negative staining of PE fluorescence.*

*3.4. WJ-MSCs condition medium induced the migration of HacaT cells.*

We used conditioned medium of isolated MSCs in 10% and 0.1% serum concentrations of 24h and 72h culture to determine wound healing capability of MSC-CM *in vitro*. Representative images showed the progression of wound closure chronologically. After 8h since scratched, the wound width in CM-72h sample was the smallest with regard to the Control and the other samples (Fig. 3).Wound closure in CM-24h, CM-72h, nonCM-72h were completed after 20h (Fig. 3F,I,R),while other samples haven't closed yet (Fig. 3C,L,O). Based on the wound width over time (used Axiovision Rel 4.8.2 software), we calculated migration speed of the HacaT cells covering the wounds (Fig. 4). The data showed that cell migration rate in CM-72h was highest, increased by approximately 7-fold, 4-fold and 2-fold as compared to the control 1, control 2 and nonCM-72h, respectively. It indicated that in serum starvation condition, MSC-CM showed significantly enhanced keratinocytes migration rate ($P<0.05$). Moreover, it suggested that increased culture time MSC in starvation serum condition also increased the efficiency of MSC-CM.

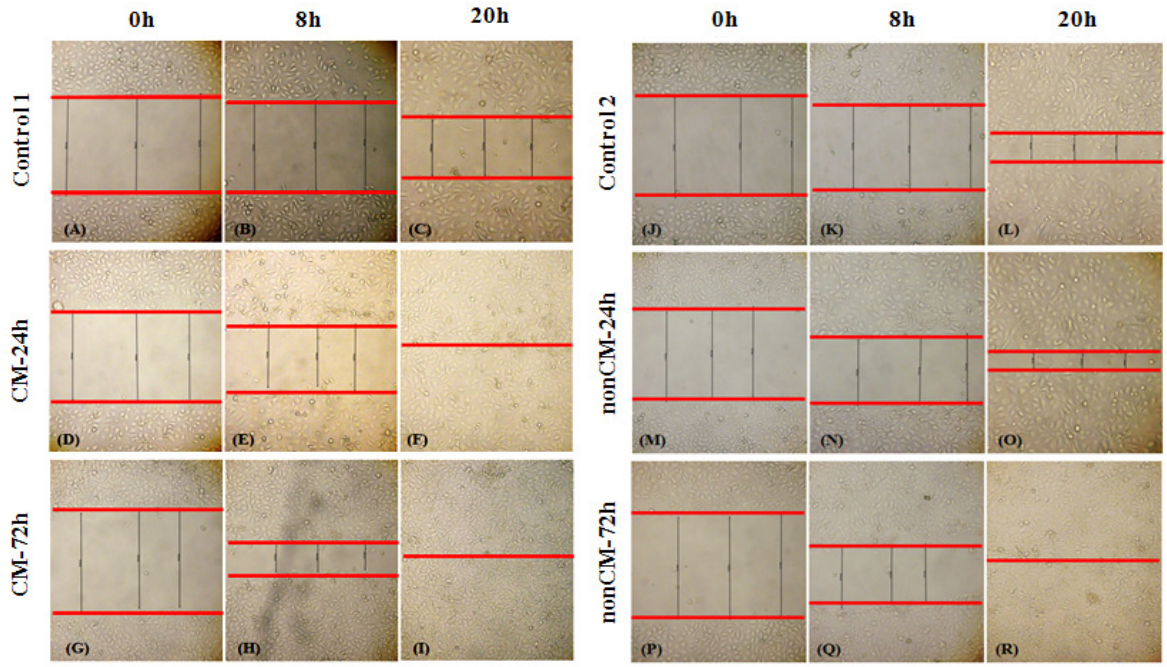


Figure 3. The process of HacaT cells closed scratch wounds throughout the time. HacaT cells were cultured in different MSC-conditioned media (CM). A cell- free area was introduced with a 200 µl pipette tip, and migration was evaluated after 0h, 8h and 20h of culture. Control 1- RPMI 1640 supplemented 0.1% FBS; Control 2 - RPMI 1640 supplemented 10% FBS; CM-24h and CM-72h were MSCs conditioned medium supplemented 0.1% FBS for 24h, 72h respectively; nonCM-24h and nonCM-72h were MSCs conditioned media supplemented 10% FBS for 24h, 72h respectively.

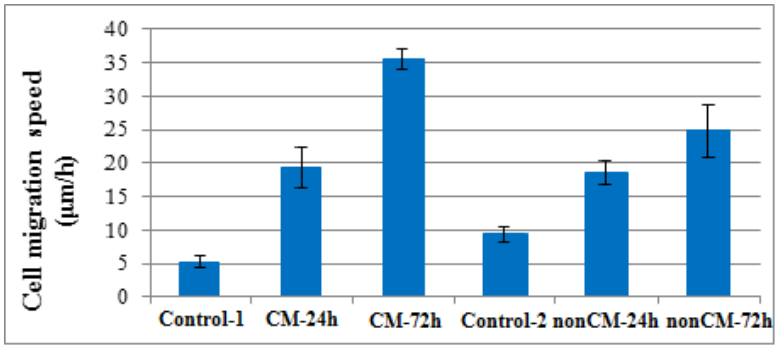


Figure 4. HacaT cell migration speed in different WJ-MSCs condition media. CM-72h induced the highest migration of HacaT cells indicated significant increase of cell migration speed in CM-72h compared to other samples (P <0.001, analysed by ANOVA followed by individual t-test).

## 4. Discussion

Isolation of human umbilical cord-derived mesenchymal (hUCM) cells has several techniques that can affect the quantity and quality of the isolated cells [15]. The most common isolation technique is based on enzymatic digestion. In general, enzymes such as collagenase, trypsin is used to digest tissue [15]. However, the use of trypsin alone for a long period may cause degradation of the extracellular matrix and disintegration of cell membranes because some cells are sensitive to exposure with trypsin but not to collagenase [15]. In this study, we proceeded to isolate MSC-derived Wharton's jelly of the umbilical cord by using enzyme collagenase type I in order to identify the optimal protocol for hUCM isolation while still maintain their growth capacities in our laboratory condition. The results showed that the primary cells are heterogeneous and the interval between primary culture and the first passage was approximately 13 day. These results are similar to the results of the previously published reports [15].

Due to the advantages in proliferative ability, differentiation and immunomodulatory potential, MSC was used commonly in the treatment many diseases. MSC–based therapy has emerged as a promising therapeutic strategy for treating non-healing wounds [13]. The paracrine factors secreted by MSCs can accumulate in the conditioned medium (CM), which has been reported to serve multiple positive functions in tissue regeneration [16]. Studies have shown that MSC-CM plays an anti-inflammatory role in corneal wound healing following chemical injury [17]. In addition, MSC-CM has been found to accelerate wound closure through enhanced epithelial and endothelial cell migration, cell infiltration, granulation formation, and angiogenesis in an excision wound model [18]. With the emergence of this approach, we described the possibility of using MSC conditioned medium as a novel and promising alternative to skin wound healing treatment in keratinocytes model because keratinocyte proliferation and migration play an essential role during the re-epithelialization process to cover the denuded wound surface [16].

As mentioned previously, the foremost mechanism by which MSCs participate in tissue repair seems to be related to their trophic factors liked growth factors, cytokines and chemokines. In in vitro condition, these molecules can be identified in the conditioned medium or used media harvested from cultured cells [10]. Reports previously demonstrated that MSC secrete VEGF, MCP-1, MIP-1α, and MIP-1β, which have significant biological effects on cell migration, apoptosis, and capillary formation [2]. In addition, MMP-2 secreted by MSC may promote directed cell

migration [19]. MSC are generally cultured in 10–20% serum which contains numerous factors that may not be present in the tissues where these cells reside. Serum deprivation alters the secretion of paracrine factors and the expression of stem cell and endothelial markers in MSC [14]. Therefore, in scratch wound assay, we collected MSC-CM in 10% serum concentration and serum starvation condition to determine the effects of these conditions on cell migration. The results suggested that MSC-CM in serum starvation condition significantly promotes keratinocytes migration compared with high serum condition. Another aspect is the timing of collection of the CM from the cells. Our results indicated that CM collected after 72h culture of MSC successfully improved the healing ability in scratch wound assay compared with 24h CM. Thus, serum reduction may be one of the ways increases the paracrine factors in MSC-CM enough for them to be used for the treatment. Serum deprivation is one method to synchronize mammalian cells culture to G0/G1 phase [20]. The healing of the wound in MSC-CM of HacaT cells were caused by neither cell dividing nor migration. This healing should due to paracrine factors secreted by MSC.

## 5. Conclusion and further work

In conclusion, we have successfully isolated MSC-derived Wharton's jelly of human umbilical cord by enzymatic method. Under serum starvation condition, MSC-CM have significantly promoted on keratinocyte cells migration. In addition, the results suggested that increased the timing of collection of the CM from the MSCs also increased the efficiency of MSC-CM. Our future goal is to optimize the MSC culture conditions to obtain more effective wound healing. Besides, we continuously determine the effect of MSC-CM on the wound healing ability in *in vivo* model for further works.

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# Phân lập tế bào gốc trung mô từ chất nền Wharton dây rốn ứng dụng trong chữa lành vết thương

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**Tóm tắt:** Tế bào gốc trung mô (MSC) là loại tế bào có nhiều triển vọng trong liệu pháp tế bào, có thể sử dụng chữa trị nhiều bệnh khác nhau trong đó có chữa lành vết thương trên da. Trong nghiên cứu này, chúng tôi thực hiện phân lập tế bào gốc trung mô từ chất nền dây rốn người theo phương pháp sử dụng enzym (collagenase). Để xác định hiệu quả của việc sử dụng môi trường nuôi cấy MSC (MSC-CM) đến khả năng chữa lành vết thương, chúng tôi tiến hành đánh giá tác động của MSC-CM thu được ở hai điều kiện nuôi cấy sử dụng nồng độ huyết thanh khác nhau (0,1 và 10% FBS) trong thời gian nuôi cấy MSC khác nhau (0, 8, 20h). Kết quả cho thấy MSC-CM trong điều kiện bổ sung huyết thanh với nồng độ thấp (0,1%) có tác dụng tăng cường đáng kể tốc độ di chuyển của tế bào keratinocyte, đồng thời việc tăng thời gian nuôi cấy MSC từ 24h lên 72h trong điều kiện trên cũng làm tăng hiệu quả tác dụng của MSC-CM.

*Từ khóa*: Chất nền Wharton, môi trường nuôi cấy MSC, thiếu hụt huyết thanh, chữa lành vết thương.