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Long bones, a slaughterhouse by-product, may serve as an excellent source for mesenchymal stem cells

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ABSTRACT

Mesenchymal stem cells (MSCs) are one of the rarest sub-populations of bone marrow resident cells having inherent ability to differentiate into mesenchyme tissues e.g. bone, cartilage and adipose tissues. The natural self-renewal ability and potential for lineage specific differentiation have made these cells an excellent material for research and therapy in regenerative medicine. But, successful isolation and *in vitro* expansion of these cells still remain the pivotal steps for majority of stem cell based applications. Various techniques have been successfully used for isolation of MSCs from laboratory animals, but those are difficult to apply for domestic species. Hence, harvesting MSCs from most domestic animals remains a real challenge. Here we have demonstrated an easy, convenient, low cost method of MSCs isolation from slaughtered animals. As a proof of concept, MSCs were isolated from bone marrow of 3 different species, namely, sheep, pig and goat. These cells expressed multiple markers and also retained their self-renewal potential, exhibited by successful sub-culturing over 30 passages. Moreover, MSCs expressed many pluripotency factors e.g. OCT4, Nanog, c-Myc, KLF2 and KLF4. This indicated that the bone marrow derived MSCs were at very early stage of commitment and therefore, possibly retained high plasticity. Since these cells are available from slaughtered animals, this circumvents the bioethical issues associated with invasive method of MSC isolation from bone marrow. This invaluable and easily adoptable method for isolation of MSCs from large domestic animal would encourage isolation process in other animals and help in future cell based researches and therapies in the field of regenerative medicine.

Key words: Bone marrow, *In vitro* culture, Mesenchymal stem cells (MSCs), Pluripotency, Self-renewal

Stem cells (SCs) are considered the most promising candidate for regenerative or transplantation medicine for having the capacity to self-renew and ability to differentiate into any kind of cell types. For the last few decades, it has become one of the most powerful tools in basic and applied researches (Pellegrini *et al.* 2014, Stoll 2014). Stem cells serve as an unlimited source for various cell types, required for therapy as well as for research. Broadly stem cells are categorized into embryonic stem cells (ESCs) and adult stem cells (ASCs). ESCs are isolated from inner cell mass of the blastocyst and ASCs are found in adult mature tissue albeit few in number. Mesenchymal stem cells (MSC) or alternatively multipotent stromal cells are a heterogeneous population of ASCs with fibroblast like morphology. These 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 major source of MSCs is bone marrow, although these cells 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).

MSCs are very rare cells (1 in 10 thousand cells) capable of forming special niche in bone marrow. In the niche, these cells remain in contact with other cells directly or indrectly through extracelluar matrices and produce soluble components that take part in maintenance of cellular homeostasis and differentiation (Baadhe *et al.* 2014). In this microenvironment niche, MSCs remain quiescent and undifferentiated for indefinite period of time unless stimulated (Chow *et al.* 2011). When the differentiation is required for regeneration of a tissue, certain pertinent signals must have to find their way into the niche to activate the quiescent MSCs. Upon stimulation by proper signal, MSCs first differentiate into progenitor cells. Progenitors have

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tremendous proliferation ability and are capable of homing to the site of injury where they differentiate into various cell types depending upon the demand and availability of local signals (Ehninger and Trumpp 2011).

The same inherent differentiation potential can be harnessed for directed differentiation of stem cells into various desired cell types e.g. bone, cartilage, fat etc. *in vitro*. The ability to differentiate *in vitro* into several different cell types and the relative ease of growing them in culture dish make 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 remains the bottleneck for research and therapy (Fekete *et al.* 2012, Harichandan and Buhring 2011, Hsu *et al.* 2012). Although various sophisticated methods for MSCs isolation are available in human and small laboratory research animal, very little information is available for the domestic animals (Fadel *et al.* 2011, Stewart *et al.* 2007). In this article we have demonstrated an easy method of procuring MSCs from slaughtered animals. We further showed that these populations of stem cells are rapidly adapted to culture dishes and grow very well in artificial media for multiple subsequent passages. Lastly, it can be concluded that the same easy and convenient method is equally apt for harvesting MSCs from multiple species including sheep, goat and pig.

MATERIALS AND METHODS

Biological samples: For this study, the femurs of sheep, goats and pigs were collected immediately after slaughter and brought to the lab in an autoclaved bag on ice.

Culture medium: For culturing MSC cells, the medium was prepared with either DMEM/F12 high glucose (4,500 mg/l) with glutamax as basal medium or 1:1 mixture of DMEM and TCM199. Basal medium was supplemented with 10× antibiotic/antimycotic solution, 10× MEM non-essential amino acid (NEAA), 1× 2-mercaptoethanol (Gibco Cat#21985) and 10% fetal bovine serum (South American type). Following mixing, the complete medium was filtered using 0.2 µm syringe filter.

Isolation of bone marrow stromal cells: The collected bones were thoroughly cleaned with 70% ethanol and were taken to the work bench, cleaned with 70% rectified spirit. A round cut was made at both ends of the bone head using a sterile hack saw and the bone was taken into the biosafety cabinet. Heads of the bone at both ends were broken to expose the marrow and most of the marrow was pushed using a sterile glass pasture pipette to a 50 ml tube containing 2 ml of PBS-ACD-A solution (PBS-ACD-A composition: 100 ml PBS solution containing glucose monohydrate 2.45 g, trisodium citrate 2.2 g, citric acid 0.8 g). The remaining semisolid marrow from the bone cavity was also flushed into the same tube with PBS-ACD-A solution. The cell mass was broken using 5 ml wide bore pipette and made a uniform cell suspension. The whole content was transferred into a 15 ml tube and centrifuged at 1,200 rpm for 5 min to precipitate the cells including red blood corpuscles (RBCs). Discarding the supernatant, the washing of cell mass was repeated in PBS-ACD-A solution in a new 15 ml tube by spinning at 1,200 rpm for 5 min. The supernatant was discarded and the cell pellet was resuspended in 1 ml sterile Gentamicin-PBS (PBS containing 10% gentamicin) by pipetting up and down followed by further addition of 4 ml gentamicin-PBS and centrifugation at 1,000 rpm for 5 min. The supernatant was discarded. The washing step was repeated in gentamicin-PBS as described above. The final washing was done with 1 ml of culture medium at 1,000 rpm for 5 min. Resuspending the cell pellet in MSC culture medium, the cell suspension was seeded into 3 sterile T25 culture flasks (Nunclon flask with filter cap, PS, sterile) containing culture media incubated at 37ºC for at least 30 min.

Culture of cells: The cell culture flasks were incubated at 37°C and 5% CO_2 with 80% relative humidity for 24–48 h. To check adherence and colonization, cells were routinely observed under microscope. Non-adherent cells were discarded from the flasks at 48 h and replenished with fresh media without disturbing the cells. Medium was changed every 48–72 h until 80% confluence was reached.

Passaging and storage of cells: For subsequent passaging of cells, the medium was aspirated out completely from the culture flask and the cells grown in monolayer were washed twice with 2 ml gentamicin-PBS by gentle rocking for 30 sec each. Following the washing, cells were treated with 0.25% trypsin-EDTA at 37°C until adherent cells started detaching off from the culture surface. The trypsin activity was neutralized by adding 1 ml complete MSC culture medium and cells were completely detached off the surface by pipetting up and down. The cell suspension was transferred into a 15 ml centrifuge tube and pelleted by centrifugation at 1,200 rpm for 5 min in room temp. 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 re-suspended 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.

Total RNA isolation: Total RNA was isolated from cultured MSCs using RNeasey Plus Mini Kit following the manufacturer's protocol. Briefly, cells were lysed using RLT buffer, and lysates were pipetted several times before passing through gDNA eliminator column. Equal volume of 70% ethanol was added to the flow-through to precipitate the nucleic acids and loaded to RNeasy spin column. The column was centrifuged and washed using RW1 buffer followed by RPE buffer. RNA was eluted using nuclease-free water and stored at –80°C until use.

Spectrophotometric quantification of RNA: For the quantification, 1 µl of RNA was used for using Nanodrop ND 1000 spectrophotometer. The reading was measured at 260 and 280 nm wavelength against nuclease free ultrapure water as a blank. Pure RNA had an A260/A280 ratio of

1.6–2.0. Concentration of RNA in sample was directly recorded from the instrument in ng/µl.

First strand cDNA synthesis: The cDNA was synthesized using thermocycler machine in a total volume of 20 µl reaction mixture using RevertAidT^{M} First Strand cDNA Synthesis Kit following supplier's instruction. For cDNA synthesis, 1.5 µg of RNA and oligo dT primers supplied with the kit were used.

Semiquantitative polymerase chain reaction: Semi quantitative polymerase chain reaction (PCR) was performed using Dream Taq Green Master Mix in a volume of 25 µl containing 25 ng cDNA and 0.2 µM (final concentration) each of forward and reverse primers (provided in the Table). Cycling was performed in ABI Veriti 96-well thermal cycler with an optimized annealing at 57°C.

Gene	Accession number	Primers	Sequence	Product size
CD90	ENSSSCG00000015122	F	GTCTTGCAGGTGGCCCGTGG	491 bp
		R	TGGGCCTTGTGGCTTCGTGT	
CD105	Z23142.1	F	CAGAACCAGGAAGCCAAGAG	551 bp
		R	GTTGGGGTCCTGGGATAAGT	
CD73	ENSSSCT00000004745.2	F	CTCACCCACGTTCGCGTCCT	489 bp
		R	GCTAGCGGCCCCTTGGCTTT	
CD44	ENSSSCT00000014523.2	F	GCCCTGAACATAGGGTTTGA	469 bp
		R	CCGTACTGGTAGCTGGGGTA	
CD34	NM_214086.1	F	GAGAAAGGCTGGGCGAAGAC	160 bp
		R	GAATGGCCGTTTCTGGAGGT	
CD45	AY444871.1	F	GGAATTACGTCCTGAAGTCCCT	153 bp
		R	ACTCCGGAGCACCATGAAAT	
Beta-actin	KU672525.1	F	CCGGGACCTGACCGACTACCTCA	486 bp
		R	GGTGGACAGCGAGGCCAGGATG	
Oct4	NM_001113060.1	F	ACAAGGAGAAGCTGGAGCCG	453 bp
		R	CGCGGACCACATCCTTCTCT	
Nanog	EF522119.1	F	ACAGGAGTGCCTGGACAGAGAAGG	386 bp
		R	CCCTCTGTCCCGCCTTCCCAA	
Klf 2	EF095721.2	F	CACTACCGCAAACACACAGG	520 bp
		R	AAACCAGGCAGTCAAAATGC	
Klf 4	EU669075.2	F	CCGCGCATGTGCCCCAAGAT	349 bp
		R	AGGGCTCCCCAGCCCCAACTA	
c-Myc	ENSSSCT00000006548	F	AGGGAGATCCGGAGTGAAAG	588 bp
		R	TTGGTGAAGCTGACGTTGAG	

Agarose gel electrophoresis: Products from semi-quantitative PCR were run on a 1.5% agarose gel electrophoresis in 1× TAE (40 mM Tris, pH 7.6; 20 mM acetic acid, 1 mM EDTA) buffer. Gel was micro-photographed using UV transilluminator.

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 T*i*-S inverted fluorescence microscope was used. The images were captured using imaging software.

RESULTS AND DISCUSSION

Present study intended to establish a simple method of harvesting mesenchymal stem cells from bone marrow of slaughtered animals. Generally, MSCs are harvested from live animals by aspiration method inserting a long wide bored bone needle into the marrow cavity. With the help of negative pressure, by drawing out the plunger, the bone marrow is aspirated. In large animals, the same practice becomes a real challenge since this invasive method demands skilled personnel. Besides, it also demands staffs for restraining the animal and to take care of the pre- and post-operative management and care. Furthermore, the invasive method of aspiration often raises an issue of animal bioethics. Collecting bones from slaughterhouse alleviates all these drawbacks associated with surgical aspiration based MSC isolation from live subjects. Moreover, the method is simple, direct, and can easily be adopted and practiced on a regular basis.

For the experiment, sheep, goat and pig species were selected for harvesting MSCs from bone marrow (Fig. 1A). The long bone (femur or tibia) was preferred as the source of bone marrow because large volumes of marrow are available in the bone cavity. Bone marrow derived stromal cells were harvested from all the species and adopted in culture medium for their expansion and subsequent passages (Fig. 1A). Mesenchymal stem cells, unlike red blood cells or haematopoetic progenitors, are adherent to tissue culture plastic (Cournil-Henrionnet *et al.* 2008, Curran *et al.* 2011) within 24 to 48 h. Here too, we found that harvested MSCs form monolayers during *in vitro* expansion in tissue culture flask (Fig. 1B-G).

It is already established that MSCs have the ability to

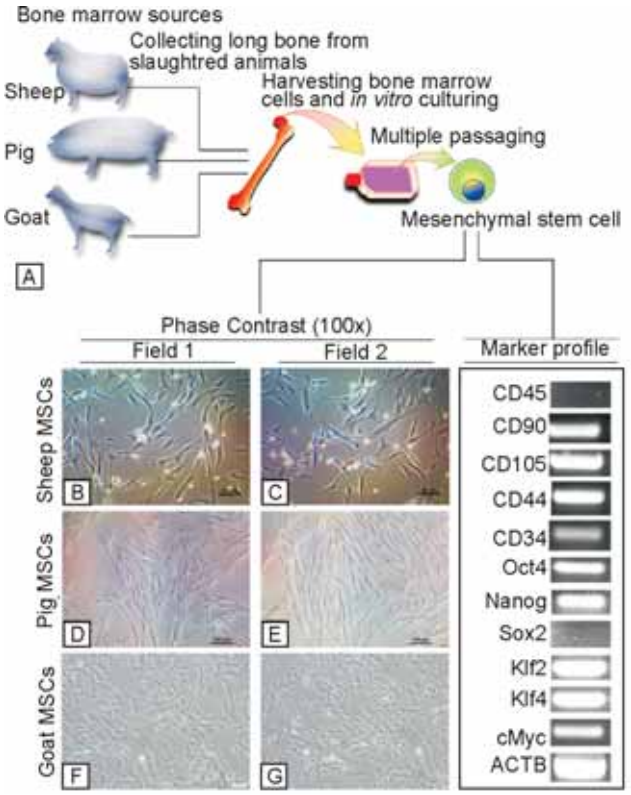

Fig. 1. Isolation and *in vitro* culturing of bone marrow derived Mesenchymal Stem Cells (MSCs) from slaughtered animals. (A) Schematic diagram showing procedure for isolation of MSCs. Long bones (preferably the femur) from slaughtered animals have been collected and brought into laboratory. The bone marrow stromal cells are isolated and cultured in flasks with cell specific culture media. (B-G) The panel of photomicrographs (100×) showing the characteristic morphology of MSCs in monolayer. B & C represent the MSCs of sheep origin; D & E showing porcine MSCs and F & G represent MSCs from goat. (H) Characterization of bone marrow derived MSCs by expression profile of various markers. The bone marrow derived MSCs are characterized by semiquantitative PCR of panel of surface markers (CD molecules) and transcription factors. These cells were found to be positive for CD90, CD105, CD73, CD44 and CD34 molecules and negative for CD45 expression. They also express transcription factors, Oct4, Nanog, Klf2, Klf4 and cMyc but negative for Sox2. βActin (ACTB) was taken as housekeeping gene for template control.

form colony similar to fibroblast. The phenomenon of forming a colony out of a single MSC, either in the form of raw unpurified bone marrow cell or ficoll-purified bone marrow mononuclear cell, when plated directly into cell culture dish is known as colony-forming unit-fibroblasts (CFU-F). Here we also found, for all the 3 species, similar CFU-F appearance upon plating onto culture flask. During the stage of expansion, individual colonies coalesce with adjacent colonies and form the monolayer as described elsewhere (Cournil-Henrionnet *et al.* 2008).

MSCs have a tendency to flatten and form spindles in monolayer culture and therefore, they exhibit a fibroblast-like appearance. This fibroblastic appearance allows MSCs to migrate. In addition, these cells are also capable of proliferation. These features help assessing the inherent growth potentials of any MSC. The characteristic long and slender shape and small cell body with a few processes indicated that the bone marrow derived MSCs have tremendous growth potentials for all the 3 species. It is found that bone marrow derived MSC cultures are highly proliferative (with a cell doubling time 1–2 days) and can be maintained successfully in culture for at least 30 passages. This extensive growth potential of isolated MSCs indicated the tremendous ability for self-renewal and maintenance of stemness.

Additionally, during initial setup of bone marrow cell culture, we noted heterogeneous cells with varying nuclei size. But with few sub-culturing, MSCs could be differentially identified from other non-MSCs. Eventually, within few passages, culture became homogeneous with MSCs. The isolated MSCs from all the species show a large, round nucleus with a prominent nucleolus, surrounded by finely dispersed fibril structure, giving the nucleus a clear appearance (data not shown).

Apart from these morphologic features, MSCs are generally characterized by their surface markers and expression of these markers is often used to differentially identify MSCs from other cell population. MSCs express on their cell surfaces few cluster of differentiation (CD) molecules e.g., CD44, CD73, CD90, CD105 etc (Cournil-Henrionnet *et al.* 2008). The semiquantitative PCR results demonstrated that bone marrow-derived MSCs are positive for CD105, CD90, CD44 and CD34 expression (Fig. 1H). Further, the absence of CD45 (Fig. 1H), the marker for hematopoietic stem cells (HSCs) (Kakiuchi-Kiyota *et al.* 2013), confirmed that the isolated cells are not HSCs. Very recently CD34 has been considered to be another MSC marker attributing for early passage number and astounding growth potential (Sidney *et al.* 2014, Simmons and Torok-Storb 1991). The expression of CD34 in bone marrow derived MSCs indicated that these harvested MSCs have remarkable self-renewal ability (Fig. 1H). Taken the expression profile of different markers together, it is evident that isolated cells were primarily MSCs.

Another reliable parameter for characterizing MSCs is their intrinsic differentiation potentials. MSCs are multipotent stem cells of mesodermal origin, having potential to give rise to adipose tissue, cartilage and bone. We also tested the osteogenic and adipogenic differentiation potential of *in house* procured bone marrow derived MSCs. The differential staining for bone (Alizarin red and ALP) and adipose (Oil-O-Red) tissue as well as molecular evidences confirm that the tested cells are indeed MSCs (unpublished data).

Sometimes, it is found that despite exhibiting homogeneous immuno-phenotype, MSCs may not possess identical differentiation ability. Accordingly, not all cells in cultures show equal potential to differentiate to all 3 lineages tested. This is due to the fact that the bone marrow stromal cells, during the course of *in vitro* culturing, become

heterogeneous. This heterogeneous cell population primarily comprised true MSCs (with full differentiation potential) and more committed MSC-progenies. The number of these more committed MSC-progenies is gradually increased with the passage number and that is why MSC differentiation potential is found to decrease moderately in late passages. This was also observed for MSCs isolated from femurs of freshly slaughtered animals (unpublished data).

In ths study it was demonstrated that MSCs express some of the pluripotency related transcription factors such as OCT4, Nanog, c-Myc, KLF2 and KLF4 (Fig. 1H). Therefore, isolated MSCs are a unique cell type with very early in their plasticity and potency. This unique ability to express pluripotency factors may have some important roles during differentiation in determining cell fate, not yet known. It is to note that SOX2 expression is not detected in these cells. Previously, Takahashi and Yamanaka (2006) described that forced expression of OCT4, KLF4, c-Myc and SOX2 are sufficient to induce pluripotency in any somatic cells. Given that it would be also interesting to look into if this exclusive feature would help reprogramme MSCs easily over other cell types for generation of induced pluripotent stem cells (iPSCs) or not.

The method would be of immense help for isolating the rare and invaluable MSCs for several downstream applications for these cells are suitable for developmental biology study, transplantation and drug screening, pharmacogenomic study and more significantly, for tissue bio-banking. However, since the cell source is a dead animal, direct and immediate use of the animal may not be possible. To overcome such issue, one has to adopt other reproductive technology such as SCNT (somatic cell nuclear transfer) to recreate the superior animal for other purposes.

In this article we have demonstrated a simple method for isolation of adult stem cell by recycling the long bones collected from slaughterhouse. These bones are easily available without subjecting the animal to surgical invasion. The method does not require highly skilled personnel for collection of cells and utilize ordinary cell culture grade chemicals. The established method is useful for isolation of cells from multiple domestic species. These bone-marrow derived adult stem cells are primarily MSCs. It is further demonstrated that these cells have exceptional ability of retaining expression of pluripotency factors and therefore, are thought to be more plastic, compared to other somatic cells lacking the expression of those factors. It is anticipated that this convenient and economic method of harvesting MSCs would assist in research and therapy in stem cell based regenerative medicine.

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