An effective protocol for isolation and culture of mesenchymal stem cells from mouse bone marrow

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Abstract

Bone marrow is known as the main source of isolated mesenchymal stem cells. This study has demonstrated a protocol for isolation and culture of mesenchymal stem cells from mouse bone marrow (mBM) with minimal requirement. The cells were harvested from the tibias and femurs, and then cultured in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum. The mBM-MSCs were isolated by media changing every 8 hours in 24 hours of culture and sub-culture. Target cells subsequently were regconized by adipogenic differentiaton posibility. The study also focused on descript a simple procedure to culture BM-MSCs and the results obtained following the procedure

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Keywords

Bone marrow, cell culture, isolation, differentiation, mesenchymal stem cells.

1. Introduction

Mesenchymal stem cells (MSCs) are referred to multipotent stem cells that have the potential to self-renew and differentiation ability into a variety of specialised cell types such as osteoblasts, osteocytes [1], chondrocytes [2], adipocytes [3]. MSCs can be isolated from various sources such as adipose tissue, tendon, peripheral blood, and cord blood [4].

MSCs have been successfully isolated and characterised from many species including mouse, rat, rabbit, dog, sheep, pig, and human [4]. Bone marrow is the most common source of MSCs [5], so target tissue to isolate high number of MSCs in animal models used was bone marrow.

Mice are one of the most commonly experimental animal model in biology in many countries over the world. The number of MSCs in mouse bone marrow (mBM-MSCs) is much lower than that of rat or human MSCs [6]. Therefore, MSCs isolating from bone marrow is more challenging in mouse than other species since, in addition mBM-MSCs are difficult to harvest and grow due to the low MSCs yield, represent 0.01% to 0.001% of nucleated bone marrow cells [8].

In bone marrow, two main stem cell populations and their progenies are haematopoietic stem cells (HSC) or mature haematopoietic cells and MSCs. BM-MSCs are usually isolated and purified through their physical adherence to the plastic cell culture plate [3]. Some procedures to isolate and culture mouse BM-MSCs have been published [8], [9]. Soleimani et al. (2009) showed a effective protocol for isolation of BM-MSCs obtaining 70×10^6 bone marrow cells from one donor, however, cells got 70% confluence after a long time, 14 days. In 2014, Huang et al. descripted an improved protocol to isolate mBM-MSCs with minimal requirement and strong survival ability though isolated cells containing haematopoietic stem cells because of the loss of haematopoietic stem cells step. In addition, the study proved that the higher number of MSCs was obtained since not filter the bone marrow.

In this study, we established an effective method to isolate and culture MSCs from mouse bone marrow. The MSCs were regconized by adipogenic differentiaton posibility when the cell were stained with Oil red O and Hematoxylin [10]. The study also focused on description of a simple method for BM-MSCs isolation and confirmation by morphology and differentiation the potential without cells markers confirmation.

2. Materials and Methods

2.1 Animals

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

2.2 Materials

Growth medium (GM). High glucose Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) supplemented with 10% Fetal Bovin Serum (FBS, GIBCO), 100 U/ml penicillin and 100 µg/mL streptomycin (Mekophar).

Adipogenic differentiation medium (AdM). 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 37°C ± 1°C for 30 minutes.

For cells detaching, 0.125% (w/v) Trypsin - 1mM Na_2-Ethylenediaminetetraacetic acid (EDTA) (Thermo) was used. For cell staining, 0.5% (w/v) Oil red O, 0.4% (w/v) trypan-blue were used. Steriled phosphate buffer saline (PBS) consisted 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 Bone marrow cells collection

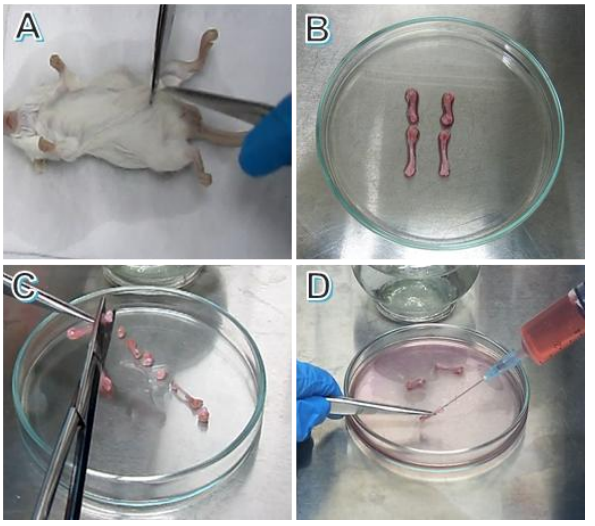

Figure 1. MSCs isolation procedure from mouse bone marrow. (A) Make an incision on stomach skin and spread towards both sides of libs. (B) Tibias and femurs after washing and removing muscle and connective tissue. (C) Cut the ends of the tibias and femurs just below the end of the marrow cavity. (D) Isolate bone marrow cells by flushing the marrow plug.

Mice were killed by cervical dislocation and then, rinse the animal skeleton in 70% ethanol. Make an incision around the perimeter of the hind limbs where they attach to the trunk and remove the skin by pulling towards the foot and cut at hook joints. Dissect the hind limbs containing femurs and tibias from the trunk by cutting at ankle joints. Store hind limbs in cooled growth medium (Fig. 1A and 1B).In biosafety cabinet, remove all the muscle and connective tissues from hind limbs in PBS pH 7.4 (Fig. 1B). After cleaning, cut the ends of the tibias and femurs just below the end of the marrow cavity using sharpened scissors. Insert a 26-gauge needle attached to a 10 ml syringe containing GM into the spongy bone. Flush the marrow plug out of the cut end of the bone with 4 ml of GM for each tibia and femur set (Fig. 1D).

Pipetting to homogenize the cells in growth medium and counting with a hemacytometer using trypan-blue. Seed the cells into T25 flask (Corning) at 1 x 10^6 cells/cm^2 density and incubate at 37°C and 5% CO_2 for 12 hours.

After 8 hours, carefully remove the non-adherent cells that accumulate on surface of the flask by replacing with fresh medium. Daily culture observating to ensure culture is free of contaminations and detect adhesion cells by inverted microscope (Optika).

2.3.2 Isolation and culture of mesenchymal stem cells from bone marrow

For isolation mBM-MSC from other cell types in bone marow, reducing the haematopoetic cell in cell culture by media changing with 2.5 ml of fresh GM every 8 hours within 24 hours of culture and sub-culture.

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

2.3.3 Adipogenic differentiation

MSCs passage 2 can be used for adipogenic differentiation. When cells reach 80% confluence, the cells were washed twice with PBS pH 7.4, exposed with differentiation medium containingreagents inducing MSCs to be committed to the adipogenesis pathway and generate adipocytes, 1µM dexamethasone, 200µM indomethacin, 1.7µM insuline, 500µM isobutyl-methylxanthine (IBMX). 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. Adipocyte-like cells should be the cells with red vacuoles and the others should be just blue at nuclei positions.

3. Results

3.1 Culture of bone marrow MSCs

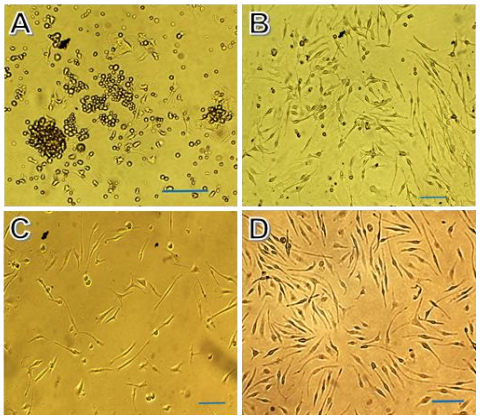

Figure 2. Morphology of mouse BM-MSCs passage 0 – 2. (A) and (B) Mouse bone marrow MSCs passage 0 after 24 hours and 7 days of culture respectively. (C) Cells after 24 culture hours of passage 1. (D) Cell culture after 10 days of passage 2. Scale bar, 100µm

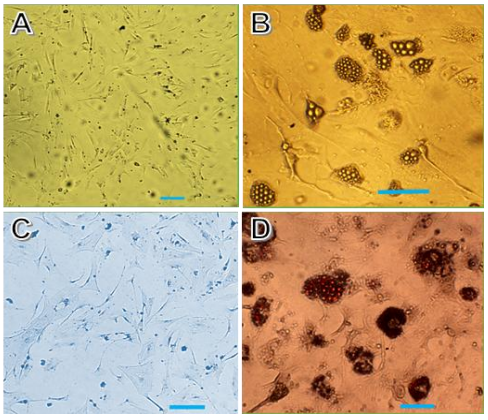

Figure 3. Adipogenic differentiation of mouse bone marrow MSCs. (A) MSCs passage 2 at day 10 after culture medium refreshing (negative control). (B) MSCs passage 2 at day 10 after 10 days of exposure of differentiation medium. (C) and (D) MSCs showed in (A) and (B) respectively were stained with Oil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

The cells from bone marrow were adherent on flask surface after 8 culture hours. On day 1, most of the cells were still round-shaped cells (Fig. 2A). On day 7, by morphology observation, fibroblast-like cells appeared more and their number increased among the others. In addition, number of cells reached 70% confluence in this day (Fig. 2B). After 24 hours of sub-culture, the fibroblast-like cells density increased in compare with the cells passage 0 (Fig. 2C). Most of cells were fibroblast-like cells in passage 2 of culture (Fig. 2D).

3.2 Identification of MSCs by adipogenic differentiation method.

On day 10 of exposure of differentiation medium, as showed in Fig. 3B, most of 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 when stained Oil red O (Fig. 3A and 3C).

4. Discussion

In this study, we successfully established a protocol for isolation and culture of MSCs derived from the mouse bone marrow. The cells have potential ability of differentiation under inducing medium containing dexamethasone, indomethacin, insuline, and isobutyl-methylxanthine.

This study focused on description of a simple method for BM-MSCs isolation. In compare with several protocols, this study has obtained the high number of bone marrow cells in a short amount of time as got reached 80% confluence after 7 days. In addition, when confirmation by adipocyte differentiation ability, most of cells were lipid accumulation cells were observated.

Previous studies have showed that authors used markers expression for MSCs confirmation but differentiation ability. In the study of Soleimani et al. (2009), the BM-MSCs were confirmed using endothelial, myeloid and hematopoietic cell lineage-specific antigens, such as CD31, Vcam-1, CD34, C-Kit, CD135, CD11 and CD45 and the results showed that these markers were not expressed in isolated cells. Huang et al. confirmed isolated BM-MSCs as MSCs positive for MSC marker CD44, CD90 and and negative for endothelial cell marker CD31, and the haematopoietic cell marker CD45. 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

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10. Oil Red O stain for in vitro adipogenesis – Lonza

Một quy trình hiệu quả để phân lập và nuôi cấy tế bào gốc trung mô từ tủy xương chuột

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Tóm tắt Tủy xương được xem là nguồn chính để phân lập tế bào gốc trung mô (MSCs). Nghiên cứu này mô tả quy trình phân lập và nuôi cấy tế bào gốc trung mô từ tủy xương chuột (mBM) với các điều kiện thí nghiệm đơn giản nhất. Các tế bào được thu nhận từ xương cẳng và xương đùi chuột, sau đó được nuôi cấy trong môi trường Dulbecco's modified Eagle's Medium bổ sung huyết thanh thai bò (FBS). Các mBM-MSCs được phân lập bằng cách thay môi trường 8 giờ một lần trong 24 giờ trong nuôi cấy và cấy chuyền. Các tế bào đích sau đó được nhận biết bởi khả năng biệt hóa của tế bào gốc trung mô thành tế bào mỡ. Nghiên cứu hướng đến việc mô tả một quy trình đơn giản để nuôi cấy BM-MSC và các kết quả thu được theo quy trình đã tiến hành.

Từ khóa Tế bào gốc trung mô, tủy xương, nuôi cấy tế bào, phân lập, biệt hóa.