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Characterization of Behavior and Niche of Bovine Marrow Stem Cells *In vitro*

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Abstract: Although the different trials of stem cell therapy progressively increased in the last decades, the successful cases are still restricted. This study use the bovine species to exit from research routine that extensively using rat and aimed to accomplish some sides of behavior of bovine marrow stem cells *in vitro*. Two groups of cell culture were prepared. The first was feeded twice weekly while the second feeded once weekly and exposed to two passage. The medium of two groups was standard containing 20% FBS. The bovine bone marrow mesenchymal stem cell (MSCs) characterized by their attachment to the plastic adherent surface of the culture vessels. The MSCs monolayer is formed after two weeks overlapped by hematopoietic cells (HCs) and colonies which increased after three weeks of the primary culture. The hematopoietic stem cells (HSCs) and progenitors showing rounded form and variable sizes. Each colony seemed to be originated from one HSCs. The bovine is related to species in which MSCs expand rapidly by increasing the density of seeded cells, but the cells start to die after propagation have been reached to overcrowding. The crowded cells of the culture groups particularly the second one showing severe depletion of MSCs associated with reduction of HSCs and colonies. Then some areas of culture vessels become free from MSCs while HSCs difficulty restore their expansion with absence of colonies and appearance of degenerated cells that may be of late developmental stages. The MSCs and stromal fibers represent important components of niche of HSCs. The HSCs are not removed as non-adherent cells in the culture through refeeding due to the adherence phenomena that obtained by bind characters of treated surface of culture vessels. The bovine HSCs possess the potentiality to perpetuate themselves even in the depletion or absence of the niche. This HSCs restoration could be carried out by minimal existence of the primitive HSCs. This study is a stage of a project which aiming to unusual method for reprogramming of bone marrow stem cells that may expose new characters of the marrow stem cells in therapy

Key words: Bone Marrow · Mesenchymal · Hematopoietic · Cell Culture · Stem Cells

INTRODUCTION

Both bone marrow mesenchymal and hematopoietic stem cells are attractive media of research in the last decades; this is due to the multipotentiality of both types of cells. The osteogenic and adipogenic differentiation of mesenchymal stem cells was already established by many researchers [1-4] achieved that mouse histone methyl transferase enhances zeste homology 2 gene in the hepatocellular differentiation of bone marrow MSCs. Neural cells were also induced to be differentiated from bone marrow stromal cells *in vitro* [5]. The effect of bone marrow stromal cells treatment for ischemic stroke including sensory and motor recovery has been reported in pre-clinical studies and clinical trials [6]. Also, *in vivo*, the purified hematopoietic stem cells can differentiate into hepatocytes and described to be the only stem cells that give rise to hepatic regeneration [7].

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The initial steps of all these application were isolation expansion and characterization of marrow mesenchymal and hematopoietic stem cells. Marrow MSCs were isolated from the bovine bone marrow based on their characteristic property of attaching to the plastic surface of culture vessels in the standard culture condition that induced DMEM media supplemented by 10% fetal bovine serum [8,9]. In a serum free medium, human bone marrow-derived mesenchymal stromal cells are isolated, propagated and characterized [10]. Biomaterial surfaces for isolation of hematopoietic stem and progenitor cells were recommended [11].

Although the successful cases are still not in a wide field of application, stem cell therapy receives a striking propaganda in treatment of diseases. This indicating that the different phenotypes of stem cells should be carefully studied and looking for new methods of stem cell manipulation and also species other than rat such as large animals which are still away of search focusing. In the present work, we select bovine to accomplish some sides of behavior of bovine marrow stem cells *in vitro*. This study is only a stage of a project aiming to adapt new method of reprogramming of bovine marrow stem cells that may present a solution for some problems of stem cell therapy.

MATERIALS AND METHODS

Sampling and Plating: About 8 of bone marrow aspirates were harvested from the iliac crest of bovine at age of 1.5-3 years immediately after slaughtering from bovine abattoir. Samples were collected using syringe with needle of epidural anesthesia. The syringe was wetted by 1000-2000 IU of heparin calcium before sampling. The amount of samples was ranged from 2-5 ml that was transmitted rapidly to the lab in a sterile bag.

The samples were washed three times by DMEM low glucose (Sigma-Aldrich, D6046) using centrifuge at 1500 rpm. The third wash was made by complete medium containing 20% fetal bovine serum (FBS), penicillin-streptomycin (100 IU and 100 μg/ml) and amphotericin B 25 ng/ml (all are Sigma-Aldrich, F6178, P4333, A2942). After the third wash, the pellets were resuspended in the complete medium and seeded in Corning cellBIND 6 well cell culture plates with treated surface. Each plate received 15 ml of cell suspension containing 1.5-2 ml of samples; each well received 2.5 ml of cell suspension. The cultures were incubated at 37C° in humidified incubator with 5% CO_2. After three days, or one week the cultures were washed slowly twice by DMEM to remove the great amount of erythrocytes which exhibit the red color of culture then feeding by complete medium.

Two groups of culture were prepared; the first group was feeded twice weekly, the second group feeded once weekly and subjected to two passages. Only two passages were applied just to know the effect of passage on the current events of the culture. The culture groups let to be long culture and followed for 8-12 weeks.

Subculture: Subculture was made using 0.25% trypsin/ EDTA in HBSS without Ca^{2+}/Mg^{2+}. The trypsin was used without dilution or diluted to 1:5 by PBS (Sigma-Aldrich, T4049, P4244). First and second passages were carried out after two weeks. Each two wells were reseeded into three wells of 6 well plates in a concentration of $15x10^3$, $5x10^4$ and $8x10^4/Cm^2$.

Lieshman´s Staining: Lieshman´s stain was applied in some selected wells after removal of medium and washing of culture by PBS and then fixed by gluteraldehyde. The stain was applied for 5 minutes then removed and the bottom of the wells are covered by coverslip and DPX and examined by the microscope.

All above methods are modified from protocols in the website of Sigma-Aldrich according to Freshney [12].

RESULTS

First Culture Group: This group is a primary culture and feeded twice weekly. After one week, the bovine bone marrow primary culture appeared to be formed of MSCs as well as HCs (Fig 1A). The MSCs exhibit their adherent character to the plastic surface of culture vessels; rather than the well surface of the culture plates is treated.

The MSCs and other bone marrow stromal cells are identified by their elongated shape with long and short processes at their ends, while the HCs are rounded. Other rounded non adherent cells including macrophages, granulocytes and adipocytes may also present.

The formation of monolayer starts by separate colonies of MSCs, each of which is made by one cell. The HCs and other rounded cells may be present in between and overlapping the MSCs that represent the developmental niche of the HCs. Sometimes minute particles of marrow tissues are aspirated during sampling and represent colonies of MSCs that appear in radiating manner around those particles (Fig 2 A, B).

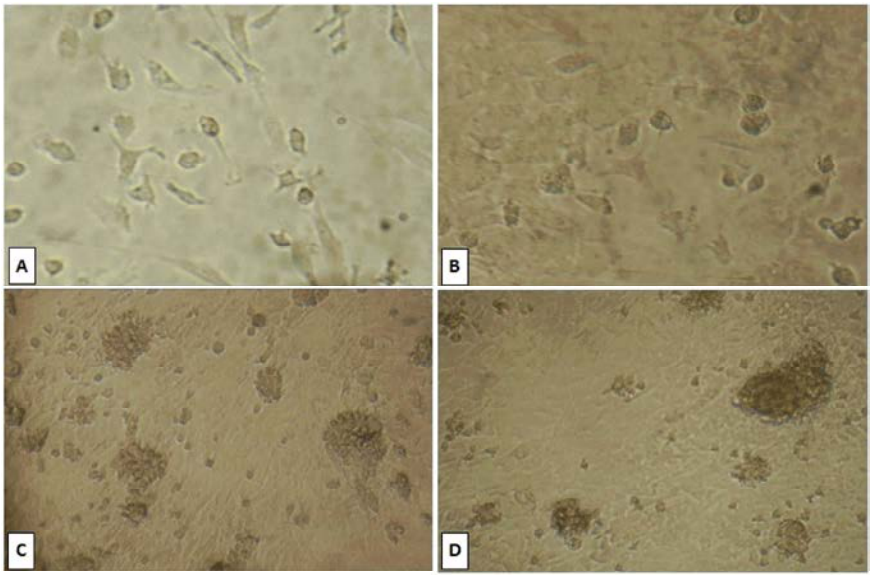

Fig. 1: Primary culture. A, after one week and B, after 2 weeks showing MSCs appear with some rounded HSCs. C and D, after three weeks showing HSCs colonies are overlapping the MSCs monolayer. A and B = x400 C and D = x250

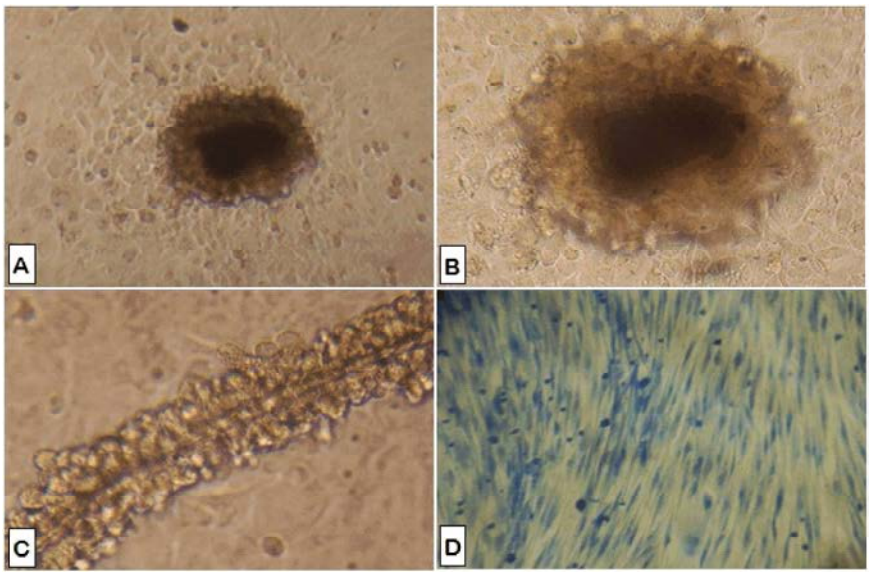

Fig. 2: Primary culture. A and B: minute particles of marrow tissue releasing stem cells to the culture. C: HSCs attracted to bone marrow stromal fibers. D: Lieshman?s stain showing rounded HSCs scattered over MSCs monolayer. A and C = x 250 B = x400 D = X 100.

After two weeks the hematopoietic colonies appeared small but well identified and overlapping the monolayer of MSCs with solitary HCs appeared scattered among the culture (Fig 2 D). By frequent renewing of the medium the non HCs rounded cells are removed. With the formation of hematopoietic colonies, the HSCs and progenitors increase in number and become representing the majority of rounded cells in the culture.

At the third week, the hematopoietic colonies persist and some of them may show larger size although the frequent feeding may remove many of them (Fig 1 C, D). This occur parallel to the well-developed monolayer of MSCs. Also, HSCs and progenitors sometimes appeared densely attached to the bone marrow stromal fibers on either sides (Fig 2 C).

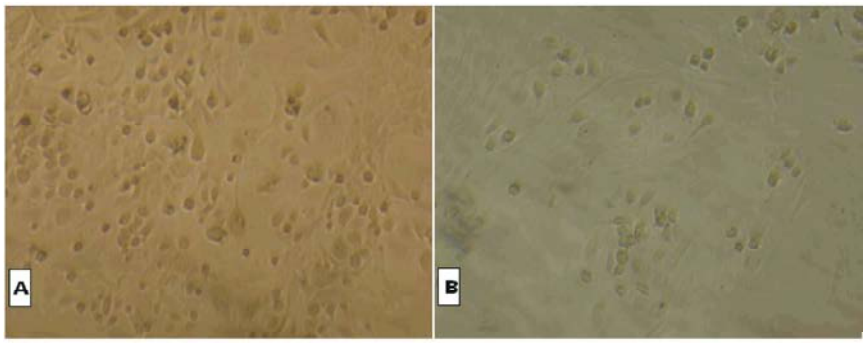

Fig. 3: A: After one week of first passage, feeded once weekly showing normal growing and few degenerated cells. B: After one week of 2nd passage, feeded once weekly. A and B is x250.

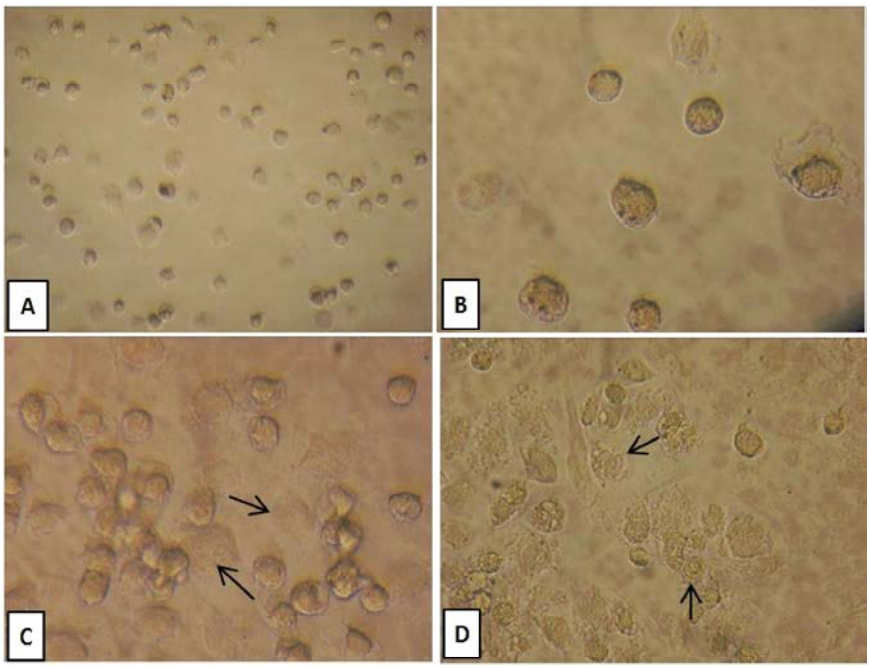

Fig. 4: A,B and C after 8 weeks of the second passage, feeded once weekly, the rounded HSCs persist while apoptotic MSCs appear as a shadow (arrows); B showing an area free from MSCs; D Primary culture after 4 weeks feeded once weekly showing few liver HSCs and many degenerated MSCs and HSCs (arrows). A= x 250 B, C, D = x400.

The propagation of bovine bone marrow MSCs in primary culture show a variation from one aspirated samples to another but the major cause seems to be the density of seeded cells, where rapid expansion is reached by increasing the density of seeded cells ($5x10^4$ - $8x10^4$), but when propagation reach to overcrowding the cells start to die. The rapid expansion of MSCs is followed by similar expansion of HSCs. Poor growth occurs by decreasing the cell density to $15x10^3/cm^2$.

Good propagation occurs by starting the first feeding after one week of seeding of the marrow sample, this gives the cells the chance to adhere in the culture vessel and grow rapidly. In long term when the cells become over crowded in the culture, many MSCs and HSCs star to die and degenerate (Fig 4D) and the cells decrease largely in the culture. By frequent feeding the HSCs can restore themselves but the MSCs not showing the same level.

Second Culture Group: This cell culture was feeded once weekly and exposed to two passages. The first passage is carried out after one week. After the first passage, the MSCs and

HSCs and colonies restore their distribution in the culture rapidly (after 1 week). At this time the second passage is carried out. Weak propagation of MSCs is

recognized (Fig 3 A, B). MSCs and HSCs as well as progenitors restore their propagation in the culture after 2 weeks and the cell density increase without significant effect of subculture except appearance of vacuoles in some cells.

After 4 week, the crowded MSCs and HSCs showing many degenerated cells (Fig 4 A, C) and decreased greatly. Discontinuation of the MSCs monolayer was recognized that some areas of some wells appeared free from MSCs. Then the majority of the MSCs monolayer seem to be completely destructed associated by severe depletion of HSCs. The HSCs do not disappear completely that after 8 weeks they can restore themselves in the culture in the form of scattered cells (Fig 4A,B). Degenerated cells (apoptosis) of both MSCs and HSCs persisted in all stages.

Although the suitable niche made by marrow stromal cells including MSCs is severely altered, the HSCs proved their characteristic perpetuation, but late developmental stages degenerated.

The stages of HSCs are characterized by small, medium and large size. The small size may be the earlier stem cells (Fig 4 B). The treated adherent surfaces of the culture vessels attracted the HSCs and prevent their complete removing from the culture.

The behavior of HSCs in the culture confirms that under unsuitable niche, they can perpetuate themselves where the criteria of hematopoiesis could be restored even by one of the early HSCs. Under the same condition, the MSCs lose their potentialities.

DISCUSSION

The present study is a stage of a project aiming to a novel address for reprogramming of bone marrow stem cells including MSCs and HSCs that may be a spot light on the major character of marrow stem cells. This project requires isolation and brief characterization of bovine bone marrow stem cells to identify which type of cell culture; the suitable culture conditions as well as the best timing for using of stem cells in injection in the future work.

Recent studies provided careful identification and analysis for a great number of markers for MSCs and HSCs in different species. Although the phenotypic identification of marrow stem cells through the different surface markers as well as the specific antigen are the most accurate methods, still some confliction of these markers and antigens are present in the different stem cells [3,10,13]. However, a general agreement for multipotentiality of these cells was reported [14].

Relatively little attention has been given to MSCs and HSCs morphology except some description that demonstrate the human MSCs to be elongated or fusiform and fibroblast like [1,10]. The identification of bone marrow MSCs of bovine, goat and rat based on their characteristic property of attaching to the plastic surface of culture vessels [2,3,8] was in accordance of the present study. Suggested colony forming unit in bovine MSCs that start the formation of adherent monolayer are noticed similar to that reviewed by Nardi and Meirelles [15]. However, this suggested colony forming unit of MSCs seems to be typically like its progenies that released by mitosis. So the term colony forming unit is not accepted for MSCs because the colony forming unit produces a new developmental stages that differ from that unit.

The bovine HCs were identified by their rounded form and different size. The other rounded non adherent stromal cells including macrophages, granulocytes and adipocytes may also be present, but these cells are not regenerated and removed from the culture by frequent feeding. Although the HCs also removed by refreshment of the culture medium, the formation of hematopoietic colonies after two weeks ensure that HSCs persist in the culture and each colony is produced by one HSC. This indicates that the rounded cells formed by colonies after 2-3 weeks are HSCs and progenitors. The HCs were described as non-adherent cells which persist in long term culture of different strains of mice [16]. In addition rapid expansion of recycling stem cells was obtained in cultures of plastic adherent cells from human bone marrow [17].

Different factors affecting the continuation as well as the level of propagation of MSCs and HSCs, these factors include the density of seeded cells and number of passages. MSCs don't maintain their stem cell characteristics indefinitely; with extensive subculture. Human MSCs senesce *in vitro* and lost their multipotential properties [18], however the cultures are largely free of hematopoietic precursors after two or three passages [17]. The bovine MSCs lose their propagation potentiality by feeding one time only per week. The senescence of stem cells and losing their growth capacity may be attributed to exhaustion of telomere driven mitosis. Telomeres are repetitive DNA sequences which protect the body of chromosome from the degenerative consequence of replication [19]. Replication shortens the telomere until a critical length at which the cell leaves the cycle [20,21]. When the monolayer is completed and the cells start to be crowded in the culture, the bovine MSCs stop the division, the suggested causes are, decreasing feeding, deviation of the PH that may be due to accumulation of the metabolic waste products of the

overcrowded cells in the culture and finally the cells could not withdraw from the cycle in the culture and have not the ability for vertical growth.

The depletion or absence of bovine MSCs from the culture is strongly affect the propagation level of HSCs and died cells related to late developmental stages of HSCs could be seen. The importance of MSCs for growth and development of HSCs was confirmed in early results. The data obtained by Ni and O'Neill [16] showed that the monolayer of stromal cells and endothelial cells that formed in long term culture stimulate the formation of hematopoietic cells rapidly. This increases the realization of the importance of environmental factors that were reviewed by Watt *et al*. [22] in regulating stem cell behavior and this is being explored by imaging stem cells *in vivo* and recreating artificial niche *in vitro*. This was proceeded by Wilson and Trumpp [23] who reviewed that the crosstalk between HSCs and cellular constituents of these niches indicating that the future successful expansion of HSCs for therapeutic use will require three dimensional reconstruction of a stem-cell-niche-unit. This niche was engineered scaffolds coated with collagen I and chondroitin sulfate. These scaffolds allowed the ovine MSCs to adhere, proliferate and migrate into the scaffolds [24]. The coated scaffold with human bone marrow stromal cells was subcutaneously implanted in mice; this implanted scaffold induced vascularized hematopoietic tissue formation [25]. This explains the attraction of bovine HSCs and progenitors to stromal fibers of bone marrow in the culture. The results of Ana-María and Jean-Paul [26] clarified one of the important roles of marrow MSCs that they suggested to improve a vascular cell adhesion molecule-1- dependent migration of primitive hematopoietic stem cells. Reviewed data indicated that the use of MSCs could provide a novel option for reducing the morbidity and mortality associating with HSCS transplantation [27].

The increased density of the seeded bovine marrow stem cells results in rapid expansion of MSCs followed by HSCs ($5x10^4$ to $8x10^4/Cm^2$). However the ovine MSCs proliferating with cell density of $2x10^3/Cm^2$. Also, human MSCs expand to much higher population doublings when plated at low density than at higher density [17].

The HSCs and progenitors of the present study are not removed as non-adherent cells through refeeding due to the adherence phenomena that obtained by binding characters of treated surface of the culture plates. This is supported by the analyzed data that concluded the HSCs characters [28].

The bovine HSCs possess the potentiality to perpetuate themselves even in the depletion or absence of MScs. This accommodation may be due to that certain cells could withdraw from the cycle then return to the cycle again [12].

This HSCs restoration could be carried out by minimal existence of the primitive HSCs. The best culture should be used in stem cell injection is the primary culture feeded twice weekly but only after 3 feeding.

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