*of the main characteristics of cortical processes on the content of phosphorus in the blood of cows is established. Thus, depending on the time of the year, the content of this element in the blood of cows of the SVR type of BOD is more by 6.6-15.7 % ($p < 0.001$) in accordance with the indices of SN cows and the weak type of VND.*

***Keywords: cows, types of the higher nervous system, Calcium, Phosphorus***

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## FUNCTIONAL ACTIVITY AND MORPHOLOGICAL PECULIARITIES OF MESENCHIMAL STEM CELLS DURING IN VITRO CULTIVATION CONDITIONS

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***Abstract.*** *The studies were conducted on 2-3-months-old males of C57BL/6 mice weighing 20-24 g. Obtaining and cultivating of mesenchimal stem cells (MSCs) were carried out in a sterile laminar box with compliance of conditions of asepsis and antiseptics. MSCs of the 2, 4, 7 and 12 passages were analyzed. Morphometric analysis was performed using a light microscopy. Morphometric parameters such as cell and nucleus area or nuclear-cytoplasmic ratio (NCR) were calculated using the Axiovision light microscope (Carl Zeiss, Germany) and ImageJ 1.45 software. Trypan blue dye used for investigation of the viability of MSC.*

*The morphological features of cells during cultivation changes: at first cells have a spindle-like shape with two long cytoplasmic processes, located bipolar. In later passages, cells have a significant number of cytoplasm processes, bipolar arrangement of processes changes to stellar. The NCR index of MSC significant decreases at the 4 passage by 12,9 % ($p \leq 0,05$), at the 7 passage - by 35,3 % ($p < 0,001$), at the 12 passage - by 76,6 % ($p < 0,001$) compared to the initial state. The proliferative activity of the MSC of the bone marrow during cultivation significantly dereases at the later passages. Cell resistance to apoptosis induced by cultivation in the serum-free medium is fairly high. The number of cells in the state of apoptosis was 14,0±1,74 at the 4 passage and was reliably increased at the 12 passage to 22,67±1,55 % ($p \leq 0,05$) during cultivation.*

***Keywords: mesenchimal stem cells, morphometric analysis, viability, apoptosis***

**Introduction.** The use of mesenchimal stem cells (MSCs) for therapeutic purposes attracts considerable attention from researchers in connection with a wide range of diseases of animals and humans, in the treatment of which they can be effectively used [13]. The mechanism of the effect of MSC in organism is not fully understood, but it is assumed that they modulate immune responses through a lot of mechanisms, engage in direct interaction with damaged cells, secrete paracrine factors that enter the intercellular fluid, blood, differentiates into cells of damaged tissues [6].

In order to obtain a sufficient number of cells for transplantation, in vitro cultivation of cells is used. During cultivation a significant number of factors affects cell culture. As result, in artificial conditions that are different from those in vivo, cells undergo a lot of mitosis. In addition, cultivation medium contains

reagents that can changes the biological properties of MSCs, and therefore their functions [3, 5].

It is known that the cell cycle of MSC during the early and late passages differs in the cell content in the phase of relative resting and proliferative pool. Studies show that the time of doubling the cell mass of MSC from bone marrow and human adipose tissue increases with the number of passages [10]. In addition, in the process of cultivation, the frequency of formation of colony forming units, secretion of the growth factors, which synthesize the cells themselves changes. It was also established that in vitro differentiated cells (in osteogenic, adipogenic, etc. directions) show different effects on the functional state of the recipient systems and organs in comparison with non-differentiated MSCs [2, 8, 12].

Some authors compared MSCs derived from different sources regarding morphology, the success rate of isolating MSCs, colony frequency, expansion potential, multiple differentiation capacity, and immune phenotype [10, 11]. No significant differences concerning the morphology and immune phenotype of the MSCs derived from these sources were obvious [1, 9]. Differences could be observed concerning the success rate of isolating MSCs, which was 100 % for BM and AT, but only 63% for UCB. The colony frequency was lowest in UCB, whereas it was highest in AT. However, UCB-MSCs could be cultured longest and showed the highest proliferation capacity, whereas BM-MSCs possessed the shortest culture period and the lowest proliferation capacity. Most strikingly, UCB-MSCs showed no adipogenic differentiation capacity, in contrast to BM- and AT-MSCs. Both UCB and AT are attractive alternatives to BM in isolating MSC: AT as it contains MSCs at the highest frequency and UCB as it seems to be expandable to higher numbers [4, 7, 8]..

MSCs from different sources (adipose tissue, bone marrow) on different passages cause a different recipient's immune response. In addition, each species of animal has its own biological characteristics, which also have their stem cells [9, 11].

The purpose of the work is to investigate the morphological peculiarities and functional activity (proliferative activity, nuclear\cytoplasm ratio (NCR), viability, Serum deprivation-induced apoptosis) of MSC of bone marrow of C57/Bl6 mices during in vitro cultivation conditions

**Materials and methods.** The studies were conducted on 2-3-months-old males of C57BL/6 mice weighing 20-24 g. All studies were conducted in accordance with the Rules of Good Laboratory Practice and Use of Experimental Animals and in accordance to Compliance with the Law of Ukraine "On the Protection of Animals from Cruel Treatment" and the "International European Convention on the Protection of Animals Used for Experimental and Other Scientific Purposes".

MSCs obtaining from bone marrow of mice.

Obtaining and cultivating of MSCs were carried out in a sterile laminar box with compliance of conditions of asepsis and antiseptics. The mice were euthanized, their femur, tibia and shoulder bones were removed, and washed three times with sterile phosphate buffer solution with the addition of 1 %

antibiotic-antimycotic solution (Sigma-Aldrich, USA). Bone marrow was washed out from the diaphyses of removed bones by using the Dulbecco's Modified Eagle's Medium (DMEM). Bone marrow aspirate was added to culture dishes filled with DMEM, 10-15 % of fetal bovine serum, 1 % of antibiotic-antimycotic solution (Sigma-Aldrich, USA) and cultured in a CO2 incubator at 37 0C and 5 % CO2. The culture medium was partially or completely changed by fresh medium every 3 days during cultivation. After formation of cells monolayer at 80-90 %, cells were removed with trypsin-ethylenediaminetetraacetic acid solution (EDTA), washed with phosphate buffer and placed in Petri dishes for cultivation. Passaging the cells provided a reduction of heterogeneity of cell culture and the development of biological material for transplantation [14].

MSCs of the 2, 4, 7 and 12 passages were analyzed.

Cells counting was performed using a light-optical microscope with an increase of 200 times in all squares and is calculated by the formula:

$$X = A \times 1000 / 0,9, \quad (1)$$

where $X$ – number of cells in 1 cm3;

$A$ – number of cells in all squares;

1000 – number of $mm^3$ in $cm^3$;

0.9 – the volume of the camera Goryaev in $mm^3$.

Calculation of the cell proliferation index was carried out according to the formula:

$$X = a/b, \quad (2)$$

where $a$ – the final concentration of the cell/$cm^3$;

$b$ – seeded cell concentration / $cm^3$.

Morphometric analysis was performed using a light microscopy. For this purpose, the cells were stained with hematoxylin and eosin dyes (Alfarus, Ukraine). Morphometric parameters such as cell and nucleus area or nuclear-cytoplasmic ratio (NCR) were calculated using the Axiovision light microscope (Carl Zeiss, Germany) and ImageJ 1.45 (National Institutes of Health, USA) software.

The viability of the bone marrow MSCs was assessed using trypan blue dye, which is unable to penetrate the cytoplasm of living cells (Shakhov VP et al., 2004). For this purpose, equal volumes of suspension of bone marrow MSCs and 0,16–0,20 % trypan blue, prepared in physiological solution, were mixed. The cells were incubated for 10 minutes at 37 ° C, and the percentage of uncolored nucleated cells from the total number of cell elements were counted in the Goriaev chamber.

Evaluation of the level of apoptosis of MSC caused by their cultivation in serum-free medium. MSC at 2, 4, 7 and 12 passages were seeded in a quantity of $2 \times 10^3$ cells in wells of a 96-well plate, and cultivated during 72 hours in a serum-free medium. Apoptotic cells were revealed by using a trypan blue dye. The method is based on the ability of inanimate cells to absorb the dye. The percentage of colored (dead) cells was calculated in the Goryaev chamber.

The statistical analysis of the obtained results was achieved by using Statistica 6.0 (StatSoft, USA) and OriginLab (OriginLab Corporation, USA) software. Normality of data distribution was determined by the Kolmogorov-Smirnov test. In order to assess the validity of the revealed changes, parametric (Student t-test for two-samples) and non-parametric (Mann-Whitney U-test for the independent groups) methods of variation statistics were used, the difference was significant at $p < 0.05$. The obtained results were presented as the mean ± SD (mean ± standard deviation).

**Results.** Cells at the second passage have a structure characteristic of fibroblasts. The form of cells is spindle-shaped with a small volume of the cytoplasm, which forms long thin processes (Fig. 1).

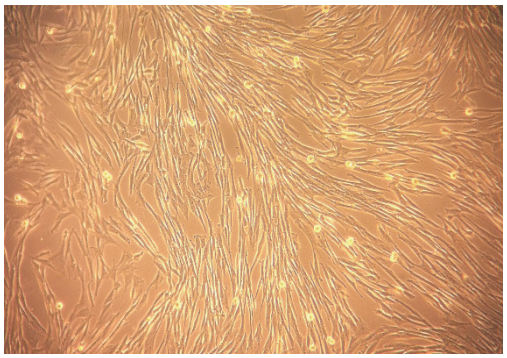

**Fig. 1 Monolayer of MSCs**

But among these cells there are cells with three cytoplasmic processes. The nucleus are elongated and contain a lot of heterochromatin. Near the nucleus in the area of enlightenment the Golgi complex are located, which is well developed in active cells (Fig. 2).

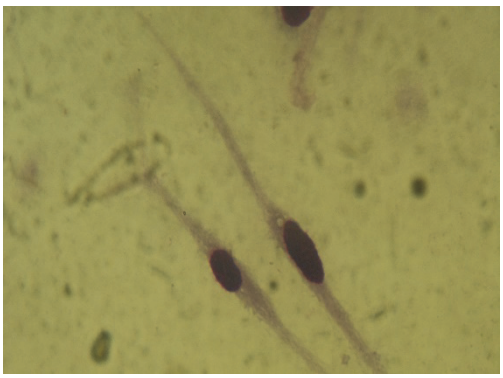

**Fig. 2. MSCs, 2-nd passage**

At the 4-th passage cells have morphology of fibroblasts. These cells are spindle-shaped, the area of the cell's cytoplasm increases and forms two or more processes, as a result of that some cells become satellite. Oval nucleus contains 1-2 nucleoli and chromatin. Near the nucleus, Golgi complex are determined as an oval enlightenment of the cytoplasm (Fig. 3.).

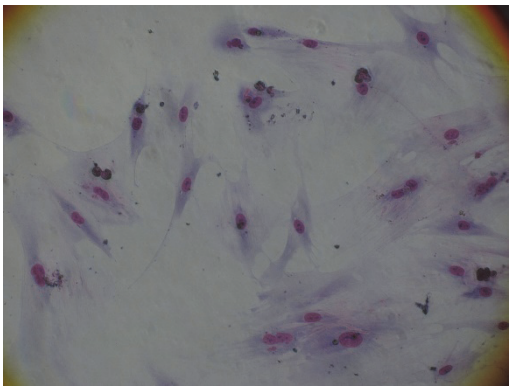

**Fig. 3. MSCs at the 4-th passage**

At the 7-th passage, the formation of a large number of cytoplasm processes is recorded. The size of cells significantly increases due to the cytoplasm area. In most cells the Golgi complex is located in the enlightened area. The oval nuclei have 1-2 nucleoli. According to an immunocytochemical study, a significant amount of actin is present in the cells (Fig. 4)..

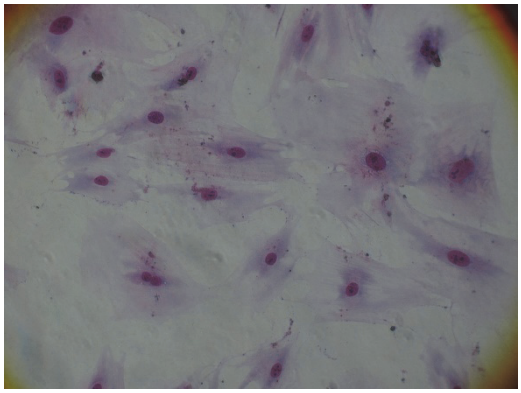

**Fig. 4. MSCs on the 7 passage: 1 – most of the cells have more than two processes, the area of the cell is enlarged due to cytoplasm, 2 – the mitosis**

At the 12 passage, a significant increase in cell area is observed, which is due to increase in the volume of the cytoplasm. Cytoplasm forms a large number of processes. Nuclei of the cells are rounded, have 1-2 nucleoli. Such cells spreads on the surface of the culture dishes (Fig. 5.).

The morphometric indices of the cells during cultivation does not remain stable. The area of cells does not change during the cultivation up to 7 passage, $\eta^2_\chi$ = 0,70 ($p$ < 0,05), but on 12 passage it is significantly lowered compared to 2 passage, $\eta^2_\chi$ = 0,80 ($p$ < 0,01) (Table 1). Such changes can relate with cell proliferation.

Unlike the nucleus, the area of MSC significantly increases at the 4 passage by 13,9 % ($p \leq 0,05$), on the 7 passage - by 32,6 % ($p$ < 0,001), on the 12 passage - by 291 % ($p$ < 0,001) compared to the initial state (2 passage). This, accordingly, leads to a significant decrease of the NCR index at the 4 passage ($p \leq 0,05$) - by 12,9 %, at the 7 passage - by 35,3 % ($p$ < 0,001), at the 12 passage - by 76,6 % ($p$ < 0,001) compared to the initial state. Consequently, the NCR index during cultivating of MSC is reduced due to an

increase of the area of the cell cytoplasm, which coincides with the morphological characteristics of MSCs at different passages.

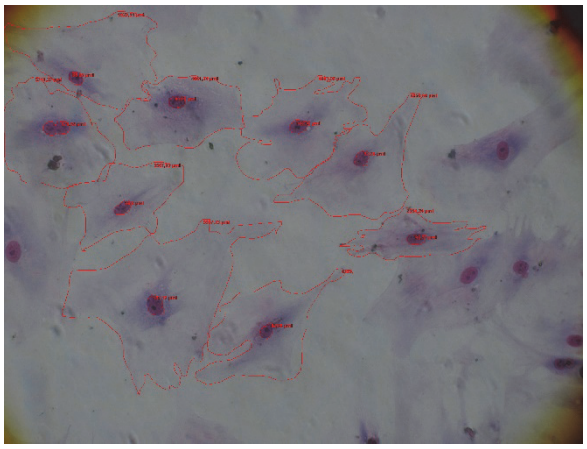

**Fig. 5. MSCs at the 12 passage with the indices of the square of the nucleus and the whole cells area**

**1. Morphological peculiarities and functional activity of MSC during in vitro cultivation conditions, *M ± SD***

| Parameters | Passages | | | |
|---|---|---|---|---|
| | 2 | 4 | 7 | 12 |
| Nucleus area ($\mu m^2$) | 154,44 ± 6,23 | 156,22 ± 4,42 | 142,44 ± 5,05 | 123,11 ± 10,507* |
| Cells area ($\mu m^2$) | 749 ± 21,16 | 853,78 ± 36,71* | 993,11 ± 36,17*** | 2304,40±280,12*** |
| NCR | 0,2598 ± 0,0068 | 0,2262 ± 0,0074** | 0,1682±0,0042*** | 0,0608±0,0066*** |
| Coefficient of proliferation | 2,86 ± 0,01 | 2,74 ± 0,3 | 2,31 ± 0,2 | 2,1 ± 0,2 |
| Viability (%) | 95,33 ± 1,55 | 96,33 ± 1,36 | 88,33 ± 1,94* | 86,33 ± 1,94* |
| Serum deprivation-induced apoptosis, % | 14,0 ± 1,74 | 19,0 ± 0,58 | 20,67 ± 1,55* | 22,67 ± 1,55 * |

*p ≤ 0.05, **- p < 0,01, ***- p < 0,001 in relation to 2-nd passage.

During cultivation of the primary material from the bone marrow unequal proliferative activity and rate of cell monolayer formation at different passages were recorded (Table 1). Formation of the monolayer depends on many soluble factors, in particular from those that synthesize cells themselves in the culture medium. During cultivation, the rate of formation of a monolayer decreases and, in our opinion, it is explained exactly by the reduction in the synthesis of soluble stimulating factors, which excretes by cells in the culture medium.

The viability of cells during cultivation reaches high rates, but with an increasing of a number of passages it is significantly reduces. This may be due to the biological aging of the cells and the influence of chemical reagents on the cells. The viability of MSC significantly decreases on the 7 passage, but

remains at a rather high level (89 ± 0,12 %, $p \leq 0,05$). At the 12 passage viability was 87 ± 0,14 % ($p \leq 0,05$).

Cell resistance to apoptosis induced by cultivation in the serum-free medium is fairly high. The number of cells in the state of apoptosis was 11,4-18,2 % during cultivation.

Thus, during cultivation of MSC changes in cell morphology parameters manifestes in their functional state. In particular, the changes in cell morphology is accompanied by a decreasing of NCR during cultivation. As a result, the coefficient of cell proliferation decreases and the percentage of apoptotic cells that show sensitivity to cultivation in serum-free medium increases.

**Conclusions**

1. The morphological features of cells during cultivation changes: at first cells have a spindle-like shape with two long cytoplasmic processes, located bipolar. In later passages, cells have a significant number of cytoplasm processes, bipolar arrangement of processes changes to stellar.

2. The NCR index of MSC significant decreases at the 4 passage by 12,9 % ($p \leq 0,05$), at the 7 passage - by 35,3 % ($p < 0,001$), at the 12 passage - by 76,6 % ($p < 0,001$) compared to the initial state.

3. The proliferative activity of the MSC of the bone marrow during cultivation significantly dereases at the later passages.

4. Cell resistance to apoptosis induced by cultivation in the serum-free medium is fairly high. The number of cells in the state of apoptosis was 14,0±1,74 at the 4 passage and was reliably increased at the 12 passage to 22,67±1,55 % ($p \leq 0,05$) during cultivation.

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## ФУНКЦІОНАЛЬНА АКТИВНІСТЬ ТА МОРФОЛОГІЧНІ ОСОБЛИВОСТІ МЕЗЕНХІМАЛЬНИХ СТОВБУРОВИХ КЛІТИН ЗА УМОВ КУЛЬТИВУВАННЯ IN VITRO

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***Анотація.*** *Дослідження проводили на 2-3-місячних самцях мишей C57Bl/6 вагою 20-24 г. Обробку первинного матеріалу та усі маніпуляції з мезенхімальними стовбуровими клітинами проводили в ламінарному боксі в умовах асептики та антисептики. Досліджували мезенхімальні стовбурові клітини 2-, 4-, 7- і 12- пасажів. Морфометричний аналіз проводили за допомогою світлового мікроскопа Axiovision (Carl Zeiss, Німеччина) та програмного забезпечення ImageJ 1.45 та обчислювали ядерно-цитоплазматичне відношення (NCR).*

*Морфологічні ознаки клітин в процесі культивування змінюються: на перших пасажах культивування клітина має веретеноподібну форму з двома довгими виростками цитоплазми, розташованими біполярно. На пізніх пасажах реєструється утворення значної кількості виростів*

*цитоплазми меншої довжини, біполярне розташування виростів змінюється на зірчасте. Показник ядерноцитоплазмового співвідношення мезенхімальних стовбурових клітин достовірно знижується на 12,9 ($p < 0.05$) на 4 пасажі, 35,3 ($p < 0,001$ на 7 пасажі, та 76,6 % ($p < 0,001$) на 12 пасажі порівняно з другим пасажем.*

***Ключові слова: мезенхімальні стовбурові клітини, морфометричні показники, ядерно-цитоплазмове співідношеня, життєздатність, апоптоз***

## ФУНКЦИОНАЛЬНАЯ АКТИВНОСТЬ И МОРФОЛОГИЧЕСКИЕ ОСОБЕННОСТИ МЕЗЕНХИМАЛЬНЫХ СТВОЛОВЫХ КЛЕТОК В УСЛОВИЯХ КУЛЬТИВИРОВАНИЯ IN VITRO

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***Аннотация****. Исследования проводили на 2-3-месячных самцах мышей C57Bl/6 весом 20-24 г. Обработку первичного материала и все манипуляции с мезенхимальными стволовыми клетками проводили в ламинарном боксе в условиях асептики и антисептики. Исследовали мезенхимальные стволовые клетки 2-, 4-, 7- и 12 пассажей. Морфометрический анализ проводили с помощью светового микроскопа Axiovision (Carl Zeiss, Германия), программного обеспечения ImageJ 1.45 и вычисляли ядерно-цитоплазматическое соотношение .*

*Морфологические признаки клеток в процессе культивирования меняются: на первых пассажах культивирования клетки имеют веретенообразную форму с двумя длинными отростками цитоплазмы, расположенными биполярно. Меньшее количество клеток имеет три отростка. На поздних пассажах регистрируется образование значительного количества отростков цитоплазмы небольшого размера. Биполярное расположение отростков меняется на звездчатое. Показатель ядерноцитоплазмового соотношения мезенхимальных стволовых клеток достоверно снижается на 12,9 ($p < 0.05$) на 4-м пассаже, 35,3 ($p < 0,001$) на 7-м пассаже, и на 76,6% ($p < 0,001$) на 12-м пассаже по сравнению со вторым пассажем.*

***Ключевые слова: мезенхимальные стволовые клетки, морфометрические показатели, ядерно-цитоплазмовое соостношение, жизнеспособность, апоптоз***