Науковий вісник Львівського національного університету
ветеринарної медицини та біотехнологій імені С.З. Гжицького

Scientific Messenger of Lviv National University
of Veterinary Medicine and Biotechnologies

ISSN 2518–7554 print
ISSN 2518–1327 online

doi: 10.32718/nvlvet9216
<http://nvlvet.com.ua>

UDC 612.014: 576.3:602.018.26.9

Morphological peculiarities and functional activity of adipose-derived mesenchymal stem cells during in vitro cultivation conditions

L.V. Kladnytska¹, A.I. Mazurkevych¹, V.T. Khomych¹, T.A. Mazurkevych¹, Z.G. Stegney¹, M.O. Maluk¹,
L.V. Garmanchuk², S.V. Velychko³, V.B. Danilov¹, Y.O. Kharkevych¹, D.V. Shelest², V.S. Velychko¹,
I.A. Stupak²

¹National University of Life and Environmental Sciences of Ukraine, Kyiv, Ukraine

²National Taras Shevchenko University, Educational and Scientific Center “Institute of Biology and Medicine”,
Kyiv, Ukraine

³Hospital of Veterinary Medicine, Kyiv, Ukraine

Article info

Received 19.10.2018

Received in revised form
19.11.2018

Accepted 20.11.2018

National University of Life and
Environmental Science of Ukraine,
Heroiv Oborony Str., 15,
Kyiv, 03041, Ukraine.
Tel.: +38-063-186-62-33
E-mail: Kladlarisa@ukr.net

National Taras Shevchenko
University, Educational and
Scientific Center “Institute of
Biology and Medicine”, Hlushkova
Avenue, 2, Kyiv, 03127, Ukraine.

Hospital of Veterinary Medicine,
Holosiivskyi Avenue, 105 b, Kyiv
03127, Ukraine.

Kladnytska, L.V., Mazurkevych, A.I., Khomych, V.T., Mazurkevych, T.A., Stegney, Z.G., Maluk, M.O., Garmanchuk, L.V., Velychko, S.V., Danilov, V.B., Kharkevych, Y.O., Shelest, D.V., Velychko, V.S., & Stupak, I.A. (2018). Morphological peculiarities and functional activity of adipose-derived mesenchymal stem cells during in vitro cultivation conditions. Scientific Messenger of Lviv National University of Veterinary Medicine and Biotechnologies, 20(92), 79–82. doi: 10.32718/nvlvet9216

The studies were conducted on 2-3-months-old males of C57BL/6 mice weighing 20–24 g. Obtaining and cultivating of adipose-derived mesenchymal stem cells (AD MSCs) were carried out in a sterile laminar box with compliance of conditions of asepsis and antiseptics. AD 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 characteristics of mesenchymal stem cells from adipose tissue during the process of cultivation changes: at the first passages of cultivation, the cells are spindle-shaped with two, at least three, long long cytoplasmic processes, 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 7 passage to 0.2189 ± 0.0122 ($P < 0.01$), and at 12 passage to 0.1111 ± 0.0086 ($P < 0.001$) compared to the 2 passage. The coefficient of proliferation of MSC from adipose tissue is significantly reduced at 12th passage. 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 ($P < 0.05$). The content of apoptotic cells that exhibited sensitivity to serum-free significantly increased at 7 and 12 passages and was respectively 21.33 ± 1.36 ($P < 0.05$) and 23.67 ± 0.97 ($P < 0.05$).

Key words: *Abrus precatorius*, *Vaccine*, *Immunoinhibition*, *Canine parvovirus*.

Introduction

It is known that mesenchymal stem cells in the bone marrow make up 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, placenta, are used in modern medicine and veterinary medicine (Ning et al., 2012). The fatty tissue is also an excellent alternative source of

mesenchymal stem cells, since it contains approximately 500 times more MSC compare to 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 (aMSC). In particular, it is known about high differential potential of MSC from fatty tissue of 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 secretion of cytokines (Arnhold and Wenisch, 2015).

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

Thus, the purpose of our work was to determine in vitro the morphological parameters and functional state of mesenchymal stem cells from adipose tissue of C57/Bl6 mice during the early and late passages.

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 adipose tissue of mice (Kladnytska et al., 2016). Obtaining and cultivating of adipose-derived MSCs (AD MSCs) 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 CO₂ incubator at 37 °C 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 (Katsube et al., 2008; Arnhold and Wenisch, 2015).

AD 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:

$$X = A \times 1000 / 0.9, \text{ where}$$

X – number of cells in 1 cm³;

A – number of cells in all squares;

1000 – number of mm³ in cm³;

0.9 – the volume of the camera Goryaev in mm³.

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

$$X = a/b, \text{ where}$$

a – the final concentration of the cell/cm³;

b – seeded cell concentration/cm³

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 Goryaev 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 2×10^3 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 $\pm$ SD (mean $\pm$ standard deviation).

Results and discussion

During cultivation the cells changes morphology. Cells at 2 and 4 passages have pronounced morphology of fibroblasts with two or three long cytoplasm processes. Cells nucleus were of the boboidal form. Near the nucleus in zone of enlightenment the Golgi complex is clearly defined, which is well developed in active forms of 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 mesenchymal stem cells of 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 adipose derived mesenchymal stem cells at different passages (table 1).

Fig. 1. adipose-derived MSCs, early passages

Fig. 2. Adipose-derived MSCs at the late passages with the indices of the square of the nucleus and the whole cells area

Table 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*

* – P < 0.05, ** – P < 0.01, *** – P < 0.001 in relation to 2-nd passage

As can be seen from the table, the area of the nucleus at the 2nd and 4th passages has not significantly changed. At the 7th passage can be seen a tendency to reduce the area of the nucleus. At the 12th passage was recorded a significant decrease in the area of the nucleus to $135.78 \pm 11.21 \mu\text{m}^2$ ($P \leq 0.05$) compared to the MSC of 2nd passage.

During the cultivation, the area of the cytoplasm at the 2nd and 4th passages has no significant differences, and at the 7th passage can be seen a tendency to increase it. At later passage was recorded a significant increase in cell area to $1416.90 \pm 151.97 \mu\text{m}^2$ ($P < 0.001$).

During the cultivating the primary material from adipose tissue, unequal proliferative activity of mesenchymal stem cells and the rate of formation of the monolayer at different passages were registered (Table 1). Formation of the monolayer 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 soluble growth factors.

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 coefficient of proliferation with the increase of a number of passages decreases and at the 12 passage

this indicator is significantly lower compare to 2 passage ($P < 0.001$), although it remains high (2.55 ± 0.01).

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

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 $23.67 \pm 0.97\%$ ($P < 0.05$).

Thus, during cultivation of MSCs from adipose tissue, there are significant changes in morphological parameters of cells, which is reflected in their functional state. In particular, the changes in cell morphology is 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 MSCs at later cultivation passes 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.

Conclusions

1. The morphological characteristics of mesenchymal stem cells from adipose tissue during the process of cultivation changes: at the first passages of cultivation,

the cells are spindle-shaped with two, at least three, long long cytoplasmic processes, 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.

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

3. The coefficient of proliferation of MSC from adipose tissue is significantly reduced at 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 significantly increased at 7 and 12 passages and was respectively 21.33 ± 1.36 ($P < 0.05$) and $23.67 \pm 0.97\%$ ($P < 0.05$).

References

- Arnhold, S., & Wenisch, S. (2015). Adipose tissue derived mesenchymal stem cells for musculoskeletal repair in veterinary medicine. *Am. J. Stem Cells.*, 4(1), 1–12. <https://www.ncbi.nlm.nih.gov/pubmed/25973326>.
- Dmitrieva, R.I., Minullina, I.R., Bilibina, A.A., Tarasova, O.V., Anisimov, S.V., & Zaritskey, A.Y. (2012). Bone marrow- and subcutaneous adipose tissue-derived mesenchymal stem cells Differences and similarities. *Cell Cycle*, 11(2), 377–383. doi: 10.4161/cc.11.2.18858. <https://www.ncbi.nlm.nih.gov/pubmed/22189711>.
- Grzesiak, J., Marycz, K., Czogala, J., Wrzeszcz, K., & Nicpon, J. (2011). Comparison of behavior, morphology and morphometry of equine and canine adipose derived mesenchymal stem cells in culture. *Int. J. Morphol.*, 29(3), 1012–1017. doi: 10.4067/S0717-95022011000300059.
- Katsube, Y., Hirose, M., Nakamura, C., & Ohgushi, H. (2008). Correlation between proliferative activity and cellular thickness of human mesenchymal stem cells. *Biochem Biophys Res Commun.*, 368(2), 256–260. doi: 10.1016/j.bbrc.2008.01.051.
- Maciel, B.B., Rebelatto C.L.K., Brofman P.R.S., Brito H.F.V., Patricio L.F.L. et al. (2014). Morphology and morphometry of feline bone marrow-derived mesenchymal stem cells in culture. *Cruz and Rosangela Locatelli-Dittrich. Pesq. Vet. Bras.*, 34(11), 1127–1134. doi: 10.1590/S0100-736X2014001100016.
- Ning, X., Li, D., Wang, D.K., Fu, J.Q., & Ju, X.L. (2012). Changes of biological characteristics and gene expression profile of umbilical cord mesenchymal stem cells during senescence in culture. *Zhongguo Shi Yan Xue Ye Xue Za Zhi.*, 20(2), 458–465. <https://www.ncbi.nlm.nih.gov/pubmed/22541119>.
- Kladnytska, L.V., Mazurkevych, A.I., & Velychko, S.V. (2016). Patent Ukrainy na korysnu model №109148. Sposib otrymannia mezenkhimalnykh stovburovykh klityn iz zhyrovoi tkanyny sobaky; zaiavnyk i vlasnyk Natsionalnyi universytet bioresursiv i pryrodokorystuvannia Ukrainy. № u201602329; zaiavl. 11.03.2016; opubl. 10.08.2016, biul. №15 (in Ukrainian).