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Vol. 13(47), pp. 4399-4409, 19 November, 2014
DOI: 10.5897/AJB2014.13751
Article Number: 0504FEE48626
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

The role of bone marrow derived mesenchymal stem cells in induced stroke

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Received 22 February, 2014; Accepted 29 October, 2014

Stroke is the third most common cause of death, and a leading cause of physical disability in adults. Recovery after a major stroke is usually limited, but cell therapy, especially by application of mesenchymal stem cells (MSCs) is emerging with fixed neurologic deficits. The aim of the current study was directed to isolation and cultivation of the bone marrow (BM) derived MSCs from young rats, as well as to study the role of intravenous administration of BM-MSCs in mature male rats as an animal model for Middle Cerebral Artery Occlusion (MCAO). MSCs are spindle in shape fibroblast-like cells and possess the ability to aggregate and form colonies-forming unit – fibroblast (CFU-F). MSCs showed positive response for CD105$^+$ (the specific marker for MSCs detection) and negative response for surface marker (CD34), characteristic for the hematopoietic cells. The immunohistochemistry study of intravenous administration of Bromodeoxyyuridin (BrdU) labeled BM-MSCs after 24 h of mechanical MCAO in mature rats, demonstrated survival, engraftment and migration of systemically delivered cells in the cerebral cortex and heart tissues. However, these cells were not indicated in the lung and liver tissues. In conclusion, intravenously administered BM-MSCs enter brain and heart, and survive of this, may provide a cell source to treat stroke and heart disease.

Key words: Middle cerebral artery occlusion, mesenchymal stem cells, rats, transplantation, bromodeoxyyuridin.

INTRODUCTION

Bone marrow derived mesenchymal stem cells (BM-MSCs), like other stem cells, have the capacity of unlimited self-renewal and they give rise to differentiated cells from various cell lineages (Doeppner and Hermann, 2010). This means, MSCs is not only differentiated into types of cells of mesodermal lineage, but also into endodermal and ectodermal lineages. MSCs have been identified as an adherent, fibroblast-like population, and

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Abbreviations: MSCs, Mesenchymal stem cells; **BM,** bone marrow.

they can be isolated from different adult tissues, including BM, umbilical cord, skeletal muscles and adipose tissue (Li and Ikehara, 2013). There are some characteristics that make MSCs safe and promising candidates for application in tissue regeneration procedures. Progenitor and pluripotent MSCs, which are located in all tissues and organs, have a connective tissue component, with the potential to form at least four tissues of mesodermal origin (Young et al., 1995), for instance MSCs have been purified from articular cartilage culture (Eslaminejad and Taghiyar, 2007). Beside, broad range of MSCs distribution, these cells are free from ethical concerns, they also possess non-immunogenic properties, have injury-seeking capabilities, and can be used as vehicles for gene therapy (Zomorodian and Eslaminejad, 2012). Accumulating evidences support the beneficial effects of MSCs transplantation: in neurodegenerative diseases (Torrente and Polli, 2008); for attenuation pain, induced by spinal cord injuries (Takikawa et al., 2013); in both patients with Ischemic Stroke (Lee et al., 2010) and animal models of stroke (Chen et al., 2001b; Doeppner and Hermann, 2010).

Stroke is a leading cause of death, along with cancer and coronary heart disease, and it is the most common cause of physical disability in adults (Kim et al*.,* 2013). Approximately 80% of all strokes are ischemic with occlusion of a cerebral artery, leading to infarction of brain tissue and consequent death of neurons and/or glia. Subsequent symptoms depend on both the location of the lesion and the cell types lost (Williamson et al., 2008). Moreover, stroke causes a greater loss of healthy life years, as measured in disability-adjusted life years, than over illnesses (Hong et al., 2011). Recently, cell-based therapy has been evaluated as a regenerative strategy for patients with fixed neurologic deficits after stroke (Kim et al., 2013). Therefore, several clinical trials have used BM-MSCs in stroke and the results from these studies have raised important issues. Specifically, different variations according the patient characteristics, cell therapy timing, dose and type of cells delivered and mode of treatment, have been noted (Bhasin et al., 2011; Savitz et al., 2011; Friedrich et al., 2012; Kim et al., 2013).

Since the BM is very an important source of adult stem cells, especially of MSCs, the aim of the current study was connected with isolation and cultivation of BM-MSCs from young rats, to maintain them through several passages, and to study the role of intravenous administration of BM-MSCs, derived from different passages in stroke.

MATERIALS AND METHODS

Isolation and cultivation of BM-MSCs

Mesenchymal stem cells (MSCs) were harvested from young albino male rats (53 to 58 days old and weighting 128 to 197 gm). Rats were obtained from the Animal Breeding House, College of Education, University of Duhok, Duhok, Iraq. The animal experiments were done in Animal Tissue Culture Laboratory, Scientific Research Center, University of Duhok. The rats were sacrificed by using chloroform, and the BM-MSCs cultures were prepared according to Peister et al. (2004) with some modification. Briefly, under sterile conditions, BM was harvested by flushing the tibial and femoral bone marrow cavities with complete culture media [Iscove's modified Dulbecco medium (IMDM) (US Biol-USA), supplemented with 15% fetal bovine serum (FBS) (Invitrogen)]. Our modification was the use of IMDM instead of RPMI-1640 medium, and 15% FBS instead of 9% FBS and 9% horse serum which were used by Peister et al. (2004). Marrow plug suspension was dispersed by Pasture pipette. The so-called obtained cells suspension was resuspended in 4 to 5 ml of phosphate buffer saline (PBS), and layered over an equal volume of Ficoll-Paque (1.077 g/ml) (US Biol –USA). Then, the cell suspension was centrifuged at 2500 to 3000 rpm for 25 min at 8°C. After density gradient centrifugation, the resulting mononuclear cells (MNCs) and stem cells populations were retrieved from the Buffy coat and placed in a sterile conical tube, then washed two times with PBS and centrifuged at 2000 rpm for 10 min.

The MNCs derived stem cells population suspension were seeded in 25 cm^3 plastic tissue culture flasks (Denemark: Nunc) with 5 ml IMDM plus 15% FBS, and cultivated in incubator with 5% CO_2 (Korea LabTecl) at 37°C in a humidified atmosphere for 21 days, until they reached confluence and were defined as passage 0. Cultures of MSCs were inspected daily and re-fed every three days by replacing half of culture medium by an equal volume of fresh medium. The MSCs were isolated on the basis of their morphology and ability to adhere to the tissue culture flask (Javazon et al., 2001). So, when the culture reached approximately 80 to 90% monolayer confluence, the cells were recovered using 0.25% trypsin-EDTA and the final product was resuspended in 1 ml of IMDM plus 15% FBS. Then, the cell number and viability were determined using 0.4% trypan blue prepared in 0.9% normal saline (Buzanska et al., 2002). The cells were re-cultured in new plastic tissue culture flasks at a density of 5 X 10^4 cells / cm^2.

Immunophenotypic analysis of BM-MSCs

At the 2^{nd} passage, BM-MSCs were trypsinized into single cell suspension and re-cultured in multi-well tissue culture plates (4-wells), containing Poly-L-Lysine (Sigma-Germany), pre-coated cover slides at a density of 1×10^3 cell/well in IMDM medium, supplemented with 15% FBS. The cultures were maintained in CO_2-incubator, in the same culture conditions until they reached confluence. The attached cells were washed with PBS and fixed with 4% Phosphate buffered formalin for 10 min, then detected by immunocytochemistry method using mouse monoclonal antibodies against human CD105 and CD34 (Abcam). This procedure was performed according to the manufacturer's instructions of these CD markers (Buzanska et al., 2002) and counter stained with Harris Hematoxylin.

Stroke induction

Animal model for middle cerebral artery occlusion (MCAO)

Adult albino male rats (n=28), weighing 270 to 300 g, were subjected to middle cerebral artery occlusion (MCAO). The animals were anaesthetized with 70 mg/kg body weight intra-peritoneal (i.p.) injection of ketamine and 5 mg/kg body weight xylazine (Gonzalez and Klob, 2003). MCAO was induced by a method of intra-luminal vascular occlusion described by (Chen et al., 1992; 2001a). Briefly, 1.5 cm incision was made in the right side of the animal neck, and then sterile small size cannula was gently inserted into the right common carotid artery and advanced toward the internal carotid

Figure 1. A. The right common and external carotid arteries were expressed carefully through incision. **B.** Insertion of the needle into the right common carotid artery gently. **C.** Insertion of nylon suture (blue colour) through the cannula to reach the origin of the MCA after withdrawal. **D.** The wound sutured by catgut suture after withdrawal of the nylon suture and the cannula. **E.** Intravenous administration of Brdu labeled BM-MSCs (through the tail vein) via 27 gauge needle slowly.

artery. After withdrawal of the needle, a length of 4.0 monofilament nylon suture (18.5 to 19.5 mm), determined by the animal weight, was inserted into the cannula and advanced toward the origin of the middle cerebral artery (MCA). Three hours after MCAO, reperfusion was performed by withdrawal of the cannula and nylon suture, the wound was sutured by catgut (Figure 1A, B, C and D). The rest of the body placed over thermo regulated operating table, fixed at 37°C.

MCAO animals groups

MCAO animals groups (n=28) were divided into four groups (n=7 for each group), as follows: groups (1, 2, 3): rats of these groups were injected intravenously after 24 h of stroke induction with MSCs at 2^{nd}, 8^{th} and 12^{th} passages, respectively. While, group (4) (first control group) were rats given MCAO alone without donor cell administration. In addition to these groups, this experiment also included second control group (5) (n=7), but these were rats without given MCAO and cell administration, that means normal rats without any treatment.

Labeling of mesenchymal stem cells

In order to study the capability of rat BM-MSCs to maintain their plasticity in different passages for survival, multiplication and migration after intravenously administration in rat animal models for MCAO, the 2^{nd}, 8^{th} and 12^{th} passages of BM-MSCs were maintained to grow in culture. When the culture reached approximately 80% monolayer confluence, the culture medium was removed and replaced with fresh culture medium (IMDM plus 15% FBS), containing 4 µg/ml Bromodeoxyuridin (BrdU) (Sigma, Germany), to label MSCs for intravenous administration in rat model of stroke (Seghatoleslam et al., 2012).

Intravenous administration of BrdU-labeled mesenchymal stem cells

The BM-MSCs were maintained with labeling medium (IMDM plus 15% FBS and 4 µg /ml BrdU) for 72 h, then this medium was aspirated, the cells were washed with PBS and trypsinized into single cell suspension. Then, for each animal from groups 1, 2 and 3, after 24 h of stroke induction, 27 gauge needle loaded with the $1X10^6$ BrdU- labeled MSCs in 500 µl culture medium were slowly injected intravenously into the tail vein within 2 to 3 min (Figure 1E). All transplantation procedures were performed under aseptic conditions and immuno-suppressors were not used in any experimental animal.

Histological study

Animals of groups 1, 2 and 3 were allowed to survive for 31 days after MSCs transplantation, after which these animals were sacrificed by chloroform, whereas the animals from the first control group (4) were sacrificed after 24 h of stroke induction (to show the eventual changes in the brain at this time point). Animals from the second control group (5) were also sacrificed by chloroform. Generally, the skin, muscles and skull bones were dissected out with suitable surgical instruments, and then the whole brain was manipulated carefully and fixed in 10% Phosphate buffered formalin at room temperature. In parallel, the animals' hearts, livers and

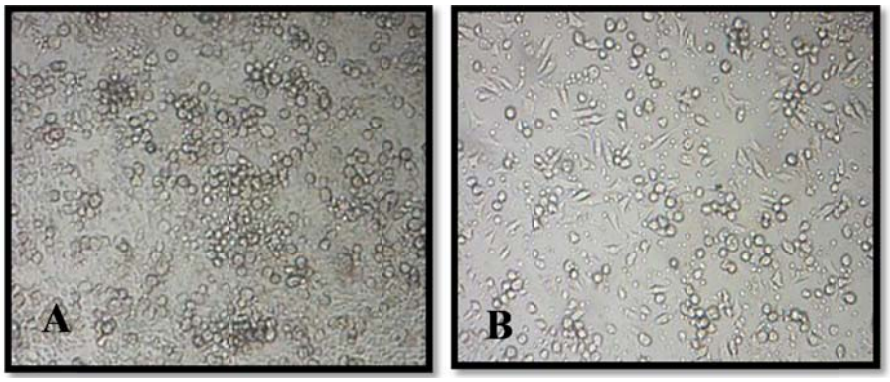

Figure 2. Primary culture of young rat BM under inverted microscope (X100). **A.** The cells after 2 to 3 days, most of BMCs are floated and the remaining of cells began to attach. **B.** after 4 to 5 days some of cells became elongated and have a fibroblast-like morphology and other cells appear as small round cells.

lungs were removed and also fixed by 10% Phosphate buffered formalin at room temperature. These organs were embedded in paraffin, in order to be ready for immunohistochemical assay. The BrdU-labeled cells were detected in sections according to manufacturer's instructions of the markers by light and fluorescent microscope.

RESULTS

BM-MSCs culture and detection

The first step in this study was connected with laboratory cultivation of BM-MSCs, obtained from young rats. The results show that in the initial BM-MSCs culture, two major types of cells were observed; hematopoietic stem cells (HSCs) and MSCs. During first hours of culturing, these cells were floating in culture medium then; some of them began to adhere progressively to the tissue culture flask surface. After two to three days of cultivation, these cells became elongated and received small uni-polar processes or fibroblast like-spindle shape (Figure 2A and B). While, the non-adherent cells (HSCs) were removed and discarded through the continuous medium changing, by replacing half of culture medium with an equal volume of fresh medium. As a result of continuous medium changing, it became clear to distinguish adherent cells with proliferation activity. The most characteristic *in vitro* feature of MSCs was their ability to aggregate and form colonies, dispensed in the culture. After 9 to 10 days from the initial cultivation, numerous colonies of different sizes appeared in the culture, each colony was derived from single adherent cells and termed as a colony forming unite–fibroblast (CFU-F) (Figure 3A). Within time of culture, cells' proliferation resulted in forming population of fibroblast-like cells, which were shown around these colonies; such cells tend to connect the adjacent colonies (Figure 3B and C). After 21 days, the adherent cells reached approximately 80 to 90% confluence and formed homogenous monolayer of spindle-like cells, which is the typical shape of the MSCs (Figure 4A). These adherent cells (passage 0) were washed and harvested. The harvested cells from primary culture of rat BM were re-cultivated at ratio of 1:3 into new flasks and incubated in the culture medium, described above. This new culture has constituted the cells of first passage. Within 2 to 3 days, the so re-cultivated cells were observed retaining their morphology and become elongated, spindle in shape, like fibroblasts. In addition, these cells were maintained in their ability to generate CFU-F. The so formed colonies were increased in size and formed homogenous mono-layers of MSCs. Gradually, the monolayer of BM-MSCs tend to appear as homogenous layer (the majority of cells were spindle–like), this aspect for instance, is represented by MSCs at different passages. The MSCs were generally expanded in culture for twelve passages, remaining undifferentiated.

The majority MSCs of the 2nd passage (Figure 4B), indicated positive response for CD105^{+}, the specific marker for MSCs detection, and they appeared in green color of fluorochrome-conjugated secondary antibody (Figure 5A, B and C). However, these cells were negative for surface marker (CD34), which is associated with HSCs, and they stained in blue color of counter stain Harris hematoxylin (Figure 5D). These results indicate that the so derived cells are MSCs, but not hematopoietic in origin.

MCAO in mature rats

MCAO was performed for the first time in Iraq, as described previously, and followed by reperfusion. To quantitate the changes that occur, the brains from animals of group 4 were examined in details after 24 h of MCAO. The gross anatomy of the rats from this group (4) revealed congestion, which was observed on the surface

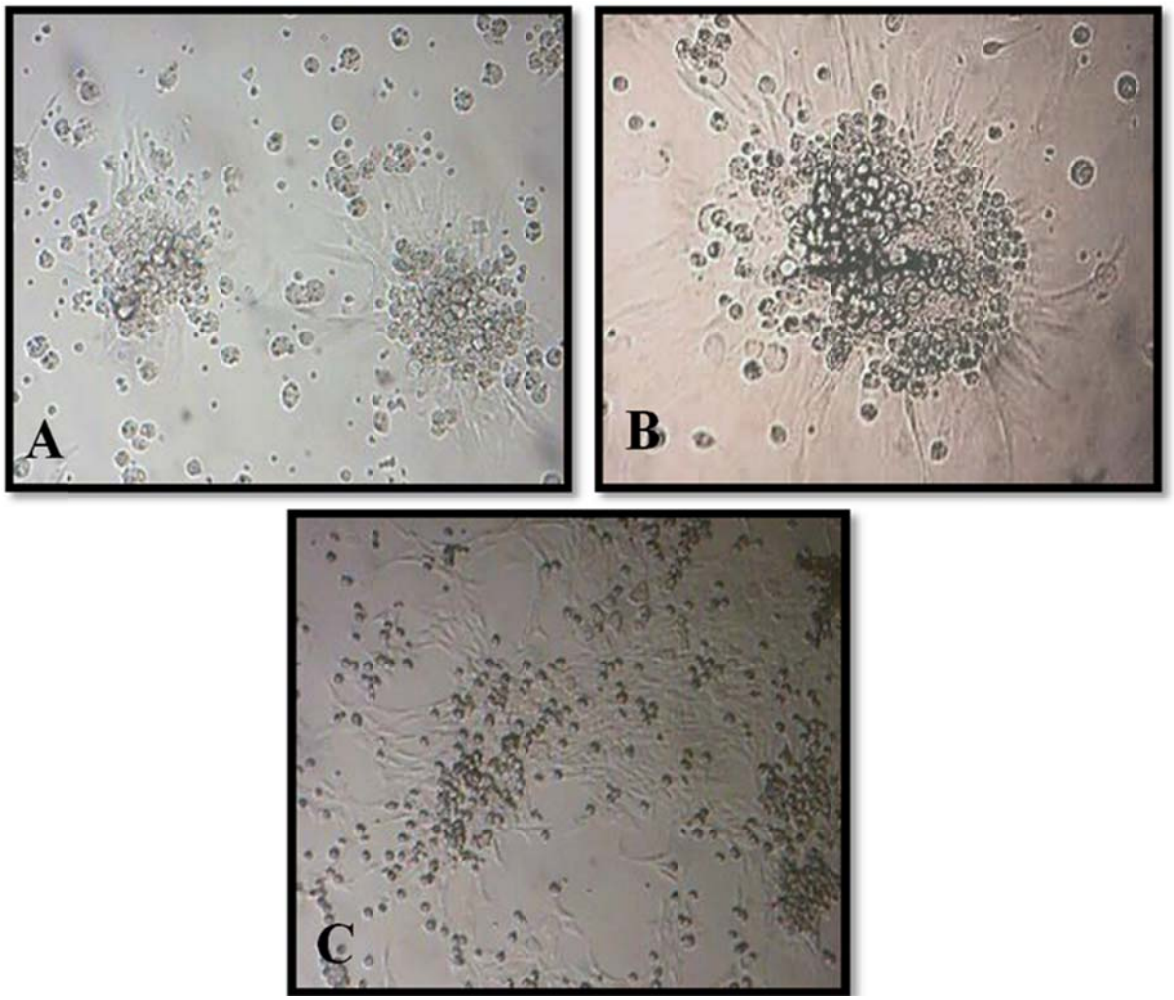

Figure 3. Illustration of the primary culture of BM-MSCs under inverted microscope. **A.** The formation of MSCs colony forming unit-derived fibroblast (CFU-F) (X100). B (X150) and C (X50): These figures show expanded MSCs colonies with numerous cells displaying fibroblast-like morphology.

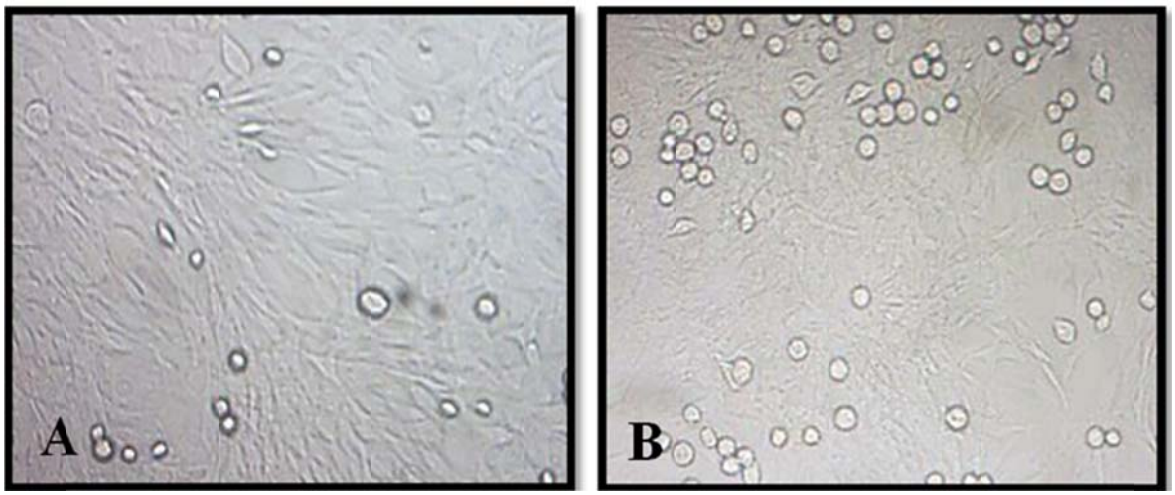

Figure 4. A, after 21 days from initial culture and B (2^{nd} passages): The formation of homogenous layer of fibroblast-like cells with small round cells (X100).

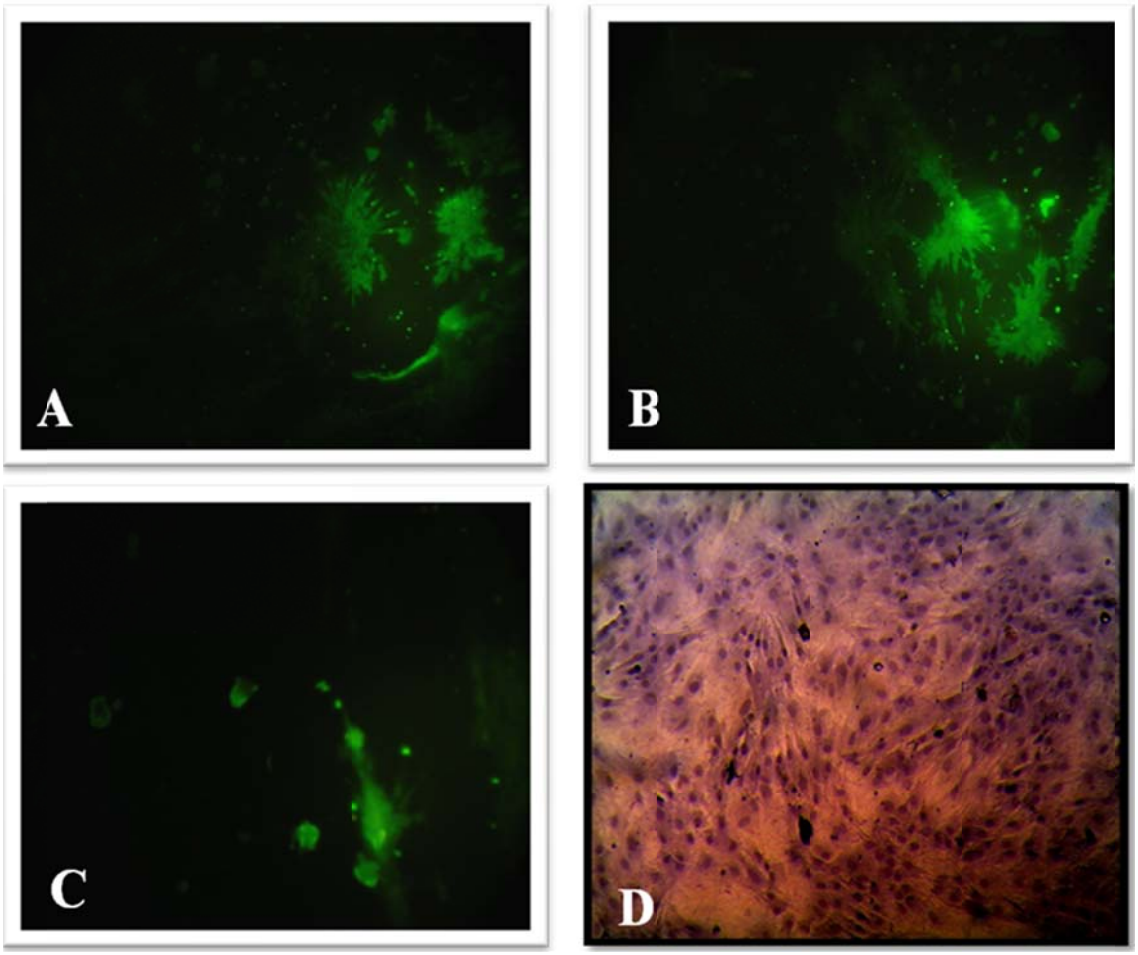

Figure 5. Immunophenotypic analysis of BM-MSCs *in vitro* at 2nd passage. **A, B** (X200), and C (X150): MSCs show positive response for CD105 (the specific marker for MSCs detection); cells revealed the colour of fluorescent conjugated secondary antibody (green colour). **D.** MSCs show negative response for CD 34 (the specific marker for Hematopoeitic stem cells detection), and stain with blue color (Hematoxylin stain) (X 100).

of the brain (Figure 6A). The congestion was noticeable in the middle cerebral artery MCA (Figure 6C). This effect of stroke induction in the experimental animals became clear in comparison with the brain of normal animals (group 5) (Figure 6B and D). Later, the experimental rats were inspected in their home cage environment, and no obvious movement abnormalities such as rotation or alteration of stereotyped behaviors were detected, that would distinguish them from controls. However, typical movements, associated with exploratory behavior, such as brief stopping, sniffing, rearing, or leaning against the cage wall were all reduced in the animals subjected to MCAO.

Histological and immunohistochemical studies

Immunohistchemical studies by light and fluorescent microscope were selected for demonstration of BM-MSCs engraftment, migration and long-term survival in the brain and other organs. Animals were allowed to survive for 31 days after intravenous administration of BM-MSCs; the examination of brain cross-sections, revealed the presence of BrdU-labeled cells as clusters. The migratory BrdU–labeled cells were located in different regions of the cerebral hemisphere, and specifically in the cerebral cortex (Figure 7A, B, C, and D). Despite the experimental groups, the microscopic examinations revealed the same ability of MSCs at different passages for migration and localization in the brain and heart tissues (Figure 8A and B). However, the investigations of liver and lung tissues revealed no BrdU-labeled cells, in addition we did not record any histo-pathological changes during examination of these tissues (Figure 8C and D).

DISCUSSION

In the present study, the culture of young male rats' BM-

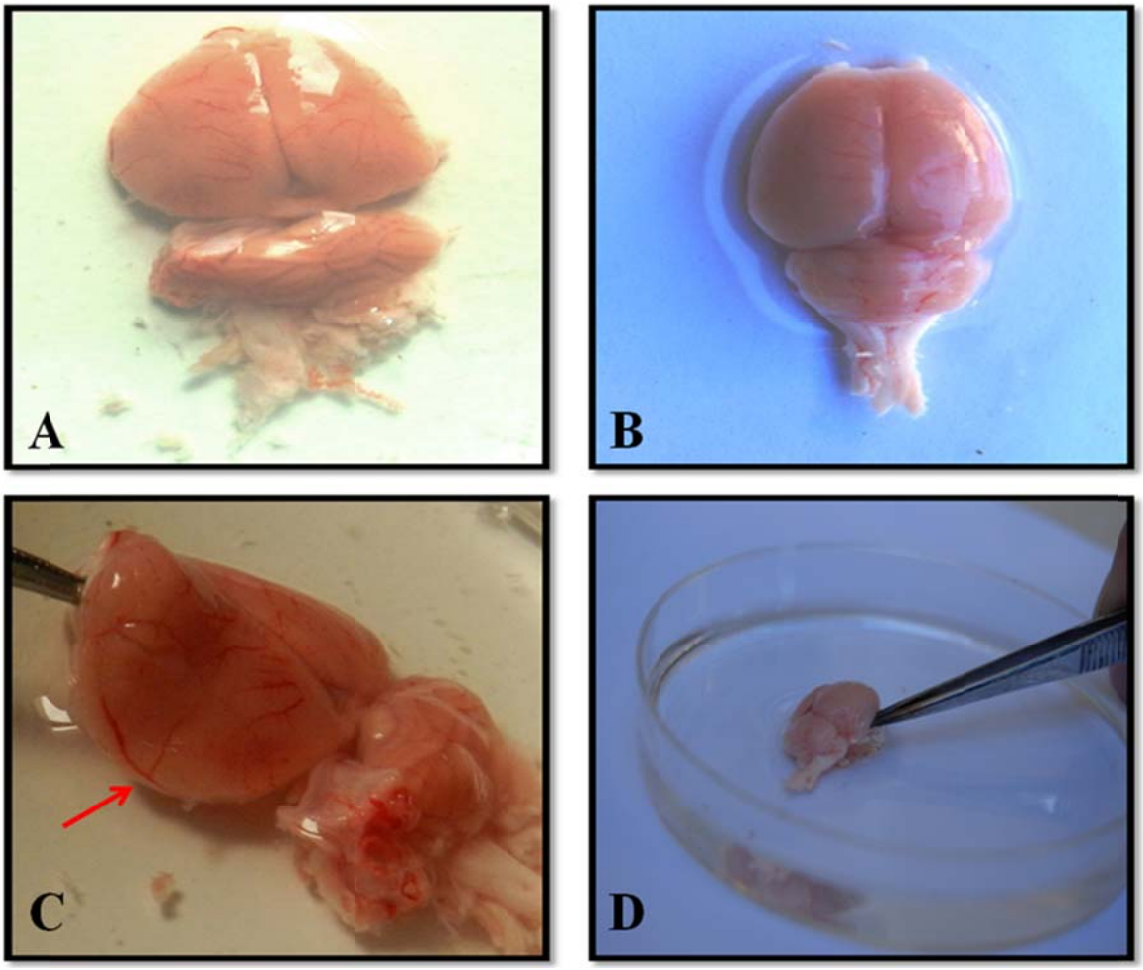

Figure 6. Photographs elucidate aspects of brain after 24 h of stroke induction (A, C left side) and show the comparison with the control brain (B, D right side), **A.** Congested blood vessels on the surface of cerebral hemispheres, **B.** The brain of control did not display any sign of congestion. **C.** The congestion of MCA (arrow) was quite obviously. **D.** In contrast the controls cerebral hemisphere did not display congestion.

MNCs and stem cells populations resulted in appearance of a population of cells with fibroblastic morphology. These cells could be considered as MSCs, as described by many researchers, such as Friedenstein et al. (1970); Pittenger et al. (1999); Javazon et al. (2001); Eslaminejad et al. (2008) and Li and Ikehara, (2013). According to Friedenstein et al. (1970) and Javazon et al. (2001), MSCs in the present study were isolated on the basis of their morphology and ability to adhere on the tissue culture flask. One of the characteristic *in vitro*-features of MSCs is their ability to generate single cell-derived colonies of adherent cells. This single precursors cells with colony–forming ability are termed CFU-F, and they are usually used as an indicator for mesenchymal progenitor potential (Bochev et al., 2008). This feature of MSCs to generate CFU-F was observed in the present study, so many CFU-F with different sizes were observed that represent varying growth rates from cells with fibroblast-like spindle shape, migrating from these colonies.

The results of the immuno-phenotypic assay of BM-MSCs indicated that the majority of adherent cells and their colonies (CFU-F) were strongly stained by CD105. As indicated by Dominici et al. (2006), this molecule is an important CD marker, known to be expressed by MSCs. However, these cells did not express CD34, which is the specific marker, used for HSCs detection (Vogel et al., 2003). In order to facilitate a more purified approach for studying MSCs biology, the international for cellular therapy proposed minimal criteria to define MSCs as follow: 1. MSCs must be plastic–adherent. 2. MSCs must express CD105, CD73, and CD90 and lack expression of CD11b, CD14, CD19, CD34, CD45, CD79 and/or HLA-DR surface molecules and 3. MSCs must differentiate to osteoblasts, adipocytes and chondroblasts *in vitro* (Dominici et al., 2006).

However, Alt et al. (2011) had reported that conventional stem cell properties such as plastic adherence and

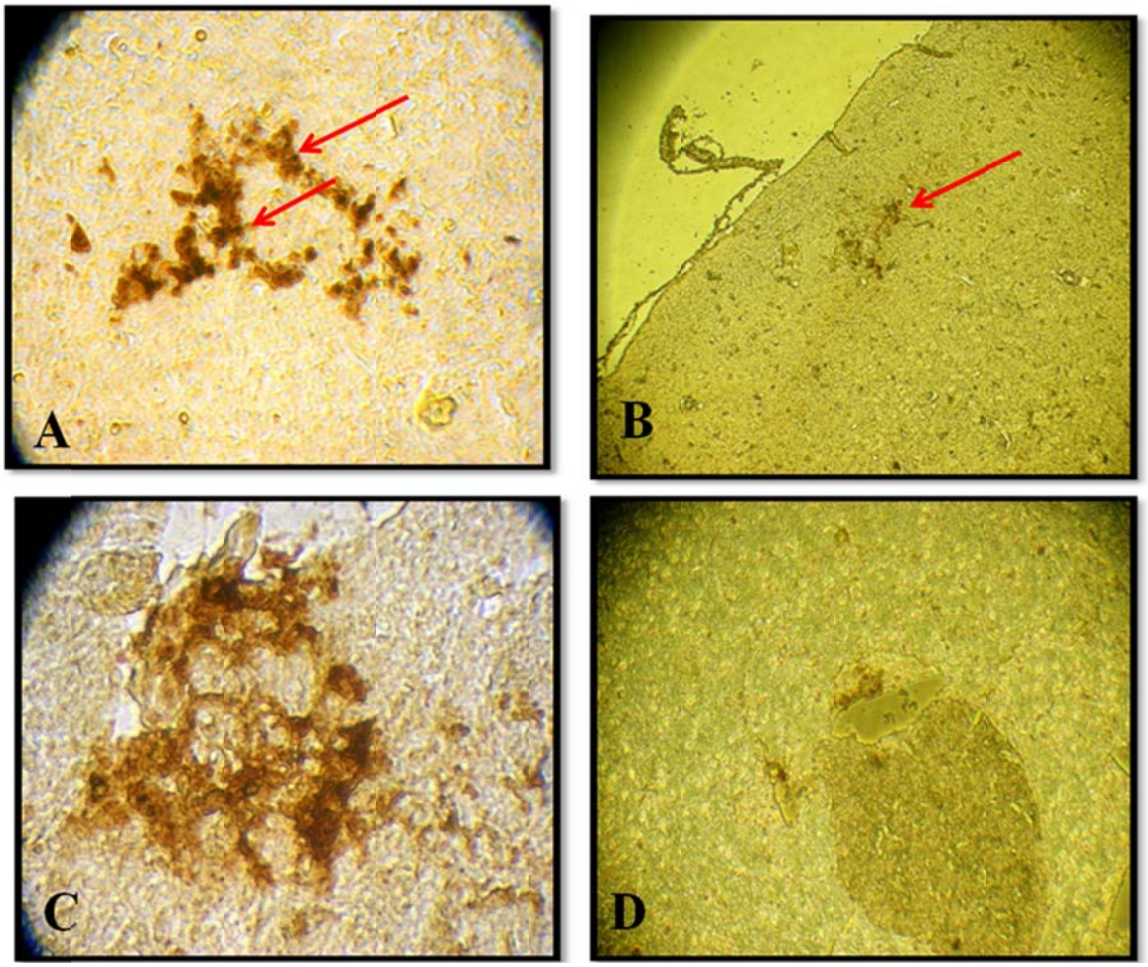

Figure 7. Photomicrographs of immunohistochemistry studies of the brain to determine the fate of BrdU-labeled BM-derived MSCs, after 30 day of cells infusion. A: BrdU –labeled MSCs appear as a clusters of cells in the brain, showing the intensity of staining after one step antigen retrieval (X200). B. Survival cells in the cortex of cerebral hemisphere injected by passage eight MSCs (X50). C. The intensity of staining reduced in cells migrated from cluster, may be this is attributed to clonal expansion (X400). D. Localization of BrdU –labeled cells in another region of the brain (X100).

the expression of CD105, CD44 and CD90 are unspecific for stem cells, unlike colony-forming capacity and differentiation capacity, which are specific properties, which differ MSCs from fibroblast.

MCAO in mature rats

Human specific disease is often random, sporadic and variable in its occurrence, and beyond a certain point, its biochemical and molecular complexities are simply inaccessible without turning to animal models, which offer reproducibility, replicability and control of confounding variable factors, essential to scientific hypothesis testing (Ginsberg, 2003). Since cerebral infarction is a heterogenous clinical entity with a variety of reasons such as etiology, localization, severity of ischemia and co-existing systemic diseases determining the outcome, these factors make clinical stroke characterization in human challenging. Many of these variables can be eliminated by employing an appropriate acute ischemic stroke (AIS), ischemic model enabling neuroscientists to focus on fundamental questions (Mehra et al., 2012). Transient occlusion of MCA by insertion of nylon suture in the right common carotid artery was preferred in this study, because this method is less invasive, cerebral artery occlusion produced is highly reproducible, and it is reversible allowing study of tissue reperfusion. This is in contrast to other methods like devascularization, which has limitations, although pia-stripping could induce cortical infarction, but mechanical damage to the underlying tissue and hemorrhagic does not permit reperfusion (Gonzalez and Kolb, 2003).

Histological and immunohistochemical assays

The immunohistochemical assays showed that after 31

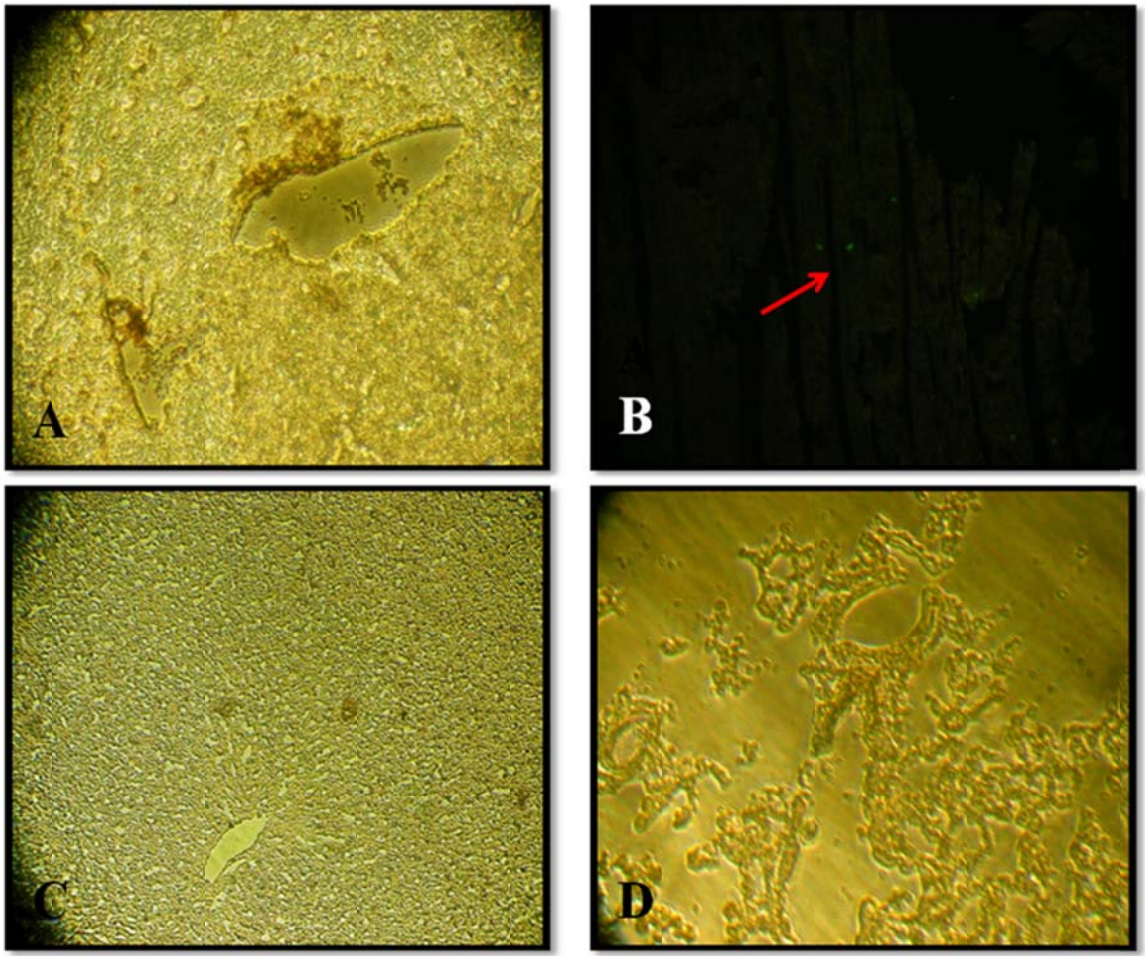

Figure 8. Microscopic examination of brain and other tissues in recipient rat of BrdU labeled cells. **A.** a number of cells reside around the blood vessel (X200). **B.** the micrograph demonstrate that some of the labeled cells reside in the heart (X100). **C.** this section of liver tissue was free from labeling cells (X100), D. Section in lung tissue of recipient rat, revealed no pathological changes that occur by the way as a result of systematic delivery of BM-derived MSCs (X200).

days from intravenous injection of BrdU, labeled MSCs at 24 h after stroke, these cells are more likely to enter into damaged brain and heart than into other organs such as liver and lung. This result was also reported by Chen et al. (2001a), but these authors demonstrated that intravenously injection of BrdU-labeled MSCs at 1 or 7 days after stroke significantly improved functional outcome, compared with non-treated rats. The most important interesting point in the present study is that the distribution of the labeled cells was not homogeneous. There were clusters of cells, which means that single progenitor cells underwent clonal expansion. This finding is in parallel with the study of Mezey et al. (2003), who observed this pattern of cells distribution during examination post-mortem brain samples from patients with lymphocytic leukemia, who had received bone marrow transplants. In this study, intravenous administration of BM-MSCs on the 2nd, 8th and 12th passages, offers an opportunity to highlight on some of MSCs characteristics, as follows: Despite the systemic delivery of BM-derived MSCs, these cells have been distinguished in the cerebral hemisphere, particularly in the cortex region. This can be attributed to the fact, that the MSCs possess the unique capacity to migrate or dock preferential to injured sites, due to expression of growth factors, chemokins and extracellular matrix receptors on the surface of MSCs (Meirelles et al., 2009; Rastegar et al., 2010).

In order to study the capability of rat BM-MSCs to maintain their survival, multiplication and migration on different passages, cells on the 2nd, 8th and 12th passages were chosen, to ensure that the delivered cells are nearly homogenous population, and hence no great difference between their migration and survival in the brain of rats would exist. These results indicated that MSCs retain their phenotype through several passages, and in this

way, increase the possibilities for clinical application of MSCs in treatment of different human diseases, which require substantial number of cells, a factor that makes the *ex vivo*-expansion of MSCs are very necessary (Hassan and El-Sheemy, 2004). Furthermore, MSCs transplantation in neurodegenerative diseases has been proven as feasible, safe and potentially effective. Although, there are doubts concerning the exact mechanisms, responsible for the beneficial outcome observed after MSCs transplantation into neurodegenerative tissue (Torrente and Polli, 2008; Li and Ikehara, 2013) have reported that MSCs are capable of secrete factors, including IL-6, IL-10, human growth factor (HGF), as well as platelet-derived growth factor (PGE2), that promote tissue repair, stimulate proliferation and differentiation of endogenous tissue progenitors, and decrease the inflammatory and immune reactions. From the current data, we can conclude that the intravenously-administered BM-MSCs enter brain and heart, and they survive, which might provide a cell source for treatment of stroke and heart diseases.

Conflict of Interests

The author(s) have not declared any conflict of interest.

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