Biological activity of multipotent stem cells of horses depending on the method of obtaining of mononuclear cell from bone marrow aspirate

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Abstract. *Experimental studies which are relating to improving the method of obtaining of the fraction of mononuclear cells from bone marrow aspirate of horses by centrifugation of cell suspension in ficoll density gradient were conducted. It was established that the optimal conditions for obtaining the fraction of bone marrow mononuclear cells of horses which are enriched with multipotent stem cells is centrifugation of bone marrow aspirate in a density gradient with = 1,076 at centrifugal force of 300 g.*

Key words: *multipotent stem cells, hematopoietic stem cells, mononuclear cells, bone marrow, density gradient.*

Obtaining the bone marrow of animals and the allotment of faction of mononuclear cells is an important precondition for providing the high biological activity of stem cells which can be intended for use in cell therapy, because the bone marrow is their main source [6]. Faction of mononuclear cells contain stem cells in sufficient quantities for their production.

Because in the samples, taken from bone marrow, mononuclear cells (fibroblasts, macrophages, adipocytes, osteoblasts, osteoclasts, endothelial cells, multipotent (MSC) and hematopoietic stem cells, etc.) constitutes a small number of the total number of its cells, therefore is searched such methods of allocation of mononuclear cells that can be characterized by high content of MSC in this fraction and / or by rapid production of biomass [1].

Today the most widely used method of excretion of MSCs from bone marrow of animals remains the planting of native cell suspension of bone marrow in cultural cup or mattresses and further selection of cells by their ability to adhesion to plastic. However, this approach of MSC excretion requires frequent change of culture medium to remove products of destruction of red blood cells, which, in turn, requires significant expenditure of time and material resources.

Recently in veterinary medicine was tested the method of MSC obtaining from bone marrow of animals, based on consideration of different densities of cells contained in it, and their ability to be separated in a density gradient [3, 4, 5]. This method is adopted from humane medicine, where it is used to obtain lymphocytes from the peripheral blood of people [7]. According to this method, obtained suspension of bone marrow cells dilute with phosphate solution and centrifuge with appropriate centrifugal force and density gradient ficol. Mononuclear cells, which, unlike the red blood cells and granulocytes, have high density are not able to go through the gradient layer, which has a higher density than the cells themselves, and therefore remain on the gra-

dient surface. This method of isolation of fraction of mononuclear cells of bone marrow successfully tested in experiments on rabbits, cats and dogs. However, such methods of selection of mononuclear fraction of bone marrow cells in horses in available sources are absent.

Considering this, the goal of our work is approbation and improving the method of obtaining of mononuclear fraction of bone marrow cells of horses which are enriched with population of mesenchymal stem cells via centrifugation of bone marrow aspirates in ficoll density gradient.

Materials and methods. Bone marrow was taken from external hill of iliac bone of clinically healthy horses aged 1–2 years. For the purpose of appropriate anesthesia and fixation of animal operation was performed under general anesthesia, followed by infiltration of soft tissues in the area of bone puncture with 2% solution of lidocaine. Premedication was conducted by intravenous administration of detomidine at a dose of 0.02 mg / kg of the animal. Propofol was used as a general anesthetic at a dose of 2 mg / kg of the animal, which was also administered intravenously [1].

Animal was fixed in the left lateral recumbent position. Place of operative access previously was investigated with palpation, identifying the dorsally, ventral, cranial and caudal boundaries of ilium.

Bone marrow was collected with a needle for bone trepanobiopsy and a syringe with a volume of 20 ml. Obtained cell suspension, diluted with phosphate buffer solution at a ratio of 1: 5 (8 cm3), impose on previously introduced in sterile tubes ficoll gradient filled to 4 cm3 (ρ = 1,074; ρ = 1,076; ρ = 1,078; ρ = 1,080; 1,082) at 300g for 25 minutes. After centrifugation the top layer of supernatant was removed without disturbing the integrity a layer of mononuclear cells located below. Then a layer of mononuclear cells was carefully transferred to a new sterile centrifuge tube and added of 10 cm3 of phosphate buffer solution. Cells were pipetted and centrifuged at 300 g for 5 min. Washing of cells was repeated twice.

Cells precipitate was pipetted in nutrient medium (DMEM) and transferred to a Petri dish (S = 9,6 cm2) in an amount of 3 million of mononuclear cells per dish. Cultivation of cell was performed in DMEM with 20 % of embryonal calf serum (ECS) with the addition of antibiotic-antimycotic (10 microliter / cm3 of medium).

After 11 days of cultivation evaluation of proliferative activity of cells was carried out in every group of cells. As control for evaluation of proliferative activity of cells was used the method of obtaining the fraction of mononuclear cells of bone marrow by its separation in gradient density with ρ = 1,074.

Results. It is known that the regulation of development, growth and division of cells that populate the bone marrow, need to exchange of information with each other, which is carried out by the production of the growth factors by resident cells. While some cells actively proliferate (for example, myeloblasts or lymphoblasts), other (multipotent stem cells) are in the so-called inactive state and activate when in the body certain pathological processes happens. In this case they participate in replacement of damaged tissues.

Violation of genetically established composition of bone marrow cells that observes ex vivo after their separation in a density gradient violate a genetically fixed scheme of intercellular interactions between cells and cause a change of cells behavior in vitro during their cultivation, which essentially depends on the combination of cells obtained after centrifuging of bone marrow aspirate in different density gradients.

The results of research are presented in Table 1 and Fig. 1. They show that parameters of centrifuging of suspension of bone marrow cells affect on their proliferative potential in vitro, which obviously is due to cultivation of

Table 1

Proliferative activity of MSCs of horses depending on density gradient at centrifugal force of 300 g (n = 3, M ± m)

Density gradient, ρ	Number of cells per Petri dish
1,074 (control)	160540+/-43233
1,076	372185+/-22584*
1,078	214423+/-20648
1,080	63883+/-52912

Note. * - P <0.05

different population of mononuclear cells obtained in different density gradients.

Centrifugation of cell suspension of horse bone marrow in gradient density with ρ = 1,076 is the most optimal for obtaining cell population with the highest proliferative activity. On the day 11 of cultivation of fraction of mononuclear cells obtained by centrifugation at above-mentioned method the number of cells that have adhesive properties was 372,185 +/- 22584 per Petri dish, which is significantly more than at centrifugation of cell suspension of horse bone marrow in gradient density with ρ = 1,074 and other density gradients. The expansion of MSCs was about 90 %, while in control samples which have been obtained by centrifugation of cell suspension of bone marrow in density gradient with ρ = 1,074, covering of surface of culture dishes was only about 45 %.

In experiments with allocation of fractions of mononuclear cells from bone marrow, using a density gradient with ρ = 1,078, cells expansion in Petri dish was about 60 % (Table. 1, Fig. 1).

The lowest level of proliferative activity of mononuclear cells was in fraction, which was obtained by separating the suspension of bone marrow cells in density gradient with ρ = 1,080. Expansion of cells was less than 20 %.

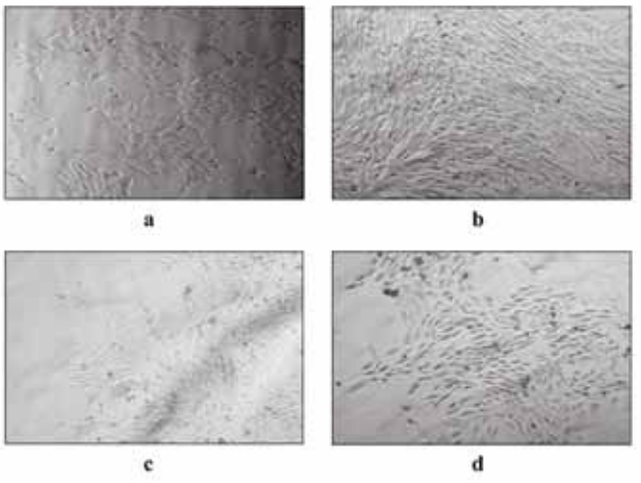

Fig. 1. Stem cells after centrifugation of suspensions of bone marrow cells at different density gradients at zero passage: ρ = 1,074 (a), ρ = 1,076 (b), ρ = 1,078 (c), ρ = 1,080 (d) (11 day of cultivation) x 100

Conclusions

1. Centrifugation of suspension of bone marrow cells of horse in ficoll density gradient is an effective method of obtaining of fractions of mononuclear cells from bone marrow aspirate.

2. Optimal conditions for obtaining of fraction of mononuclear bone marrow cells of horse which are enriched with population of mesenchymal stem cells are centrifuging of suspension of bone marrow cells in density gradient with ρ = 1,076 at centrifugal force of 300 g.

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