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Isolation, proliferation, characterization and *in vivo* osteogenic potential of bone-marrow derived mesenchymal stem cells (rBMSC) in rabbit model

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Information on isolation, characterization of rabbit MSC and its evaluation in critical bone defect (CSD) is scarcely available. Here, we attempted to isolate, proliferate, differentiate, characterize and evaluate the *in vivo* osteogenic potential of bone marrow derived mesenchymal stem cells (BMSCs) collected from New Zealand White rabbits. They were isolated and proliferated in antibiotic supplemented DMEM (Dulbecco's Modified Eagle's media). Osteogenic differentiation of rabbit bone marrow derived mesenchymal stem cells (rBMSCs) was induced by osteogenic supplements and evaluated by alizarin red staining and alkaline phosphatase activity assay and characterized by specific CD surface antigen markers through FACS (Fluorescent activated cell shorting) and RT-PCR. Day '0' cells were round/oval and floating, and on day 3-5, cell attachment with spindle/polygonal/star morphology was seen. On subsequent passages, they assumed uniform spindle shaped morphology. After culturing in respective differentiation media rBMSCs showed increased alkaline phosphatase activity, intense alizarin red staining, blue staining for Alcian blue and deep red colour on oil red O staining supporting the osteogenic, chondrogenic and adipogenic differentiation ability. *In vivo* osteogenic potential of rBMSCs was evaluated in a 30 mm critical sized defect of rabbit radius. The cellular morphology of plastic adherent cells was seen as single cell form in P0 and in P1, P2 and P3, as elongated/spindle-shape in clusters. The rBMSCs were positive for CD44, CD73 and CD105 and negative for CD34 and CD45 and could differentiate to osteogenic cells in osteogenic induction media. The *in vivo* experiments in rabbit CSD model confirmed that rBMSCs promote faster healing of critical size defects. Hence, we may suggest that rBMSCs are suitable for bone formation in fracture healing and non-union.

Keywords: Critical Size Defects (CSD), Differentiation, Fracture healing

Stem cell biology is gaining tremendous interest recently. Currently, it is playing a major role in the treatment of a number of incurable diseases via transplantation therapy. Stem cells are a specific group of cells that remain in an undifferentiated state and can be found in embryos, fetuses and adult individuals. Bone marrow stem cell is the ideal stem cell that is unspecialized and can undergo lineage specific differentiation into bone or blood cells under different signals or environment with new special function[1].

Mesenchymal stem cells (MSC) are multipotential cells capable of proliferation and differentiation into chondrogenic, osteogenic and adipogenic lineages[2,3]. Minimal criteria for defining MSC according to International Society of Cell Therapy (ISCT) are: (a) they must be plastic-adherent when maintained in standard culture conditions; (b) MSC must express some surface antigens such asCD105, CD73, CD90, CD44 and must not express CD34, CD45, CD 14, CD19, and HLA-DR; and (c) MSC must differentiate *in vitro* into osteoblast, adipocytes and chondrocytes under specific differentiating condition[4].

Although MSC is traditionally isolated from bone marrow, there are also reports on isolation of cells with MSC characteristics from a variety of tissues including umbilical cord blood, chorionic villi of the placenta, Wharton's jelly, peripheral blood, fetal liver and lung, adipose tissue, skeletal muscle, periosteum, deciduous teeth, amniotic fluid and synovium[5,6]. Bone marrow and periosteum sources are richest in young animals with their numbers diminishing, but still present in old age[5]. One of the advantages of bone marrow derived mesenchymal stem cells (BM-MSCs) as a source of cell transplantation is their low immunogenecity. BM-MSCs are immune-privileged cells that do not elicit

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immune response which could be attributed to the absence of their immunologically relevant cell surface markers[7]. BM-MSCs also are known to inhibit proliferation of T & B lymphocytes, dendritic cells and natural killer cells[7].

As MSCs comprise merely 0.01-0.0001% of total bone marrow nucleated cells, *in vitro* cell culture expansion is inevitable to get sufficient numbers for clinical applications. In this study, we attempted to isolate, proliferate and characterize mesenchymal stem cells collected from bone marrow of New Zealand white rabbit.

Materials and Methods

We used six skeletally mature New Zealand white rabbits of either sex between six months and one year old and with a mean weight of 2.86±0.38 kg (sd). They were given tap water and food *ad libitum* and kept in separate cages allowed to move freely. The experiments had been approved by the Institute Committee for Animal Care and Animal Experimentation.

Collection of bone marrow aspiration

The animals were anaesthetized by intramuscular injection of xylazine @ 5 mg/kg followed, 5 min later, by ketamine @ 50 mg/kg in the thigh muscles. The area of iliac crest on either side was prepared in aseptic manner. The bone marrow aspirate was collected with the help of an 18 G bone marrow biopsy needle from the posterior aspect of iliac crest. To collect bone marrow (BM), the biopsy needle was inserted through the skin and the musc1e with a little force. Once the needle made contact with the bone, it was advanced further by rotating it slowly until the bone cortex was penetrated. The stylet of the biopsy needle was removed and 2.5 mL of bone marrow was aspirated into a hypodermic syringe containing 0.1 mL of heparin (300 U/mL). The biopsy needle was then removed and the same procedure was followed in the contra-lateral bone to collect another 2.5 mL of bone marrow aspirate in the same syringe. Thus, a total of 5 mL of bone marrow aspirate was collected from each animal.

Isolation and culture of mesenchymal stem cells (rBMSC)

The marrow sample was diluted with equal amount of Dulbecco's phosphate buffered saline (DPBS) and was layered on density gradient medium -Ficoll-Hypaque (Sigma) in 2:1 ratio. The sample was subjected to gradient centrifuge at 3000 rpm for 30 min and the buffy coat containing mononuclear/nucleated cells were collected carefully from the interface (Suppl. Fig. 1). The buffy coat containing nucleated cells was taken in the test tube along with the growth media [Dulbecco's Modified Eagle's Medium (DMEM)] containing antibiotic (Strepto-penicillin) and subjected to centrifugation for 10 min. Then the cell pellet were diluted with the same media containing antibiotic and subjected to centrifugation again for 10 min. The cell pellets were re-suspended, counted and plated in a 25 cm² culture flask. The cells were cultured in DMEM (Gibco) medium containing 10% fetal bovine serum (Gibco) and antibiotics (penicillin G, 100 U/mL; streptomycin 0.1 mg/mL; amphotericin B 0.25 µg/mL) in a CO_2 incubator (Sanyo-MCO-20 AIC) with an atmospheric concentration of 5% CO_2, 21% O_2 and 95% humidity at 37°C. The media was first changed at the third day of culture, and then every other day. Non-adherent cells were removed subsequently. The cells achieved confluence after 10-15 days. The cell morphology was regularly checked under inverted microscope.

Expansion of rBMSC

Once the cells (MSCs) attained 80-90% confluence, they were passaged several times (up to passage 3) to increase cell population. For that, old culture media was removed and cells were washed twice with DPBS (without Ca and Mg). Trypsin (0.25%) and EDTA (0.001M) was added into the culture flask and kept at CO_2 incubator for 5-10 min for detachment of cells (confirmed by microscopic examination). Equal volume of growth media was added to the flask and mixed gently with 5 strokes, transferred into a 15 mL tube and centrifuged at 2000 rpm for 4 min at room temperature (20-22°C) and the supernatant was removed completely with care. Then the cell pellet was re-suspended in culture media and subjected to washing after which the cell suspension was counted using a hemocytometer and reseeded in flasks with pre-warmed growth medium at approximately 100000 cells per 75cm² cell culture flasks. Cells were examined regularly for its viability, morphological features and confluence under high power microscope. The cells were passages and expanded in optimal cultivation conditions in define culture medium until a pure culture was obtained.

Characterization of rBMSC

Flow cytometry analysis

Flow cytometry was performed in FACS Caliber (BD, Frankin Lakes, NJ, USA) equipment from the first to third passage using the anti-rabbit CD-105, CD-73,

CD 44 and CD-34 and CD-45 monoclonal antibody to evaluate cell expression which were marked with goat anti-mouse IgG-FITC (Santa Cruz biotechnology, INC.) For determination of surface markers of BM-rMSC, first three generations of MSCs were cultured, trypsinized, centrifuged and washed with DPBS twice. The cell concentration was determined and they were mixed with anti-rabbit CD-105, CD-73, CD 44 and CD-34, CD-45 monoclonal antibody (in PBS instead of primary antibody as negative control). After 30 min reaction in room temperature, cells were washed with PBS twice, mixed with FITC labelled goat anti-mouse IgG (secondary antibody) and kept for dark reaction for 15 min followed by flow cytometry for expression of cell surface markers.

Reverse transcription-polymerase chain reaction (*RT-PCR*) *analysis*

The expression analysis of rabbit CD105, CD44, CD45, CD34 and CD73 was done semi-quantitatively by RT-PCR using β-actin as reference as follows. Isolation of RNA from New Zealand white rabbit mesenchymal stem cell (r-MSC) after 3rd passage in culture cells (around 4 million) was done as per the protocol of Qiagen RNeasy plus mini kit. C-DNA preparation from isolated r-RNA was prepared using fermentas kit. Further, PCR amplification of a 377 bp region of CD44 gene; 533 bp region of CD73 gene; 342 bp region of CD105 gene; 531 bp region of CD45 gene; 400 bp region of CD34 gene; and a 432 bp region of β-actin gene were carried out using the following primer pairs procured from Chromous (USA): CD44: for 5′-GCCTGCCCACCATGG CTCAG-3′ rev 5′-CCCGGGAGACCCACTGCTCA-3′; CD73: for 5′-CACTCAGTCATGCCGCTTTA-3′ rev 5′-CGCTGATATCTTGCTCACCA-3′; CD105: for 5′- CCGGCGAATACTCTCTCAAG-3′ rev 5′-AGGTCAGGTTCAGGATGGTG-3′; CD45: for 5′-TCCCGCCGACACAGCTCTCA-3′ rev 5′-GGCCTG GCATTCACGTCCCA-3′; CD34: for 5′-TAGGGCTC AGTGCCTGCTGCT-3′rev 5′-GCCGTTTCTGGAGGTG GCCT-3′; and β-actin: for 5′-AAGGACCTGTACGCCA ACAC-3′rev 5′-CACCTTCACCGTTCCAGTTT-3′.

Table 1 explains the various PCR programme steps used to optimize amplification process using different annealing temperatures.

After the execution of PCR programme, the PCR product was stored at 4°C. The amplicon were checked by 1% agarose gel electrophoresis at 90 V for 45 min. The amplified products were visualized under gel-documentation system and image was captured and saved for further analysis.

Table 1—PCR Programme for amplification

Sl. No.	Steps	Temp. for amplification (°C)	Time
1	Initial denaturation	95	5 min.
2	Cyclic denaturation	95	45 s
3	Cyclic Annealing	55	45 s
4	Cyclic extension	72	45 s
5	Final extension	72	10 min.
6	Storage	4	for ever

Steps 2 to 4 were repeated 35 times

Osteogenic differentiation

Undifferentiated cells were induced towards the osteogenic lineage[1]. Putative MSC was seeded in six well plates at a density of approximately 3000 cells/cm^2 and cultured in expansion media till it reached 90-100% confluence. Osteogenic differentiation was hereafter induced by culturing the cells for 20 days in osteogenic induction medium (OS) consisting of 100 nM dexamethasone, 10 mM β-glycerophosphate, and 0.05 mM L-ascorbic acid-2-phosphate and 10% FBS in low glucose DMEM. As a negative control an equal number of wells were maintained in expansion media for 20 days. The media in both groups were completely replaced every 4 days. The cells were cultured at 37°C in a CO_2 incubator at 5% CO_2 and maximum humidified environment. The presence/ differentiation of osteoblasts was confirmed by mineralization staining (Alizarin Red S staining) and alkaline phosphatase activity.

Alizarin Red S staining

Alizarin Red S (Sigma-Aldrich), staining of calcium deposits was done by carefully aspirating the medium from each well so that the cells were not aspirated. The cells were fixed by incubating in ice-cold 70% ethanol for 5 min at room temperature, before carefully aspirating the alcohol and rinsing twice (5 min each) with water. The pH value of the Alizarin Red S solution was adjusted to a 4.1-4.3 with ammonium hydroxide prior to the procedure. The water was aspirated and 1 mL of 2% Alizarin Red S solution was added. The plate was incubated at room temperature for 10 min, before removing the Alizarin Red S solution and washing the wells five times with 2 mL water. Water was added to wells to prevent the cells from drying and the plates were visualized under bright field microscopy.

Alkaline Phosphatase assay

Alkaline phosphatase (ALP) assay is performed as a routine marker for osteogenic differentiation of

MSCs, and normally, intracellular ALP activity increases rapidly during *in vitro* osteogenesis. The assay for expression of the alkaline phosphatase in BM-rMSC was conducted after fixation of cells with ethanol using alkaline phosphatase staining kit (Sigma-Aldrich). The cell layer was washed with distilled water and incubated with the staining solution prepared according to the instruction manual at room temperature for 20 min in the dark. The colorimetric reaction was stopped by washing with dist water and the cells were stored in PBS until the analysis with bright field microscopy. ALP can hydrolyze Naphthol AS-MX phosphate to Naphthol AS-MX, and the latter combines with fast violet B salt to form the bluish purple, insoluble, granular dye deposit, which indicates sites of ALP activity.

Adipogenic differentiation

The adipogenic differentiation potential of rBMSCs was evaluated by culturing in mesencult basal medium with 10% of FBS containing isobutyl-metilxantine (0.5 mM), indomethacin (200 μM), dexamethasone (10–6 M) and insulin (10 μg/mL). After 8 days of induction, cells were stained with OIL Red O.

Chondrogenic differentiation

The chondrogenic differentiation of rBMSCs was achieved for 3 weeks with DMEM, ITS supplements 100 n*M* dexamethasone, 0.17 m*M* ascorbic acid-2-phosphate, 1 m*M* sodium pyruvate, 0.35 m*M* L-proline (all Sigma-Aldrich) and 10 ng/mL transforming growth factor-β3 (TGF-β3; PeproTeck, Hamburg, Germany). The control cells were cultured in the same medium in the absence of TGF-β3. The medium (500 μL) was changed thrice in a week. For Alcian Blue staining, cells were rinsed once with DPBS, and fixed with 4% formaldehyde solution for 30 min. After fixation, rinsed wells with DPBS and stained cells with 1% Alcian Blue solution prepared in 0.1 N HCL for 30 min and again rinsed wells 3 times with 0.1 N HCl, added distilled water to neutralize the acidity, visualized under light microscope, and captured images for analysis.

Colony forming unit-fibroblast (CFU-F) assay and colony forming unit-osteoblast (CFU-OB) assay

For CFU-F assay, 1×10^6 bone marrow nucleated cells per 60-mm dish were seeded and cultured at 37°C in 5% CO_2 for 12 days with media change every 2 days, followed by Giemsa staining. The numbers of CFU-Fs were counted under a microscope. For CFU-OB assay, 1×10^6 bone marrow nucleated cells per 60-mm dish were seeded and cultured for 21 days in differentiation medium (α-MEM containing 10% FBS, 1% penicillin/streptomycin, 50 μg/ml L-ascorbic acid and 2.0 mM b-glycerophosphate). Media was changed every 2 days. Alizarin red staining was done to identify the mineralized bone matrix of osteoblast differentiated colonies. These colonies were enumerated and designated as CFU-OB colonies.

Implantation of MSC-ceramic tissue engineering construct for repair of critical bone defect (30 mm) (*in vivo* experiment)

For the *in vivo* evaluation of bone healing potential of rBMSCs, experiment was carried out in 18 New Zealand White (NZW) rabbits by creating a non-union fracture model. For this, NZW rabbits of either sex were used. Under general anaesthesia (Xylazine-Ketamine combination) and strict surgical asepsis, a medial approach to the radius bone was performed. A 30 mm long osteo-periosteal critical bone defect was created with the help of an oscillating bone saw that was continuously cooled by irrigation with normal cold saline. In three groups of six animals each, one group (Gr-I) where critical bone defect was left as such (untreated) served as control and another group (Gr-II) with critical bone defect filled by bioceramic block (HASi) (silica coated calcium hydroxy apatite bioceramic) only without any type of stem cells and a third group wherein HASi seeded with autogenous bone marrow derived rabbit mesenchymal stem cells of third passage (rBMSCs) (Gr- III) was implanted in the critical bone defect (30 mm). The stem cell-bioceramic tissue engineering construct was secured in position by suturing the ceramic in the bone defect with Catgut #2, followed by suturing the surrounding muscles and subcutaneous tissues in layers with Catgut #1. The skin was sutured with non-absorbable suture (nylon). A custom designed wooden splint was applied on the test bone for 10 days. Regular dressing with antiseptic lotion was carried out for 5 days post-implantation. The animals were kept in an individual cages and allowed unrestricted weight bearing activity post-surgery.

Radiographs were taken using table top procedure at 14 mAs; 50 kVp and 90 cm FFD. Radiographic evaluation was performed to record the acceptance or rejection of MSCs/scaffold, the initiation and progression of healing and bone formation at the critical size bone defect site. Radiography was performed on all groups on 10, 20, 30, 40 and 60th day in mediolateral (ML) and antero-posterior (AP) views under light anaesthesia. Due to overlapping of the radius by ulna bone in AP view, the radiographic

evaluation of the defect site at different intervals mostly involved analysis of mediolateral views of the radiographs.

Results

Bone marrow collection procedure from rabbits was carried out as per procedure mentioned in the materials and methods and the protocol was repeated and standardized. The mesenchymal stem cells (MSCs) were successfully isolated, cultured and propagated from all the aspirated bone marrow samples.

On day '0' all the seeded cells were found floating in the culture medium. Majority of the cells showed round/oval shaped morphology (Fig. 1A) and by the next 3-5 days, the cells began to attach at the coated surface of plastic culture flask with the formation of primary colonies. The cells in monolayer assumed polygonal/spindle/star morphology (Fig. 1B). After attachment of cells, the media was supplemented to replenish the depleted nutrients in the old culture media. After 7-8 days of passage 0, number of adherent cells increased significantly and a colony formation unit (CFU) of long spindle cells which were uniformly distributed was observed. After 7-9 days, adherent cells increased in number and gradually the morphology changed to polygon and spindle-shape. After 12-15 days of passage 0 (P0), a colony formation unit (CFU) of uniformly distributed long spindle cells were observed. Following 1st, 2nd and 3rd passage, the cells showed attachment after 2 days with spindle shape and longer cytoplasmic extensions and were closely packed. The cells reached 80-90% confluence by 10-12 days.

After the cells became 80-90 % confluence, they were passaged using trypsin (0.25 %) with EDTA for lifting/detachment the stem cells and reseeded at 30% confluent level or 1000 cells/cm^2 to a fresh culture flask. On subsequent passages, all the cells showed attachment from day 2 of passaging and acquired spindle shape with uniform morphology (Fig. 1C). The crystal violet staining showing spindle shaped colonies of cells is shown in Fig. 1D.

In this study, we characterized the first three generation cell lines of surface antigens by flow cytometry (Suppl. Fig. 2A and B) and RT-PCR (Fig. 2 A-C, respectively) which showed positive expression of surface antigens CD44, CD73, CD105. However, this expression percentage varied in different passages tested. Greater expression was observed as the number of passages increased.

To characterize unique differentiating property of rBMSCs and mineral deposition, osteogenic differentiation was performed in monolayer culture for 3 weeks. The cells changed from a fibroblastic appearance to a more polygonal appearance and formed nodules. After 3 weeks of culture time, the induced cells were stained positive (orange red) with alizarin red S stain for mineral (calcium) deposition in their newly formed matrix (Fig. 3A). Control cultures, although becoming over-confluent after 3 weeks, retained their fibroblast like appearance, did not form cell aggregates and were stained negative for mineral deposition (Fig. 3B). Osteogenic induced cell cultures changed morphology from adherent monolayer of swirling spindle shaped cells, which was still apparent in the control cultures, to layered cell clusters surrounded by a matrix-like substance positive upon Alizarin Red S. Statistically significant (P <0.001) higher quantities of calcium deposition and alkaline phosphatase activity at the 90% level were also demonstrated in these osteogenic induced culture wells.

Osteogenic differentiation was evaluated using morphological and biochemical techniques both in cells grown on osteogenic supplement medium (OS culture) and in cells cultured in culture medium alone (control culture) which represented control cells. Proliferation and differentiation of control and

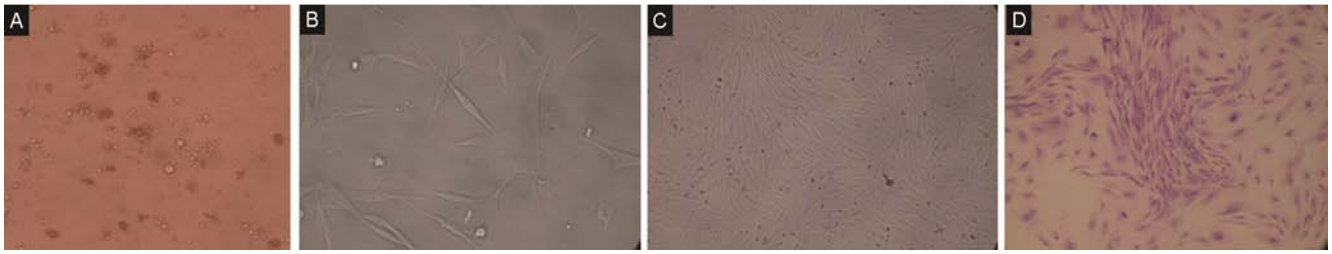

Fig. 1—Morphological observations of bone marrow derived rabbit mesenchymal stem cells (rBMSCs). (A) Day 0 showing floating of round cells; (B) Day-5 showing spindle and polygonal shaped cells; (C) P2 cells showing spindle shape with uniform morphology; and (D) Crystal violet staining of rBMSCs.

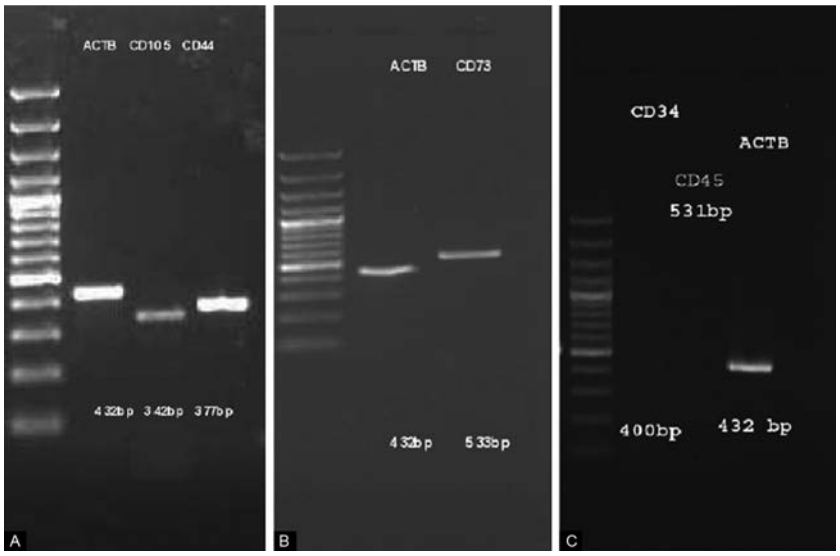

Fig. 2—Reverse transcription-polymerase chain reaction (RT-PCR) analysis. (A) Positive expression for CD 105 & CD 44; (B) Positive expression for CD 73; and (C) Negative expression for CD 34 & CD 45.

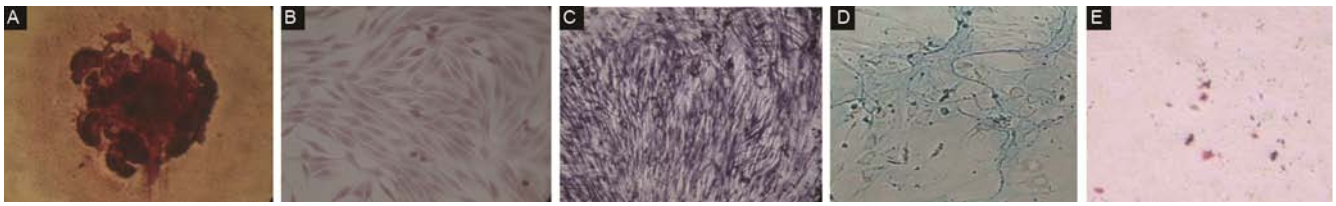

Fig. 3—(A) Alizarin red staining showing orange red calcium deposits; (B) Control culture showing absence of calcium deposits; (C) Alkaline phosphatase staining of MSCs following osteogenic differentiation showing bluish purple granular dye deposits; (D) Chondrogenic differentiation of rBMSCs showing blue staining with Alcian blue stain; and (E) Adipogenic differentiation of rBMSCs showing deep red stained oil droplets with Oil Red O staining.

OS cultures were compared using inverted microscopy. In both cultures, cells proliferated and reached almost complete confluence at day 9. In OS cultures, nodular aggregates of cells became evident at day 9 of culture and increased up to 21 days. These aggregates were characterized by deposits of amorphous material. In control cultures similar aggregates were not observed and lacked deposits. The nodular aggregates in OS cultures stained with alizarin red S, demonstrating that the amorphous deposits observed at the microscope were calcium deposits (Fig. 3). Alizarin red positive nodular aggregates present at day 21 were larger and stained more intensively, indicating that a more extensive calcium deposition had occurred. Control cultures showed only very minimal background staining. Alkaline phosphatase (ALP) activity was also observed more intense in OS culture than control culture (Fig. 3C).

Following Alcian blue staining, the cells cultured in chondrogenic differentiation media stained blue (Fig. 3D). Oil red O staining of cells cultured in adipogenic differentiation media stained deep red (Fig. 3E).

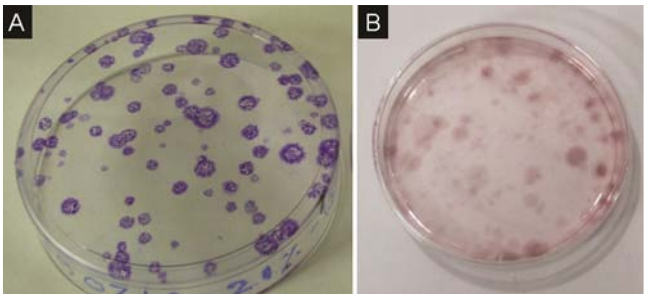

Fig. 4—(A) Colony forming unit-fibroblast (CFU-F) assay; and (B) Colony forming unit-osteoblast (CFU-OB) assays.

In CFU-F assay, 52±2.3 colonies of fibroblast were obtained from three different trials and 49±2.6 colonies of osteoblasts in CFU-O assay (Fig. 4 A and B, respectively).

The radiographic comparison between 3 groups was done to evaluate the osteogenic differentiation potential of MSCs and is shown in Fig. 5. In control

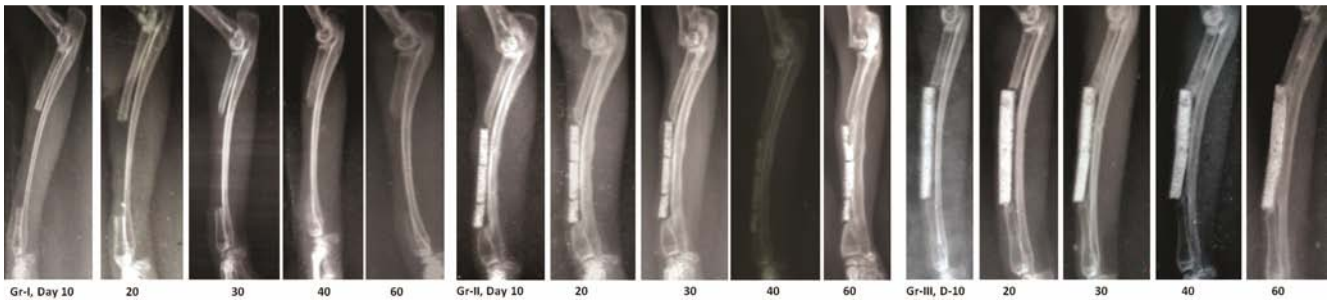

Fig. 5—Radiographs of group I, II and III showing bone healing at different time intervals.

group, the osteotomies line at one defect edge became invisible by 30th day marking the progress of bone repair near this cut end, and by 40th day a slight increase in radio-opacity was seen near this defect marking the beginning of callus formation. By 60th day, the area of radio-opacity was seen to be spread a little more with the defect remaining unbridged.

In group II, mild resorption of the graft started on 40th day and by 60 days post-implantation the resorption was moderate. There was gap between the graft and the cut ends of host bone till 60th day but there was increased bone density at the junction of graft with the ulna by 60th day.

In group III, bridging of the bone and graft at one end was seen from 30th day onwards. By 60th day, there was close attachment of the graft with the ulna and bridging between graft and cut end of host started at the other end also. The graft also underwent mild resorption by this time.

Discussion

Friedenstein *et al*.[8] have first demonstrated that fibroblast-like cells could be isolated from bone marrow due to their inherent adherence to plastic in culture. They described these cells as multipotential stromal precursor cells, which were spindle shaped and clonogenic in culture conditions, defining them as colony-forming unit fibroblasts (CFU-F). In addition to this, several studies conducted on MSCs reported that these cells are capable of differentiation into cardiomyocytes, neurons, and astrocytes *in vitro* and *in vivo* in addition to the mesoderm lineage[9-12]. In addition to bone marrow, rabbit MSCs (rMSCs) have also been isolated from adipose tissue, peripheral blood, synovium, periosteum, placenta and fetal liver[6].

Rabbit mesenchymal stem cells isolated from different compartments present different biological characteristics. The colony-forming efficiency of bone marrow derived rMSCs is higher than that of peripheral blood-derived rMSCs[13]. Bone marrow derived cells and periosteum-derived cells proliferate faster than do adipose derived rMSCs[14]. Fetal tissues present a superior growth rate, clonogenic capability and plastic adherence, as observed for fetal liver-derived rMSCs, which present a higher adhesion capacity and proliferate faster than do adult bone marrow-derived rMSCs[15], but there are ethical regulations for its use.

Several procedures were proposed for simply harvesting autologous or homologous mesenchymal stem cells, expanding them in culture, inducing differentiation and seeding them on suitable scaffolds in accordance with the targeted tissue type and implanting the construct into the patient's body[4]. Density gradient media was used most commonly for isolation of MSCs and their plastic adherent property was exploited for culture expansion.

It is generally accepted that mesenchymal stem cells (MSC) can be recognized as the adherent cells derived from bone marrow capable of extensive proliferation, with a fibroblastic profile and with the ability to differentiate into mesenchymal lineages[16]. In this study, the cells reached 80-90% confluence by 10-12 days following seeding in T25 flasks. Studies have shown that rBMSCs attained confluence after 14-18 days[17]. P1 to P2 passages showed uniform spindle shaped morphology which is in accordance with earlier report[18].

Flow cytometry analysis revealed: CD-105, CD-73 and CD 44 as positive cell expression and CD-34 and CD-45, as negative. The positive expression so obtained was weak in Flow Cytometry; however, the findings were confirmed by RT-PCR. This may be because specific monoclonal antibody to identify rabbit mesenchymal stem cells is not yet available commercially; thus, it was important to conduct such characterization using the largest number of markers that present a reaction (CD 44, CD 73 and CD105)

and that present no reaction (CD 34 and CD 45) for which RT-PCR was done. The weakest expression of markers was seen with first passage and the expression percentage gradually increased following second and was stronger in third passage in comparison to first passage. This may be because of the fact that macrophages/monocytes which may be there as contaminants no longer proliferated on repeated passages as they are already differentiated cells and only MSCs proliferated and expanded.

The hematopoietic cell surface markers CD34 and CD45 showed negative or minimal expression, which were in accordance with the literature[9]. The absence of hematopoietic cell markers and expression of membrane molecules CD-105, CD-73 and CD 44 showing mesenchymal origin of cells and the plastic adherence properties of these cells in tissue culture flasks hereby fulfil the minimum criteria for defining stem cells[4]. The surface antigens expressed by rabbit MSCs are not uniformly cited in literature and different studies are reporting that they are positive for CD 29[6], CD 44 and CD 105[18] and negative for CD34 and CD40L[6,18]. The BM-rMSCs expressed CD29, CD44, CD73, CD81, CD90 and CD166, but did not express CD34, CD45, CD117 and HLD-DR[19].This difference may be due to variation in the expression levels of rabbit MSC surface antigens. Of rabbit MSCs, 91 % expressed CD44, 27 percent expressed CD29, 10 % expressed CD14, 6 % expressed CD90, and 2 % expressed CD45, MHC I or MHC II[20]. Those surface antigens with lower expression levels may seldom be encountered.

Increase in the levels of the alkaline phosphatase activity, an intracellular enzyme necessary for mineralization, is an early marker of cells oriented towards osteogenic production. It degrades inorganic pyrophosphate to release phosphate that is needed for mineralization. Adult MSC exposed to OS medium differentiate towards osteoblastic lineage[21]. Calcium deposits in the matrix were demonstrated by alizarin red S staining. This histological staining is based on the capacity of alizarin red to specifically stain matrix containing calcium and its positive appearance is considered an expression of bone matrix deposition[22]. The co-localization of alizarin red stain with the deposit of amorphous material close to the nodular cell aggregates observed in OS cultures demonstrated that these amorphous deposits contain calcium and suggests that nodular cell aggregates are made up of cells committed to the osteoblastic lineage. In the present study, this target was reached in a simple and safe way using a cocktail of three different drugs, of which the key member was dexamethazone. Dexamethazone, as is the case with other glucocorticoids, has both a stimulatory and an inhibitory effect on osteogenic differentiation depending upon the dose. Dexamethazone is necessary for *in vitro* bone nodule formation and mineralization in marrow derived cell cultures. The increase in ALP activity in OS culture is a marker of the commitment towards osteoblastic lineage. The ALP activity increases up to a peak and decreases to control level in OS medium treated MSC[1].

Alcian blue staining of rBMSCs revealed the production of proteoglycans and hence confirmed the differentiation to chondrocytes[23]. Oil Red O staining revealed intracellular lipid filled droplets that stained deep red confirming the adipogenic differentiation potential of rBMSCs of third passage. This has indicated that rBMSCs may possess multi lineage potentiality. CFU-F and CFU-O assays revealed the proliferation capacity and colony forming characteristics of fibroblasts derived from rabbit bone marrow and osteoblasts from rBMSCs.

The *in vivo* experiments showed that the critical sized defect remained unbridged in control group with very slow progress in healing rate. In group III, the periosteal bridging between host bone and graft was noticed from 30th post-implantation day whereas the gap between graft and host bone persisted till 60th day in group II. This faster healing potential noticed in group III may be attributed to the osteogenic differentiation potential of MSCs. So also they have strong ability to induce angiogenesis by attracting host-derived vascular endothelial cells via paracrine effects. By mediating neovascularization, MSCs promote infiltration of osteoprogenitor cells, and thus enhance their subsequent mineralization and bone formation[24].

Conclusion

The present study underlines the techniques of isolation, culture, proliferation and characterization of bone marrow derived mesenchymal stem cell from New Zealand white rabbit. The simple way in which MSC can be obtained from bone marrow, and the capacity of the entire population of bone derived cells to proliferate and differentiate, make this source of MSC particularly feasible for clinical application in

restorative surgery. The possibility to greatly expand the number of MSC *in vitro* is a particularly important aspect since it suggests that in humans a limited bone marrow aspirate can supply an adequate number of cells for clinical applications. *In vivo* study in rabbit model has shown that rBMSC possess osteogenic and osteoinductive properties and tissue engineered bone construct can be used in large bone defect as well as in delayed and non union bone fracture. The technique is considered easy and useful for further research or application in regenerative medicine, cell therapy, tissue engineering, and gene therapy in animal sciences.

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