

# Isolation and Characterization of Rabbit Bone Marrow-Derived Mesenchymal Stem Cells

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## Abstract

**Background:** Stem cells are defined as cells that can self-renew indefinitely and able to differentiate into various mature cells when induced appropriately. It have many other properties bring attention to use in regenerative medicine. Stem cell therapy has attracted much interest in this 21<sup>st</sup> century, not only because of the controversy surrounding the ethics involving pluripotent stem cells, but their potential for clinical use.

**Objectives:** The aim of this study was to isolate and characterize mesenchymal stem cells from bone marrow of New Zealand white rabbits by its morphological, multipotential differentiation and immunophenotypical analysis.

**Materials and Methods:** Three rabbits were euthanized with pentobarbital, an incision was made through the skin at thigh region and all muscles related to femoral bone were removed. The two epiphysis ends were cut and the bone marrow was flushed to be cultured for series of passages.

**Results:** The bone marrow cells were shown to adhere to the plastic surface and started to form fibroblastic-like colonies after 3 to 7 days of initial seeding. Further characterization was conducted using cells from passage two onward by analyzing their surface protein expression and ability to differentiate into mesodermal lineages under a relevant inductive condition. Flow cytometer analysis showed that the adherent bone marrow cells were expressing CD44 and CD90 but not CD34 which is a standard profile of mesenchymal stem cells. Besides, the bone marrow cells which were subjected to adipogenic and osteogenic differentiation exhibited differentiation into adipocytes and osteoblasts when cultured in appropriate inductive differentiation media.

**Conclusion:** Based on our observation, the bone marrow adherent cells from New Zealand white rabbits had reflected common mesenchymal stem cells characteristics which have been confirmed via morphological, multipotential ability and immunophenotyping analysis.

**Key Words:** mesenchymal stem cells, bone marrow, new zealand white rabbit, cell morphology, multipotency, immunophenotyping

## 1. Introduction

Stem cells are defined as cells that can self-renew indefinitely and able to differentiate into various mature cells when induced appropriately [1]. It have many other properties bring attention to use in regenerative medicine. Stem cell therapy has attracted much interest in this 21<sup>st</sup> century, not only because of the controversy surrounding the ethics involving pluripotent stem cells, but their potential for clinical use [2].

Stem cells are classified according to their sources into two main types, the embryonic and non-embryonic. Embryonic stem cells (ESC) are pluripotent and can differentiate into all germ layers [3]. Non-embryonic stem cells (Non-ESC) can be sub-classified into fetal stem cells (FSCs) and adult stem cells (ASCs) [2]. Both of them are multipotential. Their potential to differentiate into different cell types appears to be more limited [3]. The capacity of these cells for potency (power) and relative

ease to isolate and expand are valuable properties for regenerative medicine [4].

Mesenchymal stem cells have been shown to adhere to cell culture flask and exhibit fibroblastic-like shape. Many studies have been demonstrated the effects of different culture protocols on the cell phenotype. The reports show little and no significant differences among the cells isolated by any protocol [5-7].

Differentiation is the process by which matured cells change to a specialized type. During differentiation, certain genes are turned on and become activated while others are switched off and become inactivated; a complex process tightly regulated, resulting in cell development of specific structures, which perform certain functions. Cultured cells can be made to differentiate into exclusive lineages by providing Selective media components that can be identified by histochemical staining and quantified by quantitative Real-time polymerase chain reaction (RT-PCR) [8]. The standard test to confirm the mesenchymal stem cells is differentiation of the cells into other specific cells such as osteoblasts, adipocytes, chondrocytes, myocytes and neurons. MSCs have been seen to even differentiate into neuron-like cells. The process of differentiation normally will occur with the aid of influencing factors and it is considered the test for multipotency of MSCs [9].

Immunophenotyping is using a flow cytometry technique to enable identification of specific cell types from complex biological samples according to the cell surface antigen expression. Mesenchymal stem cells can be identified based on the expression of specific proteins called surface antigen phenotype of mesenchymal stem cell markers. Some of these markers are present on undifferentiated MSCs and disappear during differentiation [10].

## 2. Objectives:

The aim of this study was to isolate and characterize mesenchymal stem cells from bone marrow of New Zealand white rabbits by its morphological, multipotential differentiation and immunophenotypical analysis.

## 3. Materials & methods:

### 3.1 Experimental animals

The use of animals were approved and in accordance to the guide by the Animal Care and Use Committee (ACUC), Faculty of Veterinary Medicine, University Putra Malaysia (Ref.UPM/FRV/PS/3.2.1.551/AUP-R94). Three New Zealand White rabbits, 3 to 4 months old, weighing  $1.3 \pm 0.3$  kg were used in this experiment to take stem cells derived bone marrow. All rabbits were healthy based on physical and blood profile examinations. , on 9<sup>th</sup> April 2010

### 3.2 Isolation of mesenchymal stem cells from rabbit bone marrow (BM-MSCs)

The isolation of MSCs was performed on euthanized rabbits as illustrated by Braga-Silva *et al.* [11]. This included anesthetizing the rabbits with ketamine-xylazine and subsequently euthanizing them with sodium pentobarbital (Dolethal). An incision was then made through the skin on the cranial thigh region and all muscles attached to the femur were removed to allow for a brief immersion of the femoral bone in 70% alcohol. The femoral bone was later placed in a 50 ml falcon tube containing media and both ends of the epiphysis cut using a bone cutter. Finally, bone marrow was flushed out into a 15ml falcon tube with 5ml media.

The collected bone marrow was immediately mixed with 5 ml of 83% Dulbecco's Modified Eagle's Medium Ham's F12 (DMEM F12) that contained high glucose supplemented with 15% fetal bovine serum (FBS), 1% penicillin/streptomycin (antibiotic) and 1% amphotericin B (fungi zone) (GIBCO®, USA) as previously described [12, 13]. Ten ml of previously prepared media was placed in a T75 tissue culture flask and bone marrow suspension was added. The flask was incubated at 37°C in 5% CO<sub>2</sub> for 3 days in a CO<sub>2</sub> incubator. Non-adherent cells were removed together with the old medium and replaced with a fresh medium. After 12 days of incubation, the culture reached the semi-confluent stage (P0) and the monolayer cells were washed twice with 2 ml of phosphate buffer saline (PBS) (pH 7.2). Then, two ml 0.2% trypsin in ethylene diamine tetra-acetic acid (EDTA) (Sigma, USA) was added to the flask and gently mixed for equal distribution in the tissue culture flask for 2 minutes in order to separate adhered cells from the culture flask. The cells were examined under an inverted microscope (Olympic, Japan) until the cells appeared rounded and the trypsin solution was then discarded. DMEM F12 medium containing 10% FBS was added and gently tapped to detach the cells from the flask. The trypsination process was repeated for another three consecutive sub-cultures.

The cells were harvested by discarding the medium, washing with PBS and addition of trypsin to the tissue culture flask in order to detach the cells. The trypsin solution was then replaced with 10 ml of fresh DMEM F12. The medium and cells were collected in a test tube, centrifuged (Hettich, Germany) at 1800 revolutions per minute (rpm) for ten minutes and the supernatant was decanted to allow for resuspension of the pellet in 2 ml DMEM F12. The number of cells in each culture flask was quantified using a haemocytometer (Neubaur, Haemocytometer, Hawksley and son. Ltd, England). Cell suspension (0.1 ml) was removed in a sterile manner and added to a dilution tube containing 0.8 ml of DMEM F12 and 0.1 ml of 0.4% Trypan Blue stain. The mixture was gently mixed at room temperature and a small drop of the stained cell suspension was transferred onto the haemocytometer and cover slip placed on top. A Small drop of the cell suspension was removed aseptically using a Pasteur pipette and placed on one side of the haemocytometer and examined under the inverted microscope (Leica, Auerian). The total number of viable cells in each four corners of the haemocytometer was counted. The total number of cells harvested from the tissue culture flasks was determined using the following equation:  $NC \times D \times 10^4 / Q$ , where NC=number of count vital cells (non-vital cell is stained blue), D=sample dilution (10) and Q=number of squares used in haemocytometer [14].

At 1<sup>st</sup> passage the stem cells were preserved using liquid nitrogen N<sub>2</sub>. Since freezing can be lethal to cells due to the effects of damage by ice crystals, alteration in the concentrations of electrolytes, dehydration and changes in PH, a typical freezing medium containing 90% serum and 10% Dimethyl sulfoxide (DMSO) was used, as reported by Fleming and Hubel [15] and Linch *et al.* [16].

### 3.2 Characterization of Rabbit Bone Marrow Derived Mesenchymal Stem Cells (BM- MSCs)

The isolated cells were pre-characterized by their morphology, multipotency and immunophenotyping characters of stem cells to ensure the isolated cells were mesenchymal stem cell (MSCs) in nature.

#### 3.2.1 Morphology

Morphological characterization was based on the isolated cells' ability to adhere to tissue culture flask and exhibit fibroblast-like shapes (spindle-shapes) that are considered as the normal morphology of MSCs.

#### 3.2.2 Differentiation Potential

##### 3.2.2.1 Adipogenesis

The differentiation of BM-MSCs to adipocytes was carried out according to the method explained by Tsai *et al.* [17]. Normally, adipogenic cells express lipid droplets deposition in the adipocytes which can be stained with Oil Red O solution. MSCs were harvested after complete confluence on 2<sup>ed</sup> passage (P2) and the cell suspension was plated into MSC expansion medium (DMEM F12) at a density of  $10^5$  cells in a 24-well culture dish (Lab-Tak®, USA) with 1 ml media per well. The plate was incubated at 37°C under 5% CO<sub>2</sub>. On reaching 100% confluence, the media was carefully aspirated from each well and 1.0 ml adipogenesis induction medium (DMEM-low glucose, fetal bovine serum 10%, 10 mM Dexamethasone, 0.5 M 3-isobutyl-1-methylxanthine, recombinant human insulin 10 mg/ml, 10 mM Indomethacin and penicillin and streptomycin) was added. Lipid droplets were detected using inverted microscope.

Oil Red O staining protocol was done after 21 days of differentiation after the medium of each well was carefully aspirated. The adipocytes were fixed in 4% paraformaldehyde for 40 minutes at room temperature, following which the fixative was aspirated and the cells were rinsed three times for 10 minutes each with PBS. The PBS was aspirated and 1 ml of the Oil Red O solution was added to cover each well and then incubated at room temperature for 50 minutes. Following removal of the Oil Red O solution, the cells in the wells were washed three times with 1 ml tap water. The cells' nuclei were stained with 0.5 ml Hematoxylin for 15 minutes. The cells preparation was examined under an inverted microscope (Leica, Auerian).

### 3.2.2.2 Osteogenesis

The differentiation of BM-MSCs to osteocytes was carried out according to the method described by Tsai *et al.* [17]. Osteogenic differentiation cells are known to express elevated levels of alkaline phosphatase and produce a mineralized matrix, which can be stained with Alizarin Red S stain. MSCs were harvested in 1 ml of DMEM, and the cells suspension was plated in DMEM F12 at a density of  $10^5$  cells per well in the vitronectin/collagen pre-coated 24-well culture dish. Each well contained 1-ml suspension volume per well and the dish was incubated at 37°C under 5% CO<sub>2</sub>. On reaching 100% confluence, the medium was aspirated and 1 ml osteogenesis induction medium (DMEM-low glucose, fetal bovine serum 10%, 10 mM Dexamethasone solution, ascorbic acid 2-Phosphate solution, L-glutamine and penicillin and streptomycin) was added into each well. The osteogenic induction medium was changed every 2-3 days during the 21 days of culture.

Alizarin Red S staining protocol was conducted after 21 days of differentiation. The medium was carefully aspirated from each well and the osteocytes suspension was fixed in ice-cold 70% ethanol for 1 hour at room temperature. Following aspiration of alcohol, the cells were rinsed twice for 10 minutes each with tap water. The tap water was later

aspirated and enough Alizarin Red S solution staining was added to cover the wells and incubated at room temperature for 30 minutes. The staining solution was then removed and cells were washed four times with 1-ml distal water and aspirated after each wash. Tap water (1 ml) was then added to each well to prevent the cells from drying and the dish was examined under an inverted microscope. The osteocytes containing calcium deposits stained orange red with Alizarin Red stain.

### 3.2.3 Immunophenotyping of Stem Cells

Mesenchymal stem cells derived from rabbit bone marrow (BM-MSC) were characterized based on the expression of surface molecules (CD markers) by using flow cytometric technique. Adherent rabbit BM-MSC at passage two were trypsinized; washed with PBS and centrifuged at 2500 rpm for 10 mins. Cells were then divided into five Fluorescence-activated cell sorting (FACS) staining tubes with at least  $2.0 \times 10^5$  cells per tube. Cells were then labeled with 2  $\mu$ l of un-conjugated primary antibodies of anti-CD34, anti-CD44 and anti-CD90 in separate tubes. The remaining two tubes served as unstained and isotype controls. Stained cells were incubated at room temperature for 30 mins and washed with PBS by centrifuging at 2500 rpm for 10 mins. All primary antibodies were monoclonal IgG1 anti-rabbit antibodies that were produced in mouse. Secondary staining was performed by adding 2 $\mu$ l fluorescein isothiocyanate (FITC)-conjugated anti-mouse Immunoglobulin G1 (IgG1) antibody and incubated at room temperature for 30 mins in a dark room. Upon completion of secondary staining, cells were washed with PBS and centrifuged at 2500 rpm for 10 mins for further analysis. Control tubes were left unstained with primary and secondary antibodies whilst isotype control tubes were stained with FITC-conjugated IgG1 isotype. All samples were assessed by the FACSCalibur flow-cytometer (Becton Dickinson, USA) and the data were analyzed using FACS Diva software. The relevant isotype antibody controls were used in parallel to all measurements to set negative gating. All antibodies were purchased from i-DNA biotechnology (M) Sdn Bhd Malaysia.

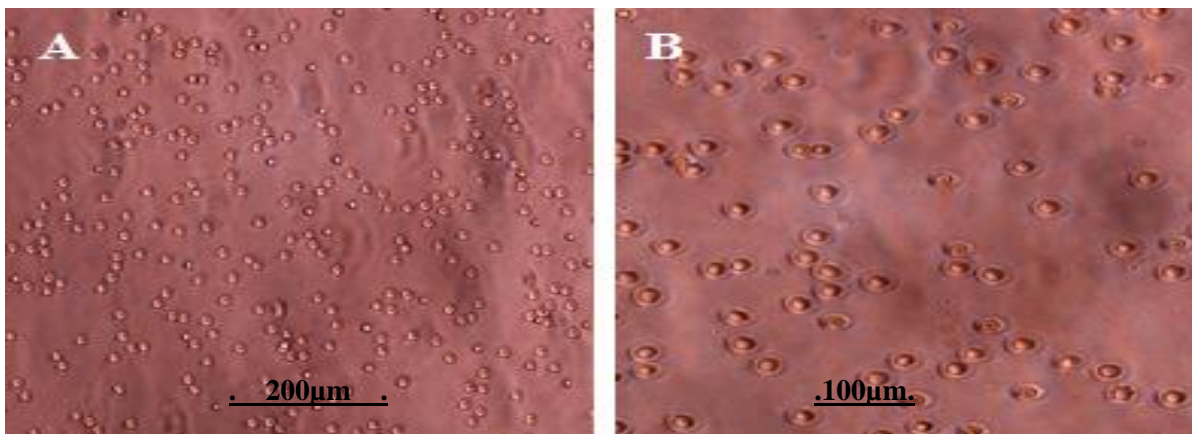
## 4. Result

### 4.1 Characterization of Rabbit BM-MSCs

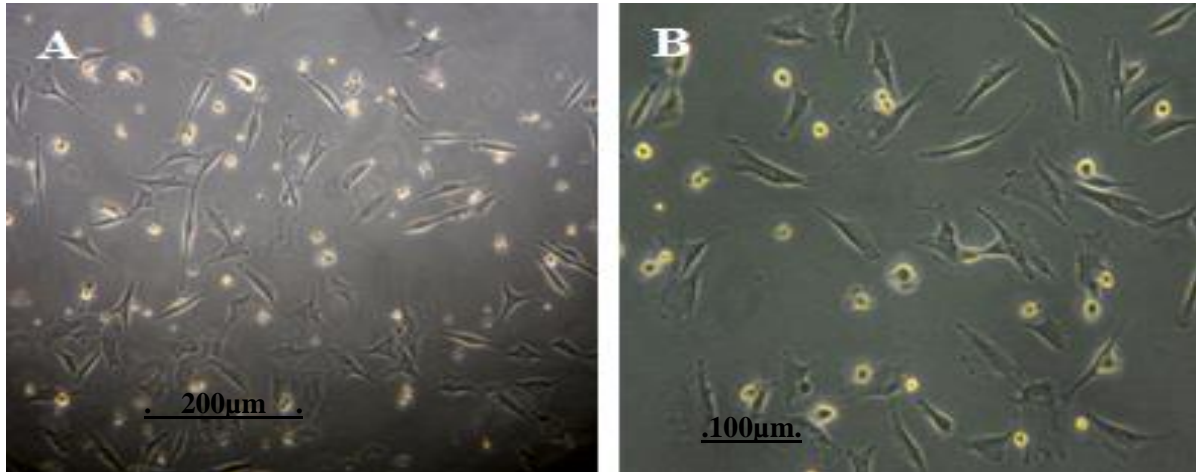
The isolated cells were pre-characterized by their morphology, differentiation capacity and expressed CD markers to ensure that the isolated cells were MSCs in nature.

#### 4.1.1 Morphology

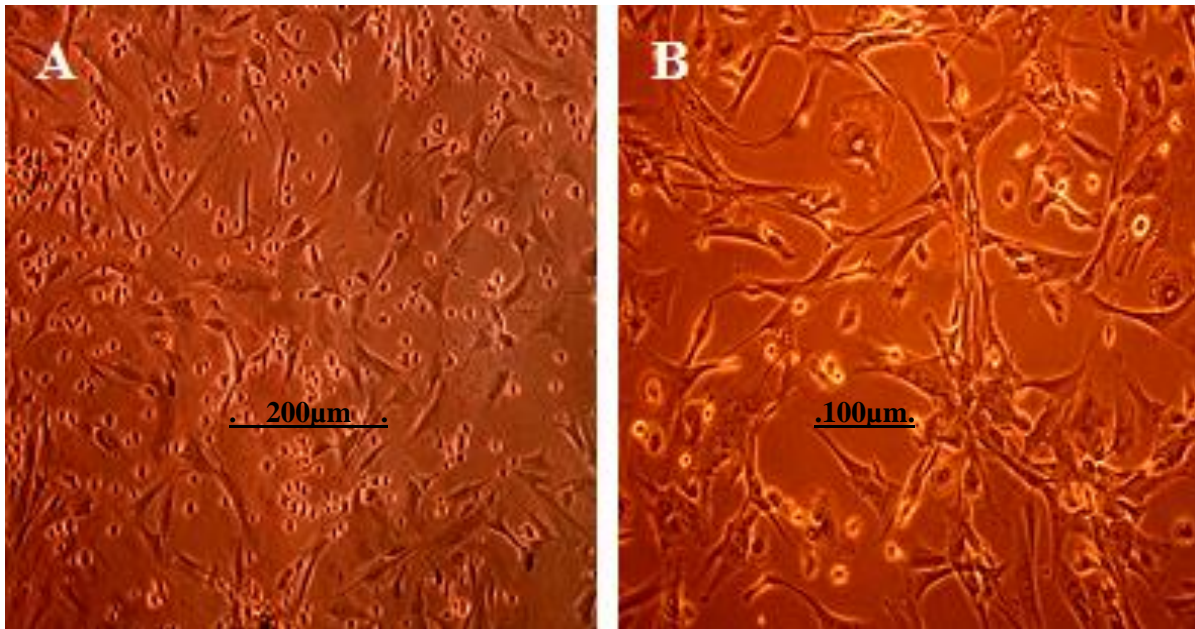
In this study, the isolated cells suspended in media were regularly rounded in shape, evenly distributing, albeit occasional cells clumping (Figure .1). Cells adhered to tissue culture flasks after 3 days -7 days of seeding, and it exhibited a fibroblast-like shape. The population became more homogeneous after subsequent passages (Figures .2-4).



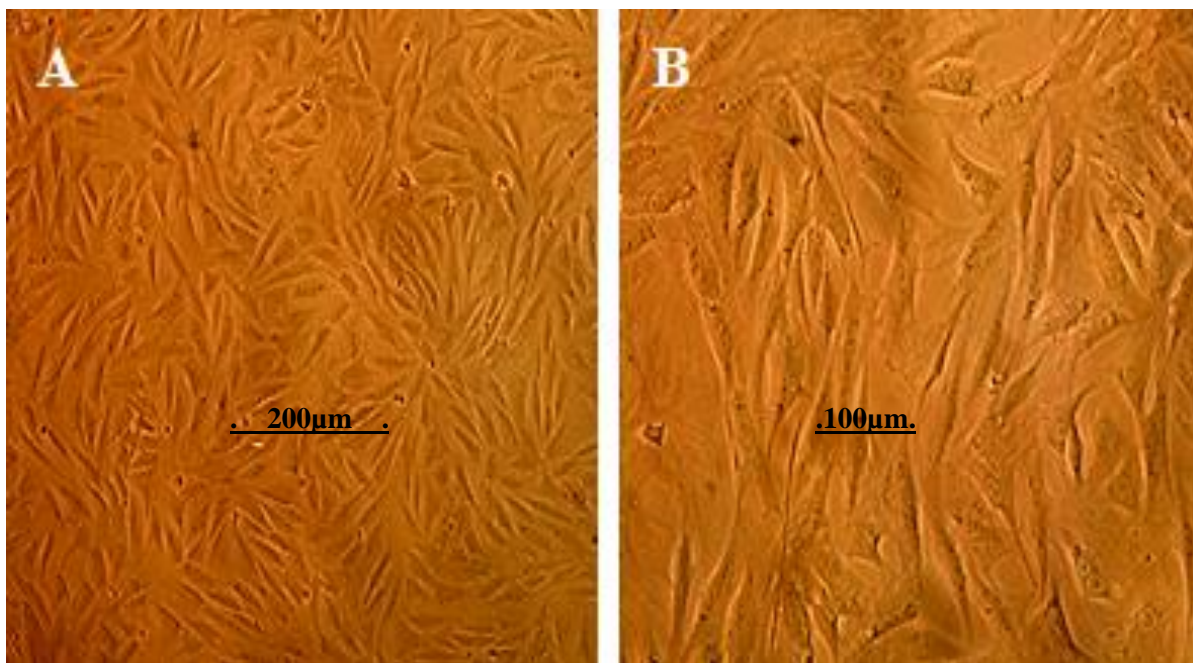
**Figure .1:** MSCs at initial passage (P0) immediately after seeding (A) as seen under the microscope at 200  $\mu\text{m}$  powered objective, and (B) 100  $\mu\text{m}$  powered objective. Cells in both figures appeared rounded.



**Figure .2:** MSCs after 3 days of initial passage (P0) showing attached fibroblastic-like shapes (A) under 200 $\mu\text{m}$  powered objective, and (B) 100  $\mu\text{m}$  powered objective. Cells in both figures appeared spear (spindle) like in shape.



**Figure .3:** MSCs at first passage (P1) showing attached fibroblastic-like shapes (A) under 200 $\mu\text{m}$  powered objective and (B) under 100  $\mu\text{m}$  powered objective. Cells in both figures appeared spear (spindle) like in shape.



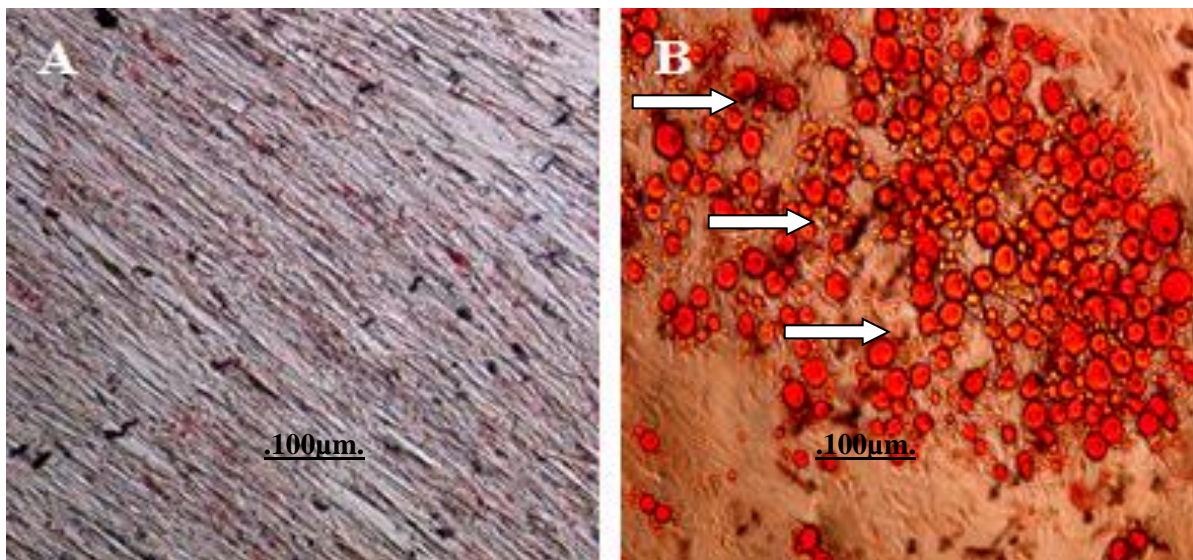
**Figure .4:** MSCs at second passage (P2) showing attached fibroblastic-like shapes (A) under 200µm powered objective and (B) under 100 µm powered objective. Cells in both figures appeared spear (spindle) like in shape and homogenous.

#### 4.1.2 Differentiation Potential

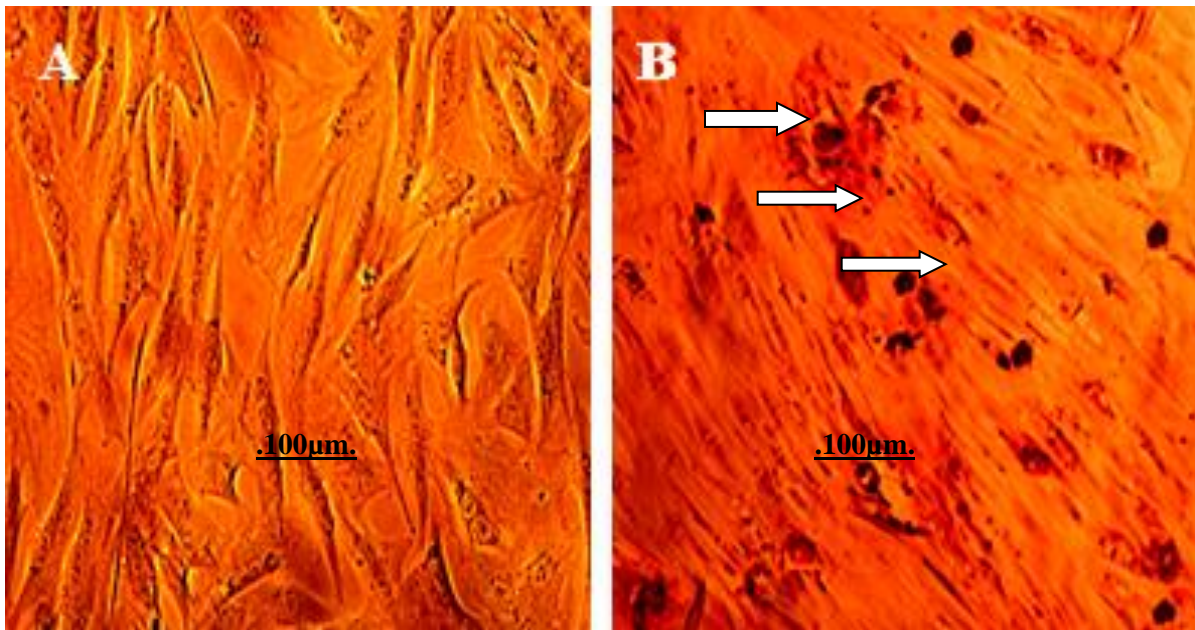
Pellet culture of rabbit BM-MSCs in adipogenesis media after 21 days showed positive deposition of fat droplets by using Oil Red Stain and the cells' nuclei were stained purple with hematoxylin stain. This indicated the differentiation of MSCs to adipocytes containing the intracytoplasmic lipid vacuoles (fat droplet). Meanwhile the cultured cells of

rabbit BM-MSCs in control media did not stain positive for Oil Red Stain (Figure .5).

The rabbit BMSCs cultured in osteogenesis media for 21 days showed positive deposition of calcium as detected by Alizarin Red S stain. This indicated the differentiation of MSCs to osteocytes. While the rabbit BM-MSCs cultured in control media did not stain positively for Alizarin Red S Stain (Figure 6). Overall, the rabbit BM-MSCs showed multi-potential ability.



**Figure .5:** Adipogenesis from MSCs as observed under 100 µm powered objective (A) showing no deposition of fat droplet for control, and (B) accumulation of intracellular lipid droplets for differentiated adipocytes using Oil Red O stain.

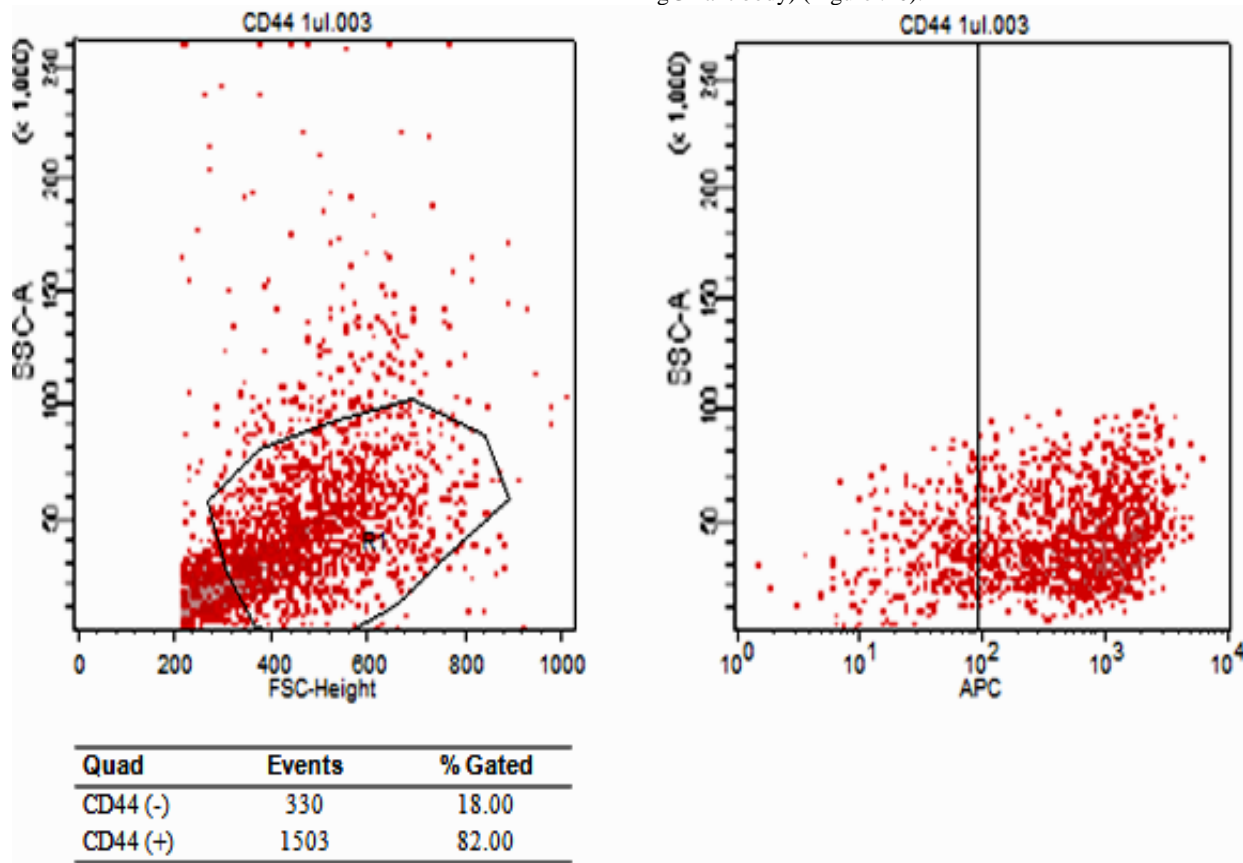


**Figure 6:** Osteogenesis from MSCs as observed under 100 µm powered objective, (A) Showing no deposition of calcium minerals for control, and (B) deposition of calcium for differentiated osteocytes using Alizarin Red S staining.

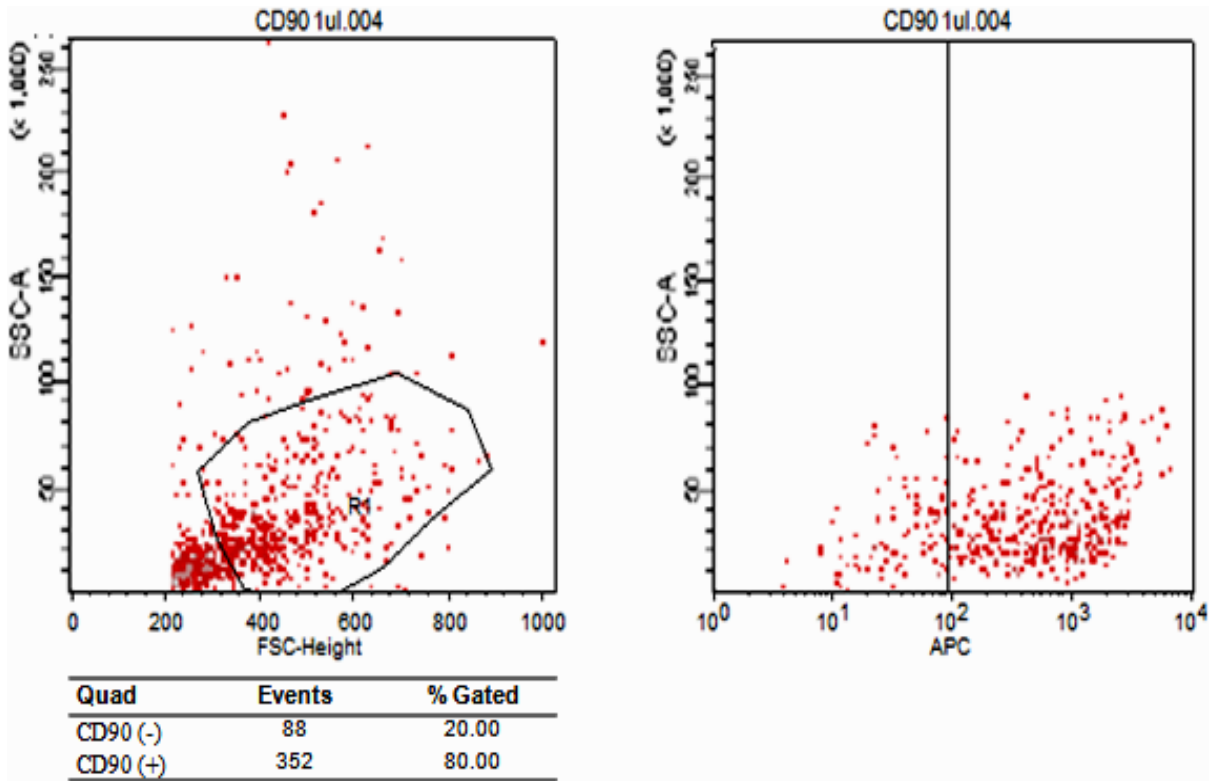
**4.1.3 Immunophenotyping of Stem Cells**

The second passage of rabbit BM-MSCs was utilized for detection of the cell surface epitopes. Those cells were positive in the expression of MSCs marker related to cell adhesion CD44 (Pgp-1, HCAM) (Figure 3.7) and

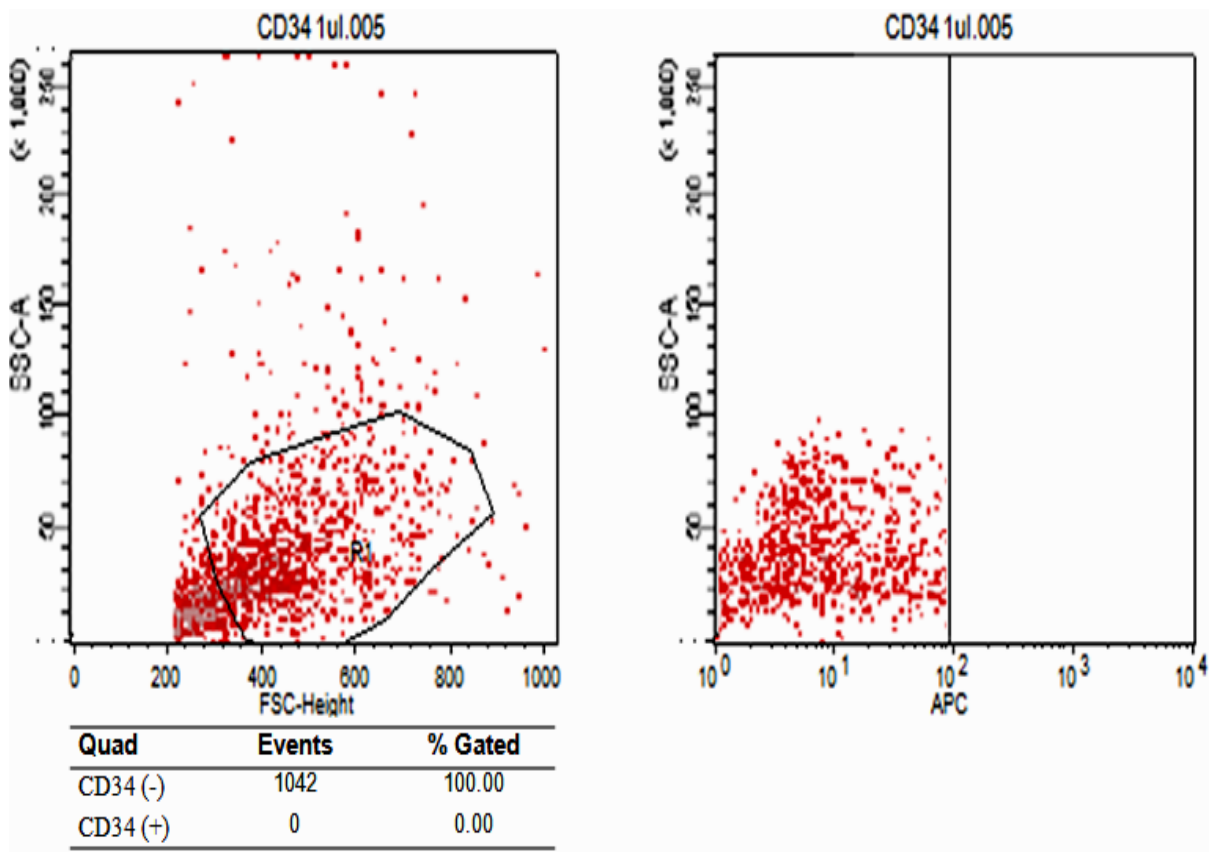
multipotency CD90 (Thy-1) (Figure 8). While the same cells showed negative in the expression of hematopoietic stem cells marker CD34 (Figure 3.9), they also showed negative even in the presence of second (2<sup>nd</sup>) antibody only (negative control), (FITC-conjugated anti-mouse IgG1 antibody) (Figure .10).



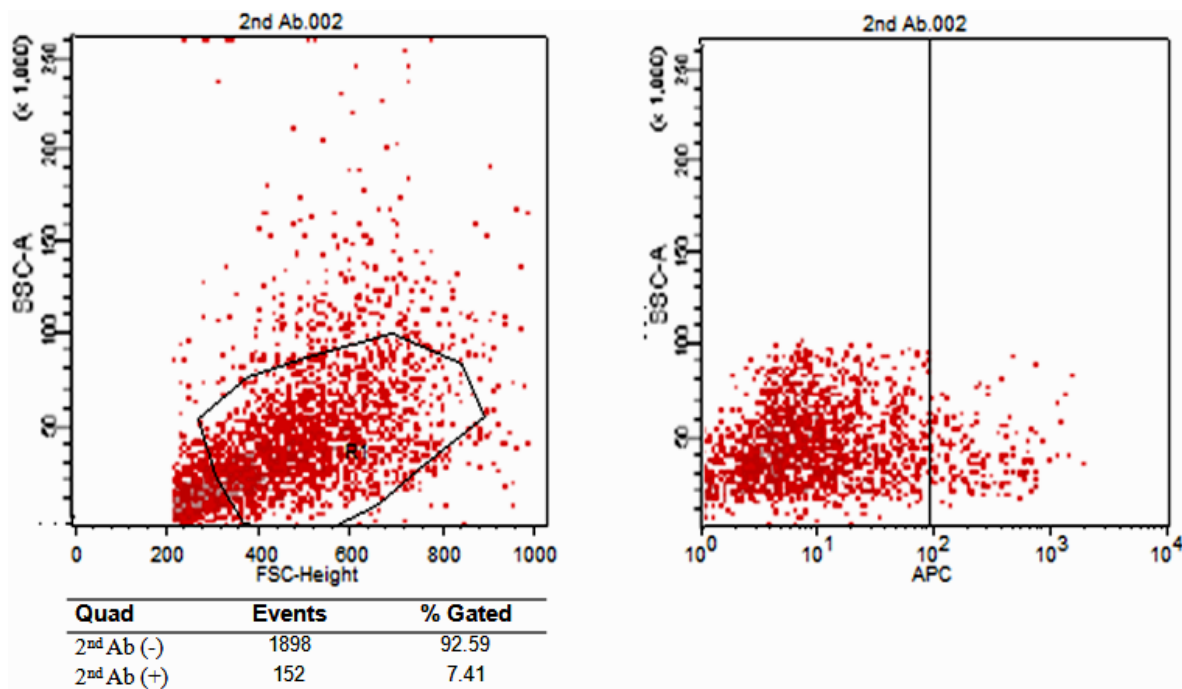
**Figure 7:** Histogram depicting stem cell marker CD44. BM-MSCs were positive for CD44.



**Figure 8:** Histogram depicting stem cell marker CD90. BM-MSCs were positive for CD90.



**Figure 9:** Histogram depicting stem cell marker CD34. BM-MSCs were negative for CD34.



**Figure 10:** Histogram depicting stem cell marker secondary antibody. BM-MSCs were partially negative for secondary antibody.

## 5. Discussion

**The Morphological Characteristic:** the present study demonstrated that BM-MSCs adhered to tissue culture flasks after 3 to 7 days of seeding and exhibited fibroblast-like shapes. BM-MSCs were isolated based on their adherence to tissue culture flasks and survival through the cultivation period. These may be possible because they maintained their proliferative capacity, as reported previously [18, 19]. Two populations of cells in primary passage (P0) were identified including small round cell (non-adherent cells) and spindle-shaped stromal stem cells (adherent cells). The small rounded cells decreased in size rapidly and disappeared with repeated passages in a culture of regular media, while the fibroblastic cells maintained their proliferative characteristics similar to those in the primary passage (P0). The cells required up to 14 days to reach 90% confluence, at the first passage (P1), and showed complete confluence at day 5 of subculture. In the second passage (P2), cells achieved confluence on day 2, and by the third and fourth passages (P3 and P4), cells became morphologically homogeneous with more than 95% showing fibroblastic shapes. All these heterogeneous and homogeneous changes in morphology observed were in agreement with previous reports [12, 18-20].

**The Immunophenotyping Characteristic:** the present result demonstrated that BM-MSCs derived from rabbits exhibited a similar expression profile as MSC surface markers (cell adhesion marker CD44 and multipotency marker CD90) and lacked hematopoietic markers (CD34). However, previous studies investigating MSCs isolated from various tissues have indicated that cell surface phenotype can vary from that of BM-MSCs [21, 22], most notably using a panel of antibodies used to define MSCs [23]. In BM-MSCs, the surface markers have clearly been defined, the success of which has depended largely on their ability to adhere to tissue culture flasks that separate them from cells of hematopoietic stem cells [24].

**The Multipotential Character:** the differentiation potential of BM-MSCs derived using two different techniques (by way of anesthesia or euthanasia) demonstrated similar differentiation capacities in comparison with most previous studies [20, 25]. The isolated cells incubated in adipogenic induction medium were able to differentiate into adipocytes

with accumulation of intracellular lipid droplets detected by staining with Oil Red O. This result is consistent with previous reports [20, 25, and 26]. Also, BM-MSCs incubated in an osteogenic induction medium were able to differentiate into osteocytes with intracellular calcium deposit as demonstrated by red coloration of Alizarin S, in agreement with what has been reported previously [20, 27, 28]. Other studies have reported that BM-MSCs are multipotent cells that can differentiate into different cell types, including adipocytes and chondrocytes [29]. Besides that, these cells have the ability to Trans-differentiate into cells of endoderm and ectoderm lineages such as hepatocytes and neurons [30].

Overall, The confirmation and identification of MSCs through morphology, multipotency and immunophenotyping characteristics have proven that the isolated cells from rabbit bone marrow were MSCs in nature, which is consistent with reported findings [12, 18-20, 23-26].

## 6. Conclusion

In general, the morphological, multipotency and immunophenotypical characteristics for the isolated MSCs from euthanized rabbit bone marrow have proven that those cells were stem cell in nature. The isolated MSCs from euthanized rabbit bone marrow illustrated similarities in morphological characteristics and multipotential ability compared with other research work which were isolated MSCs from anesthetized rabbit, even though variations existed in the sample harvesting, materials and required skills in the two methods. Euthanized rabbits were subsequently used as the method of choice for harvesting the bone marrow cells. Further comparative study should be made to study cell viability and proliferation capacity between bone marrow derived stem cells from anaesthetized and euthanized animals, moreover study the advantage and disadvantage for each method.

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their help, guidance, and inspiration in carrying out this work". Instead of the way it was written in this article.

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