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Age Dependent Changes of Biological Properties in the Multipotent Mesenchymal Stromal Bone Marrow Cells in FVB Mice

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Abstract

Objective: In the last years researchers' attention has been given to the immunomodulatory properties of multipotent mesenchymal stromal cells. The question of effectiveness of the stem cell therapy with MSCs from elderly donors is worth studying. Our aim was to compare the potency of bone marrow MSCs in aging.

Methods: BM-MSCs were obtained using the standard methods. The following methods were applied: immunophenotyping, colony-forming unit fibroblasts assay, granulocyte/macrophage colony-forming cells assay in the semi-agar cultures, directed differentiation, colorimetric method, MTT assay.

Results: The stromal progenitor cells increase in their number indicating age-associated elevated ability of the BM-MSCs to proliferation. The BM-MSCs have shown their ability for osteogenic and adipogenic differentiation along with age-associated abnormalities in the osteogenic differentiation. The BM-MSCs expressed immunomodulatory effect in dose-dependent manner regardless the donor age.

Conclusions:

1. The obtained culture cells derived from adult and old mice according to phenotypic profile and capacity for directed multilineage differentiation meet minimum criteria for the MSCs.
2. Age-dependent differences of directed osteogenic BM-MSC differentiation have been shown.
3. BM-MSCs regardless of mouse age expressed a dose-dependent inhibitory effect on the mitogen-induced proliferation of splenocytes.

Keywords

Multipotent mesenchymal stromal cells; Biological properties; Bone marrow; Age

Abbreviations:

BMCs- Bone Marrow Cells

BM-MSCs - Bone Marrow Derived Multipotent Mesenchymal Stromal Stem Cells

CFU-Fs - Colony-Forming Unit Fibroblasts

G-CSF - Colony Stimulating Factor

GM-CFCs - Granulocyte/Macrophage Colony-Forming Cells

OD - Optical Density

Pgp1 - Phagocytic Glycoprotein-1

PHA - Phytohemagglutinin

PI - Proliferation Index

Introduction

The multipotent mesenchymal stromal cells (MSCs) have been intensively studied in view of their wide application in clinic and regenerative medicine. Such interest for these cells is associated with their ability to differentiate into various tissues of mesenchymal and non-mesenchymal origin, trophic effect on the injured organs and tissues, immunosuppressive properties [1,2,3].

MSCs inhibit the proliferation and differentiation of B cells and therefore suppress the production of antibodies that allow using them in the treatment of autoimmune diseases, such as diabetes, rheumatoid arthritis, multiple sclerosis and others [1,2]. There for the possibility of using the MSCs to improve survival of allogeneic transplants [2].

The MSCs can block the differentiation of monocytes and bone marrow precursors into dendritic cells [3,4]. They can exhaust IL-2 production by T cells, leading to the reduced formation of cytotoxic $CD8^+$ T cells [5], and inhibit proliferation and cytotoxic activity of natural killer (NK) cells [6]. MSCs inhibit T cell proliferation [7], induce apoptosis of the activated T cells [8], and stimulate both the formation of T helper type 1 (Th-1) and T helper type 3 (Th-3) of the regulatory T cells as well as production of IL-10, which prevent the development of graft-versus-host reaction [9,10].

With age, the frequency of infectious, autoimmune, inflammatory diseases and malignancies increases, while the proliferative potential of the MSCs and their ability for differentiation and recovery decreases [11]. However, the literature data about the proliferative capacity of MSCs in aging are contradictory. Thus, one sources show the decrease of their proliferative capacity. These changes are referred to the age-related shortening of the telomeres, a decreased telomerase activity and / or expression, genetic mutations etc. [12,13]. At the same time there are published data indicating that reduction in the proliferative capacity of the bone marrow MSCs is not observed with age [14]. Consequently, a comparative analysis of the biological properties of the multipotent mesenchymal stromal bone marrow cells (BM-MSCs) in adult and old FVB mice was carried out to determine the capacities of the BM-MSCs in aging.

Material and Methods

The studies were conducted on adult (4-6 months) and old (18 months) male FVB "wild type" (H-2q) mice. All experiments with animals were carried out according to the Law of Ukraine "About

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protection of animals from cruelty" (from 21.02.2006), the European Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes.

Isolation and culturing of BM-MSCs: The mice were killed by cervical dislocation [15]. Bone marrow single-cell suspensions were prepared by flushing from femur with RPMI-1640 medium using technique according to Anjos-Afonso et al. [15]. Then the suspension of cells were transferred into the culture flasks 25 cm^2 ("Sarstedt", Germany) with complete medium. One vial contained 10-12x10^6 nuclear cells [16].

Colony-forming unit fibroblasts (CFU-Fs) assay: The number of bone marrow colony-forming unit fibroblasts (CFU-Fs) was measured by the bone marrow monolayer culture technique [17]. 5x10^6 of BMCs were seeded into culture flasks 25 cm^2 in 6 ml of culture medium RPMI-1640 ("Sigma", USA) containing 15% fetal bovine serum (FBS), L-glutamine (10 mM), HEPES (20 mM). Cultivation was performed for 12 days at + 37˚C under humidified air and 5% CO_2.

Granulocyte/macrophage colony-forming cells (GM-CFCs): Since the MSCs affect the differentiation of the hematopoietic stem cells [18], we assessed the number of granulocyte/macrophage colony-forming cells (GM-CFCs) in the semi-agar cultures [19]. 3x10^5 BMCs were added to 1 ml of medium McCoy 5A ("Sigma", USA) supplemented with 15% FBS, 1.6% sodium pyruvate, L-glutamine (10 mM), HEPES (20 mM), 0.94% of sodium bicarbonate, 1% GM-CSF (at a final concentration of 0.5 ng/ml, "Sigma", USA). Cultivation was conducted for 9 days at + 37˚C under humidified air and 5% CO_2.

Accordingly, on the 12th and 9th day of BMCs cultivation the number of colonies consisting of at least 50 cells was counted and divided by the total number of nuclear BMCs in the femur.

Flow cytometry: The phenotyping of BM-MSCs was performed using the monoclonal antibodies to mouse membrane antigens labelled with fluorochromes, as recommended by the manufacturer (Becton Dickinson, USA) in a working concentration of 0.5 μg per 10^6 cells. Measurements were made using a laser flow cytometer BD FACSAria (Becton Dickinson, USA) with the BD FACS Diva 6.1 software.

Directed osteogenic differentiation: Osteogenic differentiation was performed in the medium DMEM-low glucose (1g/l) ("Sigma", USA) containing 10% FBS, 50 μg/ml ascorbate-2-phosphate, 100 nM dexamethasone and 10 mM β-glycerophosphate. Replace of the medium with the appropriate fresh induction medium every 3 days for 21 days.

After 21 days the cells were fixed and stained with a 2% solution of Alizarin Red S ("Sigma", USA) for the detection of calcium deposits in the extracellular matrix [20]. The semi-quantitative analysis of the extent of mineralization of Alizarin Red S colored cultured cells was carried out by C. Gregory colorimetric method [21]. The dye bonded with calcified matrix was extracted with acetic acid; pH value was reduced to 4.1 with ammonium hydroxide. The optical density was determined with microtiterplate reader "Labsystems" at 405 nm.

Directed adipogenic differentiation: Adipogenic differentiation was perfomed in the medium DMEM-high glucose medium (4.5 g/l) ("Sigma", USA) containing 10% FBS, 5% horse serum ("PAA", Austria), 1 μM dexamethasone, 200 μM indomethacine, 500 μM isobutylmethylxanthine and 5 μg/ml insulin ("Sigma", USA). Replace of the medium with the appropriate fresh induction medium every 3 days for 14 days.

After 14 days the cells were fixed and stained with Oil Red solution (Oil Red O) to identify lipid inclusions [15]. Cells with and without lipid inclusions were counted in 10 random visual fields.

MTT assay for proliferation: To determine the immunomodulatory properties of BM-MSCs in aging we performed a joint cultivation of MSCs with syngeneic splenocytes stimulated by T-cell mitogen phytohemagglutinin (PHA).

For the cultivation the BM MSCs of third passage were used. 15 000, 30 000 and 60 000 MSCs were added to micro plate wells and incubated for cell adhesion in full culture medium for 2 hours. After 2 hours the splenocytes (10^6 /well) and the mitogen PHA (0.01 mg / ml) were added and further cultivated for 72 hours at 37˚C under humidified air and 5% CO_2.

After 72-hour culturing, the proliferative activity of splenocytes with and without addition of MSCs was assessed [22]. Two hours before the end of incubation, 0.01 ml of a 0.5% solution of 3-(4.5-dimethylthiazol) -2.5-diphenyltetrazolium bromide (MTT, "Sigma", USA) was added to each well. After 2-hour incubation at 37˚C the formed formazan crystals were dissolved in 0.15 ml in the isopropyl alcohol solution of 0.04 M HCl. The optical density of the supernatant was measured with microtiterplate reader "Labsystems" at 492 nm.

The results were expressed as the optical density (OD) and proliferation index (PI) (units): PI = OD of the mitogen-activated splenocytes + MSCs/OD of the splenocytes without MSCs. Control - PI = OD of the mitogen-activated splenocytes / OD of the splenocytes without mitogen.

Cell counting and viability assay: The cell number and viability were estimated after staining with 0.4% trypan blue and counted in the Goryaev chamber [23].

Statistics: Statistical analysis of the results was performed using parametric methods (Student's t-criterion) and nonparametric statistics (Wilkinson - Mann-Whitney test). *p* values less than 0.05 were considered statistically significant.

Results and Discussion.

The bone marrow cell structure in FVB adult and old mice: Our study has shown that total number of nuclear cells in the bone marrow does not change in the FVB mice in aging (Table 1). The relative number of CFU-Fs in old mice was 1.3 times higher compared to adult mice. The relative and absolute numbers of GM-CFCs did not change in aging. It is known that aging of the immune system is associated, first of all, with the processes occurring in the thymus. Age changes of immune system manifest themselves by thymus involution, decrease in serum levels of its hormones, disturbances of proliferation, differentiation, functional activities of T- and B-lymphocytes, macrophages and neutrophils, reduction of T-lymphocyte subpopulations and change in the ratio of regulatory lymphocytes [24,25,26,27]. Also, it is known that the IL-17 synthesized by BM T-helper cells alters proliferation of the MSCs and their production of granulocyte colony stimulating factor (G-CSF) and IL-7. The latter, in turn, activates $CD8^+$ T cells in the BM and stimulates their proliferation [28]. Our studies have revealed an increase in the number of CFU-Fs (Table 1). As we showed earlier, this is might be associated with high level of thymic hormone thymulin in old FVB mice, and consequently, the proliferative potential of BM-MSCs is

Table 1: Cellular structure of bone marrow from FVB adult and old male mice, M ± m.

Indices	Adult mice *n* = 9	Old mice *n* = 10
Total number of nuclear cells in the bone marrow, x10^6	20.5 ± 3.2	19.2 ± 1.6
Relative number of CFU-Fs/10^6 cells	31.1 ± 3.5	41.0 ± 3.5 *U
Absolute number of CFU-Fs/femus	627.3 ± 79.9	788.2 ± 81.3
Relative number of GM-CFCs/10^6 cells	12.2 ± 3.6	17.7 ± 3.6
Absolute number of GM-CFCs/femus	241.1 ± 93.3	339.5 ± 90.0
Note *U : $p<0.05$ vs adult mice (difference relevant indicators significant by Wilkinson - Mann-Whitney test).		

increased [29,30]. It should be noted that the interaction between glucocorticoids and thymic secretory component in old FVB mice is disturbed and this might affect the decrease of the thymulin level in vitro under their influence [30].

Phenotypical characteristics of BM-MSCs of adult and old FVB mice: The International Society for Cellular Therapy proposed minimal phenotypic and functional criteria to define MSCs requiring mandatory expression of CD73 (ecto-5'-nucleotidase, SH3 or SH4), CD90 (Thy-1) and CD105 (SH2 or endoglin). It should be noted that none of these molecules are strictly specific to MSCs. Therefore, in order to identify MSCs the absence of the following markers should be proved: monocyte and macrophage CD11b or CD14 markers expressed by the hematopoietic cells, CD34 of early hematopoietic cells and endothelial cells, CD45 leukocyte marker, and the markers of B cells (CD19 or CD79) and HLA-DR [31].

We performed a comparative analysis of the expression of the following surface markers in BM-MSCs in vitro: CD44 (Pgp1 - phagocytic glycoprotein-1/hyaluronate receptor which are consistently expressed by the undifferentiated MSCs), CD45 (pan-leukocyte marker), CD73 (ecto-5'- nucleotidase), CD117 (c-kit, a receptor of stem cell growth factor) and Sca-1 (stem cell antigen) and CD90 (T cell differentiation antigen expressed by proliferating MSCs). Our analysis has revealed the similar pattern of expression of surface markers in the mice of different age. Thus, the cells of the 3rd passage showed expression of the typical markers of multipotent cells such as CD44, CD73, Sca-1 and CD90 (Figure 1). However, expression of CD45 and CD117 also remained for a long time. CD45 expression at early passages in the BM-MSC culture can be explained by its heterogeneity.

Directed differentiation of BM-MSCs in aging: One of the main characteristics of the MSCs is their ability to differentiate into different cell types of connective tissue. To determine the age differences of the biological properties of MSCs, we assessed the potential of these cells to differentiate in two directions: osteogenic and adipogenic.

The first signs of impact of the osteogenic medium on BM-MSCs of different age appeared on the 7th day of cultivation. The osteogenic differentiation the BM-MSCs was characterized by the bone tissue phenotype, manifesting itself by the formation of multi-cellular aggregates and synthesized dense extracellular matrix subjected to calcification. Notably, mineralization of the extracellular matrix was more expressive in the calcification nodes and spreading to the culture area. We found that the degree of mineralization of BM-MSCs cultures derived from old versus adult mice was lower (Figure 2). Our results have been confirmed by the literature data showing that age-dependent differences in the mineralization [32].

During the adipogenic differentiation in vitro we observed certain morphological changes of cells, such as: the appearance of lipid inclusions stained with Oil Red. The first lipid vacuoles in BM-MSCs, regardless donor's age, appeared 24 hours after addition of the induction medium. We did not reveal any age changes in the number of cells containing lipid inclusions. The percentage of these cells was 97.5% and 97% in the adult and old mice, respectively (Figure 2).

In general, the obtained data indicate the decrease rate of the MSCs differentiation into osteogenic direction in aging and no age-dependent changes in adipogenic differentiation. Such differences in the differentiation potential can be explained by the aging of the stem cell niche that influences on the state of stem cells themselves and decreases the pool of stem cells in the body with aging. It is suggested that the ratio in the expression of the osteoblast-specific Distal-Less Homeobox 5 (Dlx5) and Runt-related transcription factor 2 (Runx2) and transcription factor of adipogenic differentiation Peroxisome proliferator-activated receptor gamma 2 (PPARγ2) undergo changes during aging of the immune system that result in alteration of the osteo-/adipogenic processes [33].

Age-related changes in the immunosuppressive effect of BM-MSCs on lymphocytes proliferation:

According to the published data, interactions between MSCs and various subpopulations of cells are regulated by the mechanisms of both contact and indirect interference. However, the MSCs produce their main effect on the lymphocytes via cell cycle arrest at the G0/G1. Thus immunomodulatory effects can be estimated by the influence of MSCs on the mitogen-induced lymphocytes. We have found that proliferative potential of the splenocytes significantly decreases during their mitogen activation in the presence of BM- MSCs in the intact adult mice (Figure 3). This degree of inhibition of lymphocyte proliferation with MSCs obtained from adult and old mice depended on the number of MSCs. With increasing amount of MSCs from 15 000 to 60 000, the degree of inhibition of splenocytes proliferation was increased. Thus, the splenocytes PI was twice lower 0.46 ± 0.03 and 0.27 ± 0.07 for MSCs derived from adult mice, 0.76 ± 0.09 and 0.35 ± 0.09 MSCs derived from the old mice. At the same time the inhibition of proliferation of splenocytes with BM-MSCs depended on the donor age. The PI of syngeneic splenocytes after addition of the BM-MSCs derived from adult mice was 7 times lower compared to the control (splenocytes without MSCs) and 5 times lower in the case old donor of the MSC. In summary, we have revealed the immunosuppressive effect of BM-MSCs obtained from old mice. In addition, the immunomodulatory effect of BM-MSCs obtained from adult mice was higher. The inhibition of lymphocyte proliferation by BM-MSCs obtained from adult and old mice could be mediated by soluble cytokines (such as pro-inflammatory tumor necrosis factor alpha (TNF-α) and anti-inflammatory transforming growth factor beta (TGF-β) and interleukin (IL-10)) and changes in the proportion of the T regulatory cells ($CD4^+CD25^+$) in the presence of BM-MSCs [34].

Conclusions

Multipotent mesenchymal stromal cells from bone marrow are widely used in regenerative medicine. Therefore the age-related

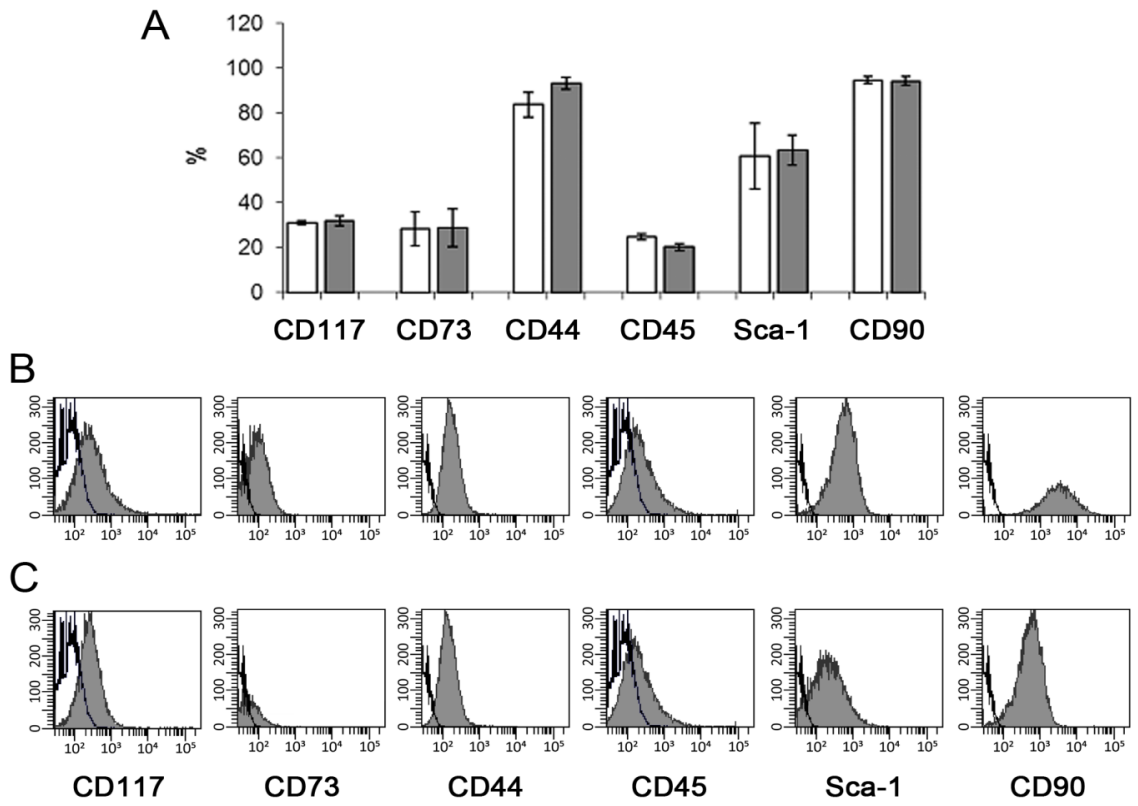

Figure 1: Flow cytometric analysis. (A) The level the expression cell-surface antigens of BM-MSCs from different ages mice (white column - BM-MSCs from adult mice, black column - BM-MSCs from old mice). (B) Flow cytometric histograms show the expression (shaded) of selected molecules (CD117, CD73, CD44, CD45, Sca-1, CD90) of BM-MSCs from different ages mice compared with controls (unshaded peaks) (cultures at the 3rd passage).

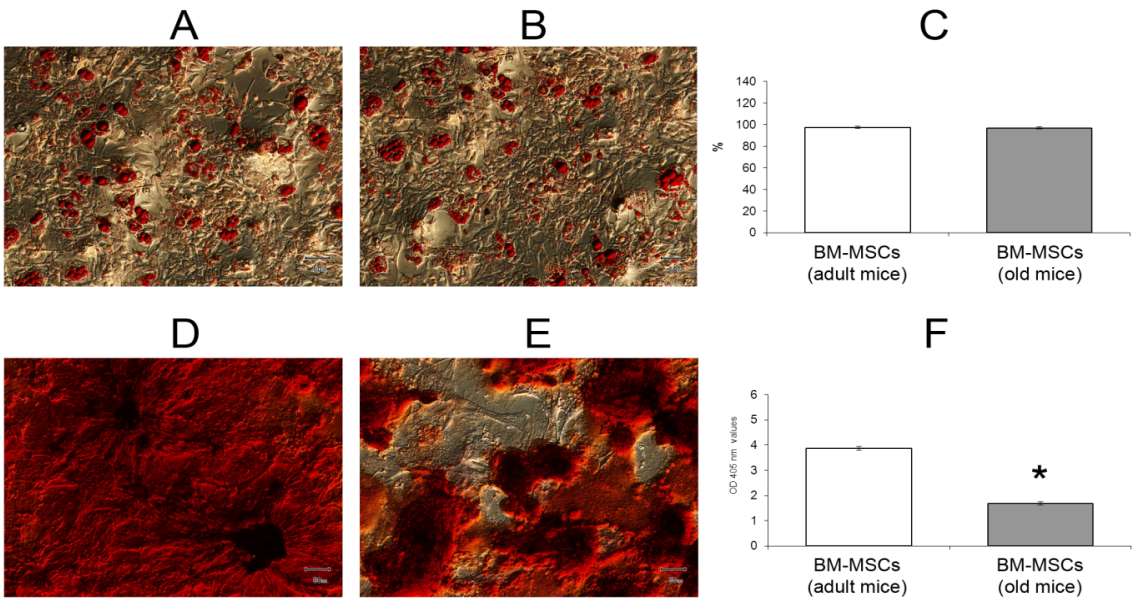

Figure 2: The differentiation potential of BM-MSCs. Lipid vacuoles are stained with Oil Red O (A) BM-MSCs from adult mice, (B) BM-MSCs from old mice, (C) Counted the number of cells with lipid droplets, calculating the percentage of the total number of cells. Calcium deposited in the extracellular matrix is stained by Alizarin Red S (D) BM-MSCs from adult mice, (E) BM-MSCs from old mice, (F) Extraction of mineralized matrix (colorimetric method), * – $p<0.05$ vs BM-MSCs from adult mice. Phase-contrast micrographs (A, B, D, E). Magnifications, x200 (A, B), x400 (D, E).

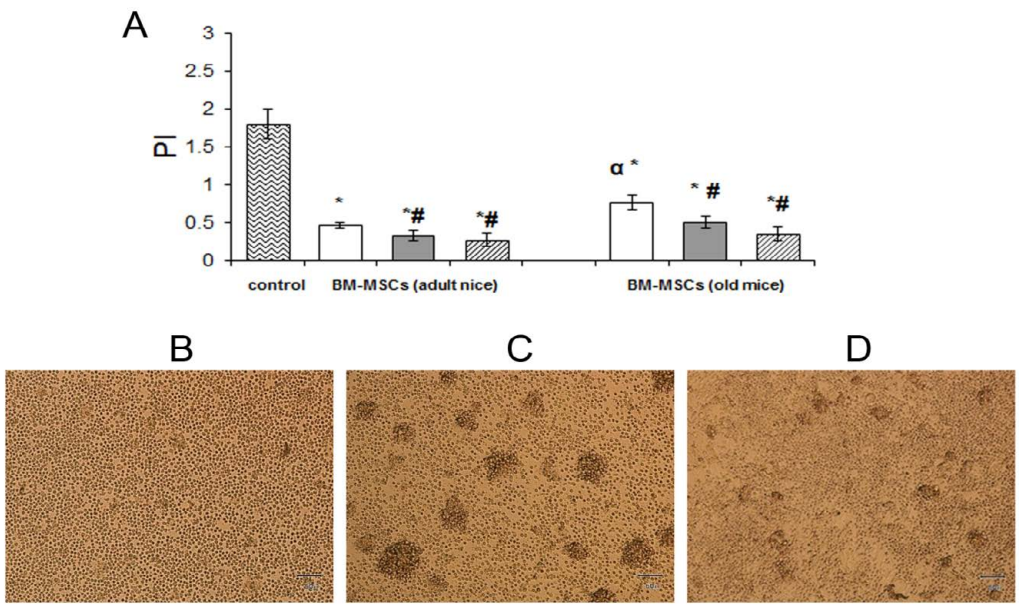

Figure 3: Immunosuppressive effects of BM-MSCs in aging. (A) Immunomodulatory effect of BM-MSCs on proliferation splenocytes in the presence of PHA: column with wavy shading – PI splenocytes (control), white column – PI splenocytes co-cultured with 15,000 BM-MSCs, black column - PI splenocytes co-cultured with 30,000 BM-MSCs, column with diagonal shading - PI splenocytes co-cultured with 60,000 BM-MSCs; * – $p<0.05$ vs control, # – $p<0.05$ vs PI splenocytes co-cultured with 15,000 BM-MSCs, α – $p<0.05$ vs PI splenocytes co-cultured with BM-MSCs from adult mice appropriate group. (B, C, D) Phase-contrast micrographs of (B) splenocytes, (C) splenocytes + PHA, (D) splenocytes + PHA + BM-MSCs. Magnifications, x100.

changes in the biological properties of these cells are of a particular interest. Aging is associated with changes in the number of bone marrow MSCs, their differentiating potential and synthesized by them the spectrum of cytokines [14,35]. Such alterations can result in reducing the functional capabilities of the organism during aging, development of age-related diseases, decrease in the proliferative and differentiating potential of MSCs. MSCs are excellent candidates of cell therapy and tissue engineering in preclinical and clinical studies [36] and may be effectiveness from old age patients.

The present study was shown, age-associated elevated ability of the BM-MSCs to proliferation, decreasing ability in the osteogenic differentiation and immune inhibitory effect. Our findings may contribute to the knowledge about the biological properties of MSCs in aging that benefit their further application in regenerative medicine.

However, the exact molecular mechanisms of the age-related changes are not completely understood and require further studying.

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