aJava

Asian Journal of Animal and Veterinary Advances

Academic
Journals Inc.

www.academicjournals.com

Asian Journal of Animal and Veterinary Advances 10 (10): 549-555, 2015
ISSN 1683-9919 / DOI: 10.3923/ajava.2015.549.555

A Comparative Analysis of Invasive and Non-Invasive Method of Bone Marrow Stromal Cell Isolation

[1]Lakshman Santra, [1]Saurabh Gupta, [1]Ajay K. Singh, [1]Amit R. Sahu, [1]Ravi Kumar Gandham, [2]Soumen Naskar, [3]S.K. Maity, [4]Jyotirmoy Ghosh and [1]Sujoy K. Dhara

[1]Division of Veterinary Biotechnology,
[2]Division of Biological Products,
[3]Division of Veterinary Surgery, Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, 243122, India
[4]National Institute of Animal Nutrition and Physiology, Bangalore, India

Corresponding Author: Sujoy K. Dhara, Division of Veterinary Biotechnology, Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, 243122, India

ABSTRACT

The conventional method of Bone Marrow Stromal Cell (BMSC) isolation from live subjects are complex due to involvement of lot of expert personnel and pre and post operational medication and cares. Moreover, the concerning ethical issues also pose lots of restrictions for isolation of BMSC's making stem cells research restricted to certain elite laboratories only. This study aims to compare between the regular aspiration (invasive) method and an alternative, straight forward and non-invasive method of BMSC harvest. The BMSCs were harvested by both invasive and non-invasive methods and cultured in MSC (Mesenchymal Stem Cell) medium. The cells were undergone visual assessment of growth dynamics as well as identification and characterization of MSC cells, based on microscopic examination. Both of these tested methods successfully yielded significant amount of BMSC that were found to be identical in morphology, growth dynamics and *in vitro* cultural properties. Unlike the invasive method that requires a live animal, the non-invasive method relies on post-slaughtered bone and therefore obviates the requirement of skill personnel and setup. Eventually, the used bone from already dead animal no longer becomes the issue of conflict with animal ethics and welfare. The ease in cell harvest and lack of ethical barrier would definitely make BMSC harvest convenient and therefore, anticipated to be well accepted in resource poor laboratories around the globe.

Key word: Bone marrow stromal cells, invasive method, non-invasive method, stemness, self-renewal, differentiation potential, domestic animal

INTRODUCTION

Bone marrow is a rich source of heterogeneous cell population that includes variety of adult cells, progenitor cells and even few stem cells. The bone marrow resident adult stem cells mainly include Hematopoietic Stem Cells (HSCs) and Mesenchymal Stem Cells (MSCs) or mesodermal stromal cells (Ehninger and Trumpp, 2011). Although, MSCs have now been isolated from various other tissues of mesodermal origin e.g., adipose tissue, muscle, bone, tendon, as well as tissues of non-mesodermal origin e.g., brain, spleen, liver, kidney, lung, pancreas, thymus etc. (Jamnig and

Lepperdinger, 2012), the major source of MSCs remains the bone marrow. These multipotent cells have the property to proliferate in-vitro, adhere to plastic surface, form colonies and differentiate into cells of mesodermal origin e.g., bone (Yamaguchi, 2014), cartilage (Ragetly *et al*., 2010) and fat cells (Jamnig and Lepperdinger, 2012; Sawant *et al*., 2012). The MSCs have inherent property of differentiating into tissues of mesodermal origin e.g., bone, cartilage, fat etc and this differentiation potential is now being harnessed for directed differentiation into various desired cell types as per need. Their fibroblast like morphology, ease in isolation and expansion and ability to differentiate *in vitro* into several different cell types made MSCs a promising cell source for regenerative or transplantation medicine (Baadhe *et al*., 2014; Curran *et al*., 2011) as well as basic and applied researches. However, harvesting MSCs from heterogenous cell population still remains one of the challenging tasks that are worth considering (Fekete *et al*., 2012; Harichandan and Buhring, 2011; Hsu *et al*., 2012). Despite of several advancement and sophistication for MSC isolation was made in case of human and small laboratory research animal, a standard and convenient method of isolating MSCs from large domestic animal (Fadel *et al*., 2011; Stewart *et al*., 2007) still need to be optimized. De Schauwer *et al*. (2014) reported the non-invasive sources of MSCs such as umbilical cord blood, umbilical cord matrix and peripheral blood. In this article we tested two different methods for MSC isolation. One is the conventional invasive method that aspirates the marrow content after inserting a wide bored needle inside the marrow cavity and hence is painful and likely to raise the ethical issue. The other one is a non-invasive method, as an aspiring alternative for procuring adult stem cells from slaughtered animal. We found that both the methods yielded sufficient stem cell population and they grew very well in cultured dishes in artificial media for several passages. However, the non-invasive method was found to be more appropriate in terms of convenience, expenses, expertise and ethical concern. We further found that this easy and convenient method is equally apt for harvesting MSCs from small ruminant e.g., sheep and goat as well as from pig too.

MATERIALS AND METHODS

Cell sources: BMSC_NI1-3 (bone marrow stromal cell isolated by non invasive method) harvested from bone marrow of slaughtered animals from division of LPT, IVRI, slaughter house, Bareilly and slaughter house, Bangalore.

BMSC_IH1-3 (bone marrow stromal cell isolated by invasive method and Histopaque) harvested from porcine sternum by invasive method at pig farm Indian Veterinary Research Institute, Izatnagar.

Culture medium: For culturing cells the MSC medium was prepared with either DMEM/F12 high glucose (4500 mg L^{-1}) with glutamax (Gibco Cat# 10565) as basal medium or 1:1 mixture of DMEM (Gibco Cat#10564) and TCM199 (HIMEDIA, Cat# AL014). All these basal medium additionally supplemented with 10X antibiotic/antimycotic solution (HIMEDIA, Cat# A002-A), 10X MEM non essential amino acid (NEAA; HIMEDIA Cat# ACL006), 1X 2-Mercaptoethanol (Gibco Cat# 21985) and 10% fetal bovine serum (South American type; Gibco Cat# 10270). Following mixing the complete medium was filtered by 0.2 μm syringe filter (MDI Cat# SYKG0601MNXX204).

Isolation of bone marrow stromal cells

Non-invasive method for BMSC_NI1-3: The collected bones were cleaned with 70% ethanol thoroughly and were taken to the clean workbench priorly wiped with 70% rectified spirit. Then

a round cut was made at the both ends of the bonehead using a sterile hacksaw. After flushing the bone and the cut surface with 70% alcohol, the bone was taken into the biosafety cabinet. After breaking the bone on both sides to expose the marrow, the marrow was pushed using a sterile glass pasture pipette to a 50 mL tube containing 2 mL PBS-ACD-A (100 mL PBS solution containing glucose monohydrate (Dextrose, SRL, Mumbai) 2.45 g, trisodium citrate (SRL Mumbai) 2.2 g, citric acid (SRL, Mumbai) 0.8 g) solution. The remaining semisolid marrow from the bone cavity was also flushed into the same tube with PBS-ACD-A solution. For making uniform cell suspension the cell mass was broken using 5 mL wide bore pipette and the whole content was transferred into a 15 mL tube and then was centrifuged at 1200 rpm for 5 min to precipitate the cells including RBC. Discarding the supernatant the cell mass was broken into uniform cell suspension using 5 mL wide bore pipette.

Then the whole content was transferred to a 15 mL tube then spin at 1200 rpm for 5 min to precipitate the cells including RBC and the supernatant was discarded. The cells pellet was resuspended in 1 mL sterile gentamycin-PBS (PBS containing 10% gentamycin, Gentalab injection, Laborate Pharmaceuticals India Pvt Ltd) by pipetting up and down followed by additional addition of 4 mL gentamycin-PBS and centrifugation at 1000 rpm for 5 min. The supernatant was discarded. The washing step may be repeated as described above. The final washing was done with 1 mL of culture medium at 1000 rpm for 5 min. Resuspending the cell pellet in MSC culture medium, the cell suspension was seeded into three T25 flasks (Nunclon flask with filter cap, PS, sterile; Cat# 136196, Genetix), priorly incubated at 37°C with culture media for at least 30 min.

Invasive method for BMSC_IH1-3: Bone marrow (~5 mL) from sternum of animal was aspirated in vaccutainer containing anti-coagulant (heparin) using biopsy needle following procedure approved by institute animal bioethics committee. Erythrocytes and granulocytes were removed from the mixture using a gradient centrifugation with ficoll for 30 min, as described previously (Horn *et al*., 2008; Peterbauer-Scherb *et al*., 2010; Schallmoser *et al*., 2008). All mononuclear cells including MSCs were trapped in an interphase ring between plasma and Ficoll Histopaque (Cat# 10831, Sigma). The interphase ring was collected, plated in tissue culture flask with MSC specific media and incubated in carbon dioxide incubator. Subsequent sub-culturing using trypsin usually allowed faster detachment of MSCs from dishes and leaving monocytes in the old tissue culture flask.

Culture of cells: The respective flasks were incubated at 37°C and 5% CO_2 with 80% relative humidity for 24-48 h. A routine observation of the individual flask under the microscope was done in order to check cell adherence and colonisation. The non-adherent cells were discarded at 48 h and fresh media was added to it without disturbing the cells. Media was changed every 48-72 h until 80% confluence was reached.

Passaging of cells: For subsequent passaging of cells, the medium was aspirated out completely from the culture flask and the monolayer was washed twice with 2 mL gentamycin-PBS by gentle rocking for 30 sec each. Following the washing step, cells were treated with 0.25% trypsin- EDTA (HIMEDIA, Cat # TCL007) at 37°C until all the adherent cells became detached off the flask. Once the cells were found detached, the trypsin activity was neutralised by adding 1 mL afresh complete medium. The cell suspension was transferred into a 15 mL centrifuge tube and pelleted by centrifuging at 1200 rpm at room temp for 5 min. After resuspending the cell pellet with medium, the cells were seeded in the ratio of 1:3.

For long term storage, cells were first pelleted and resuspended in cryopreservation medium (growth medium with 20% DMSO) under ice cold condition and placed either in -80 C freezer in cryovial for 1-2 months or in liquid nitrogen for long time storage. Image capturing and visual examination.

For routine visual assessment of growth dynamics as well as identification and characterization of MSC cells, based on microscopic examination, Nikon Eclipse Ti-S inverted fluorescence microscope (Nikon, Japan) was used. The images were captured using Nikon Elements imaging software.

RESULTS

For the current comparative study, porcine sternum and femur were used the source of bone marrow for invasive and non-invasive methods respectively as represented schematically in Fig. 1a. We have isolated altogether six cell lines. The bone marrow stromal cells BMSC_NI 1, BMSC_NI 2 and BMSC_NI 3 were isolated by non-invasive method harvested from bone marrow of slaughtered animals. The bone marrow stromal cell BMSC_IH 1, BMSC_IH 2 and BMSC_IH 3 were isolated from porcine sternum by invasive method followed by Histopaque purification and subsequent adoption to artificial culture medium. The bone marrow derived stromal cells harvested from either method grew well in culture medium (Fig. 1b-g) and were successfully maintained for several passages (Fig. 1h).

During *in vitro* expansion the bone marrow derived stromal cell formed individual colony that afterward coalesced with the neighboring colony and gave rise to cell monolayer irrespective of their isolated methods. Figure 1b-d represents the cultural characteristics of cells isolated by non-invasive method whereas; Fig. 1e-g represents the cultural characteristics of cells isolated by invasive method. In monolayer, all of these cells exhibited a fibroblast-like appearance with long and slender shape, small cell body with large, round nucleus and fewer processes (Fig. 1b-g). These rapidly proliferating cells were found to have doubling time 24-48 h with an average of 36 h (Fig. 1h).

DISCUSSION

The present study intended to establish a convenient method for regular BMSCs harvest from large domestic animal. Generally invasive method involves insertion of a long wide bored needle into the marrow cavity and with the help of negative pressure, created by drawing out the plunger, the bone marrow is aspirated. In case of large animal the invasive method becomes a real challenge since it demands skilled personnel e.g., a surgeon for anaesthesia and aspiration, veterinary nurses for taking care during the pre and postoperative measures and other staffs for restraining the animal and assistance. An alternative to the above mentioned method is non-invasive method that involves collection of bones from slaughterhouse. Non-invasive approach alleviates all the above-mentioned drawbacks associated with painful surgical aspiration based bone marrow isolation from live subjects (Carrade *et al*., 2011). Moreover, this simple and direct method can be practiced at regular basis without any effort and cost.

Mesenchymal stem cells were established to be adherent to tissue culture plastic (Cournil-Henrionnet *et al*., 2008; Curran *et al*., 2011) within 24-48 h post seeding and then form colonies similar to fibroblast. The phenomenon of forming a colony out of a single cell, either in the form of raw non-purified bone marrow cell or histopaque-purified bone marrow mononuclear cell is known as Colony-Forming Unit-Fibroblasts (CFU-F). Here we also found, for either case, similar

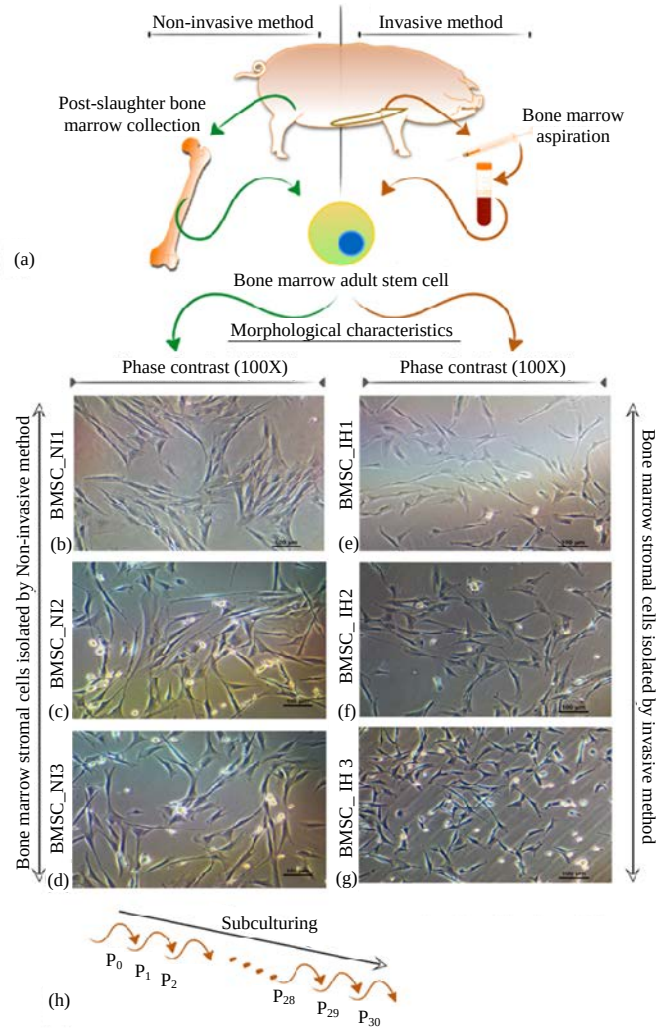

Fig. 1(a-h): Comparative analysis of non-invasive and invasive method of bone marrow stromal cell isolation (a) Schematic representation of bone marrow stromal cell (BMSC) harvest by two exclusive methods. The non-invasive method relies on salvaging the large bones of freshly slaughtered animal. The invasive method depends on painful aspiration method from live animal, followed by density gradient centrifugation. Either method was implemented for harvesting BMSCs and the morphological characteristics were analyzed, (b) Photomicrograph (100X) of BMSC_NI1 isolated from slaughtered animal following non-invasive method, (c) Photomicrograph (100X) of BMSC_NI2 isolated from slaughtered animal following non-invasive method, (d) Photomicrograph (100X) of BMSC_NI3 isolated from slaughtered animal following non-invasive method, (e) Photomicrograph (100X) of BMSC_IH1 isolated from live animal following invasive method and then purified by Histopaque column, (f) Photomicrograph (100X) of BMSC_IH2 isolated from live animal following invasive method and then purified by Histopaque column, (g) Photomicrograph (100X) of BMSC_IH3 isolated from live animal following invasive method and then purified by Histopaque column and (h) Schematic representation of sub culturing of isolated BMSCs for several passages. The BMSCs were found to be rapidly multiplying cells with doubling time varies from 24-48 h (average being 36 h)

CFU-F appearance upon plating onto culture flask. During the stage of clonal expansion, individual colonies coalesced with adjacent colonies and formed the monolayer (Cournil-Henrionnet *et al*., 2008).

BMSCs have a tendency to flatten and form spindles in monolayer culture and therefore they exhibit a fibroblast-like appearance (Spaas *et al*., 2013). This feature is an indicative of inherent growth, migration, proliferation and self-renewal potentials (Vidal *et al*., 2012). The characteristic long and slender shape and small cell body with a few processes indicated that the BMSCs, isolated by either method, have identical growth potentials as indicated by their rapid doubling time. Moreover, the presence of large, round nucleus with less cell body also indicated about their growth potential. Besides, the BMSCs, irrespective of followed harvesting methods, were found to be positive for several Cluster of Differentiation (CD) molecules attributing for mesenchymal stem cell surface markers (Agarwal, 2013). Furthermore, the osteogenic and adipogenic differentiation potential (Ahern *et al*., 2011), as tested in our laboratory, suggested that these BMSCs are a valuable cell source for future regenerative medicine.

CONCLUSION

In this article a comparative analysis is demonstrated between the invasive and non-invasive method of stem cell isolation from live and slaughtered animal respectively. Though bone marrow derived stromal cells (BMSCs) were isolated successfully by either methods, the non-invasive method was found to be much less cumbersome and hence is expedient. We believe that this effortless and convenient method would be appreciated and adopted globally.

ACKNOWLEDGMENTS

The authors gratefully thank Director IVRI, Izatnagar, for providing necessary facilities to carry out this work. The authors also acknowledge the financial support provided by Department of Biotechnology (DBT), Government of India.

REFERENCES

Agarwal, M., 2013. Expression of transcription factor CRISPLD2 during osteogenic differentiation of pig mesenchymal stem cells. M.V.Sc. Thesis, Indian Veterinary Research Institute, Izatnagar, Bareilly.

Ahern, B.J., T.P. Schaer, S.P. Terkhorn, K.V. Jackson, N.J. Mason and K.D. Hankenson, 2011. Evaluation of equine peripheral blood apheresis product, bone marrow and adipose tissue as sources of mesenchymal stem cells and their differentation potential. Am. J. Vet. Res., 72: 127-133.

Baadhe, R.R., N.K. Mekala and R. Potumarthi, 2014. Significance of biotic factors in mesenchymal stem cell fate in regenerative medicine. Curr. Stem Cell Res. Ther., 9: 489-496.

Carrade, D.D., V.K. Affolter, C.A. Outerbridge, J.L. Watson and L.D. Galuppo *et al*., 2011. Intradermal injections of equine allogeneic umbilical cord-derived mesenchymal stem cells are well tolerated and do not elicit immediate or delayed hypersensitivity reactions. Cytotherapy, 13: 1180-1192.

Cournil-Henrionnet, C., C. Huselstein, Y. Wang, L. Galois and D. Mainard *et al*., 2008. Phenotypic analysis of cell surface markers and gene expression of human mesenchymal stem cells and chondrocytes during monolayer expansion. Biorheology, 45: 513-526.

Curran, J.M., F. Pu, R. Chen and J.A. Hunt, 2011. The use of dynamic surface chemistries to control msc isolation and function. Biomaterials, 32: 4753-4760.

De Schauwer, C., K. Goossens, S. Piepers, M.K. Hoogewijs and J.L. Govaere *et al*., 2014. Characterization and profiling of immunomodulatory genes of equine mesenchymal stromal cells from non-invasive sources. Stem Cell Res. Ther., Vol. 5.

Ehninger, A. and A. Trumpp, 2011. The bone marrow stem cell niche grows up: Mesenchymal stem cells and macrophages move in. J. Exp. Med., 208: 421-428.

Fadel, L., B.R. Viana, M.L.T. Feitosa, A.C.M. Ercolin and K.C.S. Roballo *et al*., 2011. Protocols for obtainment and isolation of two mesenchymal stem cell sources in sheep. Acta Cirurgica Brasileira, 26: 267-273.

Fekete, N., M.T. Rojewski, D. Furst, L. Kreja, A. Ignatius, J. Dausend and H. Schrezenmeier, 2012. GMP-compliant isolation and large-scale expansion of bone marrow-derived MSC. PLoS One, Vol. 7. 10.1371/journal.pone.0043255

Harichandan, A. and H.J. Buhring, 2011. Prospective isolation of human MSC. Best Pract. Res. Clin. Haematol., 24: 25-36.

Horn, P., S. Bork, A. Diehlmann, T. Walenda, V. Eckstein, A. Ho and W. Wagner, 2008. Isolation of human mesenchymal stromal cells is more efficient by red blood cell lysis. Cytotherapy, 10: 676-685.

Hsu, S.H., G.S. Huang and F. Feng, 2012. Isolation of the multipotent MSC subpopulation from human gingival fibroblasts by culturing on chitosan membranes. Biomaterials, 33: 2642-2655.

Jamnig, A. and G. Lepperdinger, 2012. From tendon to nerve: An MSC for all seasons. Can. J. Physiol. Pharmacol., 90: 295-306.

Peterbauer-Scherb, A., M. van Griensven, A. Meinl, C. Gabriel, H. Redl and S. Wolbank, 2010. Isolation of pig bone marrow mesenchymal stem cells suitable for one-step procedures in chondrogenic regeneration. J. Tissue Eng. Regener. Med., 4: 485-490.

Ragetly, G.R., D.J. Griffon, H.B. Lee, L.P. Fredericks, W. Gordon-Evans and Y.S. Chung, 2010. Effect of chitosan scaffold microstructure on mesenchymal stem cell chondrogenesis. Acta Biomaterialia, 6: 1430-1436.

Sawant, A., D. Chanda, T. Isayeva, G. Tsuladze, W.T. Garvey and S. Ponnazhagan, 2012. Noggin is novel inducer of mesenchymal stem cell adipogenesis: Implications for bone health and obesity. J. Biol. Chem., 287: 12241-12249.

Schallmoser, K., E. Rohde, A. Reinisch, C. Bartmann and D. Thaler *et al*., 2008. Rapid large-scale expansion of functional mesenchymal stem cells from unmanipulated bone marrow without animal serum. Tissue Eng. Part C: Methods, 14: 185-196.

Spaas, J.H., C. De Schauwer, P. Cornillie, E. Meyer, A. Van Soom and G.R. Van de Walle, 2013. Culture and characterisation of equine peripheral blood mesenchymal stromal cells. Vet. J., 195: 107-113.

Stewart, A.A., C.R. Byron, H. Pondenis and M.C. Stewart, 2007. Effect of fibroblast growth factor-2 on equine mesenchymal stem cell monolayer expansion and chondrogenesis. Am. J. Vet. Res., 68: 941-945.

Vidal, M.A., N.J. Walker, E. Napoli and D.L. Borjesson, 2012. Evaluation of senescence in mesenchymal stem cells isolated from equine bone marrow, adipose tissue and umbilical cord tissue. Stem Cells Dev., 21: 273-283.

Yamaguchi, D.T., 2014. Ins and outs of mesenchymal stem cell osteogenesis in regenerative medicine. World J. Stem Cells, 6: 94-110.