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Comparison of Cell Dynamics and Characterization of Bone Marrow-Derived Stem Cells in Different Passages in Young and Aged Rats

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Abstract

Background: **Bone marrow-derived mesenchymal cells (BM-MSCs) isolation and culturing have a great interest in many diseases as cell therapy. The clinical use of cultured stem cells is facing debates and criticism due to the increasing number of unproven cell-based therapies and the appearance of many limitations, including the age of the donor and the number of passages.** ***Aim:*** **Due to the discrepancy in the published data, we investigated changes associated with the first four passages of cultured BM-MSCs from old and young rats, regarding morphological and ultrastructure changes, growth kinetics, and surface cell markers.** ***Methods:*** **Cells were isolated and cultured from young and old Wistar rats' long bones. The morphological and ultrastructural characters, cell surface markers, and growth kinetics of the stem cells were analyzed.** ***Results:*** **BM-MSCs in P1 and 2 showed non-significant differences between young and old cells. Morphologically, cells from P3 and P4 became larger and filled with filaments in both groups. The capacity of the cells to proliferate decreased compared to the prior passages, though, it is still better in the young population. Immunophenotypically, young source of cells showed a significant expression of MSCs surface markers across different passages reaching at least 50% of the cells by P4. While steady expression was experienced in old cells in different passages. Conclusion: the donor age directly influences BM-MSCs morphology, ultrastructure, and proliferation. We assert that cells obtained from P2 and P3 of young rats have the best proliferative index which can be used for future studies.**

Keywords: Cell dynamics; Electron microscope; Flow Cytometry; Mesenchymal stem cells

Introduction

The multiple aspects of the performance of mesenchymal stem cells (MSCs) have acquired worldwide attention over the past ~30 years. Because they are a fabulous and novel pattern for studying the biological mechanisms behind cellular proliferation and generation of various cell kinds.

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They have many therapeutic roles based on the products derived from their exosomes. Also, the cells have a role in the area of tissue engineering and gene therapy[1,2]. Mesenchymal stem cells are a diverse population of multipotent cells which was first described in 1966 as a population of adherent fibroblast-like colony-forming stromal cells that did not belong to the hematopoietic cells[3]. In 1991, the multilineage potential of MSCs has been approved[4]. Mesenchymal stem cells could be obtained from many sources like bone marrow, fatty tissue, peripheral blood, dental pulp, periodontal ligaments, and neonatal tissues (umbilical cord, placenta, and amniotic fluid). These sources greatly affect the phenotype, growth kinetics, potency, differentiation, surface markers expression, and cytokines profile of the cells. Though, there are no fixed markers for mesenchymal stem cells. International Society for Cellular Therapy in 2006 has created standards, that these in vitro cells must attain specific properties to be recognized as mesenchymal stem cells[5 6]. The cells must express the basic identification markers CD73, CD90, and CD105. They should not express CD34, CD14, CD45, CD11b, CD19, CD79a, CD31, lack human leukocyte antigen complex-2 and co-stimulatory molecules, like CD86, CD40, or CD80. In addition, mesenchymal stem cells must have transcription factors octamer binding transcription factor-4 (OCT-4) and homeobox protein NANOG[1]. Bone marrow is a very vigorous and diversified microenvironment, settled with different types of cells. Marrow cavities, from the early postnatal period onwards, contain heamatopoietic tissue and stromal microenvironment, known as active red bone marrow. Active red bone marrow formed of cords of hemopoietic stem cells with developing blood cells and megakaryocytes, mesenchymal stem cells, osteoblasts, osteoclasts, macrophages, mast cells and few adipocytes. Despite the great numbering of hematopoietic stem cells, bone marrow includes a relatively low numbering of bone marrow mesenchymal stem cells (0.001–0.01%). Moreover, the inactive yellow bone marrow is the chief form in the medullary cavity of bones in the adults, in these bones, the red bone marrow has been replaced consistently by adipocytes [7, 8]. Bone marrow mesenchymal stem cells appear to carry an embryological imprint due to their mesodermal origin. Primary isolated BM-MSCs have spherical appearance and with subsequent passages they take fibroblast-like shape. Ultrastructural analysis of BM-MSCs revealed that the cells have polymorphic nuclei, euchromatin and conspicuous nucleoli. Their cytoplasm contained well-developed dilated cisternae of rough endoplasmic reticulum near to the nucleus, well developed Golgi apparatus, mitochondria, and aggregation of glycogen granules. BM-MSCs possess numerous branched projections of their cytoplasm and cell membrane that contains cell surface receptor mediating cell-matrix adhesion proteins and integrins [9, 6]. The cell morphology and proliferation are regulated by integrin activation which are stimulated by signaling cascades that activate the genes responsible for cell growth. The spread of cells occurs following the activation of protein kinase C (PKC), they accumulated focal adhesion kinase (FAK) and actin filaments at the edges of the cells which forms a stable cytoskeleton, maintains the cells adherence and growth [10]. The growth ability of BM-mesenchymal stem cells was still having obstacles even with standardized protocols and the use of specified proliferation materials. Many researchers have used differentiation media to help cultured cells grow into fat cells, bone cells, and cartilage cells[11]. Following a limited number of passages,

mesenchymal stem cells enter a state of cellular senescence, recognized by noncurable growth arrest with diverse pathological and physiological consequences. Long-term culture can also cause potency and stability impairment of MSCs, chromosomal changes, and a rise in the frequency of cells vulnerable to cancer cells. In the cell cycle, MSCs senescence led to an increased frequency of cells that stay in the G0/G1 phase of the cell cycle and a concurrent, considerable decrease in S and G2/M cells over passaging [12,13]. Considering the heterogeneity of BM-MSCs and the association between the animals' age with the cellular senescence-associated morpho-functional changes, we have designed our study to evaluate the isolated and long-term expanded heterogenous BM-MSCs that were obtained from young and old Wister albino male rats in the terms of growth kinetics, ultrastructural morphology, and cell surface markers profile. This study suggested that the age of the donor cells can be a factor that affects the growth of cultured cells.

Materials And Methods

Animals

Twelve healthy male Wistar albino rats were used in this study. Animals were maintained under standardized conditions in line with the ethics of the Research Committee in the Faculty of Medicine, Suez Canal University, Egypt. Animals were randomly divided into two groups (six animals each). Group I: old group: rats aged 15 months old and weighing between 300 - 350 g and Group II: young group: rats aged 4 months old, weighing between 100-150 g.

Isolation and seeding of BM-MSCs[14,15]

Rats were sacrificed with cervical dislocation and immersed for 2 minutes in 70% ethanol and then were brought to a sterile dish. Under sterile conditions, in the Molecular and Cellular Medicine Center of Excellence, the whole skin was removed from the limbs. By using sterile micro-dissecting scissors and surgical scalpels, the soft tissues were carefully removed from tibias and femurs. Femurs were separated from the tibias and iliac crests from each leg and the feet were removed. Bones were transferred to the Tissue Culture Unit in a 100-mm sterile culture dish and immersed with 10- 15 mL of complete Dulbecco's Modified Eagle Medium and low glucose. The two ends of the bones were excised using micro-dissecting scissors. Through pushing complete DMEM by a 5 mL syringe, the bone marrow was washed out from the femurs and tibias at one end and received from the other end in a falcon tube. The complete culture media was low glucose DMEM (Biowest, France) supplemented with Fetal Calf Serum 10% (FCS) (LSP, UK) and 1% Penicillin/ Streptomycin (Biowest, France). The process was repeated until the bone cavity becomes pale. The isolated cells were centrifuged at 2000 rpm for 5 minutes, then the supernatant was removed. The pellet washed twice with phosphate buffer saline (PBS) and centrifuged at 2000 rpm for 5 minutes. Finally, the cells were seeded in 25 cm^2 flask and nourished with complete culture medium. Flasks were maintained under 5% CO_2 infusion, at 37° C inside a humidified incubator. The cells were kept for 3 to 4 days before the first media change. When adherent cells grown to ~80% confluence, they were defined as passage zero (P0) cells and the splitting of cells began. At ~80% confluent flasks, trypsin 0.25% including 0.02% ethylene diamine tetra acetate (EDTA) 10x (Biowest, France) was incubated with the cells 1-2 minutes. The action of the trypsin/ EDTA was then blocked by adding 10 ml of complete culture medium. The cells were centrifuged two times with PBS and the pellet was serially passaged using a 1:2 splitting ratio. Three samples

were taken from each passage, before the secondary cultivation, to do testing for cell viability and counting, immunophenotypic changes, and ultrastructural changes. The cells were maintained in culture and expanded across four passages (from P0 to P4).

Morphological characterization of old and young BM-MSCs

- Phase contrast inverted microscopy

The changes in morphology and confluency of the seeded cells were evaluated using inverted phase contrast microscopy Olympus CKX31 (magnification power x10 & 40) and photographed with Leica DM IL LED camera, from passage zero (P0) to passage 4 (P4).

- *Transmission electron microscopy (TEM)*

Ultrastructural morphology was assessed by transmission electron microscopy during primary culture and in the second, third, and fourth passages at Al Azhar University Mycology and Biotechnology Center, Egypt. The media was removed and replaced with buffered 2.5% glutaraldehyde in 0.1 M cacodylate buffer with a pH of 7.2–4 and at room temperature. This fixation was stand for 2 h at 4°C. Post fixation and at room temperature, the buffer of 1% osmium tetroxide was added for 30 min. Then in an ascending hexylene glycol series (70%, 90%, 100%) the dehydration of the samples was done, and then the samples were covered with 100% epoxy resin. Leica ultra-cut (UCT) (100 nm) was obtained after breaking down the dishes with a hammer. Sections were taken on nickel grids and finally, were stained with alcoholicuranyl acetate and Reynold's lead citrate. Ultrastructural examination and photography were taken out by transmission electron microscope (JEOL JEM 1010, JEOL Ltd, Tokyo, Japan) at an acceleration voltage of 70 kV[12].

Flow Cytometry: cell surface markers[16]

The immunophenotypic evaluation of stem cell surface markers was carried out at the Oncology Diagnostic Unit, Faculty of Medicine, Suez Canal University. At each splitting of seeded cells, a trypan blue exclusion test was done to count the viable and dead cells, 50,000 only of BM- MSCs were labeled with fluorochrome conjugated mouse anti-rat antibodies. These antibodies were specific for CD73 APC (BD Biosciences), CD34 PE (phycoerythrin), CD90 FITC (Fluorescein isothiocyanate) and CD45 PerCP. The cells were incubated in the dark with the labeled antibodies for 20 minutes and at 4°C. Unstained BM-MSCs were used as a control. Then, at room temperature, the tubes were centrifuged at 150x g for 5 minutes. PBS was used to wash the cell pellets at 2-8°C in a dark room and then centrifuged. Resuspension of pellets was done using 2 ml of PBS and then the cells were passed through a 70 mm filter and analyzed for cell surface marker profile using Partec-CyFlow® ML instrument. Data collection, analysis as well as presentation were carried out using FloMax®software.

Growth Kinetic

Harvested cells in each passaging were counted using a Hemocytometer slide after staining with trypan blue. The growth kinetic curves of cultured cells from each passage were plotted, using those counts.

a- *Doubling Time*

The initial seeding number of cells and the harvested cells were recorded and computed into a doubling time (hrs.). It was calculated using Patterson Formula:

$$T_d = \frac{T \log 2}{\log(N_t/N_0)},$$

T_d is the doubling time (h), T is the time cells need to proliferate from N_o to N_t (h). N is the cell count[17].

b- *Cell Density*

When the seeded MSCs reached confluency, the cell density was recorded with each passage to evaluate the expansion of cells. The density was measured as mean confluent cell count for each passage per cm^2 growth area [18].

c- *Population proliferation rate*

The daily increase of cell counts of cultured BM-MSCs in each passage was calculated using the following formula: Daily proliferation rate (cell count before splitting- cell count at start of seeding)/number of days in each passage [19].

Statistical Analysis

All quantitative data were recorded and analyzed using SPSS version 25 (IBM Inc.). Values for all measurements were shown in mean ± standard deviation, for each BM-MSCs passage and for average passages. Data analysis was performed by sample t-test (independent), One-way ANOVA, and post-hoc Bonferroni test. Significant values were at value $p < 0.05$.

Results

Morphological characters of BM-MSCs in young and old rats

- *Inverted microscopy:*

Morphological assessment of the cultured BM-MSCs revealed variable results from P0 to P4. After three to four days of primary cultures, one layer of fibroblast-like cells was seen adherent to the plastic floor of the flasks. P0 showed nearly similar outcomes between the young and the old BM-MSCs, however, cells from the young rats reached better confluence within 3-4 days of post-primary media isolation and the old cells became confluent after 8-12 days (Fig. 1). After 7-12 days of passaging, young cells in P1 revealed increased confluency reaching about 100% of fibroblast-like cells and were split into P2, however, 40-50 % confluency in old cells were present in the same period (Fig. 2A, B). Old cells reached confluency after about 14 days and were split into P2. Dishes from P2 showed nearly 70-80% confluency after 14 days of passaging, in both groups, of fibroblast-like cells with rounded open face nuclei that contain multiple nucleoli (Fig. 2C, D). The old and young cells were split and started a new culture as P3. Both groups in P3 showed different cell morphology and lesser cellular confluency. Cells appeared more rounded and contained a larger amount of filamentous cytoplasm. Nuclei were rounded vesicular and contained one or two nucleoli. Old and young cells reached 70-90% confluency after 8-10 days of passaging and were split into P4 (Fig. 3A, B). Cells of P4 also showed lesser confluency of multipolar granular and vacuolated cells with large vesicular nuclei with multiple nucleoli, after 7 days of passaging (Fig.3C, D).

- *Transmission electron microscopy*

The ultrastructural changes at different Passages of 0, 1, 2, 3, and 4 were assessed in young and old rat stem cells by electron microscopy. The ultrastructure of the stem cells in all passages showed mainly rounded to oval euchromatin nuclei with narrow-margined heterochromatin (Fig. 4 and 5). The cells appeared heterogeneous with centrally located nuclei during P0 in both age groups (Fig. 4A, B). Many eccentric irregular nuclei in all the passages were evident in both age groups. Many cells with double nuclei were noticed in the young age group during P1 (Fig. 4C, D). The cytoplasm in both age groups from passages P0 to P2 exhibited all the subcellular organelles; plenty of mitochondria, well-formed rough endoplasmic reticulum (RER), and numerous secretory vesicles (Fig 4). Passage 3, in old animals' cells, indicated that part of the peripheral cellular zone of some cells lacked subcellular organelles and exhibited disorganization of the

remaining organelles along with the heterochromatic appearance of many nuclei. On the other hand, the cells of the young age group during P3 had normal and well-arranged cytoplasmic organelles (Fig. 5A, B). During P4, BM-MSCs of young animals showed intact well-arranged cytoplasmic organelles apart from dilated RER (Fig. 5C). However, many cells of old animals during the same passage had massively vacuolated cytoplasm with lost or masked cytoplasmic organelles (Fig. 5D). Cytoplasmic processes were noticed in almost all the cells from P0 to P3. They were more numerous and finer in the young age group (Fig. 4, 5) and became rudimentary during P4 (Fig. 5C), on the other hand, the cytoplasmic processes were hardly seen in the cells of old animals during P3 and P4 (Fig. 5B, D). Regarding the mitochondria, they were abundant, dark colored, rounded to oval, and spread on one side of the nucleus from Passages 0 to 2 in both age groups (Fig. 4, 5) however they became smaller and scarce in P4 in young animals and in P3-4 in old animals (Fig. 5). In P4, in both age groups, vacuoles were scanty, small, and located at the periphery in the young age group but were abundant in the whole cytoplasm at the old age group (Fig. 5).

Cell surface markers profile expression in the adherent MSCs from young and old rats: The expression of cell surface markers among different passages of BM-MSCs, from the two groups, was evaluated by the flow cytometry (Fig. 6 B and C). Two MSCs-specific markers (CD73 and CD90), and two HSCs markers (CD45 and CD34) were evaluated. We found that CD90 and CD73 were highly and significantly expressed across passages in young MSCs reaching at least 50% of the cells by the fourth passage with progressive decreased expression of CD45 and CD34. While the old MSCs showed a steady increase in the expression of CD90, and CD73 in different passages (Fig. 6A).

Doubling time, Cell density, and population proliferation rate

The growth curve of young BM-MSCs samples showed significantly higher cell counts compared to old BM-MSCs samples across different passages, as shown in (Fig. 7). As shown in (Fig. 7E), Young BM-MSCs showed a highly significant increase in the daily proliferation rate compared to old BM-MSCs in different passages ($P<0.001$). On calculating the cumulative cell count, young BM-MSCs showed a highly significant increase in the daily proliferation rate compared to that of old BM-MSCs in all study passages, except for P1, ($P<0.001$) as shown in tables (1). Except for P0, the young BM-MSCs showed an increase in the *mean cell density*/ cm^2. This result was significant ($p < 0.05$) in comparison with that of old BM-MSCs across passages (P1-P4), based on cell seeding and cell splitting counts. Mean cell density/cm2 of old, and young BM-MSCs were shown in (Fig. 7D). On counting the *doubling time* in hours (Td), young BM-MSCs showed significantly prolonged doubling time, compared to old BM-MSCs in P2DT, and P3DT. However, in P1DT, and P4DT, young BM-MSCs showed significantly shorter doubling time compared to old BM-MSCs (Fig. 7C). The prolonged P3DT of young BM-MSCs was mostly attributed to the higher significant output of cell count of young BM-MSCs compared with old BM-MSCs as shown in growth curves of the BM-MSCs samples (Fig. 7). Duplication of a larger population of cells, consumes more time, with a result of longer DT for young MSCs than old MSCs.

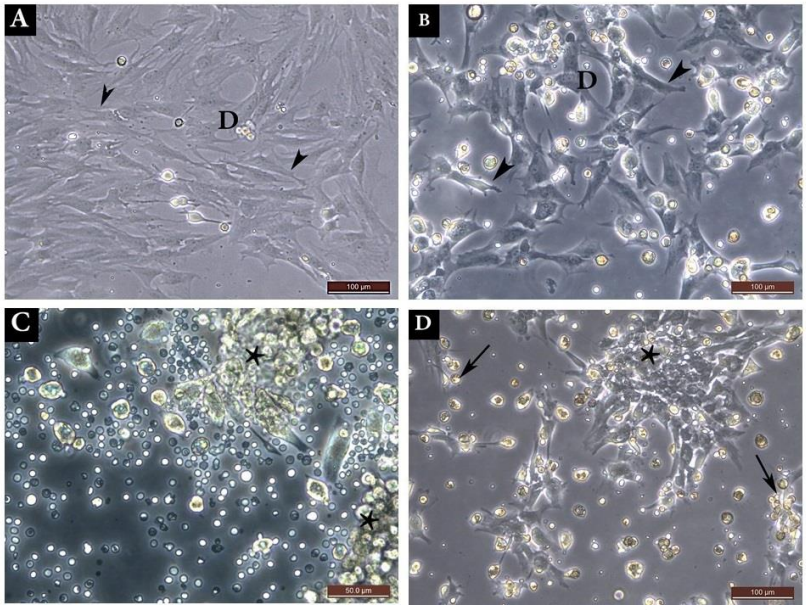


Figure 1: Phase contrast photomicrographs from passage zero (P0) cultured rats BM-MSCs with 50 -80 % confluency. *A and C represent cultures from young rats' cells that were variable in size and exhibited the characteristic heterogeneous population with predominant fibroblast-like morphology (arrowheads) and dividing cells (D). Other patches showed rounded cells and colonies (*). B and D represent cultures from old rats, they showed rounded cells (arrows), colonies (*), fibroblast-like cells (arrowheads), and dividing cells (D). (Inverted microscope, X 100, C: X 400)*

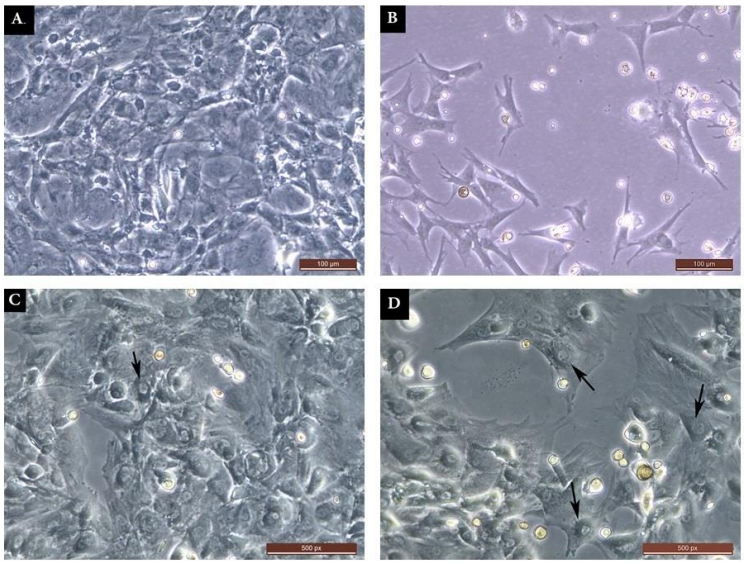


Figure 2: Phase contrast photomicrographs from passage one (P1, A & B) and passage two (P2, C & D) cultured rats BM-MSCs. *(A) shows 90-100% confluency of fibroblast-like and spindle-shaped cells from young rats after 7 days of passaging. (B) shows 40-50% confluency of fibroblast-like cells from old rats after 10 days of passaging. P2 after 14 days of passaging, (C) from young rats, and (D) from old rats. Both showed 70-80% confluency of fibroblast-like cells with rounded open-face nuclei which contained multiple nucleoli and perinuclear granules (arrows). (Inverted microscope, X 100)*

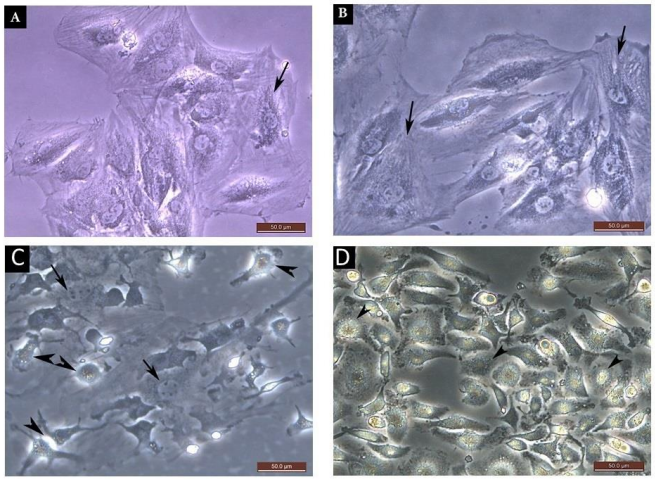


Figure. (3): Phase contrast photomicrographs from passage three (P3) and four (P4) cultured rat BM-MSCs, young and old, after four and seven days of passaging, respectively. *They showed 30-50% confluency of cells. P3 (A, B) cells became more rounded and contained a larger amount of cytoplasm that was crowded with filaments (arrows). Nuclei were rounded, vesicular, with one or two nucleoli and surrounded with granules. P4 (C, D) cultured rat BM-MSCs were more multipolar. (C) showed culture from young rats with most of the cells containing cytoplasmic vacuolations that mask their nuclei (arrowheads). Few cells had granular cytoplasm with large vesicular nuclei that possessed multiple nucleoli (arrows). (D) showed cells from old rats, some had long granular cytoplasmic processes and rounded vesicular nuclei; others had cytoplasmic vacuolations (arrowheads). (Inverted microscope, X 400)*

Discussion

The potential use of BM-mesenchymal stem cells in disease applications has been investigated in a variety of research using animal models. Preclinical research is essential for clinical trials, so it is necessary to build up a culture system of animal MSCs through easy isolation and expansion in the tissue culture unit. Our study tried to reach some parameters that may affect culture outcomes. We applied a direct seeding method of bone marrow culturing, from the tibia and femur of Wister albino rats from two age groups. Our results revealed donor age-dependent morphological and ultrastructural changes, and proliferation profile differences of BM-MSCs after long-term ex-vivo cultivation, starting from P0 up to P4. These outcomes might become applicable to be used for improving the growth and differentiation abilities of cultured BM-MSCs in future studies. In P0, 1, and 2 there were no differences in the morphological and ultrastructural results between the two different age groups of BM-mesenchymal stem cells, yet the growth and proliferation of the young cells were faster than that in the old cells. Primary cultures from young cells reached 80% confluency after 3-4 days and 8-12 days in old cells. In later passages, P3 and P4, cells from both age groups became larger, rounded, and filled with cytoplasmic filaments. Young cells had normal and well-arranged cytoplasmic organelles and few vacuolations; however, the old cells had a massively vacuolated cytoplasm that masked the organelles. The capacity of the cells to grow and proliferate decreased compared to the prior passages, nevertheless, it's still better in the young population than in the old ones. The previous findings were further supported by many studies, for instance, Patil GA & Moghe SA, 2021 who documented that age has a significant direct effect on MSCs cell growth, differentiation, and senescence parameters in rats from two age groups (young at about 6 weeks and old at about 56 weeks) [20].

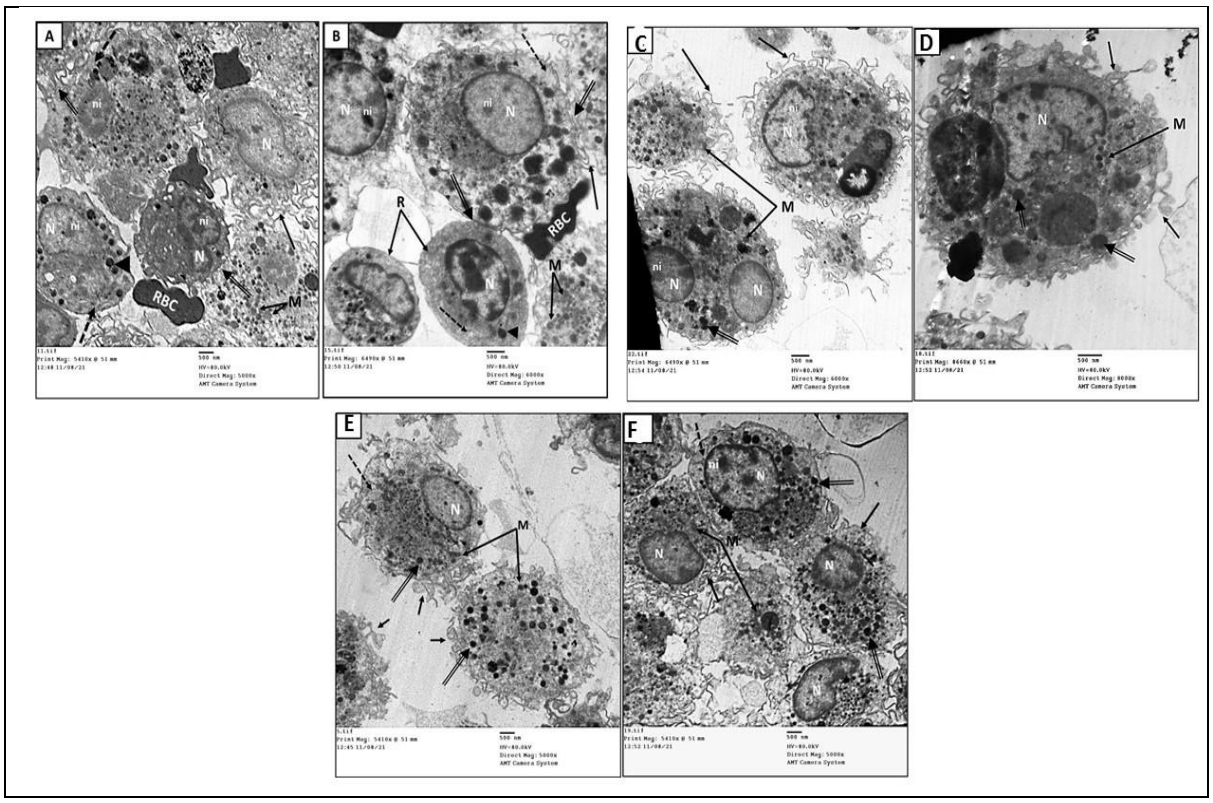


Figure. (4): Transmission electron microscopy of young and old rat BM-MSCs during passage zero (P0), passage one (P1) and passage two (P2). *P0 (A: young, B:old) cells appeared heterogeneous in both age group. Most of the cells had euchromatic nuclei (N) with a thin rim of heterochromatin at their periphery and small nucleoli (ni). Many scattered rounded to oval mitochondria (M), many course cytoplasmic processes (arrow), secretory vesicles (arrowhead), well-formed cell junction (double arrow) and rough endoplasmic reticulum (dashed arrow) were evident. Some rounded (R) cells without any cytoplasmic processes were noticed in the old age group. P1 (C: young, D:old) cells exhibited many cytoplasmic processes (arrow) in both age group which were more numerous and finer in young age group. Most of the cells had euchromatic nuclei (N) and small nucleoli (ni) with marginization of heterochromatin. Indentation of some nuclei were noticed in both groups. In addition, some cells in the young age group processed two nuclei. Many scattered rounded to oval mitochondria (M), and secretory vesicles (double arrow) were evident. P2 (E: young, F:old) cells had abundant cytoplasmic processes (arrow) in both age groups. Most of the cells had euchromatic nuclei (N) and small nucleoli (ni) with marginization of heterochromatin. some cells exhibited irregular nuclei. Many scattered rounded to oval mitochondria (M), rough endoplasmic reticulum (dashed arrow) and secretory vesicles (double arrow) were noticed. (all images magnification X 6000, D X 8000).*

Old cells' frequency in primary culture, doubling time of cells, and the cells' outcome were significantly decreased compared to young rats. Also, Yang YH et al., 2018 results approved that the aged human mesenchymal stem cells gradually the characteristic spindle and fibroblast-like shape, causing eminent abnormal morphology as well as the doubling rate of cells decreased with subsequent passaging [21]. In addition, Ridzuan et al., 2016 reported that BM-MSCs contained two diverse types of cell shapes; the first is small spindle-shaped and fibroblast-like cells, which is characterized by rapid proliferation. And second is a large, flattened cell that has a slower proliferation rate with when increased number of passages, the large-flattened cells gradually became dominated, a characteristic of cellular senescence[18]. These results were further confirmed with the doubling time values. Cells from P1–P3

had preserved a steady doubling time; however, the doubling time began to rise at P4 and reached the maximum at P5, and then the cells were unable to be further ex panded beyond P5.

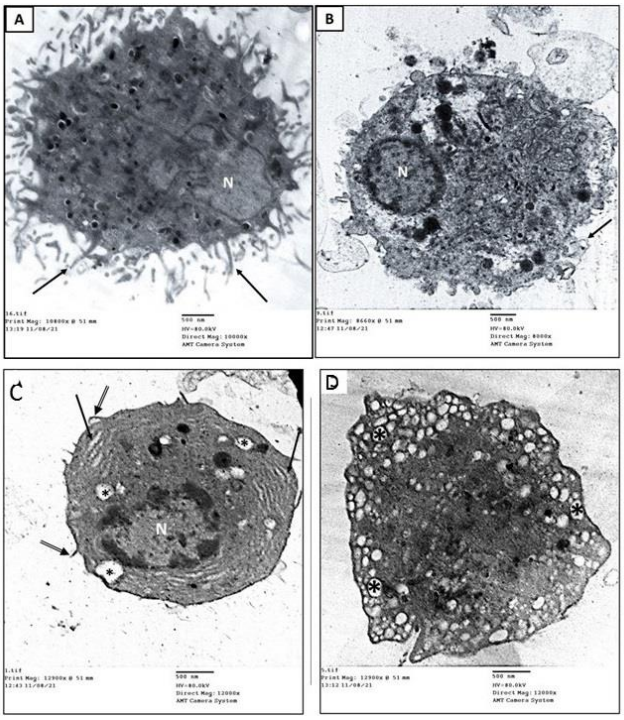
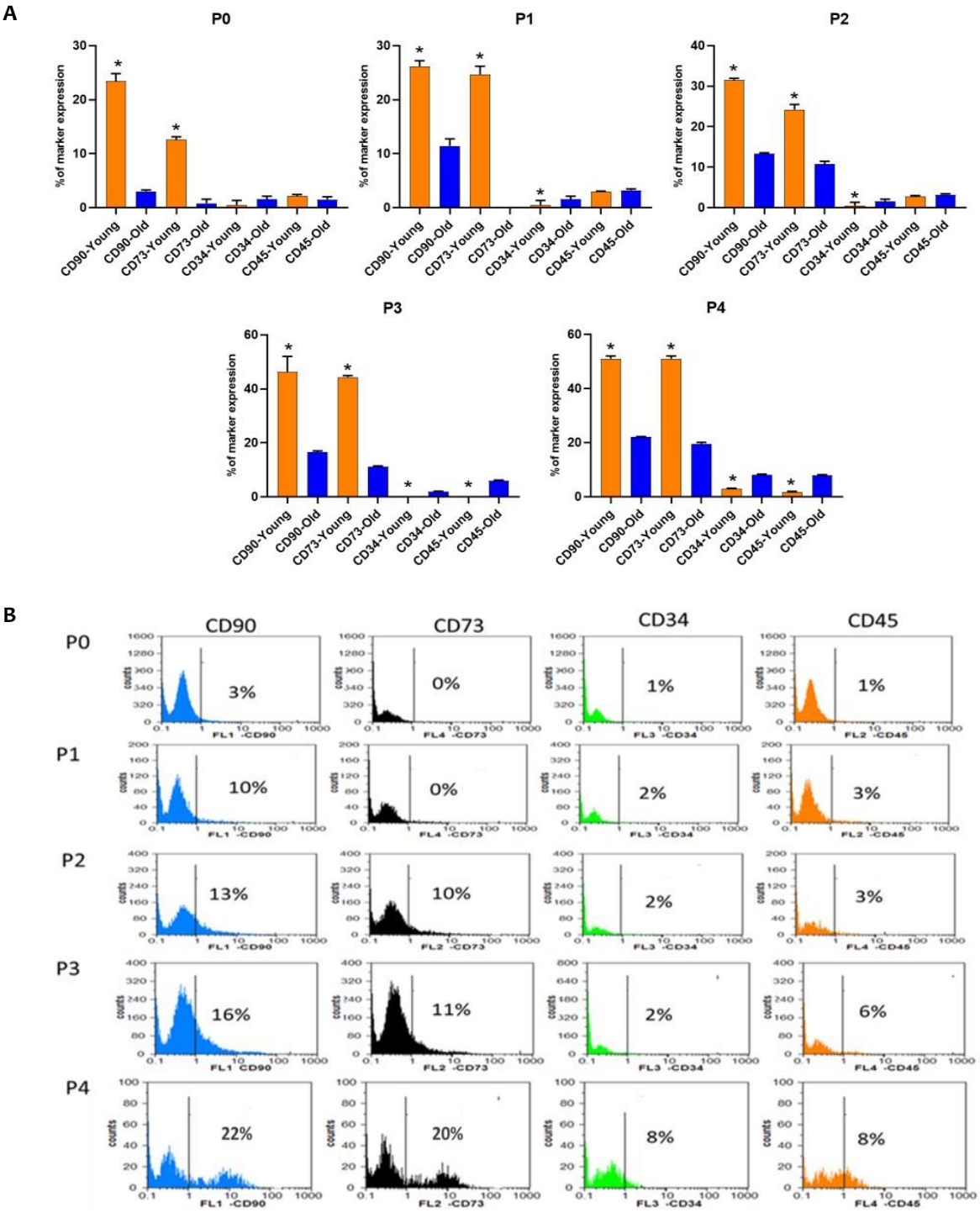


Figure 5. Transmission electron microscopy of passage three (P3) and passage four (P4). *P3 (A): Young cells had abundant fine course cytoplasmic processes (arrow), eccentric irregular nucleus (N), and intact cytoplasmic organelles. P3 (B): old cells had few rudimentary cytoplasmic processes (arrow), small heterochromatin eccentric nucleus (N), and more or less disorganized cytoplasmic organelles. P4 (C): Young cell appeared rounded with heterochromatic nucleus (N), wide rough endoplasmic reticulum (arrow), rudimentary cytoplasmic processes (double arrow), and some vacuolations (*). P4 (D): old irregular cells had vacuolations with masked and disorganized cytoplasmic organelles. (A&B X 8000, C&D X 12000)*

On the other hand, a more expanded time was stated by Yusop et al., 2018 who found that rat BM-MSCs showed a significant increase in the doubling time of cells only when they had completed nearly P10, they could proliferate up to P50-100[22]. Moreover, inconsistent with our results, Asumda & Chase, 2011 and Babenko et al., 2021 stated that the increase in the donor age directly affects the proliferative capacity of BM-MSCs [23, 16]. They attributed this result to the diminished telomerase activity and increase in the activity of β-galactosidase associated with cell aging. Also, Hall et al., 2010 confirmed that the old age cell environment has been identified to inhibit the regeneration of adult stem cell[24] Alternatively, Siennicka et al., 2021 stated that the in vitro aging process has resulted from the multiple cell divisions and telomeres shortening, however, the age of transplanted cells had no effect on the phenotype or function of MSCs with long-term cultivation of adipose tissue-derived stem cells[25]. On ultrastructural examination, Babenko et al., 2021 detected a heterogenous group of cells. Cells obtained from young rats clearly expressed multiple extended mitochondria, endoplasmic reticulum, abundant polysomes, several

multilamellar structures (autophagosomal/lipofuscinic formations), and exosomes. However, cellular organelles in cells obtained from old rats were not expressed clearly like in young cells[16]. Several researchers tried to understand why the prolonged culturing of stem cells leads to their senescence. These results can be explained by the alteration in the cells' mitochondrial morphology, decreased antioxidant capacities of the cells, raised reactive oxygen species, and the most important obstacle was genomic instability (chromosomal abnormalities, variation of telomere length, and epigenetic instability) are a probable cause of decreased proliferation, morphological and ultrastructural changes of stem cells[26-28].



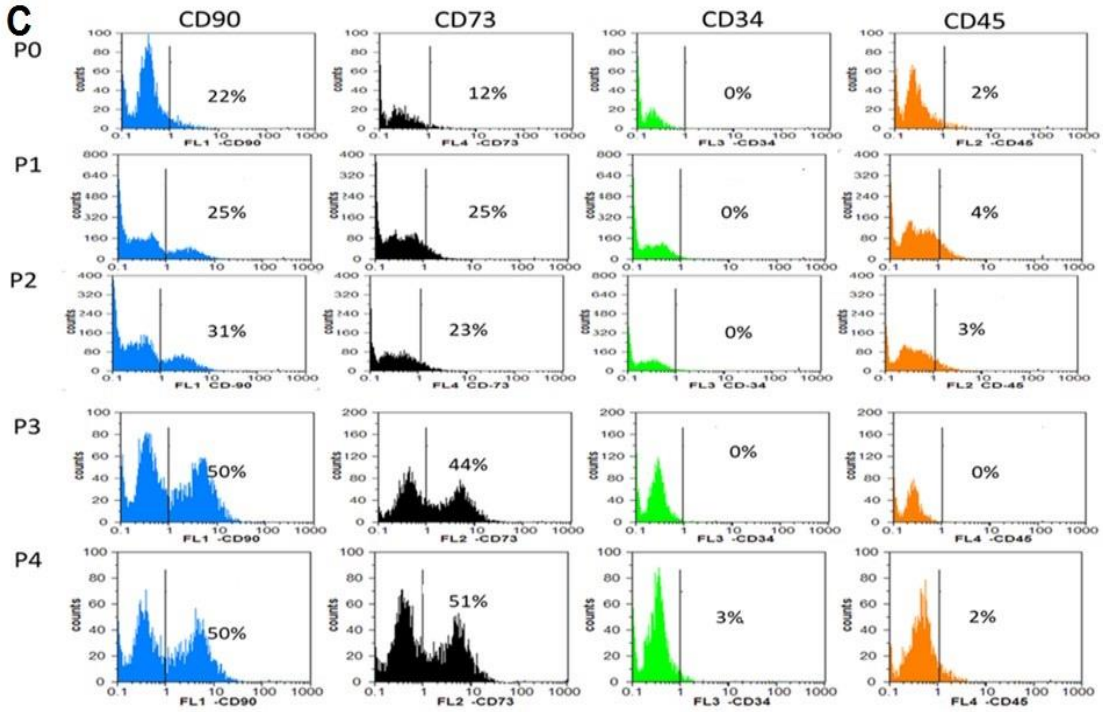


Figure 6. A: difference in the expression level of CD90, CD37, CD45 and CD34 in young and old MSCs cells in different passages. **p-value<0.05 young compared to old BM-MSCs. B: CD90, CD73, CD34 and CD45 expressions in old mesenchymal adherent stem cells across different passages. C: CD90, CD73, CD34 and CD45 expressions in young mesenchymal adherent stem cells across different passages.*

In the present work, we moreover verified the age effect of the transplanted cells on the expression of surface antigen markers of BM-MSCs. We demonstrated that the young source of cells showed significant expression of MSCs surface markers (CD90 and CD73) across passages reaching at least 50% of the cells by the fourth passage. While steady expression was detected in the old population in different passages. In accordance with our results, Khong et al., 2019 described the expression of surface markers in human BM-mesenchymal stem cells from two age groups and reported that all cells were positive for CD73 and CD90 and were negative for CD34 and CD45[29]. In addition, the influence of age on stem cell growth and proliferation and so the surface marker expression has been proved by Ahamad et al., 2021 and Iwata et al., 2021[30,31]. Aged BM-MSCs showed a significant decrease in the expression of all surface markers when compared to young cells. However, Fafián-Labora et al., 2015 declared that there is no statistically significant differences in the mesenchymal markers, CD29 and CD90, among MSCs aging groups and they added that the chronological age mainly influences directly on the expression of proliferative markers Ki67 and CD117 [32].

Conclusion

In conclusion, according to our morphological, ultrastructural, immunophenotypical, and growth kinetic assessment, we recommend that the most proper passage of BM-MSCs for cell transplantation is P2-P3 obtained from a young group of rats.

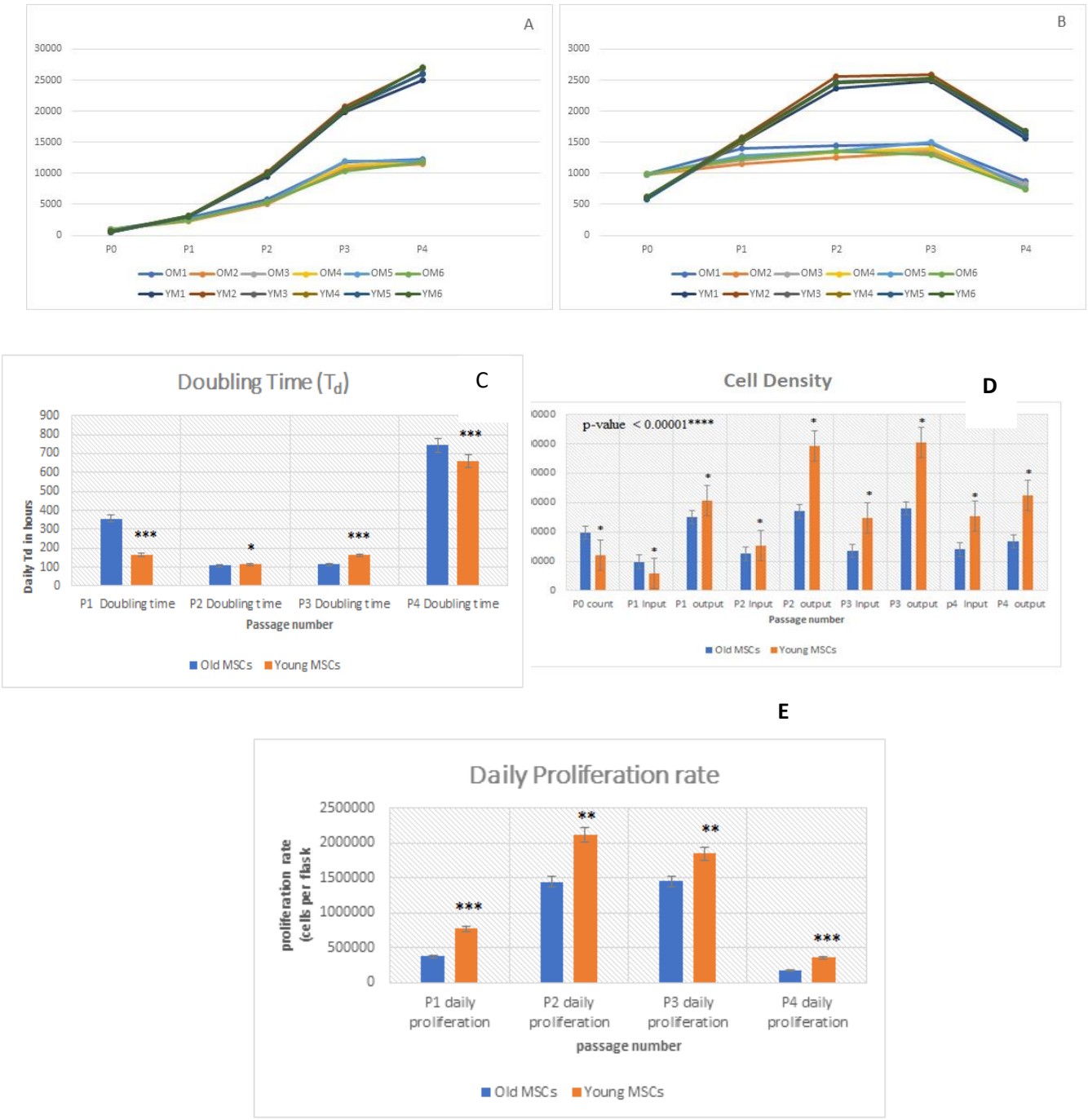


Fig. (7): A &B: A stacked Line chart showing a comparison of growth curves of old, and young rat bone marrow-derived mesenchymal stem cells (BM-MSCs) in serial passages between zero and four. *A: means of cumulative cell counts ($x10^4$) in growth curves of each sample of old, and young cells over time up to passage four. B: means of cell counts ($x10^4$) in growth curves of old, and young cells for each sample across four passages. C: Bar chart showing BM-MSCs doubling time in hours (Td) of old and young rats across the four passages. *** Very highly significant difference between old and young BM-MSCs in P1, P3 and P4 ($p < 0.000$). * Significant difference between old and young BM-MSCs in P2 ($p < 0.038$). D: Bar chart showing mean cell density/ cm^2 of old and young rats across the five passages based on cell input and total cell output showing highly significant difference between young and old MSCs, a p-value is < 0.00001. Also, a significant difference was found between young and old BM-MSCs on and across each passage, $p < 0.05$. E: Bar chart showing BM-MSCs daily proliferation rate of old and young rats across the four passages that were based on passage timing and final increase of cell count.*

**** Very highly significant difference between old and young MSCs in P1 and P4 (p <0.000). ** Highly significant difference between old and young BM-MSCs in P2 and P3 (p <0.001 & 0.003).*

Abbreviations

BM-MSCs: Bone marrow mesenchymal stem cells
MSCs: Mesenchymal stem cells
P: Passage
OCT-4: Octamer-binding transcription factor-4
PKC: Protein kinase C
FAK: Focal adhesion kinase

Declarations

Ethical considerations: Animals were acclimatized for a week before starting the experiment and were handled in agreement with the guidelines for the care and use of laboratory animals, NIH, Bethesda. Before starting the experiment, we received approval from the Research Ethics Committee of the Faculty of Medicine, Suez Canal University (ID: 4233). Availability of data and materials: They will be obtainable only on reasonable matters. Conflicts of interest: We declared that there are no conflicts of interest. No informed consent or reporting checklist: the study was carried out on experimental animals. Publication consent: All authors agreed on the publication. Funding resources: The research received a fund from the Science and Technology Development Fund (STDF), as research support call 9 and Grant/Award Number: 34856. Ministry of Higher Education and Scientific Research, Egypt.

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Author Contributions

All the authors had shared in design, conception, and manuscript writing. *RAG* did stem cell isolation and culture and interpretation of TEM results. *MFM* did stem cell isolation and culture, and interpretation of BM- mesenchymal stem cells growth kinetics. NMA interpretation of flow cytometry results. *AMA* did stem cell isolation and culture. *SSM* did stem cell isolation and culture and morphological assessment of BM-mesenchymal stem cells. *NRA* did data analysis of BM-mesenchymal stem cell growth kinetics. *NI* did stem cell isolation and culture, a morphological assessment of BM- mesenchymal stem cells. *EAM* conceived the study and shared it in its coordination.

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