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BIOLOGICAL PECULIARITIES OF ADIPOSE TISSUE-DERIVED MESENCHYMAL STEM CELLS AT DIFFERENT PASSAGES OF CULTIVATION

L. V. KLADNYTSKA[1],
Doctor of Veterinary Sciences, Associate Professor
Department of Biochemistry
and Physiology of Animals named after Academician M. F. Gulyi
https://orcid.org/0000-0002-9360-0587
E-mail: kladlarisa@ukr.net
A. Y. MAZURKEVYCH[1],
Doctor of Veterinary Sciences, Professor
Department of Surgery and Pathophysiology
named after Academician I. O. Povazhenko
https://orcid.org/0000-0002-2409-7703
S. V. VELYCHKO[2],
Candidate of Biological Sciences
https://orcid.org/0000-0003-1842-5114
L. V. GARMANCHUK[3],
Doctor of Biological Sciences, Professor
https://orcid.org/0000-0002-1527-2346
M. O. MALYUK[1],
Doctor of Veterinary Sciences, Associate Professor
Department of Surgery
and Pathophysiology named after Academician I. O. Povazhenko
https://orcid.org/0000-0003-3019-6035
T. A. MAZURKEVYCH[1],
Doctor of Veterinary Sciences, Associate Professor
Academician V. G. Kasyanenko Department of Anatomy,
Histology and Pathomorphology
https://orcid.org/0000-0002-1294-5939
V. V. KOVPAK[1],
Doctor of Veterinary Sciences, Associate Professor
Gynecology and Biotechnology of Animal Reproduction
https://orcid.org/0000-0003-2419-1246
E-mail:kovpak8887@gmail.com
T. V. KOZYTSKA[4],
Candidate of Veterinary Sciences, Associate Professor
https://orcid.org/ 0000-0002-7554-0808

Yu. O. KHARKEVYCH[1],
Candidate of Veterinary Sciences, Associate Professor
Department of Surgery and Pathophysiology named after Academician I. O. Povazhenko
https://orcid.org/0000-0002-7877-8272
R. R. BOKOTKO[1],
Candidate of Veterinary Sciences, Associate Professor
Department of Surgery and Pathophysiology named after Academician I. O. Povazhenko
https://orcid.org/0000-0002-6217-5266
T. L. SAVCHUK[1],
Candidate of Veterinary Sciences, Associate Professor
Department of Surgery and Pathophysiology named after Academician I. O. Povazhenko
https://orcid.org/0000-0002-7351-5684
E-mail: kladlarisa@ukr.net
[1]National University of Life and Environmental Sciences of Ukraine,
15 Heroyiv Oborony st., Kyiv 03041, Ukraine
[2]Hospital of Veterinary Medicine, Holosiivskyi Avenue, 105 b, Kyiv 03127, Ukraine
[3]Taras Shevchenko National University of Kyiv, Educational and Scientific
Center Institute of Biology and Medicine, 2 Hlushkova Avenue, Kyiv 03127, Ukraine
[4]Bogomolets National Medical University, 13 Shevchenko blvr., Kyiv 01601, Ukraine

Abstract. *The studies were conducted on 2–3-months-old males of C57BL/6 mice weighing 20–24 g. Obtaining and operating with adipose tissue-derived mesenchymal stem cell (MSC) culture was performed in a sterile laminar box under conditions of asepsis and antiseptics. The adipose tissue-derived MSC 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 were calculated using the Axiovision light microscope (Carl Zeiss, Germany) and Image J 1.45 software. Trypan blue dye used for investigation of the viability of MSC.*

The morphological characteristics of adipose tissue-derived MSC during the process of cultivation changes: at the first passages of cultivation, the cells are spindle-shaped with two, at least three, long cytoplasmic processes, which are located bipolar. Near the nucleus, the Golgi complex is clearly visible – a sign of active cells. At later passages, cells have a small cytoplasmic processes and the bipolar arrangement of processes changes by stellar arrangement. Golgi complex is also clearly visualized. The indicator of the nuclear-cytoplasmic ratio in MSC from adipose tissue is significantly reduced at the 7th passage to 0.2189 ± 0.0122 ($P < 0.01$), and at the 12th passage to 0.1111 ± 0.0086 ($P < 0.001$) compared to the 2nd passage. The coefficient of proliferation of adipose tissue-derived MSC is significantly reduced at 12th passage. The viability of MSC from adipose tissue with an increasing of a number of passages significantly reduces and at the 12th passage of cultivation reaches 84.67 ± 1.36 ($P < 0.05$). The content of apoptotic cells that exhibited sensitivity to serum-free cultivation significantly increased at the 7th and 12th passages and was 21.33 ± 1.36 ($P < 0.05$) and 23.67 ± 0.97% ($P < 0.05$), respectively.

Keywords: *adipose tissue mesenchymal stem cells, nucleus, nuclear-cytoplasmic ratio, coefficient of proliferation, viability, apoptosis, early passages, late passages*

Introduction

It is known that mesenchymal stem cells (MSC) in the bone marrow are from 0.001% to 0.01% of the total fraction of mononuclear cells, and bone marrow aspiration is an invasive procedure and has a significant effect on the donor after the surgical period (Dmitrieva et al., 2012). Therefore, other sources of stem cells, in particular, umbilical cord blood and placenta, are used in modern human and veterinary medicine (Ning et al., 2012). The adipose tissue is also an excellent alternative source of MSC, since it contains approximately 500 times more MSC in compare with bone marrow. It should be noted that the process of obtaining of adipose tissue is quite simple and does not harm the body. Some data are already known about biological properties of adipose-derived MSC (AD-MSC). In particular, it is known about high differential potential of MSC from adipose tissue in animals of different species, their immunomodulatory property. Some authors emphasize that they exhibit stronger immunomodulatory effects, due to the fact that they are characterized by a higher level of cytokine secretion (Arnhold & Wenisch, 2015).

Analysis of recent researches and publications

Certain specific morphological features of stem cells, which were determined by light microscopy, are known. In particular, it was found that MSC with high proliferative activity were thick, and those that had low proliferative activity were thin, even if these MSC were cells of early passages. The diameter of the nucleus of MSC from the dog and the horse is determined. Also the individual morphological parameters of the feline MSC in early passages were investigated (Grzesiak et al., 2011; Maciel et al., 2014). It was investigated that MSC from the umbilical cord at the 15th passage age due to the decrease in metabolism and proliferation activity (Katsube et al., 2008).

There are reports about some morphological characteristics of stem cells. In the study of MSC from horses and dogs, it was found that in the first passages of cultivation, mitochondries are localized pericentrically and there are endosomal vesicles in the cell cytoplasm (Otsu et al., 2009). They were mostly found in the perinuclear zone, but before cell proliferation they moved to the opposite pole. In the non-proliferation cells, there were endoplasmic vesicles that moved intensively inside the cell. It was found that the cultivation process affects the change of morphological parameters of stem cells. In particular, it was investigated that MSC from dogs and horses changed their length and diameter, as evidenced by the authors of the publication. According to these studies, stem cells at the 19–20th passages changed their adhesive properties in the direction of reduction, were more flattened and "took up more space". The number of cells decreased to 1.9 × 106 per cup, indicating a change in their proliferative activity. Their functional state also changed, which was confirmed by a decrease in viability to 56% (Grzesiak et al., 2011; Otsu et al., 2009).

A team of other researchers found that stem cells from the same culture had morphological and functional differences. In particular, in stem cell culture, there are cells of different thickness, and the proliferative activity of stem cells depends on their thickness. Thicker cells had higher proliferative activity. Cells,

being small in thickness, expressed genes associated with cell aging and had low proliferative activity (Nagano et al., 2019).

Parallel studies indicate that the morphological parameters of MSC in different passages of cultivation have significant differences. It was found that MSC from feline bone marrow had a spindle-shaped shape, a significant amount of cytoplasm, a length of 106.97 ± 38.16 and 177.91 ± 71.61 μm in the first and third passages, respectively. The cell width was 30.79 ± 16.75 μm and 40.18 ± 20.46 μm in the first and third passages, respectively. The length of feline bone marrow MSC in the first passage was 16.28 μm after 24 hours of cultivation and 21.29 μm after 120 hours of cultivation, and in the third – significantly increased and amounted to 26.35 μm after 24 hours of cultivation and 25.22 μm after 120 hours of cultivation (Maciel et al., 2014).

Thus, the **purpose** of our work was to determine in vitro the morphological parameters and functional state of MSC from adipose tissue of C57Bl/6 mices during the early and late passages.

Materials and methods of research

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 BrutalCruel Treatment" and the "International European Convention on the Protection of Animals Used for Experimental and Other Scientific Purposes".

MSCs obtaining from mice adipose tissue of mice (Kladnytska et al., 2016). Obtaining and processingprocessing of adipose tissue were carried out in a sterile laminar box with compliance of conditions of asepsis and antiseptics. The mice were euthanized, their adipose tissue removed, and washed three times with sterile phosphate buffer solution with the addition of 1% antibiotic-antimycotic solution (Sigma-Aldrich, USA). Adipose tissue 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 CO_2 incubator at 37 °C and 5% CO_2. 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 adipose derived mesenchymal stem cells (AD- MSCs) provided a reduction of heterogeneity of cell culture and the development of biological material for transplantation.

AD- MSCs of the 2nd, 4th, 7th, and 12th passages were analyzed.

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

$$X=A\times1000\div0.9 \quad (1)$$

X = A x 1000 / 0,9, where
X – number of cells in 1 cm^3;
A – number of cells in all squares;
1000 – number of mm^3 in cm^3;
0.9 – the volume of the Goriaev chamber camera Goryaev in mm^3.

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

$$X=a\div b \quad (2)$$

X = a/b, 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 Carazzi's hematoxylin Karatci 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 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 in MSC caused by their cultivation in serum-free medium. The MSC at the 2nd, 4rd, 7th and 12th passages were seeded in a quantity of 2×10^3 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 Goriaev 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 $P < 0.05$. The obtained results were presented as the mean ± SD mean error (mean M ± standard deviationm).

Results of the research and their discussion

The cells changed morphology during cultivation. Cells at the 2nd and 4th passages have pronounced morphology of fibroblasts with two or three long cytoplasm processes. Cell nucleus was bean-shaped. Near the nucleus in the zone of enlightenment, the Golgi complex is clearly defined, which is well developed in proliferative cells. A small number of cells with an oval cytoplasm and a round nucleus were recorded (Fig. 1).

At the later passages, the morphology of MSC from adipose tissue has changed. MSC had more processes, they had smaller length, the area of the cell, which was adhered to the culture plastic, was increased. A Golgi complex was registered near the nucleus, indicating that cell proliferation remain at the high level (Fig. 2).

This is confirmed by morphometric indices and functional activity of AD-MSC at different passages (Table 1).

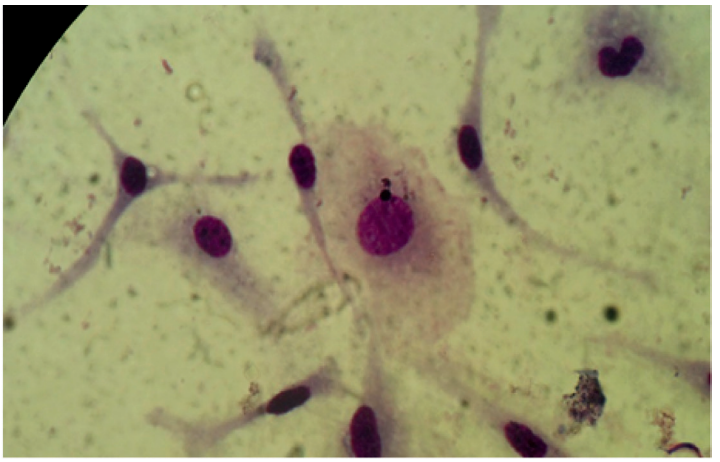

Fig. 1. Mesenchymal stem cells of mouse adipose tissue culture, 2nd passage. Painting according to Carazzi's hematoxylin and eosin. Micropreparation, × 900.

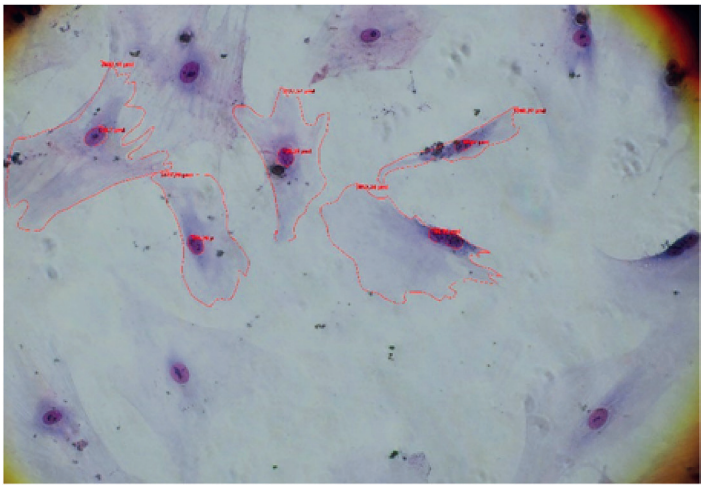

Fig. 2. Mesenchymal stem cells from mouse adipose tissue culture at the 12th passage of cultivation with the calculation of the nucleus area and cell area using the program Axio visine. Pappenheim staining. Micropreparation, × 320.

As can be seen from the Table 1, the area of the nucleus at the 2nd and 4th passages did not significantly changed. At the 7th passage, there can be seen a tendency to reduce the area of the nucleus. At the 12th passage, a significant decrease to 135.78 ± 11.21 μm^2 ($P < 0.05$) was recorded in the area of the nucleus compared to the MSC of the 2nd passage.

During the cultivation, the area of the cytoplasm has no significant differences at the 2nd and 4th passages, and a tendency to increase it can be seen at

1.The morphometric indices and functional activity of adipose-derived mesenchymal stem cells at different passages (M ± m, n = 5)

Parameters	Passages			
	2	4	7	12
Nucleus area (μm^2)	161.11 ± 5.65	161.56 ± 5.48	151.67 ± 3.51	135.78 ± 11.21*
Cells area (μm^2)	759.56 ± 28.42	748.11 ± 25.90	841.56 ± 46.96	1416.90 ± 151.97***
NCR	0.2689 ± 0.0046	0.2756 ± 0.0042	0.2189 ± 0.0122**	0.1111 ± 0.0086***
Coefficient of proliferation	2.92 ± 0.02	3.02 ± 0.03	2.79 ± 0.09	2.55 ± 0.01***
Viability (%)	96.33 ± 1.36	96.67 ± 0.97	93.67 ± 0.97	84.67 ± 1.36*
Serum deprivation-induced apoptosis, %	14.33 ± 1.94	18.67 ± 0.77	21.33 ± 1.36*	23.67 ± 0.97 *

Note: * P < 0.05, ** P < 0.01, *** P < 0.001 in comparison with the 2nd passage.

the 7th passage. At later passage, a significant increase in the area of the cell to 1416.90 ± 151.97 μm^2 (P < 0.001) was recorded.

During the cultivating, the primary material from adipose tissue, unequal proliferative activity of MSC and the rate of the monolayer formation at different passages were registered (Table 1). The monolayer formation depends on many soluble factors, in particular, from those that synthesize cells themselves in the culture medium. Since the primary material was processed with new technique, it can be assumed that a significant amount of cytokines and growth factors have been introduced into the culture medium from adipose tissue, which cause a high rate of proliferation. This coincides with the studies of many authors, which emphasize the fact that adipose tissue contain a lot of soluble growth factors.

During cultivation, the rate of a monolayer formation decreases and, in our opinion, it is explained exactly by the reduction in the synthesis of soluble stimulating factors, which are excreted by cells in the culture medium. The coefficient of proliferation with the increase of a number of passages decreases, and at the 12th passage, this indicator is significantly lower in compare with the 2nd passage (P < 0.001), although it remains high (2.55 ± 0.01).

The viability of MSC from adipose tissue during cultivation reaches high rates, but with an increase in a number of passages, it significantly reduces. At the 12th passage of cultivation, viability reaches 84.67 ± 1.36% (P < 0.05), but remains high. In our opinion, this may be due to the biological aging of the cells and the influence of chemical reagents on them.

Cell resistance to apoptosis induced by cultivation in the serum-free medium decreases with the increase of a number of passages. A significant increase in the number of apoptotic cells has been registered at the 7th passage – 21.33 ± 1.36 (P < 0.05), and at the 12th passage their number has increased to 23.67 ± 0.97% (P < 0.05).

Thus, during the cultivation of MSC from adipose tissue, there are significant changes in morphological parameters of cells, which are reflected in their func-

tional state. In particular, the changes in cell morphology are accompanied by a decrease in the nuclear-cytoplasmic ratio by increasing the area of the cytoplasm. Also, a significant decrease in the cell proliferation coefficient and the viability of MSC at later cultivation passages were determined. The content of apoptotic cells that exhibited sensitivity to cultivation in serum-free medium was significantly increased at the 7th and 12th passages.

Considerable attention was paid to the morphology of MSC. It is known that today there are two main morphological forms of MSC: small, round or spindle-shaped, rapidly proliferating cells and large spreading cells (mature, slowly proliferating cells). That is, differences were observed depending on the rate of MSC proliferation (Grzesiak et al., 2011; Cheng et al., 2012). At the same time, such patterns were inherent in MSC of different origin and no significant differences depending on the species and tissue origin of MSC were found (Martin et al., 2002; Webb et al., 2012).

Several researches have been conducted to study age-related changes in the morphology and functions of human MSC both in vivo and in vitro. Most authors focused on the influence of donor age on the morphofunctional properties of MSC. It was found that MSC from older donors have significantly worse characteristics, including proliferative activity, rate of population doubling, the efficiency of colony formation and the ability to differentiate (Baxter et al., 2004; Miyoshi, 2016; Yang et al., 2018; Yamazaki et al., 2020).

In the cultivation of MSCs of various origins in the second passage, the cells adapted to in vitro conditions, formed a monolayer at the bottom of the culture vessel, dead cells and cells that did not belong to the mesenchymal stems were eliminated. The shape of the vast majority of cells was spindle-shaped with a small volume of cytoplasm, which formed long thin outgrowths. During further cultivation, the morphology of the cells changed in the direction of increasing cell size and cytoplasm size, the appearance of spread cells with a large number of cytoplasmic outgrowths. This coincides with the data of the vast majority of researchers. Thus, Maciel et al. (2014) when culturing MSC from feline bone marrow when comparing cells of the 1st and 3rd passages, an increase in length from 106.97 ± 38.16 μm to 177.91 ± 71.61 μm and width from 30.79 ± 16.7 μm to 40.18 ± 20.48 μm.

Morphofunctional properties of MSC from bone marrow and adipose tissue cultures at different passages of cultivation in combination with other obtained data testified in favor of their gradual replication aging. It was manifested by an increase in cytoplasmic volume $r = 0.73$ ($P = 0.01$); decrease in the nuclear-cytoplasmic ratio $r = -0.87$; ($P = 0.001$); increasing the number of growths of the cytoplasm; decrease in the proliferation coefficient ($P < 0.01$) and cell viability ($r = -0.70$; $P < 0.05$); gradual decrease in the number of cells in the phases G2/M and S of the cell cycle ($P < 0.001$); increased sensitivity to apoptosis induced by the absence of growth factors $r = 0.81$; ($P = 0.001$); increasing the level of secretion of IL-6 ($P < 0.01$). The number of diploid cells decreased, although in all cases it was not less than 95%. It is known that significant MSC aneuploidy is associated with an increased risk of malignant transformation (Ben-David et al., 2018; Saalbach & Anderegg, 2019). The data obtained in the complex indicate in fa-

vor of replicative aging of cultures and determine the feasibility of using in subsequent experiments MSC cultures from adipose tissue and bone marrow in the early stages of cultivation (Kladnytska et al., 2020). Similar results were obtained by Yang et al. (2018) in the study of morphological features and proliferative activity of bone marrow MSC at the 4th and 8th passages of cultivation. The problem of replicative aging is an important obstacle to the application of MSC of late cultivation passages. Therefore, there are attempts to delay the development of this process (Yang et al., 2018). For example, Heo et al. (2016) indicate a significant delay in the aging of MSC cultures when cultured on plates coated with poly-L-lysine, other authors – when neutralizing IL-6 (O'Hagan-Wong et al., 2016).

Conclusions and future perspectives

1. The morphological characteristics of mesenchymal stem cells from adipose tissue during the process of cultivation changed: the cells at the first passages of cultivation are spindle-shaped with two, at least three, long cytoplasmic processes, located bipolar. Near the nucleu,s the Golgi complex is clearly visible – a sign of active cells. At later passages, cells have a small cytoplasmic processes and the bipolar arrangement of processes changes by stellar arrangement. The Golgi complex is also clearly visualized.

2. The indicator of the nuclear-cytoplasmic ratio in MSC from adipose tissue is significantly reduced to 0.2189 ± 0.0122 ($P < 0.01$) at the 7th passage and at the 12th passage to 0.1111 ± 0.0086 ($P < 0.001$) compared to the 2nd passage.

3. The coefficient of proliferation of MSC from adipose tissue is significantly reduced at the 12th passage.

4. The viability of mesenchymal stem cells from adipose tissue with an increasing of a number of passages significantly reduces and at the 12th passage of cultivation reaches 84.67 ± 1.36 ($P < 0.05$).

5. The content of apoptotic cells that exhibited sensitivity to serum-free medium significantly increased at the 7th and 12th passages and was 21.33 ± 1.36 ($P < 0.05$) and $23.67 \pm 0.97\%$ ($P < 0.05$), respectively.

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Анотація. *Дослідження проводили на самцях мишей C57BL/6 вагою 20–24 г віком 2–3 місяці. Обробку первинного матеріалу та роботу з мезенхімальними стовбуровими клітинами культури жирової тканини проводили в стерильному ламінарному боксі з дотриманням умов асептики та антисептики. Культивування проводили у CO_2 інкубаторі за температури 37 °С та вмісту CO_2 5%. Проводили дослідження мезенхімальних стовбурових клітин культури жирової тканини 2, 4, 7 та 12 пасажів. Морфометричний аналіз проводили за допомогою світлової мікроскопії. Морфометричні параметри, такі, як площа клітини, ядра визначали за допомогою світлового мікроскопа Axiovision (Carl Zeiss, Німеччина) та програмного забезпечення ImageJ 1.45. Для прижиттєвого фарбування мезенхімальних стовбурових клітин використовували барвник трипановий синій для дослідження їхньої життєздатності.*

Морфологічна характеристика мезенхімальних стовбурових клітин культури жирової тканини в процесі культивування змінюється: на перших пасажах культивування клітини мають веретеноподібну форму з двома, щонайменше трьома довгими цитоплазматичними відростками, розташованими біполярно. Біля ядра добре видно комплекс Гольджі – ознака активних клітин. За пізніх пасажів клітини мають велику кількість коротких цитоплазматичних відростків із зірчастим розташуванням. У них також чітко візуалізується комплекс Гольджі. Показник ядерно-цитоплазматичного співвідношення в мезенхімальних стовбурових клітинах культури жирової тканини значно знижується на 7 пасажі до 0,2189 ± 0,0122 ($P < 0,01$), а на 12 пасажі – до 0,1111 ± 0,0086 ($P < 0,001$) порівнюючи з 2 пасажем. Коефіцієнт проліферації мезенхімальних стовбурових клітин із жирової тканини значно знижується на 12 пасажі до 2,55 ($P < 0,001$). Життєздатність мезенхімальних стовбурових клітин із жирової тканини за пізніх пасажів культивування значно знижується й на 12 пасажі культивування досягає 84,67 ± 1,36 ($P < 0,05$). Вміст апоптотичних клітин, які виявляли чутливість до безсироваткового культивування, достовірно збільшувався на 7 та 12 пасажах і становив відповідно 21,33 ± 1,36 ($P < 0,05$) та 23,67 ± 0,97% ($P < 0,05$).

Ключові слова: *мезенхімальні стовбурові клітини культури жирової тканини, ядерно-цитоплазматичне співвідношення, коефіцієнт проліферації, життєздатність, апоптоз, ранні пасажи, пізні пасажи*