

THE INFLUENCE OF LOW-MOLECULAR FRACTION FROM CORD BLOOD (BELOW 5 kDa) ON FUNCTIONAL AND BIOCHEMICAL PARAMETERS OF CELLS *IN VITRO*

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The influence of a low-molecular fraction (below 5 kDa) from the cattle cord blood (CBF) on functional activity of phagocytes, human embryonic fibroblasts, mesenchymal stromal cells and BHK-21 clone 13/04 and PK-15 cells was studied. The low-molecular fraction added to culture medium increases the growth rate of cell cultures. The incubation of leukoconcentrate in the CBF-containing medium results in an increase in phagocytic indices of neutrophils in the presence of a phagocytosis inhibitor – sodium iodoacetate, leading to a significant increase in intracellular glucose content and alkaline phosphatase activity as compared to the control and the reference drug Actovegin®.

Key words: cord blood low-molecular fraction, Actovegin®, inhibitors of phagocytosis, leukocytes, glucose, alkaline phosphatase, human embryonic fibroblasts, mesenchymal stromal cells, BHK-21 clone 13/04, PK-15 cells.

Drugs based on cord blood are successfully used at present in complex therapy of diseases of different genesis. This is preconditioned by the fact that cord blood contains a unique balanced complex of specific placental factors, which determine the fetus growth in ontogenesis and regulate its metabolism. Besides, drugs based on low-molecular components of cord blood, which do not possess antigenic and pyrogenic features, exclude side effects in the form of allergic reactions and also the risk of virus diseases and arouse particular interest.

It was shown in the *in vivo* and *in vitro* studies conducted earlier by the authors [1–3] that the low-molecular fraction (below 5 kDa) from the cattle cord blood (CBF) possessed apparent immunomodulating and reparative effects. In particular, it was determined that injection of CBF accelerated healing of burns and gastric ulcers [4]. It was shown in the experiments *in vitro* that the low-molecular CBF stimulated the functional activity of human leucocytes [1, 3].

The impact of the low-molecular fraction (below 5 kDa) from the cattle cord blood on the functional condition of different cell cultures and metabolism of human leucocytes was studied in this work in the *in vitro* experiments.

Materials and Methods

The extraction of the fraction containing components with molecular weights below 5 kDa from the cryodestructed whole cattle cord blood was performed by ultrafiltration [5] using a membrane module Sartorius company (Germany). The peptide content in CBF, determined by the method [6], was 9–10 mg per 100 g of dry weight. Actovegin® (40 mg/ml), the drug produced by Nicomed company (Austria), was used as a reference drug.

The following cell cultures were used in the work: human embryonic fibroblasts (HEF) on the 4th–6th passages, mesenchymal stromal cells (MSC), obtained from rat bone marrow, BHK-21 clone 13/04 line (kidney of newly-born Syrian hamster) and PK-15 line (pig kidney). A standard cultivation condition for cell cultures was addition of 10% of fetal bovine serum (HyClone) to the media: for HEF – medium 199 (FSUE IPVE of M.P. Chumakova of RAMS, Russia), for MSC – mixture (1:1) DMEM and F12 (Sigma-Aldrich, USA), for BHK-21 clone 13/04 and PK-15 – DMEM (Sigma-Aldrich, USA). Seeding concentration were: for HEF – $8 \cdot 10^4$, for MSC – $0.5 \cdot 10^4$ – $0.6 \cdot 10^4$, for BHK-21 clone 13/04 – $6 \cdot 10^4$, for PK-15 – $7 \cdot 10^4$ – $9 \cdot 10^4$ cells per 1 ml of medium.

Proliferation of cultures was evaluated by relation of the number of gathered cells to that of seeded ones (index of proliferation). The cell number was counted by the standard laboratory method in the Goryayev chamber.

Adhesion properties of cell cultures were evaluated by two indices: cell attachment and the rate of cells spreading. The cell attachment was determined by counting cells which were not attached 24 h after seeding [7]. The culture spreading rate was determined by calculation of spindle-shaped cells 1.5; 3; 5 and 24 h after seeding. Cell monolayer was fixed in the above-mentioned terms, stained by Karachi hematoxylin and 0.1% eosin solution. The evaluation was performed visually by light microscopy.

To determine mitotic activity of culture, cell monolayer was fixed every 24 h and stained by Karachi hematoxylin. Mitotic activity was determined by the total number of dividing cells per 1,000 calculated ones. The index of mitotic activity (mitotic index – MI) was expressed in per mille (‰) [8]

Leukoconcentrate was obtained from human donor blood by sedimentation of erythrocytes in dextran solution [9]. Phagocyte activity was investigated by the method [10]. To estimate phagocytic activity we used a one-day inactivated *Staphylococcus aureus* culture, strain no. 209 ($2 \cdot 10^9$ cell/ml), as a phagocytosis item. The following parameters were chosen as indices for estimation of phagocytic activity: percentage of phagocytizing neutrophils and monocytes (phagocyte index, PI), average number of engulfed bacteria per one phagocyte (phagocytic number, PN) characterizing engulfing capacity of cells after 45 or 120 minutes of incubation, and index of phagocytosis completeness (IPC) characterizing digestion activity of phagocytes. The index of phagocytosis completeness was calculated as the following fraction: phagocytic number after 45-minute incubation/phagocytic number after 120 minutes incubation. To inhibit phagocyte activity of neutrophils, colchicine (100 μ M) [11] or sodium iodoacetate (1 mM) [12] was added to suspension of leucocytes. CBF and Actovegin, the reference drug, were added in concentrations of 0.15 and 1.50 mg/ml, respectively [3].

To determine glucose content donor blood leucocytes ($2 \cdot 10^7$ cells/ml) were preliminarily incubated for 15 min at 37 °C in glucose-containing medium (20 mM) with addition of CBF (the final concentration was 0.15 mg/ml [3]) or Actovegin® (the final

concentration was 1.50 mg/ml [3]). To inhibit glucose transporter GLUT-1, cytochalasin B (10 μ M) (Sigma-Aldrich, USA) was added to suspension of leucocytes [13]. The method of gel filtration on a column was used to remove extracellular glucose, which is contained in the incubation medium of cells. The cell suspension was further filtered through the column filled up by Sephadex G-25 (Pharmacia, Sweden).

The authors added 30 μ l of 1% Triton X-100 solution to cell suspension after filtration and determined glucose content by the glucose oxidase method using a kit of reagents to determine glucose content in biological liquids of Filicid-Diagnostika Company (Ukraine) [10]. Glucose content was expressed in μ mol/ 10^9 cells.

To study activity of alkaline phosphatase, leucocytes ($3.5 \cdot 10^7$ cell/ml) were preliminarily incubated for 30 min at 37 °C in medium with CBF (0.15 mg/ml) or with Actovegin (1.50 mg/ml) in the presence or absence of *Staphylococcus*. Activity of alkaline phosphatase was determined by spectrophotometry [14]. The choice of incubation period is based on the experimental studies [15] and corresponds to the maximum enzymatic activity during the development of the phagocytic reaction. Alkaline phosphatase activity was determined spectrophotometrically according to the manual of the kit reagents for the determination of alkaline phosphatase activity in biological fluids (Phyllis-Diagnostics, Ukraine) and expressed in μ kat/l of cell lysate (corresponding to $3.5 \cdot 10^9$ cell/l) [15]. The experimental data were statistically processed by the non-parametric Mann–Whitney test. The results are presented as $M \pm m$. Significance level was 0.05.

Results and Discussion

The growth rate of cell cultures was studied to assess the CBF influence on their functional condition. Influence of different CBF concentrations on proliferative indices of cell cultures was estimated in the first stage of our work. CBF added to HEF in the concentration of 56 μ g/ml stimulated proliferation by 47.6% after 96 h of growth and in the concentration of 224 μ g/ml – by 78% (Fig. 1). CBF in the concentration of 56 μ g/ml stimulated MSC proliferation after 72-h cultivation by 13.4%, but in the concentrations of 112 and 224 μ g/ml – by 23% in comparison to the control. At addition of CBF in the concentration of 56 μ g/ml the number of BHK-21 clone 13/04 cells exceeded the control by 89% after 96 h of incubation, in the concentration of 224 μ g/ml

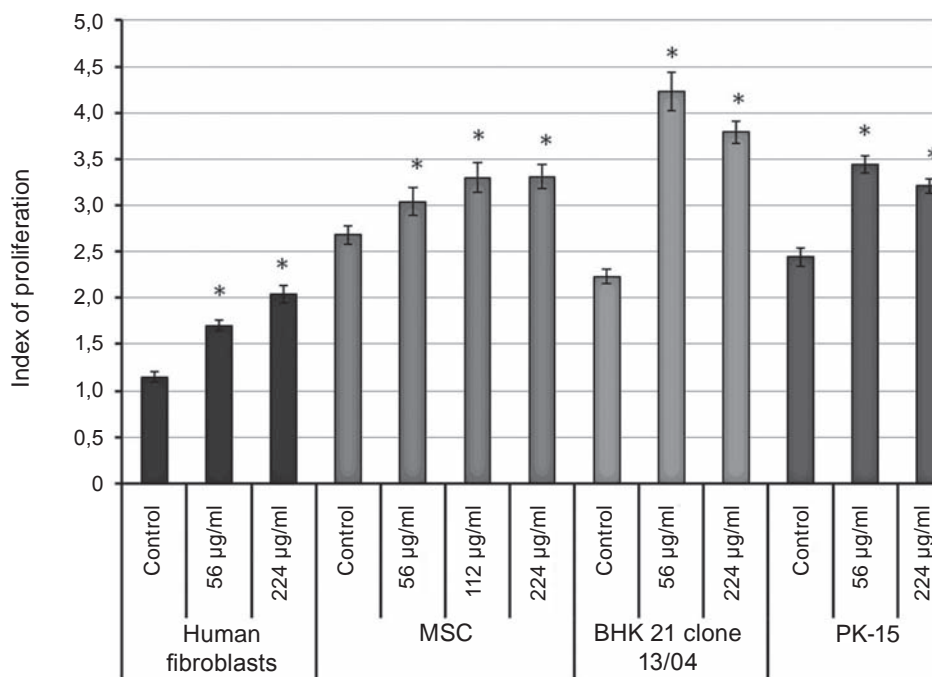


Fig. 1. The proliferative indices of cell cultures after addition of CBF in different concentrations to cultivation media. *Differences are significant in comparison with the corresponding control, $P \leq 0.05$

– by 70%, but in the case of PK-15 line – by 40.5% and 31.5%, respectively.

The performed studies allowed us to establish the stimulating influence of CBF on proliferation of cell cultures and to reveal the fact that cells of the cultures studied in this work have different sensibility to CBF addition to cultivation media.

The study of separate growth stages of cell cultures, namely, adhesion and mitotic division of cells, showed the following. The cell attachment of different cultures at CBF addition to the cultivation media remains unchanged (Table 1).

We studied the spreading rate in HEF culture; its cells take a distinct spindle shape when the process is completed. CBF addition in the concentration of 224 µg/ml increased the cell spreading rate. After 3-h cultivation the number of spindle-shaped cells in this variant was higher than in the control by 15.4%, but after 24-h cultivation – by 13.6%.

Thus, the increase of HEF spreading rate at CBF adding may be related both to the impact on energetic metabolism, which leads to improvement of cells provision with ATP energy, and to stimulation of this process by growth factors.

After reaching their maximal spreading and formation of characteristic morphology, the cells become sensitive to mitogen action [16]. We studied CBF influence on mitotic activity on BHK-21 clone

13/04 line. MI in the variant of CBF addition in the concentration of 56 µg/ml was 30.67 ± 2.18 after 24-h cultivation, 56.00 ± 2.00 after 48-h cultivation, and 40.40 ± 1.66 after 72-h cultivation, which exceeded the control values in the above-mentioned terms by 64, 55 and 20.5%, respectively. The results obtained show an earlier start of cell mitotic division and increase of culture mitotic activity under CBF influence. The increase of mitotic activity was also determined for MSC. MI on the peak of mitotic activity (72-h cultivation) under CBF influence was 18.5 ± 1.7 that exceeded the control by 28%.

Generalizing the obtained results on CBF influence on the cell culture growth rate, we may draw the following conclusions: CBF accelerates processes of culture adhesion, activates the start of culture mitotic division and stimulates their mitotic activity.

To determine mechanisms of CBF influence on the functional activity of cells the CBF influence on

Table 1. Cell attachment after 24 h of cultivation, %

Culture	Control	with CBF
HEF	59.0 ± 3.0	63.0 ± 0.6
BHK-21 clone 13/04	90.2 ± 0.4	89.0 ± 0.7

cell metabolism was studied using inhibitors. CBF impact on phagocyte indices of donor blood leucocytes was determined in our earlier studies *in vitro* [1]. It was shown that CBF stimulated phagocytic activity of frozen-thawed neutrophils and monocytes in 10-fold less concentration (0.15 mg/ml) as compared to Actovegin drug (1.5 mg/ml).

The phagocyte activity of donor blood neutrophils was studied *in vitro* in the presence of a phagocytosis inhibitor, colchicine with the aim to reveal mechanisms of the influence of low-molecular CBF and the reference drug Actovegin. The results showed that CBF or Actovegin addition did not significantly affect the indices of phagocyte activity of cells decreased under the inhibitor influence. Considering the fact that colchicine disrupts polymerization and reorientation of cytoskeleton micro-tubes, our results permit assuming that CBF and Actovegin do not renew damaged structures of plasma membrane or cytoskeleton during phagocytosis (Table 2).

It is known that activation of leukocytes induces glycolysis and engagement of cytoplasmatic glycogen pool. Taking into account that glycolysis is one of the main sources of energy, which is necessary for the basic phagocytosis stages, in the next series of experiments we incubated leukocyte concentrate in the presence of a glycolysis inhibitor sodium iodoacetate to clarify mechanisms of CBF and Actovegin influence on energetic metabolism. After adding this inhibitor to the medium of leukocyte incubation, a significant decrease in all the phagocyte indices of neutrophils was observed. CBF or Actovegin addition to the incubation medium containing the inhibitor promoted an increase in the phagocyte activity of neutrophils (Table 3).

It may be presumed that this effect is attributed to competition of sodium iodoacetate with low-molecular components of CBF and Actovegin for binding with active centers of key enzymes of glycolysis. On the other hand, taking into account the data on mechanism of Actovegin action targeted to the activation of glucose transport to cells [17], we studied CBF influence on this process. Our results showed that after cells incubation in the medium with CBF or Actovegin glucose content in leucocytes significant increased 13 and 8.7 times as compared to the appropriate control (Fig. 2).

The obtained results allow us to presume that CBF intensifies energy metabolism of leucocytes through increase in the flow of energetic substrate, the basic one for this type of cells. To determine mechanisms of the CBF effect on glucose accumulation in cells, a series of experiments with using cytochalasin B was conducted. As known, cytochalasin B, in addition to its inhibitory effect on cytoskeleton, is capable of inhibiting the transport of glucose into cells by blocking the glucose transporter family GLUT, namely GLUT-1 and GLUT-2 [18-22]. It should be noted that the major glucose transporter for leukocyte lineage is GLUT-1 [23, 24]. Fig. 2 shows that after treatment of cells with cytochalasin B CBF does not lead to an increase in intracellular glucose content as compared with the control (Cyto B). Incubation of leukocytes in CBF-containing medium without inhibitor contributed to the 13-fold increase in the index relative to the control. Based on this, it can be assumed that the mechanism of action of CBF in glucose accumulation by leukocytes associated with its effects on GLUT transporter family via enhancing their activi-

Table 2. Impact of CBF and Actovegin in the presence of colchicine on indicators of phagocyte activity of neutrophils of leukocytes concentrate

Experimental conditions	PI, % (45-min incubation)	PI, % (120-min incubation)	PN, abs. unit (45-min incubation)	PN, abs. unit (120-min incubation)	IPC, rel. unit.
Control	59.44 ± 2.18	57.77 ± 1.24	15.09 ± 1.11	11.69 ± 0.84	1.30 ± 0.03
Medium with colchicine	18.04 ± 1.69*	17.36 ± 1.03*	3.07 ± 0.82*	6.08 ± 1.15*	0.47 ± 0.05*
Medium with colchicine and CBF	20.03 ± 1.38*	19.54 ± 1.97*	4.76 ± 1.03*	7.38 ± 1.18*	0.61 ± 0.04*
Medium with colchicine and Actovegin	22.56 ± 1.80*	20.04 ± 1.54*	3.95 ± 1.02*	6.42 ± 1.29*	0.58 ± 0.05*

* Significant difference in comparison to the control values ($P < 0.05$)

Table 3. Impact of CBF and Actovegin in the presence of sodium iodoacetate on indices of phagocytic activity of neutrophils in leukocyte concentrate

Experimental conditions	PI, % (45-min incubation)	PI, % (120-min incubation)	PN, abs. units (45-min incubation)	PN, abs. units (120-min incubation)	IPC, rel. unit
Control	66.11 ± 3.31	59.69 ± 1.75	11.08 ± 1.29	9.20 ± 1.31	1.23 ± 0.05
Medium with Na iodoacetate	44.98 ± 1.61*	45.46 ± 3.71*	3.74 ± 0.64*	6.30 ± 0.55*	0.58 ± 0.05*
Medium with Na iodoacetate and CBF	56.17 ± 3.01 [#]	51.71 ± 4.44	8.75 ± 1.01 [#]	7.30 ± 1.02	1.22 ± 0.04 [#]
Medium with Na iodoacetate and Actovegin	59.63 ± 2.83 [#]	59.58 ± 2.71 [#]	9.22 ± 1.14 [#]	7.13 ± 0.72	1.28 ± 0.04 [#]

* Significant difference in comparison with the control values ($P < 0.05$); [#] Significant difference in comparison with the values of leucocytes incubated with inhibitor ($P < 0.05$)

ty, increasing the amount on cell surface or influencing transporter affinity for glucose.

Alkaline phosphatase (AP, 3.1.3.1) is a marker of the functional usefulness of leukocytes. It is contained in specific secondary granules of phagocytes and plays an important role in phagocytosis [25]. It is known that AP is localized in cytoplasm, affects oxidative processes in cells by cleavage of ATP and ADP, dephosphorylation of NADP and NADPH, as well as indirectly regulates glycogen synthesis by dephosphorylation of glycogen [26]. In the light of this information, the activity of this enzyme in lysates of leukocytes was studied. Cells were pre-incubated in media containing CBF (final concentration 0.15 mg/ml [3]) or Actovegin (final concentration 1.50 mg/ml [3]).

It was shown that AP activity in the presence of CBF during phagocytosis significantly increased 1.7 times in comparison with the control (Fig. 3, A). Besides, the reference drug did not influence this enzyme activity.

It should be noted that CBF addition to the incubation medium affects the level of enzymatic activity only in leucocytes stimulated by *Staphylococcus* (Fig. 3, A, B). An increase in AP activity after adding CBF points to stimulation of the functional activity of phagocytosing cells and correlates with the data on glucose accumulation by leucocytes. These data promote the understanding of mechanisms of CBF influence on the phagocyte activity of neutrophils studied earlier by the authors [1].

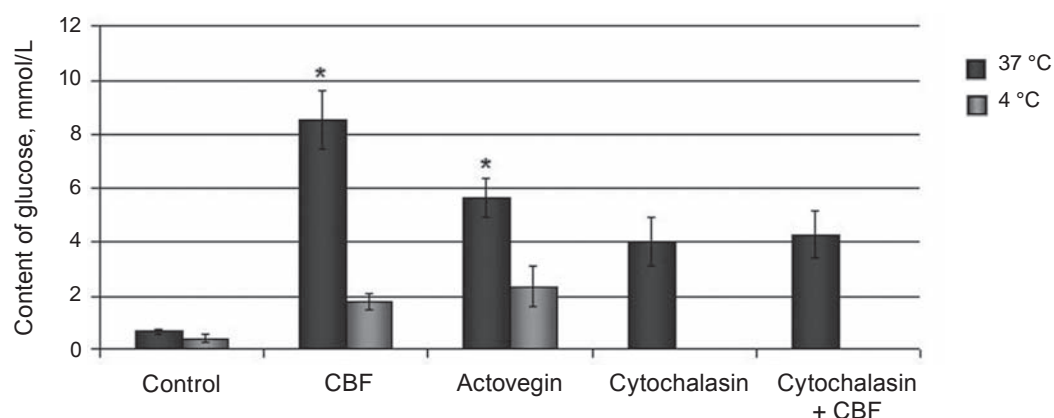


Fig. 2. Glucose content in cells of leukocyte concentrate after incubation in the medium containing CBF or Actovegin. * Significant difference in comparison with the control values, $P < 0.05$.

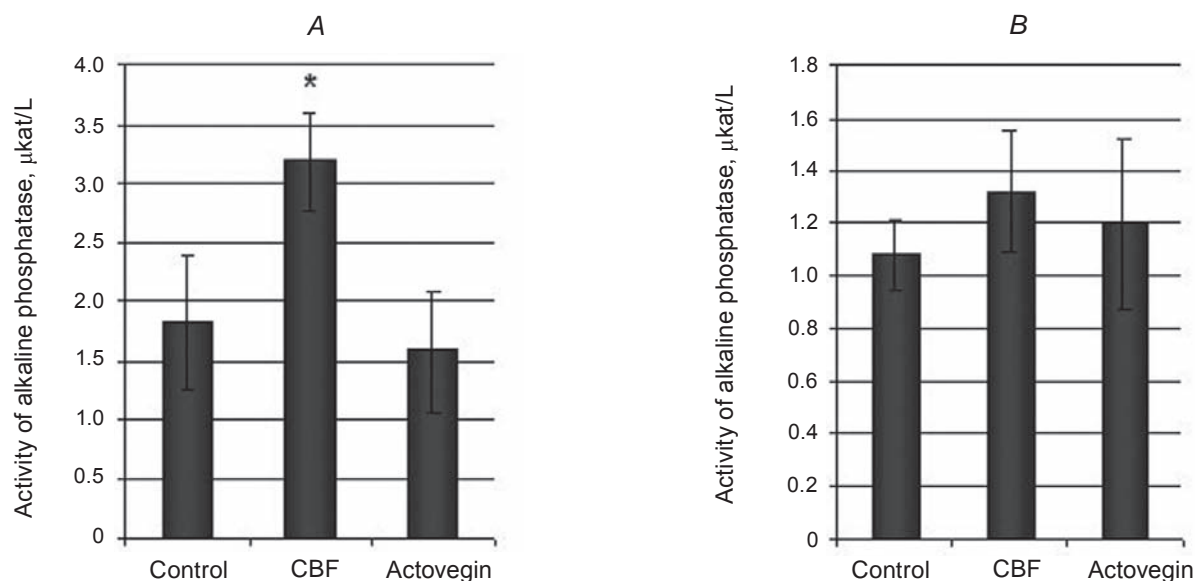


Fig. 3. Influence of CBF and Actovegin on alkaline phosphatase activity of leucocytes during of phagocytosis (A) and at rest (B). * Significant difference in comparison with the control values, $P < 0.05$

Thus, as a result of the performed studies it was determined that CBF addition to cultivation medium accelerated the processes of cell culture adhesion, activated the start of mitotic division and stimulated mitotic activity, increasing the rate of their growth. Studying biochemical indices in the process of phagocyte reaction of leucocytes, we observed the increase in AP activity and intracellular concentration of glucose in the medium containing CBF. Thus, the study of the action mechanism of the CBF allows us to suggest that the stimulatory effect of the fraction occurs due to the direct participation of its components in the energy metabolism reactions in the cell, which is one of the factors of increase in the functional status indices of cell cultures HEF, MSC, BHK-21clone 13/04 and phagocytic activity of leucocytes.

ВПЛИВ НИЗЬКОМОЛЕКУЛЯРНОЇ ФРАКЦІЇ З КОРДОВОЇ КРОВІ (ДО 5 кДа) НА ФУНКЦІОНАЛЬНІ ТА БІОХІМІЧНІ ПОКАЗНИКИ КЛІТИН У ДОСЛІДАХ *IN VITRO*

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Досліджено вплив низькомолекулярної (до 5 кДа) фракції з кордової крові (ФКК) великої рогатої худоби на функціональну активність фагоцитів, ембріональних фібробластів людини, мезенхімальних стромальних клітин та клітин ВНК-21 clone 13/04 і РК-15. Встановлено, що додавання ФКК в середовище культивування збільшує швидкість росту клітинних культур.

Інкубація концентрату лейкоцитів у середовищі, що містить ФКК, стимулює збільшення фагоцитарних показників нейтрофілів у присутності інгібітора фагоцитозу йодоацетату натрію, призводить до значимого збільшення внутрішньоклітинного вмісту глюкози та активності лужної фосфатази порівняно з контролем та дією препарату «Актовегін».

Ключові слова: низкомолекулярна фракція кордової крові, «Актовегін», інгібітори фагоцитозу, лейкоцити, глюкоза, лужна фосфатаза, ембріональні фібробласти людини, мезенхімальні стромальні клітини, ВНК-21 clone 13/04, РК-15.

ВЛИЯНИЕ НИЗКОМОЛЕКУЛЯРНОЙ ФРАКЦИИ ИЗ КОРДОВОЙ КРОВИ (ДО 5 кДа) НА ФУНКЦИОНАЛЬНЫЕ И БИОХИМИЧЕСКИЕ ПОКАЗАТЕЛИ КЛЕТОК В ЭКСПЕРИМЕНТАХ IN VITRO

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Изучено влияние низкомолекулярной (до 5 кДа) фракции из кордовой крови (ФКК) крупного рогатого скота на функциональную активность фагоцитов, эмбриональных фибробластов человека, мезенхимальных стромальных клеток, линий ВНК-21 clone 13/04 и РК-15. Установлено, что добавление ФКК в среду культивирования увеличивает скорость роста клеточных культур. Инкубация концентрата лейкоцитов в среде, содержащей ФКК, повышает фагоцитарные показатели нейтрофилов в присутствии ингибитора фагоцитоза йодоацетата натрия, приводит к достоверному увеличению внутриклеточного содержания глюкозы и активности щелочной фосфатазы по сравнению с контролем и препаратом сравнения «Актовегін».

Ключевые слова: низкомолекулярная фракция кордовой крови, «Актовегін», ингибиторы фагоцитоза, лейкоциты, глюкоза, щелочная фосфатаза, эмбриональные фибробласты человека, мезенхимальные стромальные клетки, ВНК-21 clone 13/04, РК-15.

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