A simple non-enzymatic protocol for isolation and culture of mesenchymal stem cells from adipose tissues

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Abstract

Mesenchymal stem cells (MSCs) are multipotent stem cells – ability to differentiate into several cell types. Previous studies have showed a high number of these cells can be found in adipose tissue. This study has established a simple protocol for isolation and culture of MSCs from mouse adipose tissue using minimal requirements. With non-enzymatic approach, we successfully isolated and cultured the MSCs. Isolated MSCs were identified through morphology and ability of differentiation into adipocyte-like cells under the presense of stimuli in the culture medium.

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Keywords

adipose tissue, mesenchymal stem cells, non-enzymatic

1. Introduction

Mesenchymal stem cells (MSCs) are known as multipotent stem cells that have the potential to self-renew and differentiate into a variety of specialised cell types such as osteoblasts, chondrocytes, adipocytes, and neurons. MSCs can be isolated from various sources such as adipose tissue, tendon, peripheral blood, and cord blood [1], [2], [3].

MSCs have been successfully isolated and characterised from many species including mouse, rat, rabbit, dog, sheep, pig, and human [4]. Mice are one of the most commonly used experimental animals in biology and immunology in many countries over the world.

MSCs were initially isolated from bone marrow (BM-MSCs), however, subsequent research has shown that other adult tissues also contain MSCs [5], such as adipose tissue-derived MSCs (AdSCs) which have great potential for novel cellular therapies to repair damaged tissues and in angiogenic therapy. In addition, it was reported that haematopoietic cell contamination is observed during the culture and expansion of BM-MSCs. Subsequently, to isolate pure MSC from bone marrow, haematopoietic cell depletion process is a must, leading to low yield of MSCs. Compared with other tissues, adipose tissue contains a high number of MSCs that can be easily expanded *in vitro* and differentiate into various cell types [6]. One gram of adipose tissue yields approximately 5000 MSCs, whereas the yield of bone marrow-derived MSCs is 100 to 1000 cells/ml of marrow [5].

In previous studies, AdSCs were isolated using the enzymatic method in which the adipose tissue were treated with collagenase for releasing MSCs which were then collected for cell culture [7], [8]. Recently, AdSCs isolation and culture using enzyme (collagenase type I or tpe II) procedure become a standard protocol for several laboratories [8], [9]. But the type of collagenase and time of sample digestion vary among studies leading to an effect on the cell properties. To overcome this obstacle, it was said that AdSCs were isolated and cultured by culture of adipose tissue instead of single cells [9], [10], [11] applied for rat and human AdSCs.

There was no need to use enzyme to digest adipose tissue if your laboratory are lack of collagenase and doubt about how to do the experiments. Since it does not need any enzyme and subsequent chemicals, this non-enzymatic procedure was inexpensive and easy to handle.

However, there are not much studies in AdSCs isolation from mouse by non-enzymatic method. In this study, we established a non-enzymatic method to isolate and culture mouse MSCs from adipose tissue. The cells were determined by their differentiation potential into adipocyte-like cells which are characterized by intracellular accumulation of lipid droplets stained with Oil red O [12]. The study focused on description a simple method for

AdSCs isolation and confirmation by morphology and differentiation potential without cells markers confirmation.

2. Materials and Methods

2.1 Animals

4-6 week-old mice was purchased from Pasteurs Institute in Ho Chi Minh City.

2.2 Materials

Growth medium. Dulbecco's Modified Eagle's Medium/ Ham's F12 (DMEM/F12) (GIBCO) completed with 15% Fetal Bovin Serum (FBS) (GIBCO) and 100 U/ml penicillin, 100 µg/mL streptomycin (Mekophar).

Adipogenic differentiation medium. DMEM supplemented with 5% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 1µM dexamethasone, 200µM indomethacin, 1.7µM insuline, 500µM isobutyl-methylxanthine (IBMX). The media should be pre-warmed before use by placing into a water bath (Memmert) set at 37ºC ± 1ºC for 30 min.

For cell detaching, 0.125% (w/v) Trypsin - 1mM Na_2-Ethylenediaminetetraacetic acid (EDTA) (Thermo) was used. 0.5% (w/v) Oil red O, Hematoxylin, 0.4% (w/v) trypan-blue were used for cell staining. Steriled phosphate buffer saline (PBS) consists of 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4, pH 7.4. All salts in PBS purchased from Merck.

2.3 Procedure

2.3.1 Cells isolation and culture

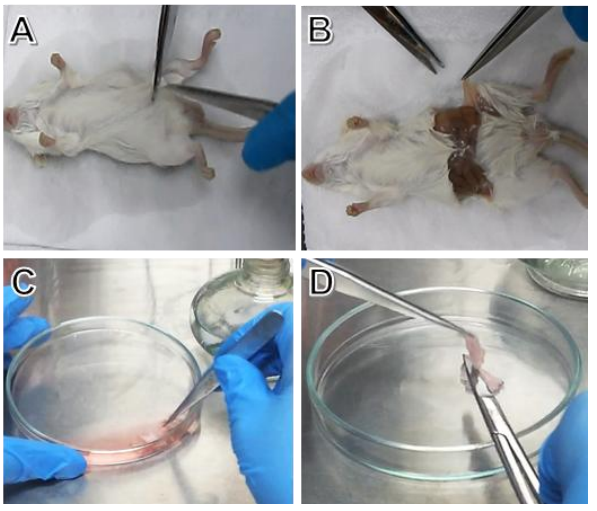

Figure 1. Mouse MSCs isolation from adipose tissue procedure. (A) Make an incision on stomach skin and spread towards both sides of libs. (B) Collect the adipose tissues. (C) Wash thoroughly in PBS pH 7.4. (D) Cut adipose tissue to 5 mg of fragments and explant into 24-well plate.

In order to isolate adipose tissue, mice were killed by cervical dislocation. Then, rinse the animal skeleton in 70% ethanol, make an incision on stomach skin and spread it towards both sides of libs (Fig. 1).

Flank adipose tissues were collected and stored in cool PBS pH 7.4 supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin. In biosafety cabinet, the tissues were washed thoroughly in PBS pH 7.4. The tissues were cut into small pieces (about 5mg) which were washed with FBS (Fig. 1). Each piece of tissues was explanted into wells of 24-well plate. The surface of each specimen was then covered with 50µl of pre-warmed FBS. Care was taken to avoid specimen floating.

The samples were incubated for 24 hours at 37ºC and 5% CO_2. Then, 300µl growth medium was added to the wells. Daily culture observating to ensure culture is free of contamination and detect adhesion cells by inverted microscope (Optika). After 72 hours, carefully remove the non-adherent cells and adult adipocytes that accumulate on surface of the wells by replacing with fresh growth medium. Medium was replaced twice per week until the cells reached confluence and the tissue pieces were removed from wells after one week of culture.

2.3.2 Cells subculture

When reach 80% confluence, the cells were washed with PBS pH 7.4, detached by using 0.125% (w/v) trypsin-EDTA for 2 minutes at room temperature. Complete growth medium was then added to inactive trypsinizing activity. Counting the cells with a hemacytometer using trypan-blue, seeding the cell at 1 x 10^4 cells/cm^2 density and incubate at 37ºC and 5% CO_2. Changing medium every 3 days after the first detaching. Until cells reach 80% confluence, implementing the second cell detaching.

2.3.3 Adipogenic differentiation

MSCs passage 2 can be used to differentiate into adipocytes. When cells reach 80% confluence, wash the cells twice with PBS pH 7.4, expose the cells with differentiation medium containing reagents inducing MSCs to be committed to the adipogenesis pathway and generate adipocytes. The cells were maintained in the medium for 10 days with medium replacement of every 3 days. After 10 days, the cells were stained with Oil red O, Hematoxylin and MSCs determination by inverted microscope. The cells in complete culture medium were used as negative control. Adipocytes should be the cells with red vacuoles and the others should be only blue at nuclei positions.

3. Results

3.1 MSCs culture from mouse adipose tissues

On day 1 of culture, there was no adherent cells observed on well surface (data not showed). On day 2-6, adherent spindle-shaped cells appeared (Fig. 2A). On day 7, the spindle-shaped cells reached about 60-80% confluence (Fig. 2B). The cells were then passaged on day 10 and showed

more homogeneous with fibroblast-like shape (Fig. 2C). Spindle-shaped cells had increased at passage 2 of culture (Fig. 2D). We succeeded in isolation and culture cells from adipose tissue without enzyme digestion step.

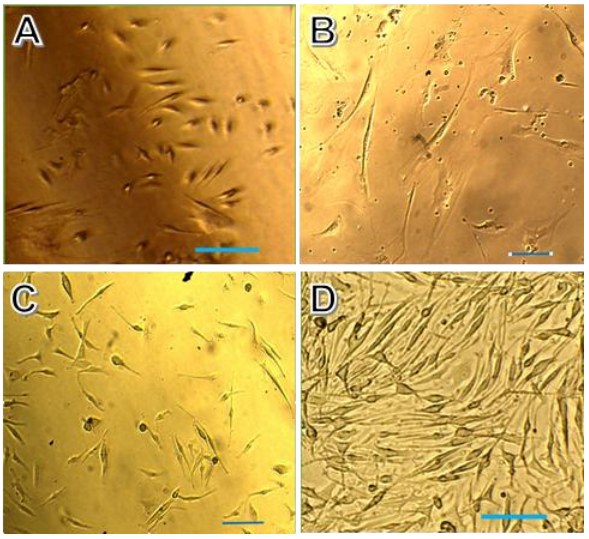

Figure 2. Morphology of mouse AdSCs passage 0 – 2. (A), (B) Mouse AdSCs passage 0 on day 3 and day 7 of culture, (C) Cells after 24 culture hours of passage 1, (D) Cells after 7 days of passage 2. Scale bar, 100 µm.

3.2 Adipogenic differentiation

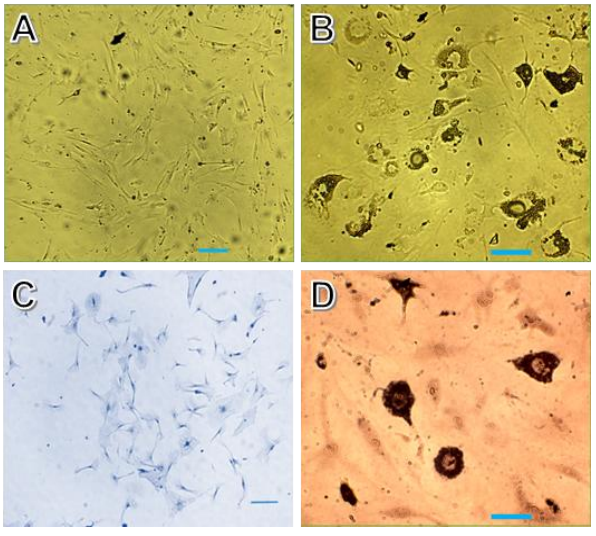

Figure 3. Adipogenic differentiation of mouse AdSCs. (A) Mouse MSCs passage 2 at day 10 after culture medium refreshing (negative control). (B) MSCs in differentiation medium. (C and (D) MSCs showed in (A) and (B) respectively were stained with Oil red O and Hematoxylin, Scale bar, 100µm.

MSCs confirmation were taken by potential of differentiation into adipocyte. After 2 days of exposure of differentiation medium, MSCs can be regconized by morphology under microscope. Most of the cells were differentiated into adipocyte-like cells and they graduately change from fibroblast-like shape to adipocyte-like shape (data not shown).

On day 10 of exposure of differentiation medium, as showed in Fig. 3B, adipocyte-like cells appeared with lipid-rich vacuoles which are stained red with Oil red O (Fig. 3D). In contrast, the control cells still remained their morphology and had no lipid accumulation in their cytoplasm (Fig. 3A and 3C).

4. Discussion

There were numbers of studies in AdSCs isolation and culture applied for rat and human adipose tissue by tissues culture. But there was not very much study applied for mouse since MSCs isolating is more challenging in mouse than other species. In this study, we are able to establish a well-known set-up protocol for isolation and culture of MSCs derived from mouse adipose tissues. The protocol is simple and does not require enzyme treatment and easy to handle on mouse model.

This study focused on description a simple method for AdSCs isolation and culture. In compare with several protocols of enzyme method, this study obtained the high number of cells in quite short time as got reached 80% confluence after 10 days. In addition, when confirmation by adipocyte differentiation ability, most of cells were lipid accumulation cells were observated.

Previous studies showed that authors used markers expression for MSCs confirmation but differentiation ability. In the study of Sung et al. (2008) [13], the AdSCs were confirmed using monoclonal antibodies conjugated to FITC, PE, or PE-Cy5 against CD3, CD11b, CD19, CD29, CD34, CD44, CD105, CD45, CD117, TER-119, CD86, Sca-1, H-2k^b, and I-A^b. The FACS results showed positive expression for CD29, CD44, CD105, and Sca-1, and negative expression for CD3, CD11b, CD19, CD34, CD45, CD117, TER-119, CD86, H-2k^b, and I-A^b. Andreeva et al. [7] confirmed isolated AdSCs as MSC populations are positive for CD29 and CD44 markers, and negative for CD11b, CD45 and CD34 markers. Compare with those studies, this one loss of markers expression for MSCs confirmation. However, with differentiation ability, one of important abilities of stem cell, isolated cells were MSCs with no doubt, but the purity level of isolated cells could not be accessed and requires further investigation.

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Quy trình phân lập và nuôi cấy tế bào gốc trung mô từ mô mỡ chuột bằng phương pháp không enzyme

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Tóm tắt Tế bào gốc trung mô (MSCs) là tế bào đa tiềm năng – khả năng biệt hóa thành nhiều loại tế bào có chức năng khác nhau. Các nghiên cứu trước đây đã tìm thấy một lượng lớn tế bào gốc trung mô trong mô mỡ. Nghiên cứu này thiết lập một phương pháp đơn giản để phân lập và nuôi cấy MSCs từ mô mỡ chuột với các điều kiện thí nghiệm đơn giản nhất. Với phương pháp không enzyme, chúng tôi đã phân lập và nuôi cấy thành công MSCs từ mô mỡ chuột. Các tế bào MSC được nhận biết thông qua hình thái và khả năng biệt hóa thành tế bào mỡ.
Từ khóa Tế bào gốc trung mô, MSC, mô mỡ, không enzyme