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Comparison between three different protocols for isolation and culture of mouse bone marrow derived mesenchymal stem cells

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Abstract:

Bone marrow derived mesenchymal stem cells (BM-MSCs) represent a promising source for cell therapy, As they can differentiate into bone, cartilage, fat, tendon and many other organ progenitor cells. Although the culture of MSCs has been studied for over 30 years. The identification of standard protocol for isolation and characterization have yet to be developed. A comparison study was made on three different isolation techniques, which include: direct plating, red blood lysis buffer strategies, and density gradient (DG) method (using two different gradient media: Ficoll and Percoll) to find the optimal isolation and culture condition for adequate amount of MSCs for clinical use.The results demonstrate that direct plating of whole bone marrow (BM) suspension provides a suitable alternative protocol for isolation of BM-MSCs with minimal requirements, which mainly based on the frequent medium change in primary culture. The BM crud cell suspension were isolated by aspiration from albino mice and cultured in fresh complete isolation media (CIM) RPMI-1640/10%FBS. Adherent cells from BM cells suspension were MSCs which then expanded by complete expansion media (CEM) α-MEM/ 10% FBS. By concluding, direct plating of whole bone marrow represent the alternative method for isolating of mesenchymal stem cells from small specimen of bone marrow. By concluding, the present study was designed to investigate the optimal technique for the isolation of BM-MSCs for use in tissue engineering and regenerative medicine.

Key words: *stem cells, mesenchymal stem cells, isolation protocol, mouse bone marrow, bone marrow stem cells.*

Introduction:

Bone marrow derived mesenchymal stem cells (BM-MSCs) have the ability to renew and differentiate themselves into multiple lineages: bone, cartilage, fat, tendon, muscle (1). MSCs were fist described by Friedensteinet al. (2). Who found that MSCs can be isolated by physical adhere to culture plates, able to form colonies with cells have fibroblast shape in vitro. Stem cell therapy utilizing MSCs are the focus of a multitude of clinical studies currently underway. They are expected to change patient treatment profoundly, generating and regenerating tissues and organs instead of just repairing those (3). Although MSCs have been isolated from many sources, such as peripheral blood, fat, skin, vasculature, muscle, trabecular bone and umbilical cord(4). Bone marrow (BM)is still considered the most popular source of MSCs (5). The ability and efficiency of various techniques to purify MSCs from a heterogeneous cells population is an important factor in the successful characterization and application of stem cells.Since, untreated bone marrow contains a high proportion of erythrocytes and may interfere with theformation ofMSCscolonies (6). A variety of protocols describing the isolation of MSCs from BM has used density gradient centrifugation (The common media used include Ficoll, Percoll or dextran) (7) and hemolysis (red blood cell lysis) (8) to remove erythrocytes for the efficient isolation of the mononuclear cell fraction. Although both these techniques were firstly developed for the isolation of white blood cells such as lymphocytes, they can also be used for the isolation of BM-MSCs because they are contained within the mononuclear cell fraction (8).several studies have used either or both of these techniques for the isolation of BMSCs; they reported that BMSCs were more efficiently isolated by these techniques (9). As bone marrow derived mesenchymal stem cells(BM-MSCs) are a rare population in the bone marrow (0.01 - 0.1% of whole marrow), it is possible that initial colony formation directly affects the total yield of BM-MSCs. Inefficient

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colony formation lead to the reduced potentials of BM-MSCs because previous studies have shown that BM-MSCs lose their differentiation abilities depending on the duration of in vitroculture (6).Instead of using density gradient centrifugation and hemolysis, some studies performing clinical-scale isolation and expansion of MSCs follow a direct plating strategy to separate plastic adherent from non-adherent cells (10). Accordingly, the present study was designed to investigate the optimal technique for the isolation of BM-MSCs for use in tissue engineering and regenerative medicine.

Material and Methods:

The care of animals was in accordance with ethics committees in Iraqi Center for Cancer and Medical Genetic Research (ICCMGR), Al-Mustansiriyah University. Briefly, young (4-6 weeks old) male albino mice were used to derive BM-MSCs cultures using a described technique by (11)with modification:

-Prepare Crude Bone Marrow Cell suspension

Mouse MSCs were harvested from the BM of the femurs and tibiae, Utilize pools of 4-6 miceweresacrificed by cervical dislocation. Femurs and tibiae were dissected from the surrounding tissues. The epiphyseal growth plates were removed and the BM was collected by flushing with syringe through a 25 gauge needle containing 1 ml of prewarmed complete growth medium RPMI-1640 (US-Biological-USA) containing 100U/ml penicillin, 100µg/ml streptomycin and 10%FBS. This operation was continued until all bones were demarrowed for three mice for each culture. Single cell suspensions were prepared by gently mixing the cells with a pipette.

- Isolation of bone marrow –mesenchymal stem cells

Three different methods (with modification) were used for mesenchymal stem cells isolation from bone marrow:

1- Primary explant culture method (direct plating)

According to the original working of Friedenstein (2) utilizing adherent cultures of untreated whole bone marrow, after preparation of mouse bone marrow uniform cell suspension. The cells suspension was centrifuged at 1000 rpm for 5 minutes at 18ºC, Supernatant containing thrombocytes and erythrocytes was discarded and the cell pellet was resuspended with complete growth medium RPMI-1640 with 10%FBS.

2- Red Blood cells (RBCs) lysis buffer strategies

The crud suspension centrifuged at 2000 rpm for 5 minutes at 18ºC. The supernatant were discarded, cell pellet resuspended in 5ml of 1X RBC lysis buffer (Geneaid-Korea) and incubated for 3 minutes on ice. Then, the tube was placed in cooling centrifuged at 2000 rpm for 5 minutes at 18C°, and the pellet was resuspended with complete growth medium RPMI-1640 with 10%FBS.

3- Density gradient centrifugation

Another method for isolation MSCs from BM have used density gradient centrifugation to enrich the mononuclear cell fraction. Two different Common media were used include Ficoll and Percoll(8).the crud bone marrow suspension were carefully overlaid on Ficoll-paque (BAG-Germany) density: 1.077 g/ml or Percoll 60% gradient (density: 1.077 g/ml) (Sigma-Alderch-USA) the specimens were centrifuged on a cooling centrifuge for 20 minutes at 2000 rpm at 18°C. After density gradient centrifugation, the resulting mononucleated cells (MNCs) were retrieved from buffy coat layer by pipetting and washed two to three times with PBS at 2000 rpm for 10 minutes at 18°C. The final product was resuspended with isolation culture medium RPMI -1640.

-MSCs analysis

The crude BM cell suspension and MSCs were counted and analyzed at each passage for viability. Following the recommendations by Freshney (12). For counting, 0.1ml of cells suspension mixed with 0.1ml of trypan blue solution and 0.8ml of culture medium and a sample of cells counted using an improved Double Naubauer Ruling Counting Chamber.

- Culturing and Expansion of BM-MSCs

The cell suspension were seeded in plastic tissue culture flasks 25cm2 with 5ml culture medium RPMI-1640 supplemented with 10% FBS and antibiotics at a plating density of 16 × 106 cells/ml for direct and hemolysed methods, and 1 × 106 cell/ml density gradient protocol. For the first two methods culture: two mouse were used, where 5-6 mice were used for the third method.The culture was incubated at 37Cº in control medium for growth until complete confluent monolayer cell culture was reached.

For direct plating and RBC lysis methods, the MSCs were isolated based on their ability to adhere to the culture plates. After 24 hours, red blood cells and other non-adherent cells were removed and washed 2-3 with PBS then fresh medium was added to allow further growth. This feeding process then lasted twice in a week until we made the first passage of MSCs. On the third day of density gradient-centrifuged cells, change media was performed by discarded exhausted media with fresh one to speed up cell growth. The adherent MSCs grown to 80% confluency in 4-5 days were defined as passage zero (P0) cells. And was expanded by passaging. The P0 MSCs were washed with PBS and detached by incubating with 1ml of 0.25% trypsin-EDTA solution (US-Biological-USA) for 2 min at 37ºC. In order to dislodge the cells, the flask gently rock, then 5ml of culture media which converted to MEM plus 10% FBS was added to inactivate the trypsin, the single cells suspension were centrifuged at 2000 rpm for 10 min, the cells pellet were resuspended in 1ml complete medium plus MEM 10% FBS and counted manually using a Hemocytometer .

The cells suspension was plated as P1 in two plastic tissue culture flasks 25cm2 at densities of 1 X 106 cell/ ml, complete medium was changed to remove non adherent cells at 48 h after seeding and every 3 days thereafter, when the first passaged become nearly confluent the cells were recultured in similar conditions. This study used bone marrow mesenchymal stem cells at passage 3, in a good growth state.

Results:

1- Explant culture method (Direct plating)

The nucleated cell yield obtained from native BM isolations reached 40 ± 2 × 106 cells per donor mouse (two mice were used in this culture) with viability ranging from 98% to 100% (Fig.1A). Non adherent cells were carefully removed after 6 and 24 hours by washing the adherent cells with complete media RPMI-1640 for three times and 5ml of fresh complete isolation media (CIM) RPMI-1640/10%FBS were replaced. Then adherent cells (passage 0) were received fresh complete medium every 72 hours and the culture were observed via inverted microscopy for their morphology. The initial culture (time 0) of the bone marrow suspension, cells were found to be round and glistening contained a heterogeneous cell populations, including Erythrocyte, B-lymphocytes, granulocytes, monocytes, adipocyte, very small embryonic-like cells (VSEL) mesenchymal, hematopoietic, and endothelial stem cells etc. (Fig. 1B). Mesenchymal stem cells were isolated based on the ability to form adherent monolayer in culture and the lack of adherence of other cells in the bone marrow stroma such as HSCs, erythrocyte, adipocytes and macrophages. Hence, change media performed after 6 hours, were many of MSCs had attached to the base of the tissue culture flask (Fig. 1 C). These adherent cells were remained attached to the bottom of the flask, formed high numbers of scattered plastic adherent colonies (at 24 hours) (Fig. 1 D). Some of adherent cells assumed spindle morphology, a characteristic oval body with the flattened ends. When they slowly elongate, others appeared as individual small size spindle-shaped cells (Fig. 2 A, B). While the floating round cells remained suspended in the medium and were mostly eliminated from the culture with subsequent media changes. The adherent cells tend to be locally confluent, growing in distinct colonies at about 48 hours (Fig.3A). Selective removal of cells in the suspension allowed the growth of adherent uniform spindle morphology cells formed colony-forming units fibroblast (CFU-F) were a large number of cells grew in a radial manner in neat rows (Fig. 3 B), and the density increased significantly which fused to form the confluent monolayer (Fig.3 C, D).

Culturing and sub culturing

Upon reaching 80-90 % confluence before day 5 of primary isolation, the MSCs were subjected to passaging, Because CFU-F adherence was sensitive to trypsinization, 80-90% were detached after treatment of adherent cells with trypsin (without affecting viability), and re-seeded at the density of 106 cells/ml at the split ratio of 1:2 using complete expansion media (CEM) α-MEM/ 10% FBS. However, as the culture progressed with trypsinization, the round non-adhesive cells gradually decreased and eventually disappeared by week 1. Fibroblast-like cells remained homogeneous morphologically (Fig.4A). These cells showed prolonged proliferative capacity without any morphological changes for more than 3 passages (Fig. 4B). The total number of adherent MSCs in the end of first passage (70-80% confluency) were reached 9.8 × 106 cells/ ml. However, morphologically three distinct-cell subpopulation of MSCs can be distinguished in direct plating culture: major fibroblast-like cells, minor round cells, very small number of large, flat cells (Fig. 4 A, B, C).

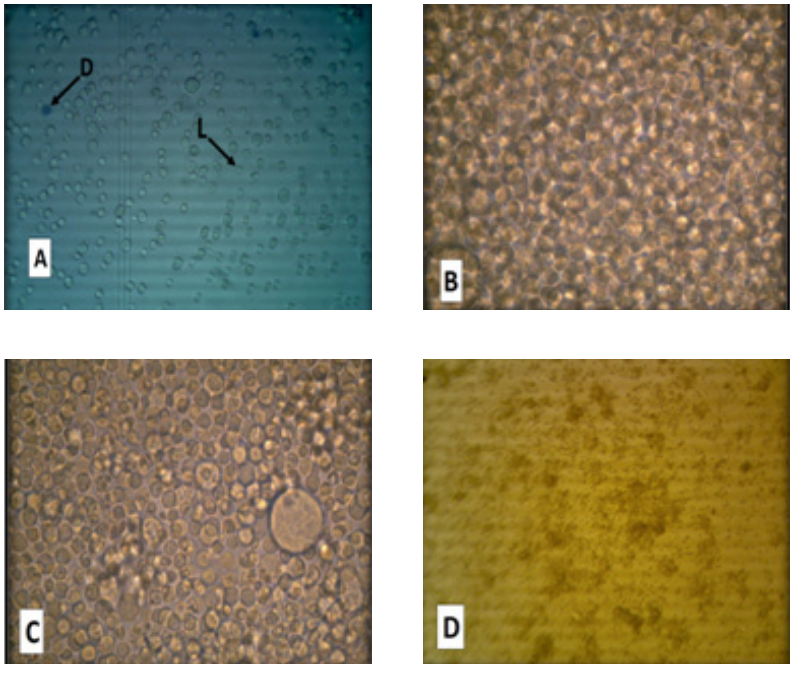

Figure (1): morphology of primary cells cultured in CIM (RPMI-1640). (A) Number and viability of isolated mouse BM-crud suspension cells for direct plating method examined under light microscope, the black arrow pointed with abbreviation: D = dead cells stained with trypan blue, and L= Live cells do not stained (X40). (B) Cultured whole BM by direct plating methods(X40) at time (0). (C) Cultured cells after 6 hours –many of MSCs were adhered firstly and others remain floated(X40). (D) BM-MSCs forms many colonies (CFU) after 24 hours(X 10). (B,C, and D were revealed under inverted microscop).

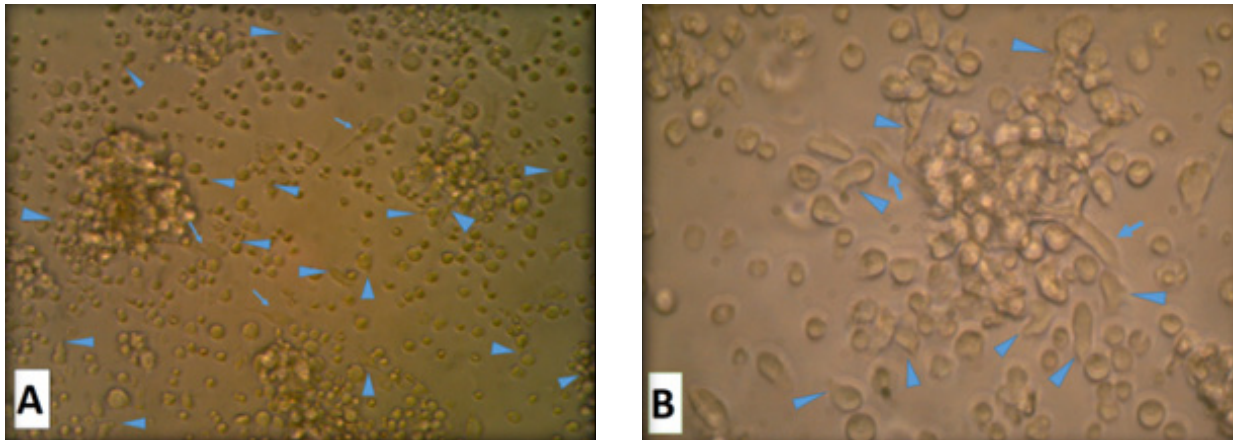

Figure (2): BM-MSCs primary cultured by direct plating at 24 hours assumed spindle morphology when they slowly elongate arrowhead, others appeared as individual small size spindle-shaped cells arrow. Examined under inverted microscope. A (X20), B (X 40).

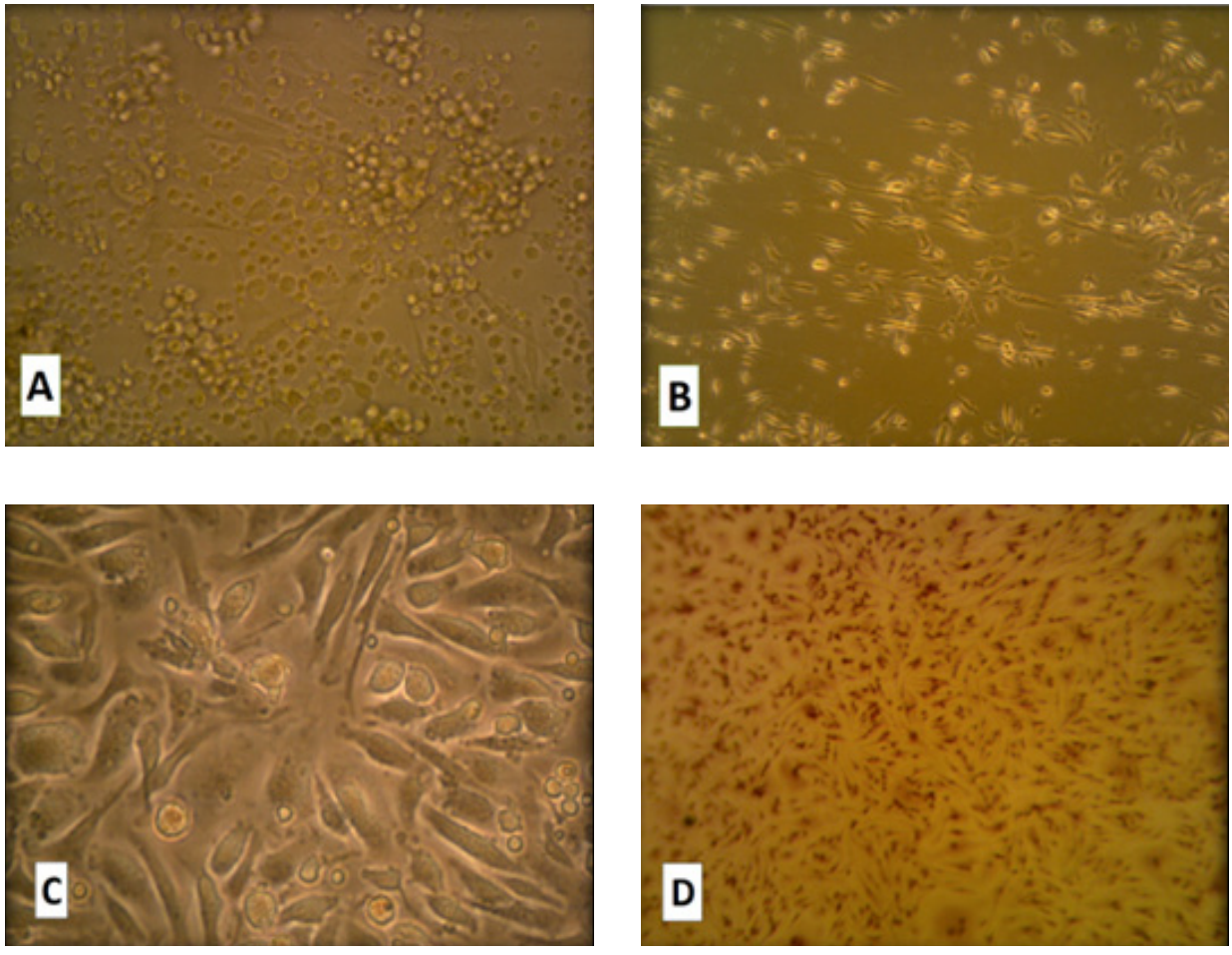

Figure (3): (A) adherent BM-MSCs cells tend to be locally confluent, growing in distinct colonies at about 48 hours revealed by inverted microscope (X40). (B) Large number of MSCs grew in a radial manner in neat rows(X20). (C): displayed the confluent monolayer of BM-MSCs, the density of spread cells increased significantly which fused to form the monolayer (X40), (D): H&E staining monolayer of BM-MSCs primary culture (X10).

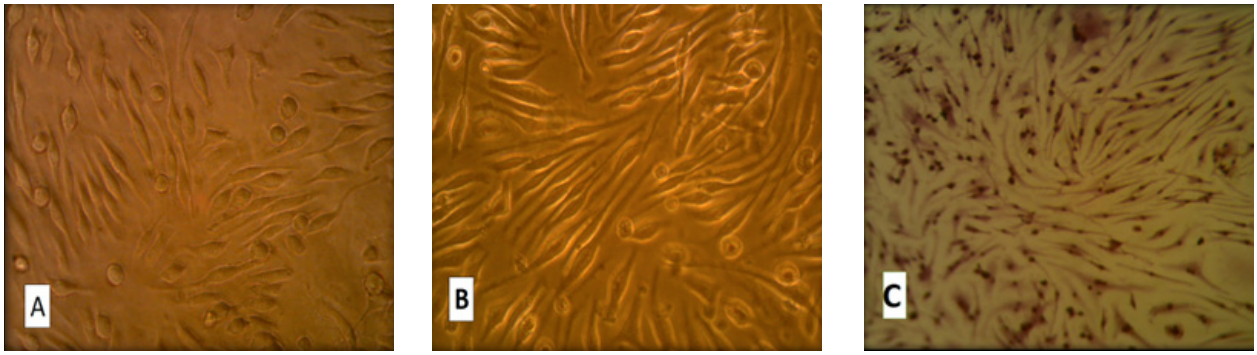

Figure (4): Expansion of BM-MSCs which cultured in CEM (MEM/ 10% FBS) revealed under inverted microscope (X40). (A): BM-MSCs confluent monolayer at 3 dayes of the first passage were progressed with trypsinization. (B): third passages of BM-MSCs after 3 dayes. (C): H&E staining of BM-MSCs culture at the fist passage examined under light microscope (X40).

2- Red Blood cell (RBC) lysis buffer strategies

After the crud suspension of mice bone marrow mononuclear cells (BM-MNCs) (40 ± 2 × 106 cells/ mouse) treated with 1X ammonium chloride-based red blood cells (RBC) lysis buffer for 3 minutes, Live/dead viability of MSCs was determined by the trypan blue staining test. The number of MNCs was counted and compared to that of before treating; the viability rates for lysed BM-MNCs decreased to 83.2% ± 1.3 from that of initially prepared (Fig. 5). The number of cells that adhered to the plastic culture flasks after 24 hours were less than that observed in direct plating when seeded at the same density 16 × 106 cells/ ml. furthermore, cells appeared shrinkage with addition features of apoptotic: chromatin condensation and nuclear fragmentation (Fig.6 A, B). After 6-7 days the cell culture confluency reached 70% and trypsinized to first passage, the adherent cells were appeared heterogonous morphology of three subpopulation: major were large, flat cells and had medium content of granules, minor population of small fibroblast-like cells and round cells (Fig. 7). The number of MSCs harvested at first passage, as determined by hemacytometer count was 7.2×106 cells /ml.

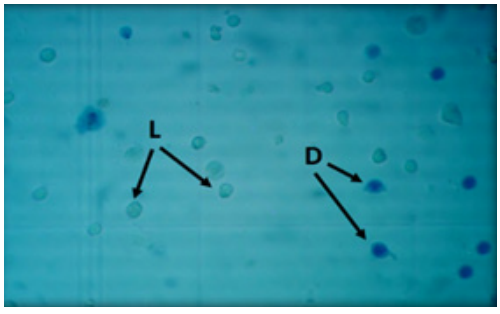

Figure (5): the viability test of whole BM-cell suspension after treated with ammonium chloride-based red blood cells (RBC) lysis buffer method. Explain the number and viability of isolated cells. The black arrow pointed with abbreviation: D = dead cells stained with trypan blue and L= Live cells do not stained. Revealed under light microscope (X40).

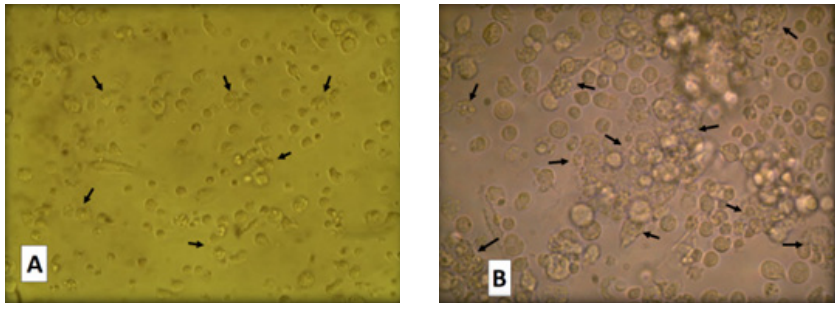

Figure (6): the morphology of BM-MSCs isolated by RBC lysis strategies and cultured in CIM (RPMI-1640)/ 10% FBS. Revealed under inverted microscope. (A, B): initial culture after 24 hours, cells appeared shrinkage with addition features of apoptotic: chromatin condensation and nuclear fragmentation (arrow). (X10), (X40) respectively.

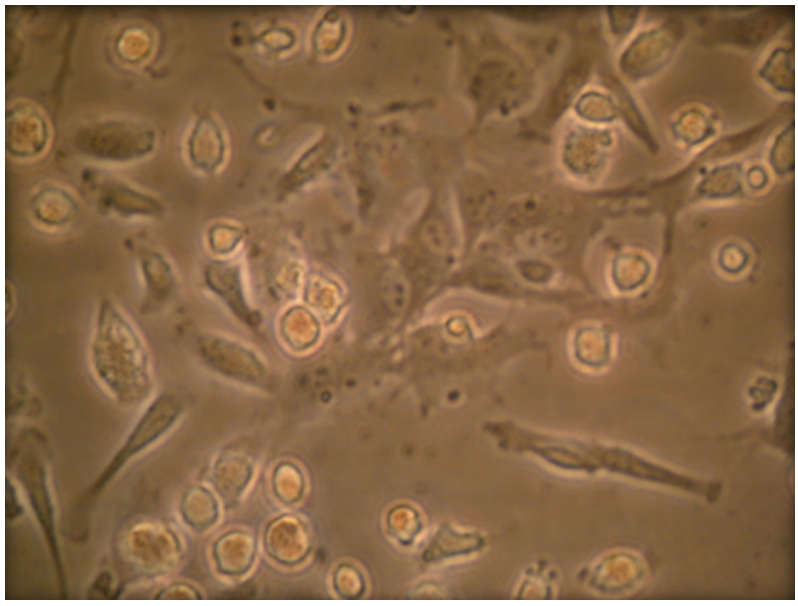

Figure (7): in vitro characterization of BM-MSCs isolated by lysis buffer method, reveled by inverted microscope. Adherent MSCs show heterogonous morphology of three subpopulation: major were large, minor population of small fibroblast-like cells and round cells (X40).

3- Density gradient centrifugation protocol (DGC) (Ficoll and Percoll media)

In this study many Attemptswas failed to isolate mouse BM-MSCs from 2-4 mice. Therefore, 9-10 mice were subjected to tibias and femurs bone marrow flushing. However, the crude cell suspension was hand-layered on top of the DG media: Hypaque (Ficoll or Percoll= 1.077g/ml) (Fig. 8 A). Under centrifugation, the cell suspension component will start to sediment through the density gradient into separate zones. Each zone consists of particles with the same sedimentation rate (Fig. 8 B).

The bone marrow mononuclear cells (BM-MNCs) were collected by removing the buffy coat. The viability of the cells was checked immediately for both gradient media: The viability of nucleated cells isolated by Ficoll was 53.8 ± 2.7% and 40.2± 3% for Percoll (Fig. 9 A, B).

These results indicates that Ficoll gradient media allowed recovery of more nucleated cells from the BM than Percoll gradient media. However, after 24 hours of initial culture (16 × 106 cells/ ml), the cells with adherent capacity were attached to the base of the tissue culture flask. With significant differences, more cells were attached in the Ficoll groups than in the Percoll groups. After 9 days, adherent cells were forms little small colonies (confluency 50%) from major giant cells appearance, small number from fibroblast- like spindle-shaped cells and oval cells(Fig. 3-10).

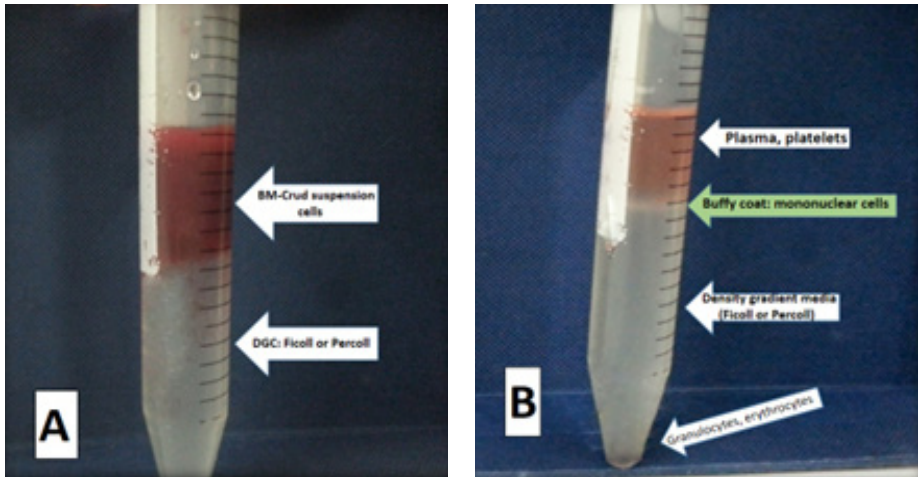

Figure (8): Mouse BM-MNCs isolation by DGC using Ficoll-Hypaque with density= 1.077g/ml. (A): the whole BM suspension cells was layered carefully on top of the Ficoll without disturbing the solutions. (B): After gradient centrifugation, cell suspension component will start to sediment into separate zones. Our goal of this process was the buffy coat (green arrow), which represent the BM-MNCs. Those MSCs are belong. (Percoll was used at the same way).

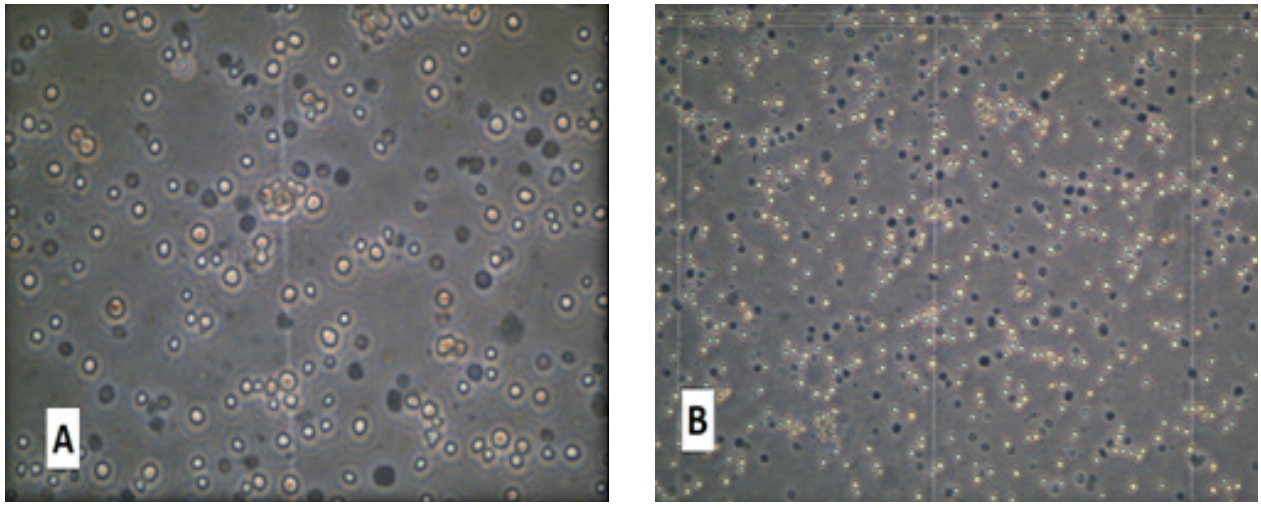

Figure (9): The viability test of BM-MNCs isolated by DGC method using (A): Ficoll (X40) and (B): Percoll (X10) Revealed under light microscope. Explain that the number of dead MNCs isolated by this methods were higher than that of direct plating and lysis buffer methods. Moreover, in Percoll than that of Ficoll media.

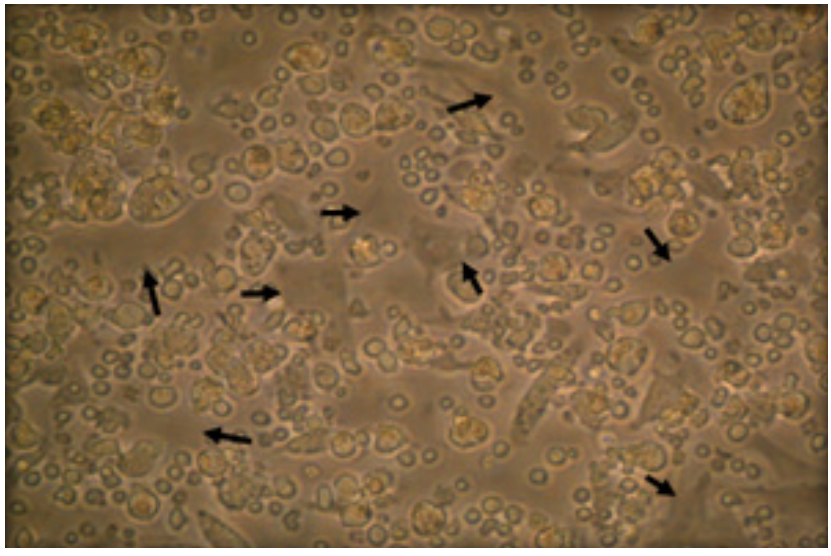

Figure (10): The morphology of BM-MSCs primary culture, which cultured in RPMI-1640+10% FCS revealed under inverted microscope (X40). The cellular composition demonstrate in vitro MSCs aging represented by the majority of flattened cells in tissue culture plate and eventually pass into apoptosis.

Discussion:

The results demonstrate that direct plating of whole bone marrow suspension provides a suitable alternative for isolation of MSCs with relatively little hematopoietic contamination.Andmore suitable for very small amounts of tissue. BM, as small-limited tissues especially with small donor like mice, rats, rabbits ….etc. (13). While DGC is, the most widely used method for isolating MSCs from human BM and mononuclear cells from peripheral blood (14). Hence, this study was failed to gain MSCs from 2-3 mice by DGC and yielded Unsatisfactory MSCs number and viability from 9-10 mice indicated that DGC method required up to 10 mice, therefore, Pittenger (15) demonstrated that the bones of up to 10 rats or 20 mice could be processed as a single preparation, Which take a long time for BM suspension cell preparation.However, it is a well-known problem that DGC causes a significant reduction of BM-MNCs to only 15–30% of the initial content. Could be up to 50%. A profound cell loss during DGC may hence directly attenuate therapeutic efficacy. Different methods have different defects and virtues. With direct plating many investigator were proven primary tissue culture plate heavily contaminated with heamatopoietic progenitors such as macrophage and fibroblasts. (16). Which the low number of BM-MSCs (1 per 106 BM- MNCs) and the large number of hematopoietic cells may explain. To solve

this problem in present study:
Firstly: Based on Initial experiments carried out indicated that RPMI-1640 medium inhibited the growth of hematopoietic cells in cultures. Therefore, it was used for the initial medium (CIM) to isolate BM-MSCs (17). Secondly: Frequently changed media, when performed in 6 and 24 hours of initial culture also get rid of floating heamatopoietic progenitors and reduce the chance for contamination. Since, MSCs have higher rates of adherence to plastic surfaces, and then other cells such are hematopoietic do not have such characteristic and removed with media changing. Thirdly: Passaging of cultured BM-MSCs also helped to eliminate heamatopoietic contaminants and yield purer MSC cultures (18). Better quality MSC cultures were also obtained by discarding cells that remained attached to flasks following trypsinization. These firmly adherent cells are probably contaminating fibroblasts and have been reported by other laboratories (18,19). Since MSCs were showed to be, more sensitive to trypsin and easily uprooting from tissue culture plate. Which represent another distinction for MSCs. The marrow stroma is a heterogeneous mixture of cells including adipocytes, endothelial cells, reticular cells, fibroblastic cells and smooth muscle cells, which provide growth factors, cytokines and chemokines as well as a physical matrix (20). To achieve the balancing between the two BM-MSCs culture systems: Friedenstein (21) in (1976), a monolayer of homogeneous, undifferentiated MSCs grown in the absenceof hematopoietic cells. And Dexter et al. (22) developed another BM culture system, for the study of hematopoiesis, in which a monolayer of stromal cells is grown in the presence of hematopoietic cells. Chang media performed in 6 hours of initial culture not as Nadri (23) were non-adherent cells were carefully removed after 3-4 hours and repeated every 8 hours for up to 72 hours of initial culture. Although, the majority of cells were attached after 30 minutes only. This process will allow the factors that support long –term maintenance of MSCs; as such, the likely source of these factors is the hematopoietic cell fraction (24).The functionality of BM-MNCs are significantly influenced by red blood cell contamination, the content of apoptotic cells. Different washing steps. The centrifugation speed. Even the choice of the density gradient medium. Both density gradient centrifugation and hemolysis RBCs (red blood cell lysis) treatment remove erythrocytes form BM- crud suspension. Since, contamination with RBCs influences the functionality of BMCs used for cell therapy, impair the mitochondrial function of BM-MNCs. Moreover, it is well established that mitochondrial function and metabolism are important determinants of stem cell self-renewal and differentiation. Reduced the viability, invasion, and CFU capacity of the applied BMCs (25). Hemolysis of RBCs results in high levels of free heme were leads to cellular injury. Moreover, free hemoglobin was shown not only to directly induce apoptosis in cultured endothelial cells but also to scavenge nitric oxide (NO) that plays a crucial role for stem cell maintenance, differentiation, and neovascularization capacity (56) and increases mitochondrial function in endothelial cells (27). Thus, reduced NO levels in the BM-MNCs suspension might have contributed to impaired benefit of BMCs associated with mitochondrial dysfunction. After centrifugation over Ficoll or Percoll, bone marrow was separated into several fractions such as plasma, mononuclear cells, granulocytes, and erythrocytes. Since BM-MSCs belong to the mononuclear cells fraction in the bone marrow. However, most of the current clinical studies still use the conventional adherence technique for the isolation of BM-MSCs because the fact that the characteristics of BM-MSCs varies with the isolation techniques and does not require any special reagents. Recently, it was described that Ficoll DGC even depleted cells with a high regenerative potential, such as MSC (28) and very small embryonic-like cells VSEL (59), and irreversibly impaired cell function by decreasing expression of chemokines receptors. Excessive cell loss during DGC is a consequence of density medium-related cytotoxicity (29).The DGC method is a time-consuming process by nature of the excessive handling involving at least three washing steps that make the system open (exposure to the environment) and more likely to result in bacterial contamination (30). With many risks: toxicity, osmotic pressure changes and penetration into particles. Which explain that after centrifugation, many suspended cells were found in the medium at 72 hours. This could be due to the density of cells, which was changed slightly in RPMI-1640. DGC using Ficoll and Percoll are associated with potential cell injury, as demonstrated by a significant reduction in tritiated thymidine incorporation and loss of lymphocyte viability following culture with Ficoll-Hypaque. As apoptotic cells could be exerting influence on numerous biological processes through release of microparticles to induce apoptosis in neighboring cells (31).Moreover, Mareschi and colleagues (32) were reported that the telomere length on MSCs isolated from whole BM was longer than MSCs isolated from Percoll and Ficoll methods. Thus, by cultivating the cells from whole BM help to isolated more immature MSCs. In vitro aged MSCs are reportedly bigger they exhibit more podia and spread further, MSCs from young donors exhibit the spindle-type morphology in very early cultivation and a gradual loss of these features over cultivation time.This study avoid the addition of glucose and growth factors into CEM (α-MEM/ 10% FBS), Since BM-MSCs were failed to spread, migrate or proliferate when cultured in high glucose media (33). This study confirmed that directly plated BM offers a more advantageous method in terms of initial cell number, minimal manipulation, hematopoietic contamination, and cellular growth. Capelliet al. (34) and Lucchiniet al. (35) few years ago described, that whole BM direct plating methods represent a good procedure for MSC expansion for clinical use compared to MSCs obtained by DGC.

References:

1. Reddy, CH. U. (2011). Wisdom Tooth A Source of stem cells. Asian J. Oral Health &Allied Sciences. 1:83-84.
2. Friedenstein, A. J.; Chailakhjan, R. K. &Lalykina, K. S. (1970). The development of fibroblast colonies in monolayer cultures of guinea pig bone marrow and spleen cells. Cell Tissue Kinet. 3:393-403.
3. Peran, M.; Marchal, J. A.; Serrano, f. r.; Alvarez, P. &Aranega, A. (2011). Tras differentiation: Why and how?. Cell Biol. Int. 35:373-379.
4. Lu, L. L.; Liu, Y. J.; Yang, S. G.; Zhao, Q. J.; Wang, X.; Gong, W.(2006). Isolation and characterization of humanumbilical cord mesenchymal stem cells with hematopoiesis-supportive function and other potentials.Haematologica. 91: 1017-26.
5. Vaananen, H. K. (2005). Mesenchymal stem cells. Ann. Med.37:469-79.
6. Agata, H.; Yamazaki, M.; Uehara, M.; Hori, A.; Sumita, Y.; Tojo, A. &Kagami, H. (2012). Characteristic differences among osteogenic cell populations of rat bone marrow stromal cells isolated from untreated, hemolyzed or Ficoll-treated marrow. Cytotherapy.14:791-801.
7. Fluks, A. J. (1981). Three-step isolation of human blood monocytes using discontinuous density gradients of Percoll. J Immunol Methods.41:225–233.
8. Polisetti, N.; Chaitanya, V. G.; Babu, P. P. &Vemuganti, G. K. (2010). Isolation, characterization and differentiation potential of rat bone marrow stromal cells. Neurol. India. 58:201-208.
9. Peterbauer-scherb, A.; Van Griensven, M.; Meinl, A.; Gabriel, C.; Redl, H.&Wolbank,S. (2010). Isolation of pig bone marrow mesenchymal stem cells suitable for one-step proceduresin chondrogenic regeneration. J. Tissue Eng.Regen. Med.4:485-90.
10. Leboy, P. S.; Beresford, J. N.; Devlin, C, & Owen, M. E. (1991). Dexamethasone induction of osteoblast mRNAs in rat marrow stromal cell cultures. J. Cell Physiol.146:370-8.
11. 11- Meirelles, L. D. & Nardi, N. B. (2003). Murine marrow-derived mesenchymal stem cell: isolation, in vitro expansion, and characterization. Brit. J. Haematol. 123:702–711.
12. Freshney, R. (1987). Culture of Animal Cells: A Manual of Basic Technique. In: Wiley-Liss, New York.
13. Lennon, D. P.;Edmison, J. M. & Caplan, A. I. (2001). Cultivation of rat marrow-derived mesenchymal stem cells in reduced oxygen tension: effects on in vitro and in vivoosteochondrogenesis. J. Cell. Physiol.187:345-55.
14. Huang, Y.; Cal, J.; Juanlv, F.; Xie, H.; Yang, Z.; Huang, Y. & Deng, L. (2013). Species variation in the spontaneous calcification of bone marrow-derived mesenchymal stem cells. Cytotherapy. 15: 323-329.
15. Pittenger, M. F. (2008). Mesenchymal stem cells from adult bone marrow. Methods Mol Biol. 449:27–44.
16. Phinney, D. G.; Kopen, G.; Righter, W.; Webster, S.; Tremain, N. & Prockop, D. J. (1999). Donor variation in the growth properties and osteogenic potential of human marrow stromal cells. J. Cell. Biochem. 75:424-436.
17. Lanotte, M.; Metcalf, D. & Dexter, T. M. (1982). Production of monocyte/macrophage colony-stimulating factor by preadipocyte cell lines derived from murine marrow stroma. J. Cell Physiol.112:123-127.
18. Polisetti, N.; Chaitanya, V. G.; Babu, P. P. &Vemuganti, G. K. (2010). Isolation, characterization and differentiation potential of rat bone marrow stromal cells. Neurol India. 8:201-208.
19. Soleimani, M. & Nadri, S. (2009). A protocol for isolation and culture of mesenchymal stem cells from mouse bone marrow. Nature protocols. 4:102-110.
20. Almeida-Porada, G.; Porada, C. D.; Tran, N. &Zanjani, E. D. (2000). Cotransplantation of human stromal cell progenitors into preimmune fetal sheep results in early appearance of human donor cells in circulation and boosts cell levels in bone marrow at later time points after transplantation. Blood. 95: 3620–3627.
21. Friedenstein, A. J.; Gorskaja, J. F. &Kulagina, N. N. (1976). Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Exp Hematol. 4:267-274.
22. Dexter, T. M.; Allen, T. D.; &Lajtha, L. G. (1977). Conditions controlling the proliferation of haemopoietic stem cells in vitro. J. Cell Physiol. 91:335–344.
23. Nadri, S.; Soleimani, M.; Hosseni, R. H.; Massumi, M.; Stashi, A. &Izadpanah, R. (2007). An efficient method for isolation of murine bone marrow mesenchymal stem cells. Int. J. Dev. Biol. 51:723-729.
24. Baddoo, M.; Hill, K.; Wilkinson, R.; Gaupp, D.; Hughes, C.; Kopen, G. C.; & Phinney, D. G. (2003). characterization of mesenchymal stem cells isolated from murine bone marrow by negative selection. J. Cellular Biochem. 89:1235–1249.
25. Mantel, C. &Broxmeyer, H. E. (2008). Sirtuin 1, stem cells, aging, and stem cell aging. CurrOpin Hematol.15:326 –331.
26. Meguro, T.; Chen, B.; Parent, A. D. & Zhang, J. H. (2001). Caspase inhibitors attenuate oxyhemoglobin-induced apoptosis in endothelial cells. Stroke. 32:561– 566.
27. Quintero, M.; Colombo, S. L.; Godfrey, A. &Moncada, S. (2006). Mitochondria as signaling organelles in the vascular endothelium. Proc. Natl. Acad. Sci. 103:5379–5384.
28. Ahmadbeigi, N.; Soleimani, M.; Babaeijandaghi, F.; Mortazavi, Y. &Gheisari, Y. (2012). The aggregate nature of human mesenchymal stromal cells in native bone marrow. Cytotherapy. 14: 917–924.
29. Luttmann, W.; Bratke, K.; Kupper, M. &Myrtek, D. (2006). Immunology (The Experimenter Series). Academic Press. 264 p.
30. Griesel, C.; Heuft, H. G.; Herrmann, D.; Franke, A.; Ladas, D. &Stiehler, N. (2007). Good manufacturing practice-compliant validation and preparation of BM cells for

the therapy of acute myocardial infarction. Cytotherapy. 9: 35-43.
31. Thum, T.; Bauersachs, J.; Poole-Wilson, P. A.; Volk, H. D. & Anker, S. D. (2005). The dying stem cell hypothesis: murine modulation as a novel mechanism for progenitor cell therapy in cardiac muscle. J. Am. Coll. Cardiol. 46:1799–1802.
32. Mareschi, K.; Rustichelli, D.; Calabrese, R.; Gunetti, M.; Sanavio, F.; Castiglia, S.; Risso, A.; Ferrero, I.; Tarella, C. & Franca Fagioli, F. (2012). Multipotent mesenchymal stromal stem cell expansion by plating whole bone marrow at a low cellular density: A more advantageous method for clinical use. Stem Cells Int. 2012: ID 920581, 10 pages. doi.org/10.1155/2012/920581
33. Yameen, Z.; Leavesley, D.; Upton, Z. & Yin Xiao, Y. (2009). Multilineage differentiation potential of bone and cartilage cells derived from explant culture. J. The Open Stem Cell. 1:10-19.
34. Capelli, C.; Salvade, A. &Pedrini, O. (2009). The washouts of discarded bone marrow collection bags and filters are a very abundant source of hMSCs. Cytotherapy.11:403–413.
35. Lucchini, G.; M. Introna, M. & Dander, M. E. (2010). Platelet-lysateexpanded mesenchymal stromal cells as a salvage therapy for severe resistant graft-versus-host disease in a pediatric population. Biol. Blood & Marrow Transplan. 6:1293–1301.

دراسة مقارنة لثلاثة طرق لعزل وزراعة الخلايا الجذعية اللحمية المشتقة من نخاع عظم الفأر

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الخلاصه:

تعد الخلايا الجذعية اللحمية المشتقة من نخاع العظم مصدرا واعدا في العلاج الخلوي. لقابليتها على التمايز الى خلاياانسجة العظم، الغضروف،الخلايا الدهنية والوتر والكثير من الخلايا المولدة للأعضاء. رغم ان زراعة الخلايا الجذعية اللحمية درست خلال الثلاثين السنة الماضية الا انها لم توفر نمط قياسي لعزل وتمايز هذه الخلايا لحد الان لذلك تم في هذه الدراسة مقارنه ثلاث طرق مختلفة لعزل الخلايا الجذعية المشتقة من نخاع العظم وهي: العزل بواسطة الطريقة المباشرة، استعمال الدارئ المحلل لكريات الدم الحمر، وباستعمال الوسط المتدرج الكثافة وذلك لإيجاد الطريقة والظروف المثلى للعزل والزراعة لحصد كميات ملائمة من الخلايا الجذعية اللحمية. أوضحت النتائج ان الطريقة المباشرة لزراعه معلق نخاع العظم الخام تمثل أفضل طريقه لعزل الخلايا اللحمية، والتي تقوم أساسا على التغيير المتكرر للوسط الزرعي في المزرعة الأولية. تم جمع عالق نخاع العظم الخام من الفئران البيض وانمائها في الوسط الزرعي العازل(RPMI-1640/10%FBS). كانت الخلايا الملتصقة من معلق نخاع العظم في اوعية الزرع هي الخلايا الجذعية اللحمية والتي تم ادامتها بالوسط الزرعي (MEM/ 10% FBS). وعلية صممت الدراسة الحالية لايجاد الطريقة المثلى لعزل الخلايا الجذعية اللحمية من نخاع العظم المستخدمة في هندسة الانسجة والطب التجديدي.