ORIGINAL ARTICLE

Generation and Characterization of Human Osteoarthritis Cartilage-derived Mesenchymal Stem Cells by Modified Sample Processing and Culture Method

Satar Jabbar Rahi Algraittee[1,2,3], Hamza Lawal[1,4], Mohadese Hashem Boroojerdi[1,2], Vahid Hosseinpour Sarmadi[1,2], Maryam Maqbool[1,2], Fahrudin Che Hamzah[5], Shalini Vellasamy[6], Cini Mathew John[7], Ling King Hwa[8], Rajesh Ramasamy[1,2]

[1] Stem Cell & Immunity Research Group, Immunology Laboratory, Department of Pathology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[2] Stem Cell Research Laboratory, Genetics & Regenerative Medicine Research Centre, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[3] Department of Medical Microbiology, College of Medicine, University of Kerbala, 46001 Kerbala City, Iraq
[4] Department of Biochemistry, Faculty of Sciences, Bauchi State University, Gadau, Itas-Gadau LGA, Bauchi State 751105 Nigeria
[5] Department of Orthopedics, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[6] Department of Microbiology, Faculty of Medicine and Biomedical Sciences, MAHSA University, 42610 Jenjarom, Selangor, Malaysia
[7] Department of Physiology and Pharmacology, Faculty of Medicine, University of Calgary, 3330 Hospital Dr NW, Calgary, AB, Canada T2N 4N1
[8] Medical Genetics Laboratory, Department of Biomedical Science, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

ABSTRACT

Introduction: Mesenchymal stem cells (MSCs) can be isolated from different tissue sources, and show a high differentiation capacity towards osteogenic, adipogenic, chondrogenic, neurogenic and myogenic lineages upon a specific induction. Although the retrieval of MSCs from normal tissues is very straightforward, yet it could be challenging in degenerative conditions that limit the expansion of stem cells such as osteoarthritis. Thus, this study aimed to establish human MSCs culture from osteoarthritic cartilage (OA hC-MSCs) by optimising the sample processing and culture techniques. **Methods:** Human osteoarthritis knee cartilage samples were obtained (2-4 g) from 8 patients with a mean age of 62.75 years old during the joint replacement surgery. A conventional culture method carried along with the modified method where the period of enzyme digestion and serial plating culture procedure were incorporated. **Results:** The modified culture method has significantly increased the number of single cells twice after the sample processing. The time taken to form colonies and achieve confluence was also reduced when samples subjected to the modified method. The number of cell yields after passage 0 for the conventional and modified methods were 3.05±0.31 and 6.10±0.42 million cells, respectively. The adherent cells generated under these two conditions comply with criteria for MSCs in term of immunophenotyping and mesodermal differentiation. **Conclusions:** The current modified method enhances the production of MSCs and could be opted for samples that known to have reduced or defective stem cell pool which may impede the in vitro cell expansion.

Keywords: Mesenchymal stem cells, Cell Expansion, Osteoarthritis, Cartilage, Degenerative disease

Corresponding Author:
Rajesh Ramasamy, PhD
Email: rajesh@upm.edu.my
Tel: +603-89472377

INTRODUCTION

Osteoarthritis (OA) is a degenerative bone disease which affects millions of people in the world. It is a complex disease where the changes in tissue homoeostasis of articular cartilage and subchondral bone gradually lead to a predominant destructive process in affected joints (1). Although, OA regarded as a degenerative disease, yet the current scientific evidence suggestive of the involvement of impaired immune response. This has brought the possibility of synovitis, and the immune system could be active players in OA development and progression (2). In OA condition, the regenerative potential of articular cartilage is challenged by an excessive wear-off which is accompanied by subchondral bone sclerosis

and synovial inflammation (3). It would be ideal to understand the pathology of OA from the aspect of cartilage stem cell biology as the regenerative potential of articular cartilage is compromised by excessive tissue destruction and synovial inflammation.

Mesenchymal stem cells (MSCs) are multipotent non-haematopoietic stem cells with the capacity to differentiate into mainly mesodermal lineages (4, 5). Many studies have shown that MSCs can be isolated from different tissue sources such as periosteum (6), synovium (7), skeletal muscle (8), bone marrow (9) and adipose tissue (10). Upon an appropriate induction, MSCs can be triggered to differentiate into osteogenic, adipogenic, chondrogenic, neurogenic and myogenic cells (11). Although MSCs could harvest from various anatomical locations and tissue origins, yet the characteristic of MSCs is assumed to be similar due to the natural self-renewal and differentiation abilities. However, it has been noticed that the tissue-specific MSCs are more inclined towards the differentiation of a particular tissue lineage as the cartilage-derived MSCs are superiorly chondrogenic compared to the MSCs that derived from other sources (12).

However, based on limited studies available, the chondrogenic potential of OA-MSC is reported to be reduced in advanced OA (13-15). The impact of OA on MSCs could be varied based on the function of MSCs. Although, OA impaired the chondrogenic differentiation of MSC, yet such compromise was not noticed in MSC exerted immunosuppression. According to Hangmann et, al MSC from OA-affected joint effectively maintained the regulatory T cell proportions in lymphocyte to control the inflammatory milieu (16), and Eljawahri et al. and Huang et al. revealed that OA synovial membrane derived MSCs are capable of suppressing lymphocyte immune response (17-20). However, these are very limited observational studies that indicated the manifestation of MSC-related deficiency in OA patients, yet a detailed investigation in deciphering the reported disorder via mechanistic approach is still lacking.

For successful cell-based therapy and regeneration, ensuring a sufficient number of MSCs is crucial. Nonetheless, an optimised protocol for culturing the diseased or impaired cartilage-derived MSCs is not as straightforward as normal tissues. Many factors, including the sample processing procedures and culture protocols, have an impact on the generation and expansion of MSCs (21-23).

The majority of the previous studies on the culture of MSCs from different tissue sources disclosed the isolation of MSCs by standard collagenase digestion method with limited incubation period (24-26). However, there might be a potential loss of MSCs during the filtration (filter-trapped) and subsequently during changing the media after the primary culture as discarded media can be an abundant cell supply. Therefore, alternative sources are necessary to be optimised to overcome these caveats. We hypothesised that the increase of enzyme digestion period and use of discarded media from the first primary culture during the culturing process would increase the number of hC-MSCs colonies in OA cartilage tissue. We intended to maximise the yield of MSCs from the OA cartilage samples by modifying two crucial steps in the conventional culture method. It will provide new insights in propagating MSCs from diseased individuals and support the application of autologous cell-based strategies in the regeneration of musculoskeletal tissues.

MATERIALS AND METHODS

Study subjects

Human osteoarthritis knee cartilage samples were obtained (~ 2-4g) from 8 patients with mean age of 62.75 years old and ranged around 53-75 years old. The samples were harvested from donors following the joint replacement surgery at Hospital Serdang, Selangor, Malaysia. All samples collected within 24 hours with a written informed consent from the patients. The research study was approved by Medical Research and Ethics Committee (MREC) [KKM/NIHSEC/P16-1102], Ministry of Health, Malaysia and Institutional Regulatory Board, University Putra Malaysia [UPM /TNCPI/RMC/ 1.4.18.1JKUPMF1].

Sample collection and isolation

The specimens were taken from the condyle of the femur (Figure 1A) within 24hours following joint replacement surgery. The samples were transported in an icebox and processed within 3 hours. During the sample processing, cartilage fragments were disinfected using 70% ethanol for 1 minute and then soaked in 1x PBS containing 2% streptomycin and penicillin (Gibco, Invitrogen) and weighted. Cartilage tissues from each donor were divided into two aliquots (~1-2g each) and meticulously minced using surgical scissors before proceeding with the two isolation procedures namely conventional (group 1) and modified enzymatic digestion-culture methods as described below.

Conventional and modified enzymatic method

The cartilage tissue fragments were transferred into a mixture of enzymatic buffer, 20ml of 0.4% collagenase type II (Gibco BRL, Invitrogen, USA) and 0.01% DNAse I (Sigma-Aldrich, USA) for both groups (group 1 and 2). For group 1, the specimen was incubated at 37oC for 1 hour accompanied by gentle mechanical agitation at 2000rpm. In contrast, the specimen for group 2 was incubated at 37oC for 24 hours without any agitation (Figure 1B). Upon incubation, both groups were filtered using 70µm and 40µm cell strainers (Becton Dickinson, USA) respectively. The filtrate was then centrifuged for 10 minutes at 2000rpm. The cell pellet was harvested and washed with 1x PBS buffer and resuspended in 1ml of DMEM/F12 complete medium [1% penicillin

and streptomycin (Gibco BRL, Invitrogen, USA), with 0.5% fungizone (Gibco BRL, Invitrogen, USA), 0.1% gentamicin (Gibco BRL, Invitrogen, USA) and 40ng/ml of recombinant human basic fibroblast growth factor (bFGF) (Peprotech,USA)]. The cell viability and count were determined by trypan blue exclusion staining. Cells were cultured in a T25 plastic culture flask (Becton Dickinson, USA) and incubated in 5% CO2 humidified incubator at 37oC. Importantly, cell culture media from group 1 were discarded and changed for the first time after the cell attachment was observed, and subsequently, the media was changed every 3 days until cells reached confluency. On the other hand, in group 2, after the outgrowth of adherent cells was noticed, 3ml of the supernatant was aspirated and added to a new second T25 flask that contains 2ml of fresh DMEM/ F12 complete media. Subsequently, once colonies of adherent cells noticed, 3ml of supernatant from the second T25 flask was added into a new third T25 flask, and the previous steps were repeated until third T25 flask. After the initial serial replating method, all flasks periodically replaced with fresh media every three days until the adherent cells achieved confluence. Finally, the cells were harvested from both groups by treating the cells with 0.05% TrypLE Enzyme (Gibco BRL, Invitrogen, USA) for 3-5minutes, and counted using a hemocytometer by trypan blue exclusion method. The schematic diagram of sample processing and culture method shown in Figure 1.

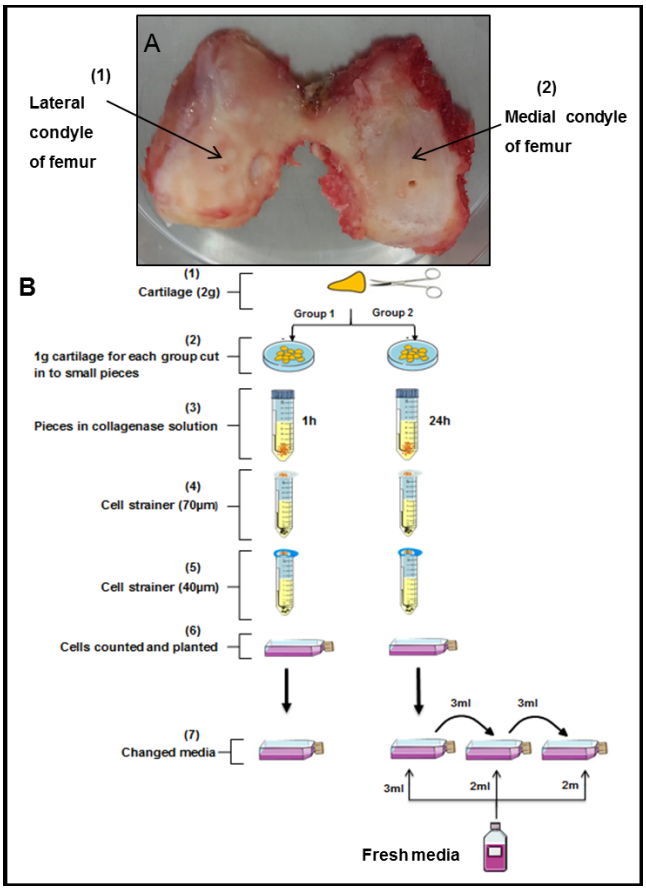

Figure 1: (A) The schematic presentation of sample harvesting and processing. (1) Lateral condyle of femur used for the harvesting of cartilage sample. (2) Medial condyle of femur showing full thickness cartilage wear. (B) Enzymatic digestion process for cell isolation from human OA cartilage and grouping. (1) Human cartilage sample was obtained from osteoarthritis patients and divided into two groups (1g for each group). (2) The tissues cartilage was meticulously minced. (3) Cartilage pieces were digested in collagenase solution after 1hour digestion in group 1(Conventional method) and 24hours in group 2 (modified enzymatic). (4, 5) Precipitated tissues were removed by 70µm and 40µm filters. (6) Cell pellets were transferred into T25 plastic culture flask. (7) After adherent cells, the media were discarded and changed for group 1. For group 2, 3ml of supernatant transferred into a new flask and replaced by 3ml of MSCs complete media.

Expansion and Cryopreservation

The adherent cells collected after reaching 70-80% confluency via trypsinisation method using 0.05% trypsin-EDTA (Gibco, Invitrogen) at 37oC for 3 minutes. The harvested cells were centrifuged at 2000rpm for 10 minutes and then sub-cultured further at 12,000 cells/cm2 seeding density in the T25 flask. The sub-cultured cells expanded till passage 6, and particularly, only passage 3 cells were utilised for the subsequent experiments or cryopreserved in freezing media containing 90% fetal bovine serum (Gibco, Invitrogen) and10% DMSO (Sigma-Aldrich).

Immunophenotyping

The adherent cells at passage 3 from both groups were subjected to the Immunophenotyping by direct immunofluorescence staining and analysed by flow cytometer (BD LSRFortessa). The trypsinised cells at 2x105 cells/ml were labelled with fluorochrome-conjugated anti-human antibodies against MSCs positive markers (CD105, CD90, CD73, CD44, CD29 and HLA-ABC) as well as MSC negative markers (CD45, CD34, HLA-DR, CD80, CD86, and CD14) antibodies for 15 minutes at 4oC and resuspended in 1ml of 1xPBS. The cells were then transferred into Fluorescence Activated Cell Sorting (FACS) tubes to be analysed by flow cytometry (BD LSR FORTESA, San Jose). A minimum of 10,000 cells was acquired and then analysed with FCS Express 6 Plus software (De Nova software). All antibodies were purchased from Becton Dickinson except anti-CD 105-PE, which was purchased from R&D system.

Mesodermal Differentiation

The mesodermal differentiation potential of OA hC-MSCs was tested at passage 3 using StemPro osteogenesis, adipogenesis, and chondrogenesis differentiation kits (Gibco, Invitrogen, USA). Adherent cells were seeded at 5000 cells/cm2 in 24-well plate using MSCs complete media; expanded at 37oC in 5% CO2 humidified the air until a monolayer of confluence cells attained. Adipogenesis and osteogenesis were induced using adipogenic and osteogenic differentiation media respectively with a control undifferentiated culture. The differentiation media were changed every after 3 days for 21days, whilst the control cells were maintained in MSCs complete media throughout the experiment. For the osteogenic differentiation assay, the cells were fixed with 70% ethanol for 60 minutes

and stained for 30minutes using Alizarin Red solution. On the other hand, adipogenic differentiation was performed by fixing the cells in 4% of paraformaldehyde for 30minutes after which they were stained with Oil Red O solution. For the chondrogenic, a particular 2D micro-mass culture method was performed to induce differentiation into chondrocytes. Technically, to induce chondrogenesis, MSCs at a density of 16 million cells/ml were prepared as a cell suspension in MSCs complete media. Five microliters of cells suspension were planted at the centre of 24 well plate to perform the classical stain. The micro-mass culture was cultivated for 2 hours under light and humid conditions, then after, STEMPRO Chondrogenesis differentiation Basal Medium and 10% STEMPRO Chondrogenesis Supplements were added and incubated at 37oC with 5% CO2. The media change was maintained every 2-3 days for 21 days of the cultivation period. Cells were rinsed with 1x PBS and fixed using 4% formaldehyde for 30 minutes. Finally, the fixed cells were stained with 1% Alcian Blue. All cells were observed and analysed using phase contrast microscope.

RESULTS

Modified process and culture method enhanced the growth of OA hC-MSCs

The outcomes of the conventional and modified culture methods were assessed based on a number of single cell yield after the sample processing; the time is taken to form adherent colonies, the period for achieving cellular confluency and the total cell yield after the passage 0. The mononuclear cell yield from the modified method (group 2) was two folds high (4.07±0.65 x10^6/cm2) as compared to the conventional enzymatic digestion (group 1) (2.02±0.32x10^6 cell/cm2) (Table I). In group 2, a serial plating method was employed where 3 ml of the supernatant was transferred to a new flask, and this step was repeated for a total of two cycles. It took a shorter period to form colonies using the modified method as the first colony was observed on day 7-10, whereas the conventional method needed at least 12-13 days. Moreover, the modified method expedited the cell growth and the cell confluency could detect on day 20-23 as compared to the 25-27 days in the conventional method. Interestingly, after the final cell harvesting for both groups, the difference in cell yields between group 1 and group 2 (3.05±0.31 and 6.1±0.42, $p<0.05$) was statistically significant.

Table I: Comparison of two approaches to generating OA cartilage MSCs

Variables	Group 1	Group 2	p-value
N	4	4	-
Nucleated cell yield after the collagenase digestion (X10^6/cm)	2.02± 0.32	4.07± 0.65	≤ 0.05
Days of first colony formation	12-13 days	7-10 days	≤ 0.05
Days in culture achieving confluence	25-27	20-23 days	≤ 0.05
Cell count after trypsinisation P0(X10^6 /cm)	3.05 ± 0.31	6.1 ± 0.42	≤ 0.05

Adherent cells of OA cartilages assumed a spindle-like morphology

When single cells plus the remnant of tissue fragment from group 1 is cultured in complete medium, an outburst of colony observed around the tissue explant after 12-13 days of culture. The primary cell culture contained an array of cells with different morphology as rod-like cells, spindle-like cells and cell with polygonal shapes were observed. When the explants were removed during the medium change and the adherent cells were left to expand until confluence, the morphology of cells turned out to be relatively homogeneous (Figure 2A1–A3). On the other hand, cells which were isolated using the modified method, spindle or polygonal fibroblast-like cell growth in a dispersed pattern was observed after just 7-10 days in culture. However, the morphological differences were diminished substantially when cells reached 90% confluence (Figure 2B1–B3). At the third passage (P3), cells from both conventional (Figure 2C1-C3) and modified methods (Figure 2 D1-D3) assumed an MSCs like morphology with no significant differences in morphology between these two groups.

Adherent cells of OA cartilages express the common surface antigens of MSCs

To verify whether the adherent cells formed using OA cartilage samples are MSCs, surface protein analysis by immunofluorescence conjugated antibody labelling (immunophenotyping) was conducted. Only cells from passage 3 onward were subjected to the immunophenotyping as any very early passages might contaminate with other haematopoietic cells that potentially affect immunophenotyping profile. It has been found that adherent cells were grown in both conventional and modified methods express MSCs positive markers namely CD29, CD44, CD73, CD90, CD105, and HLA-ABC. They did not express markers that known negative for MSCs such as CD14, CD34, CD45, CD80, CD86 and HLA-DR (Figure 3). Both MSCs derived from conventional and modified methods exhibited a similar immunophenotyping profile which is adhered to the commonly accepted international standard.

Adherent cells of OA cartilages undergone adipogenic, osteogenic and chondrogenic differentiation

Mesodermal differentiation is considered as the most important criterion to define MSCs minimally. During the 21 days of adipogenic induction, OA hC-MSC manifested numerous lipid vacuoles, which were

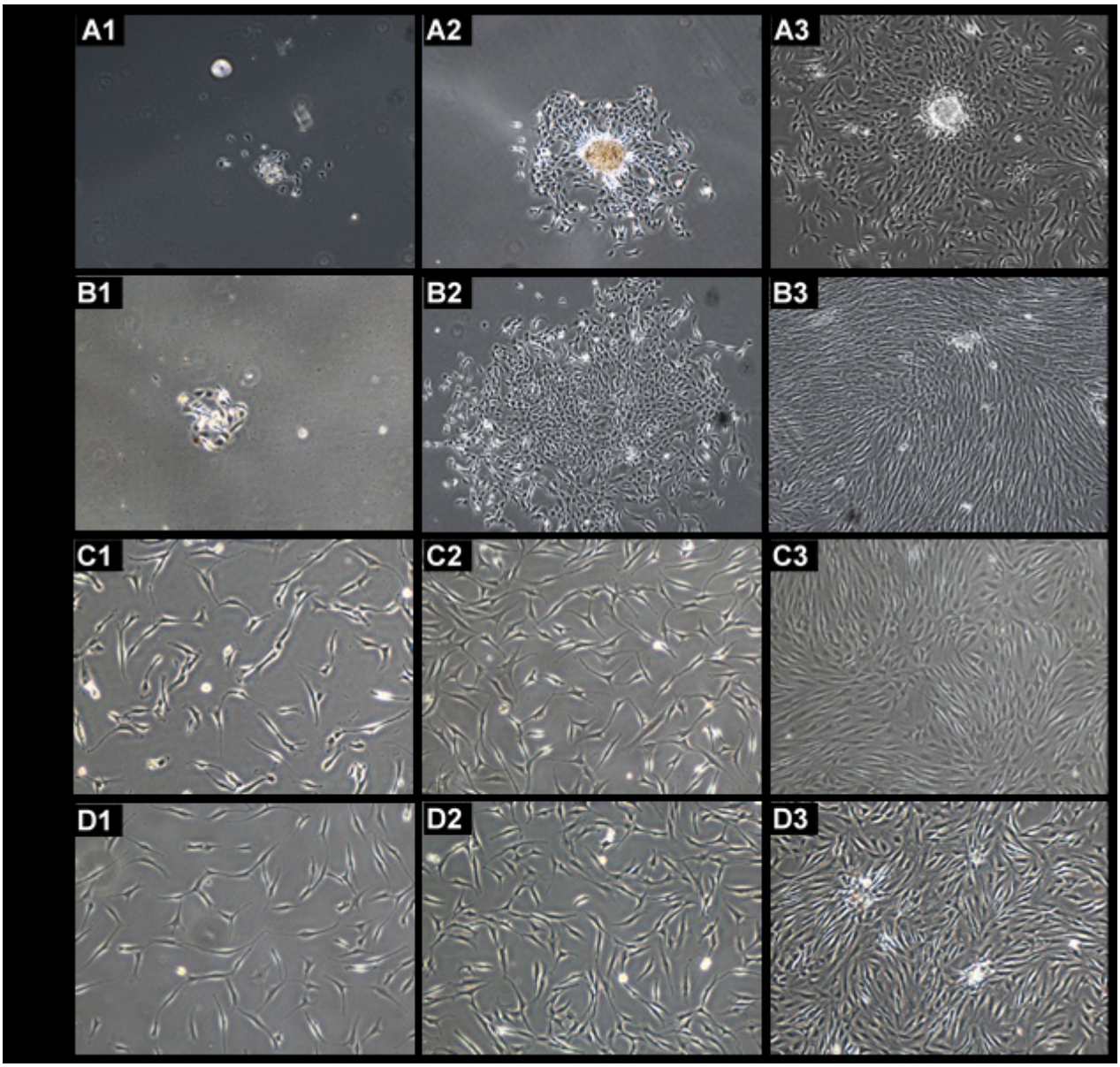

Figure 2: The morphological examination of different passages of adherent cells. (A) Primary cells (Passage 0) isolated using the conventional enzyme digestion method at 12 days (A1), 15 days (A2), and 20 days (A3). (B) Primary cells (Passage 0) isolated using the modified enzyme digestion method at seven days (B1), 15 days (B2) and 20 days (B3). (C) Passage 3 cells isolated using the conventional enzyme digestion method at day 1(C1), day 3 (C2) and day 4 (C3). (D) Passage 3 cells isolated using the modified enzyme digestion method at day 1 (D1), day 3 (D2) and day 4 (D3). Microscope magnification: 40X (A, B, C, D).

stained by a positive Oil Red O (Figure 4A). When the cells cultured in the osteogenic inductive medium and stained with Alizarin Red S, the calcium deposition as an indicator of osteogenic differentiation showed a bright red colour staining (Figure 4 B). Unlikely, osteo and adipo differentiations where the cells are in monolayer culture, the chondrogenic differentiation needed a complex 3-D environment to ignite the differentiation process. After 21 days of culture in the cell pellets, cells undergo chondrogenesis showed proteoglycans production and intense staining with Alcian Blue stain (Figure 4C). Importantly, the control (non-treated cells) did not show spontaneous differentiation of adipocytes, osteocytes, and chondrocytes.

DISCUSSION

Mesenchymal stem cells (MSC) are adult stem cells and reside in most of the solid organs and connective tissues as these are stem cells supply scaffold and stromal compartment to harness the parenchymal cells. Unlikely other adult stem cells, MSCs possess an inherent immunomodulatory and anti-inflammatory functions on almost all type of immune cells which make them an ideal candidate to treat diseases with inflammatory features such as OA and rheumatoid arthritis (27, 28).

Since OA is a multifactorial disease, hence the pathogenesis of OA also could be viewed from the point

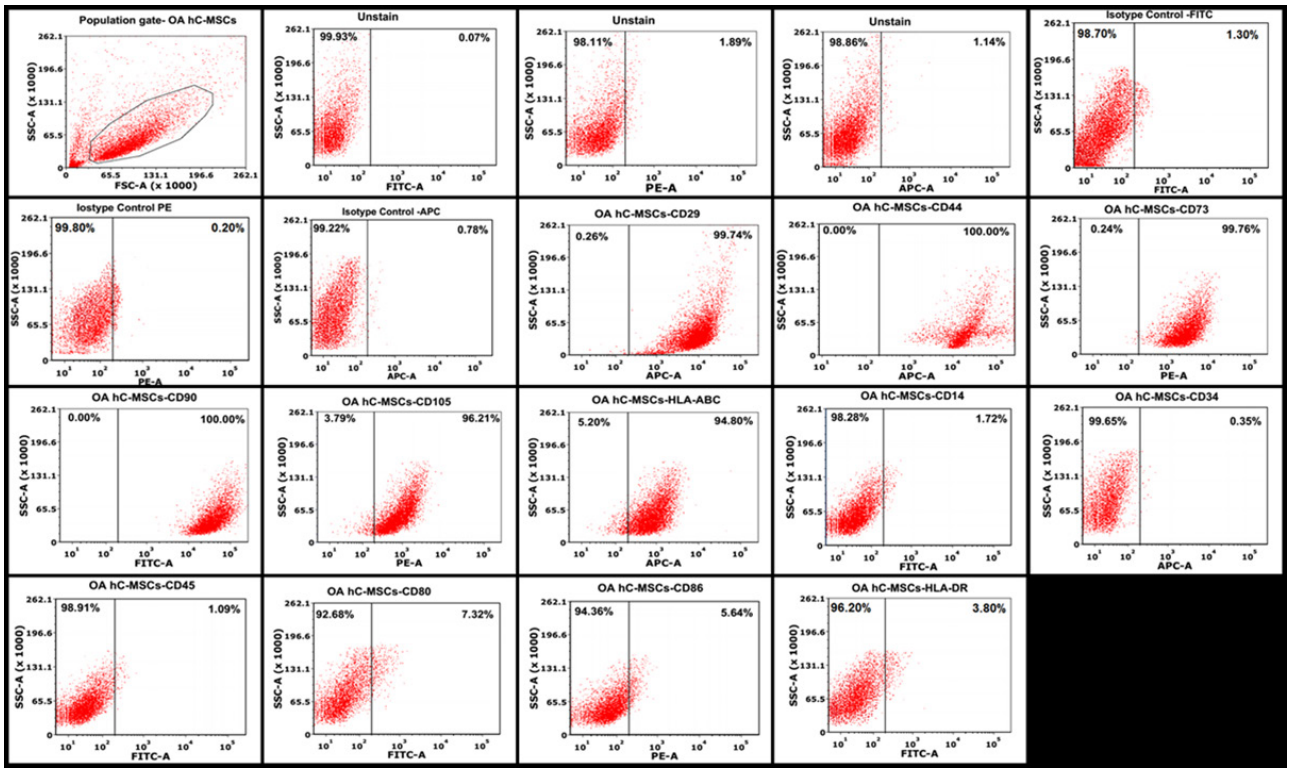

Figure 3: The immunophenotyping of OA hC-MSCs. The passage three adherent cells were harvested and stained for a panel of MSCs cell surface markers. Approximately, 2x105 cells were stained with fluorochrome-conjugated mouse anti-human monoclonal antibodies for flow cytometer acquisition. Unstained cells and isotype-matched controls were used to set the negative population. Approximately 95% of cells positive for the common MSCs' markers; more than 92% negative for other haematopoietic and immunological markers. Data are representative of an individual experiment.

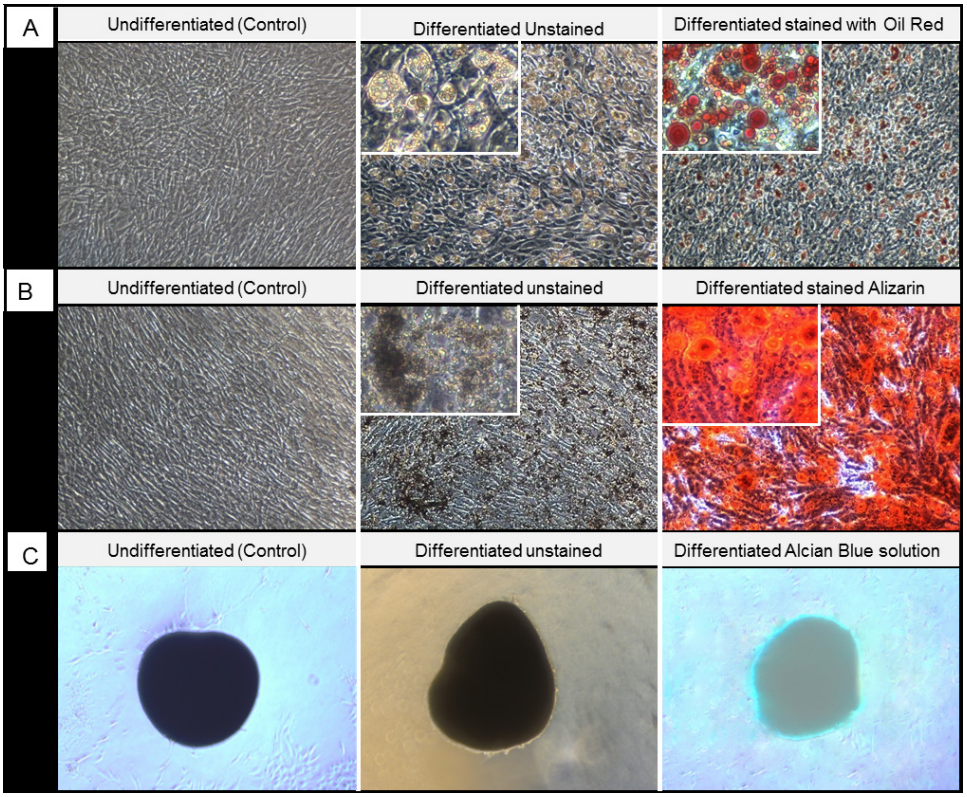

Figure 4: The mesodermal differentiation of OA hC-MSCs. (A) The histochemistry images of undifferentiated (control), unstained and stained adipogenic differentiated cells. The red colour stain indicates accumulation of fat droplets as observed in the adipogenic lineage cells. (B) Histochemistry images of undifferentiated (control) cells, unstained and stained osteogenic differentiated cells. The red colour stain indicates accumulation of calcium deposits as observed in the osteogenic lineage cells. (C) Histochemistry staining of chondrogenesis. Chondrogenic differentiation was verified by 1% Alcian Blue solution. Formation of proteoglycans (chondrocytes) was observed in differentiated MSCs. Microscope magnification: 40X (A, B, C). Microscope magnification of inserts: 200X (A,B).

of tissue resident stem cells' inadequacy in recuperating the excessive cartilage damages. In physiological condition, the functioning chondrocytes generated from the cartilage progenitor cells which could be the progenies of tissue-resident mesenchymal stem cells. However, there are several factors could jeopardise the execution of tissue resident stem cells' functions particularly cellular differentiation. To date, not much of scientific data are available on OA-derived MSCs and its contribution to the development and progression of OA. Since OA commonly diagnosed in elderly individuals (45-75 years old), it could postulate that the tissue-resident stem cell pool might deplete, or the self-renewal capacity declined due to senescence. This scenario could hamper the productivity of MSCs to produce a sufficient number of mature chondrocytes to recuperate the tissue loss due to degeneration/disease process. Even, if the capacity of MSCs to repopulate the chondrocytes is not affected, yet the physical microenvironment in OA synovial might be non-conducive for full implementation of MSCs' activities. Likewise, the normal functions of tissue-resident MSCs in OA vicinity could impair as the OA synovial fluid is fully packed with an array of pro-inflammatory cytokines and other inhibitory factors (27, 28). Thus, it is crucial to decipher the characteristic of OA-MSCs to understand the mechanism of disease onset, and it's progression.

However, many stumbling blocks impede the functional exploration of MSCs that are derived from diseased conditions, especially in degenerative diseases. In the case of OA, it is crucial to understand the biology of MSCs in diseased environment whether such pathology was inflicted by an aberrant stem cell function or the pathological/inflammatory microenvironment limits the regenerative capabilities of stem cells. To study the biology of MSCs in pathological conditions, an optimised in vitro culture system that enables harvesting of sufficient number stem cells within the permitted timeframe is inevitable. Thus, the present study has modified the conventional culture method of processing and growing MSCs to maximise the cell yield in a limited period. To state, two principal changes were made in the conventional method. These were the period of collagenase digestion was prolonged up to 24 hours, and two rounds of serial plating were employed to increase the colony formation of MSCs.

The initial seeding number of primary culture plays an essential role in colony formation as the number of cells plated had directly increased the frequency colonies formed. Since the stroma of articular cartilage is mainly composed of collagen, thus the digestion with collagenase II enzyme releases cells from the collagen matrix within the minced cartilage fragment. In the same fashion, the selection of optimal reaction time between the enzyme and tissue as well should be given careful consideration. The size of tissue fragments influences the optimal exposure time of enzyme as tissue fragment with a higher surface area (small fragments) could be easily digested as compared to the relatively big chunks. In line with this, the present study showed that the one hour incubation time with collagenase enzyme in the conventional method was not sufficient to harvest most of the cells from the cartilage tissues. It could be possible that stem cells in the cartilage tissue retained or trapped within the collagen stroma due to weak digestion. However, a prolonged enzyme incubation up to 24 hours had significantly increased the number of single cells released from the cartilage tissue, which was twice the amount of conventional method. Others as well noticed a similar pattern where 3 hours enzyme incubation period neither able to isolate MSCs from the synovial tissue as the majority of the cells lost to the undigested synovium tissue (29, 30). Similarly, using a synovial tissue, Sugita et al. had shown that the floating undigested tissue fragments might not firmly attached to the culture dish and jeopardised the chance for outgrowth of the synovium-derived MSCs. However, upon 16 hours of enzyme incubation, the same study reported 5-folds of cell yield (29). This implies the notion that a more extended period of incubation time is required to minimise the loss of cells or entrapment due to a short incubation period. In contrast, Han et al. suggested that the digestive enzymes may degrade the cell membrane; results in the complete or partial loss of their ability to stably adhere, and hence constituting further damage to the cells (31). However, prolonged incubation of collagenase for 24 hours did not significantly affect the membrane integrity of the cells.

The conventional culture method discards the culture supernatant during the medium change. On average, approximately 3 to 7 days were allotted for the passage 0 cells to form adherent colonies. Although, MSCs are known as adherent cells, yet the percentage of the stem cells or stem cell precursors that able to initiate adherent colonies in primary culture is still elusive. Not necessarily that all stem cells/precursors can adhere at instance to the culture flask as some studies had shown the presence of residual stem cells/progenitors in the non-attached cell suspension, the supernatant (32, 33). Therefore, a serial plating method was employed to re-use the culture supernatant to maximise the colony formation. With two rounds of a serial plating method, the time taken to reach the cell confluency reduced with an escalated number of final cell yield upon trypsinisation.

Apart from a volumetric increase of MSCs' number, the modified enzymatic digestion method as well generated a comparable quality of MSCs with desired and uncompromised characteristics of morphology, immunophenotyping, and differentiation capability. The cells revealed similar osteogenic, adipogenic, and chondrogenic differentiation property as of control MSCs. Moreover, the cells manifested an analogous immunophenotyping property to the standard BM-

MSCs, where they are positive for CD44, CD29, CD73, CD105, CD90 and HLA-ABC and negative for CD45, CD14, CD80, CD34 and CD86.

The greater production of MSCs through this reported modified method will warrant a timely and adequate number of MSCs for autologous usage. However, the following aspects should be taken into deliberation to consolidate the current findings. The effect of prolonged incubation of collagenase should decipher at both membrane and gene levels. Although no significant differences noted in term of the growth of MSCs, yet the tendency of MSCs to undergo apoptosis was uncertain when prolonged incubation of collagenase was employed. It would be possible that the longer incubation period inflicts epigenetic changes in the stem cells in which may cause early senescence and apoptosis. Despite these limitations, the current methodology provides new insight into expanding MSCs derived from degenerative diseases for autologous applications.

CONCLUSION

The present study had explored the feasibility and success of a modified sample processing and culture method to enhance the growth of OA hC-MSCs. As compared with the conventional method, the modified method yielded two folds of single cells for initial seeding; shorten the period of colony formation and cell confluency; increased the total number of cell yields. The modified method did not affect the characteristic of OA hC-MSCs as stem cell generated from these two methods are in accordance with the criteria delineated by International Society for Cell Therapy (ISCT). In conclusion, this study has developed more productive and effective protocol for generating OA hC-MSCs.

ACKNOWLEDGEMENT

The research was funded by the Institute grant scheme, Putra Grant - Putra Graduate Initiative (IPS), GP-IPS/2014/9432500

REFERENCES

1. Loeser RF, Goldring SR, Scanzello CR, Goldring MB. Osteoarthritis: a disease of the joint as an organ. Arthritis & Rheumatism. 2012;64(6):1697-707.
2. Kapoor M, Martel-Pelletier J, Lajeunesse D, Pelletier J-P, Fahmi H. Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. Nature Reviews Rheumatology. 2011;7(1):33-42.
3. van den Berg WB. The role of cytokines and growth factors in cartilage destruction in osteoarthritis and rheumatoid arthritis. Zeitschrift fur Rheumatologie. 1999;58(3):136-41.
4. Sekiya I, Vuoristo JT, Larson BL, Prockop DJ. In vitro cartilage formation by human adult stem cells from bone marrow stroma defines the sequence of cellular and molecular events during chondrogenesis. Proceedings of the National Academy of Sciences. 2002;99(7):4397-402.
5. Sekiya I, Larson BL, Vuoristo JT, Cui JG, Prockop DJ. Adipogenic differentiation of human adult stem cells from bone marrow stroma (MSCs). Journal of Bone and Mineral Research. 2004;19(2):256-64.
6. Fukumoto T, Sperling J, Sanyal A, Fitzsimmons J, Reinholz G, Conover CA, et al. Combined effects of insulin-like growth factor-1 and transforming growth factor-β1 on periosteal mesenchymal cells during chondrogenesis in vitro. Osteoarthritis and Cartilage. 2003;11(1):55-64.
7. De Bari C, Dell'Accio F, Tylzanowski P, Luyten FP. Multipotent mesenchymal stem cells from adult human synovial membrane. Arthritis & Rheumatism. 2001;44(8):1928-42.
8. Cao B, Zheng B, Jankowski RJ, Kimura S, Ikezawa M, Deasy B, et al. Muscle stem cells differentiate into haematopoietic lineages but retain myogenic potential. Nature cell biology. 2003;5(7):640-6.
9. Prockop DJ. Marrow Stromal Cells as Stem Cells for Nonhematopoietic Tissues. Science. 1997;276(5309):71-4.
10. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, et al. Human adipose tissue is a source of multipotent stem cells. Molecular biology of the cell. 2002;13(12):4279-95.
11. Adamo A, Sesй B, Boue S, Castaco J, Paramonov I, Barrero MJ, et al. LSD1 regulates the balance between self-renewal and differentiation in human embryonic stem cells. Nature cell biology. 2011;13(6):652-9.
12. Amable PR, Teixeira MVT, Carias RBV, Granjeiro JM, Borojevic R. Protein synthesis and secretion in human mesenchymal cells derived from bone marrow, adipose tissue and Wharton's jelly. Stem cell research & therapy. 2014;5(2):53.
13. Dudics V, Kunstбr A, Kovбcs J, Lakatos T, Gйher P, Gцmцr B, et al. Chondrogenic potential of mesenchymal stem cells from patients with rheumatoid arthritis and osteoarthritis: measurements in a microculture system. Cells Tissues Organs. 2009;189(5):307-16.
14. Scharstuhl A, Schewe B, Benz K, Gaissmaier C, Bьhring HJ, Stoop R. Chondrogenic potential of human adult mesenchymal stem cells is independent of age or osteoarthritis etiology. Stem Cells. 2007;25(12):3244-51.
15. Murphy JM, Dixon K, Beck S, Fabian D, Feldman A, Barry F. Reduced chondrogenic and adipogenic activity of mesenchymal stem cells from patients with advanced osteoarthritis. Arthritis & Rheumatology. 2002;46(3):704-13.
16. Hagmann S, Gotterbarm T, Mьller T, Baesig AM, Gantz S, Dreher T, et al. The influence of bone marrow-and synovium-derived mesenchymal stromal cells from osteoarthritis patients on

regulatory T cells in co–culture. Clinical & Experimental Immunology. 2013;173(3):454-62.
17. Djouad F, Fritz V, Apparailly F, Louis-Plence P, Bony C, Sany J, et al. Reversal of the immunosuppressive properties of mesenchymal stem cells by tumor necrosis factor α in collagen-induced arthritis. Arthritis & Rheumatology. 2005;52(5):1595-603.
18. El-Jawhari JJ, El-Sherbiny YM, Jones EA, Mcgonagle D. Mesenchymal stem cells, autoimmunity and rheumatoid arthritis. QJM. 2014:hcu033.
19. Huang Q-Q, Sobkoviak R, Jockheck-Clark AR, Shi B, Mandelin AM, Tak PP, et al. Heat shock protein 96 is elevated in rheumatoid arthritis and activates macrophages primarily via TLR2 signaling. The Journal of Immunology. 2009;182(8):4965-73.
20. Jones E, Churchman S, English A, Buch M, Horner E, Burgoyne C, et al. Mesenchymal stem cells in rheumatoid synovium: enumeration and functional assessment in relation to synovial inflammation level. Annals of the rheumatic diseases. 2009.
21. Sekiya I, Larson BL, Smith JR, Pochampally R, Cui JG, Prockop DJ. Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality. Stem cells. 2002;20(6):530-41.
22. Prockop DJ, Gregory CA, Spees JL. One strategy for cell and gene therapy: harnessing the power of adult stem cells to repair tissues. Proceedings of the National Academy of Sciences. 2003;100(suppl 1):11917-23.
23. Colter DC, Sekiya I, Prockop DJ. Identification of a subpopulation of rapidly self-renewing and multipotential adult stem cells in colonies of human marrow stromal cells. Proceedings of the National Academy of Sciences. 2001;98(14):7841-5.
24. Shimomura K, Ando W, Tateishi K, Nansai R, Fujie H, Hart DA, et al. The influence of skeletal maturity on allogenic synovial mesenchymal stem cell-based repair of cartilage in a large animal model. Biomaterials. 2010;31(31):8004-11.
25. Lee D-H, Joo S-D, Han S-B, Im J, Lee S-H, Sonn CH, et al. Isolation and expansion of synovial CD34– CD44+ CD90+ mesenchymal stem cells: comparison of an enzymatic method and a direct explant technique. Connective tissue research. 2011;52(3):226-34.
26. Tong CK, Vellasamy S, Tan BC, Abdullah M, Vidyadaran S, Seow HF, et al. Generation of mesenchymal stem cell from human umbilical cord tissue using a combination enzymatic and mechanical disassociation method. Cell Biology International. 2011;35(3):221-6.
27. Van den Berg W. The role of cytokines and growth factors in cartilage destruction in osteoarthritis and rheumatoid arthritis. Zeitschrift fьr Rheumatologie. 1999;58(3):136-41.
28. Romagnani S. Human Th17 cells. Arthritis research & therapy. 2008;10(2):206.
29. Sugita N, Moriguchi Y, Sakaue M, Hart DA, Yasui Y, Koizumi K, et al. Optimization of human mesenchymal stem cell isolation from synovial membrane: Implications for subsequent tissue engineering effectiveness. Regenerative Therapy. 2016;5:79-85.
30. Gittel C, Brehm W, Burk J, Juelke H, Staszyk C, Ribitsch I. Isolation of equine multipotent mesenchymal stromal cells by enzymatic tissue digestion or explant technique: comparison of cellular properties. BMC veterinary research. 2013;9(1):221.
31. Han Y-F, Tao R, Sun T-J, Chai J-K, Xu G, Liu J. Optimization of human umbilical cord mesenchymal stem cell isolation and culture methods. Cytotechnology. 2013;65(5):819-27.
32. Di Maggio N, Mehrkens A, Papadimitropoulos A, Schaeren S, Heberer M, Banfi A, et al. Fibroblast Growth Factor-2 Maintains a Niche-Dependent Population of Self-Renewing Highly Potent Non-adherent Mesenchymal Progenitors Through FGFR2c. Stem Cells. 2012;30(7):1455-64.
33. Mehrkens A, Di Maggio N, Gueven S, Schaefer D, Scherberich A, Banfi A, et al. Non-adherent mesenchymal progenitors from adipose tissue stromal vascular fraction. Tissue Engineering Part A. 2013;20(5-6):1081-8.