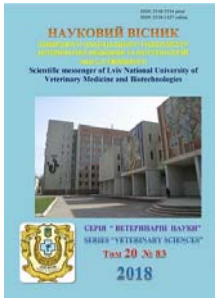

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The system influence of allogeneic adipose tissue derived mesenchymal stem cells on the functional state of immune organs

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The studies were conducted on 2–3-months-old males of mice weighing 20–24 g. Our work was to study the functional state of the organs of the immune system of C57Bl/6 mice after introduction of allogeneic MSCs of adipose tissue origin. Obtaining and cultivating of MSCs were carried out in a sterile laminar box with compliance of conditions of asepsis and antiseptics. C57Bl/6 mice adipose tissue cultured in a CO_2 incubator at 37 °C and 5% CO_2 in DMEM with 10–15% of fetal bovine serum, 1% of antibiotic-antimycotic solution (Sigma-Aldrich, USA). The following groups of animals were formed: 1 group – intact (control group); 2 group – animals, to whom 0.5 ml of 0.9% NaCl solution (placebo) were injected into the caudal vein; 3 group – animals, to whom were injected 10^4 of allogeneic MSCs from adipose tissue in 0.5 ml of phosphate buffer solution into the caudal vein. The weight index, content of lymphoid cells of thymus and spleen in C57Bl/6 mice investigated after the introduction of MSCs on 7, 18 and 25 days. To assess the content of lymphocytes in lymphoid organs, the latter were weighed. Whole thymus and 50 mg of spleen were triturated and filtered through the kapron tissue. After that, the tissue homogenate was applied to the gradient of ficoll-urografin (density 1.077) in a ratio of 3:2. The test tubes were centrifuged at a rate of 1500 rpm for 30-40 minutes. After centrifugation the layer of lymphocytes which was above the gradient was collected by a Pasteur pipette and washed twice with an arbitrary amount of Hanks' solution by centrifugation at a rate of 1500 for 10 minutes. 1 ml of Hanks's solution was added to lymphocytes after washing. Lymphocytes were counted in the Goryaev chamber. Calculation of the cells of lymphoid organs was performed on 1 mg of tissue. The administration of allogenic adipose derived mesenchymal stem cells affects on the central and peripheral organs of the immune system. Administration of allogenic adipose derived mesenchymal stem cells cause a significant increase in the content of lymphoid cells in the thymus at 7, 18 and 25 days by 71, 57 and 53% respectively ($P < 0.05$) compared to the control. Weight index of the thymus directly correlates with the content of lymphoid cells and its value was $r = 0.57$, $P < 0.01$ on 7 day and 18 day $r = 0.50$, $P < 0.05$. Quantity lymphoid cells in the spleen significant increase at the 7 and 18 days of the immune response by 33 and 19%, respectively ($P < 0.01$, $P < 0.05$) compared to the control under administration of allogenic adipose derived mesenchymal stem cells. On the 25th day of experience, the content of lymphoid cells in spleen and spleen index values return to normal. Weight index of the spleen directly correlates with the content of lymphoid cells – $r = 0.91–0.94$ ($P < 0.001$).

Key words: *mice, weight index, lymphoid cells, thymus, spleen, allogenic mesenchymal stem cells, adipose tissue.*

Ефект системного впливу алогенних мезенхімальних стовбурових з жирової тканини на показники функціонального стану імунних органів

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Дослідження проводили на самцях мишей С57BL /6 вагою 20–24г віком 2–3 місяці. Маніпуляції з отримання первинного матеріалу та культивування МСК здійснювали в стерильному боксі з дотриманням усіх правил асептики й антисептики. Абдомінальну жирову тканину мишей С57BL /6 культивували за температури 37 °С, 5% CO_2, 95% вологості у CO_2 інкубаторі у середовищі DMEM, з додаванням 10–15% фетальної сироватки бичків, 1% антибіотика-антимікотика (Sigma-Aldrich, USA). Для проведення досліджень було сформовано наступні групи тварин: 1-ша група – інтактні (контрольна група); 2–га група – тварини, яким у хвостову вену вводили 0,5 мл 0,9% розчину NaCl (плацебо); 3-тя група – тварини, яким у хвостову вену вводили 10^4 алогенних МСК з жирової тканини в 0,5 мл фосфатно-буферного розчину. Досліджували ваговий індекс, вміст лімфоїдних клітин тимусу та селезінки мишей С57Bl/6 за введення МСК з жирової тканини. Для оцінки вмісту лімфоцитів в лімфоїдних органах, останні зважували (тимус повністю), а селезінку – по 50 мг, потім розтирали та фільтрували через капронову тканину. Після цього гомогенат тканини наносили на градієнт фікол-верографіну (щільність 1,077) у співвідношенні 3:2. Пробірки з вмістом центрифугували зі швидкістю 1500 об/хв., протягом 30–40 хвилин. Після центрифугування над шаром градієнта залишається плазма і лімфоцити (не менш 90%), які збирали пастерівською піпеткою і двічі відмивали довільною кількістю розчину Хенкса шляхом центрифугування при швидкості обертання 1500–1800 об/хв. протягом 10 хвилин. Після відмивання до лімфоцитів додавали 1 мл розчину Хенкса і підраховували їх кількість в камері Горяєва. Розрахунок клітинності лімфоїдних органів проводили на 1 мг тканини. Трансплантація алогенних мезенхімальних стовбурових клітин з жирової тканини чинить вплив на центральні і периферичні органи імунної системи. За впливу алогенних МСК з жирової тканини відбувається достовірне підвищення вмісту лімфоїдних клітин тимусу на ранніх і пізніх етапах імунної відповіді на на 7, 18 та 25 добу на 71, 57 і 53% відповідно ($P < 0,05$) порівняно з контролем. Кількість лімфоїдних клітин у селезінці достовірно зростала на 7 та 18 добу імунної відповіді на 33 та 19%, відповідно ($P < 0,01$, $P < 0,05$) порівняно з контролем при введенні алогенних мезенхімальних стовбурових клітин, одержуваних з жирової тканини. На 25-ту добу показники вмісту лімфоїдних клітин та індексу селезінки повертаються до норми. Індекси ваги тимуса і селезінки прямо корелюють з вмістом лімфоїдних клітин в цих органах.

Ключові слова: *миші, ваговий індекс, лімфоїдні клітини, тимус, селезінка, алогенні мезенхімальні стовбурові клітини, жирова тканина*

Introduction

Important biological features of MSCs, in particular, the ability to migrate to the inflammation site, low immunogenicity, immunomodulatory activity, and the ability to stimulate hemopoies make them potentially active regulators of reparative processes (Kladnytska et al., 2014).

At the present stage of the development of biological sciences, different approaches develops for the use of MSCs in the treatment of various diseases, and a number of preclinical and clinical trials have already been conducted, the results of which have shown the effectiveness of the use of these cells (Haghighat et al., 2011; Reich et al., 2012; Arnhold and Wenisch, 2015; Kathrine et al., 2017).

As an alternative source of MSC is adipose tissue, which contain stem cells in a higher percentage than bone marrow. Obtaining of adipose tissue is less traumatic procedure for the donor than the obtaining of bone marrow both during the process of obtaining the primary material and during the postoperative period (Marx et al., 2014; Kladnytska et al., 2017).

Regardless of the origin of MSCs, they have pronounced immunosuppressive activity: they block in vitro differentiation of naive CD4 + T cells in Th17 and suppress the synthesis a lot of cytokines like IL-17, IL-22, interferon-gamma and TNFα (Bartholomew et al., 2002; Hryshchenko and Tomchuk, 2013).

Despite the large number of publications confirming the immunosuppressive properties of MSC, there are works that deny such effect on immune responses (Di Nicola et al., 2002; Aggarwal and Pittenger, 2005; Djouad et al., 2005).

Thus, it was found that transplantation of MSC stimulates antibody production and increases the cellularity of the bone marrow of recipients. With increasing the number of introduced cells, a significant increase in thymus cellularity and decrease cellularity of the spleen was recorded. The authors suggest that a significant dose of the MSC creates suppressive microenvironment for lymphoid cells, which is accompanied by inhibition of the immune response (Le Blanc et al., 2003; Batten et al., 2006; Lu et al., 2009).

Opposite, a small number of transplanted cells, by virtue of homing, is collected in the bone marrow niches, contributing to hematopoiesis, where the myeloid sprout can act as a spectacular factor in natural immunity (Nikolskaya et al., 2012).

So, it is not well known about the effect of MSC on the response of the immune organs, in particular on the functional state of thymus and spleen. Taking into account such controversial data on the influence of MSCs on the organs of the immune system, these issues require further research.

The purpose of our work was to study the functional state of the organs of the immune system of C57Bl/6 mice after introduction of allogeneic adipose MSCs.

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

Obtaining and cultivating of adipose MSCs (aMSCs) were carried out in a sterile laminar box with compliance of conditions of asepsis and antiseptics. The mice were euthanized, samples of abdominal adipose tissue were washed three times with sterile phosphate buffer solution with the addition of 1% antibiotic-antimycotic solution (Sigma-Aldrich, USA). Then samples of adipose tissue were chopped into pieces of 1–3 mm^3 and placed 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 mo nolayer at 80–90%, cells were removed with trypsin-ethylenediaminetetraacetic acid solution (EDTA), washed with phosphate buffer and placed in Petri dishes for futher cultivation. Passaging the cells provided a reduction of heterogeneity of cell culture and the development of biological material for transplantation (Kladnitska et al., 2016). For transplantation were used MSCs of the 4 passage.

MSCs administration to mice

The following groups of animals were formed: 1 group – intact (control group); 2 group – animals, to whom 0.5 ml of 0.9% NaCl solution (placebo) were injected into the caudal vein; 3 group – animals, to whom 10^4 of allogenic aMSCs in 0.5 ml of phosphate buffer solution were injected into the caudal vein.

Estimation of weight index of thymus and spleen of mice after introduction of aMSCs

Indicators of the weight of peripheral lymphoid organs relative to the body weight (weight index) of animals were evaluated at 7, 18 and 25 days after the introduction of aMSCs. The mice were pre-weighed for weight control. At each study period in each group 3 animals were euthanized and the weight index of lymphoid organs and their cellularity were studied.Euthanasia of animals was carried out with using of carbon dioxide, lymphoid organs – thymus and spleen – were removed and determined its mass. Indices of lymphoid organs in relation to the weight of the animal were calculated according to the formula:

Weight index (%) = weight of the lymphoid organ / weight of the animal * 100.

Evaluation of cellularity of the thymus and spleen after the introduction of aMSCs

To assess the content of lymphocytes in lymphoid organs, the latter were weighed. Whole thymus and 50 mg of spleen were triturated and filtered through the kapron tissue. After that, the cell homogenate was applied to the gradient of ficoll-urografin (density 1.077) in a ratio of 3:2. The test tubes were centrifuged at a rate of 1500 rpm for 30–40 minutes. After centrifugation the layer of lymphocytes which was above the gradient was collected by a Pasteur pipette and washed twice with an arbitrary amount of Hanks' solution by centrifugation at a rate of 1500 for 10 minutes. 1 ml of Hanks's solution was added to lymphocytes after washing. Lymphocytes were counted in the Goryaev chamber. Calculation of the cells of lymphoid organs was performed on 1 mg of tissue.

Results and discussion

The functional state of the organs of immunogenesis largely depends on the ratio of the processes of proliferation and apoptosis of the immune cells, that are practically not studied after administration MSC.

After the introduction of allogeneic aMSCs at the 7, 18 and 25 day, the content of lymphoid cells in the thymus was significantly increased compared with control animals at 71, 57 and 53% respectively (table 1).

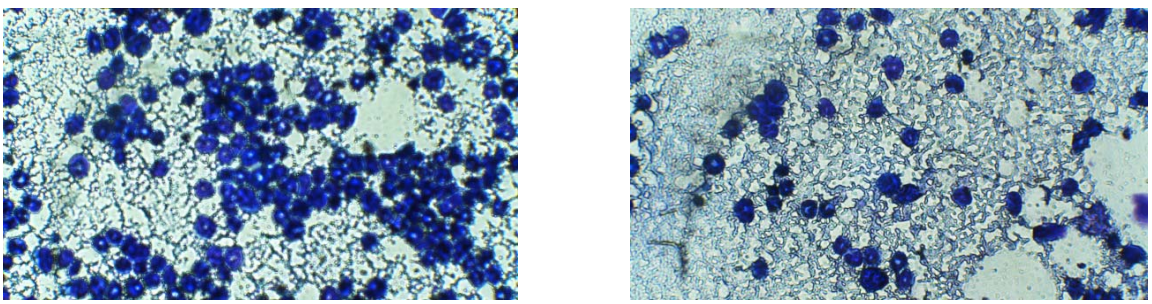
Table 1

The content of lymphoid cells and the weight index of thymus of C57BI / 6 mice after administration of allogeneic aMSCs, M + m, n = 9, $x10^6$ / mg,%

Groups of an animals / Terms of study	Intact (n = 6) ($x10^6$ / mg)	Administration of 0.89% NaCl, placebo (n = 9) ($x10^6$ / mg)	Administration of aMSCs (n = 9) ($x10^6$/мг)	Weight index of thymus after administration of aMSC,%
7 day	1.4 ± 0.1	1.9 ± 0.2	2.7 ± 0.1* ^{v}	0.19 ± 0.03* ^{v}
18 day	1.4 ± 0.1	1.3 ± 0.1	2.2 ± 0.1* ^{v}	0.16 ± 0.01* ^{v}
25 day	1.7 ± 0.1	1.4 ± 0.1	2.6 ± 0.3* ^{vv}	0.17 ± 0.01* ^{v}

*– P < 0.05, compared to a group of intact animals; v – P < 0.5, compared to placebo group

Compared with placebo group the content of lymphoid cells in the thymus was significantly increased at 42, 69 and 86% respectively. The increase of cellularity of thymus is due to the activation of proliferation of residental thymocytes due to antigenic stimulation by MSC, consistent with studies by Huang Y., Johnston P., Zakari A. et al. (Huang et al., 2009). The thymus contains T-lymphoblasts, ripening and mature lymphocytes, supporting and secretory cells of the thymus stromal component (Figure 1).



A B

Fig. 1. The cellularity of thymus on the 7 day of the study: A – intact group, B – after the introduction of aMSC, (smear-imprint, x 400)

A positive correlation between content of lymphoid cells and weight index of thymus was established at 7 days after the introduction of aMSC. Weight index of the thymus directly correlates with the content of lymphoid cells and its value was r = 0.57, P < 0.01 on 7 day and r = 0.50, P < 0.05 on 18 day of experience.

Under the influence of MSCs from adipose tissue, the indicator of the weight index of the spleen was significantly increased until the 18 day of the experiment (table 2). At the 25 day the weight index of spleen was not significantly differ from that in experimental group and placebo animals, but only observed a tendency to increase it.

Table 2

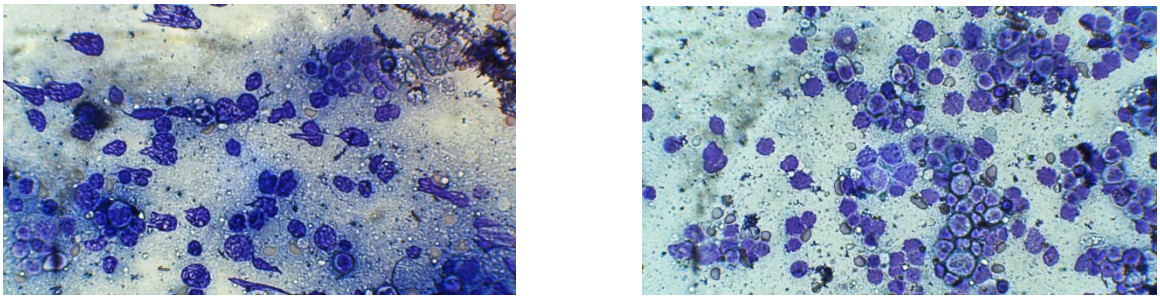
The content of lymphoid cells and the weight index of spleen of C57BI / 6 mice after administration of allogeneic aMSCs, M + m, n = 9, x10^6 / mg,%

Groups of an animals / Terms of study	Intact (n = 6) (x10^6 / mg)	Administration of 0.89% NaCl, placebo (n = 9) (x10^6 / mg)	Administration of aMSCs (n = 9) (x10^6/мг)	Weight index of spleen after administration of aMSC,%
7 day	2.7 ± 0.1	2.9 ± 0.1	3.6 ± 1.1**^{vv}	0.79 ± 0.04* ^{v}
18 day	2.7 ± 0.1	2.8 ± 0.4	3.2 ± 0.1*^{v}	0.79 ± 0.04* ^{v}
25 day	2.7 ± 0.1	2.5 ± 0.1	2.9 ± 0.1	0.46 ± 0.03

* – P < 0.05, ** – P ≤ 0.01, compared to a group of intact animals; v – P < 0.5, compared to placebo group

The spleen, as the peripheral organ of the immune system, is also involved in the process of forming an immune response to the antigen. After the administration of MSC, the content of lymphoid cells in the spleen significantly exceeded the parameters of cellularity of spleen of intact animals (table 2). Spot-imprint contains erythroid cells, neutrophil granulocytes, monocytes and lymphoid cells.

The number of lymphoid cells significantly increased by 33% and 24% compared to intact animals and the placebo group on the 7 day of the study. On the 18 day of experiment the cellularity of spleen under the influence of MSCs from adipose tissue was significantly higher at 19 and 14% respectively. At the 25 day of the experiment, the lymphoid cell count was higher by 7 and 15% within the tendency.



A B

Fig. 2. The cellularity of spleen on the 7 day of the study: A – intact group, B – after the introduction of aMSC, (smear-imprint, x 400)

The weight index of the spleen directly correlates with the content of lymphoid cells in it r = 0.91 (P < 0.001), r = 0,94 (P < 0.001), r = 0.92 (P < 0.001) on the 7, 18 and 25 day of experience respectively. Such changes indicate a direct reaction of the spleen to the introduction of allogeneic stem cells from adipose tissue.

Discussion

Thus, the administration of allogeneic MSCs isolated from adipose tissue of the C57Bl/6 mice causes systemic effects on the thymus and the spleen. As a result of antigenic stimulation by allogeneic stem cells, there is an increase in mitotic activity of thymocytes and splenocytes. Despite numerous publications that reveal the immunological properties of cells and confirm the presence of immunosuppressive effects, the results of individual scientific studies show that MSCs under certain conditions can be eliminated by cells of the immune system of the animal recipient, since they have signs of foreignness (Huang et al., 2009).

The increase of the content of lymphoid cells of the thymus and spleen after the introduction of aMSCs of our mind may be due to the heterogeneity of introduced culture cultures, insufficient number of introduced cells for the implementation of immunosuppressive effect, as well as low concentration of immunosuppressive factors synthesized by MSCs.

Conclusion

1. The administration of allogenic adipose derived mesenchymal stem cells affects on the central and peripheral organs of the immune system.

2. Administration of allogenic adipose derived mesenchymal stem cells cause a significant increase in the content of lymphoid cells in the thymus at 7, 18 and 25 days by 71, 57 and 53% respectively (P < 0.05) compared to the control.

3. Weight index of the thymus directly correlates with the content of lymphoid cells and its value was r = 0.57, P < 0.01 on 7 day and 18 day r = 0,50, P < 0.05.

4. Quantity lymphoid cells in the spleen significant increase at the 7 and 18 days of the immune response by 33 and 19%, respectively (P < 0.01, P < 0.05) compared to the control under administration of allogenic adipose derived mesenchymal stem cells.

5. On the 25th day, the content of lymphoid cells in spleen and spleen index values return to normal.

6. Weight index of the spleen directly correlates with the content of lymphoid cells in its r = 0.91–0.94, P < 0.001.

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