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Research Article

Conditioned Medium of Induced Mesenchymal Stem Cells as an Activator of Differentiation in The Osteogenic Direction

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Abstract: In the prevailing era, the need for new biologically active substances of peptide nature and their therapeutic impacts are of great interest of the contemporary pharmacology. Hence, this study intends to assess the osteoinductive potential of the conditioned medium concentrate on native mesenchymal stem cells. To gratify the study's aim, this study was carried out on a culture of rat mesenchymal stem cells. To stimulate the differentiation of Mesenchymal stem cells in the osteogenic direction, this study used a complete nutrient medium containing dexamethasone, ascorbic acid, sodium ß -glycerophosphate in the concentrations recommended in the guidelines. Subsequently, the resulting concentrate sample was examined using HPLC-MS / MS mass, spectrometric analysis. Part of the previously obtained concentrate of the conditioned medium was used, to assess the proliferation and differentiation of rat Mesenchymal stem cells in the osteogenic direction by staining for alkaline phosphatase. Given the results of the study, it can be concluded that that most of the identified proteins of the conditioned medium concentrate belong to the group of proteins regulating cellular processes, and groups of proteins were also, found that relate to the organization of the extracellular structure, the processes of cell development, or are directly responsible for the differentiation of Mesenchymal stem cells in the osteogenic direction. Along with this, the concentrate of the conditioned medium does not affect the proliferation rate of Mesenchymal stem cells. Thus, the resulting concentrate of the conditioned medium, can be further used, for the development of therapeutic preparations with osteoinductive and regenerative potential.

Keywords: Mesenchymal Stem Cells, Secretion, Conditioned Medium, Differentiation, Osteoinduction and Alkaline Phosphatase.

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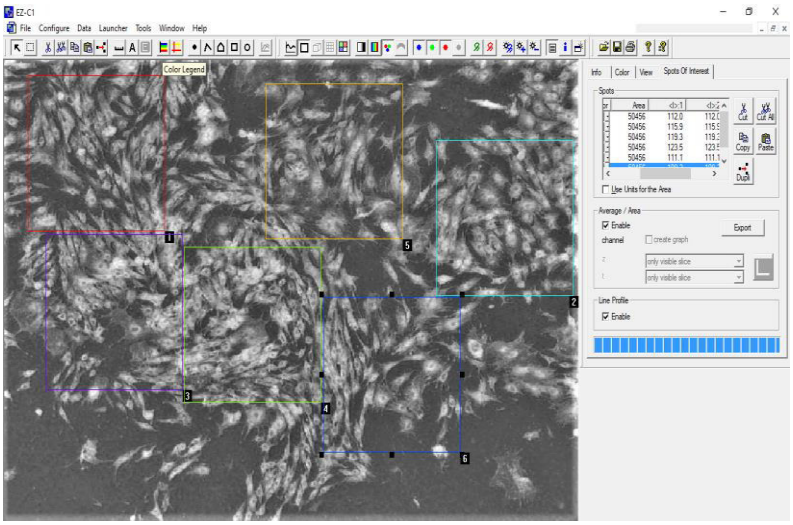
1. INTRODUCTION

Today, the search for new biologically active substances of peptide nature and their therapeutic effects remains an urgent task for modern pharmacology [1,2]. Peptides refer to amino acid polymers. They are typically considerably smaller compared to proteins and do not contain adequate activity on their own. They normally convey a small portion of a complete protein. Studies show that they can be signaling molecules interacting with specific receptors, as in peptide hormones and cytokines[3,4]. Mesenchymal stem cells are regarded as multipotent adult stem cells in multiple tissues, such as the fat tissue, umbilical cord, as well as bone marrow[5,6]. Mesenchymal stem cells are able to self-renew through dividing and may differentiate into numerous tissues, such as bone, cartilage, muscle and fat cells, and connective tissue[7,8]. Mesenchymal stem cells (MSCs) have a wide range of properties, including multipotency, high proliferative activity, and differentiation potential. They secrete a complex of bioactive substances - a secret: cytokines, chemokines, adhesion molecules, lipid mediators, growth factors, hormones, vesicles. Mesenchymal stem cells are relatively easily isolated and cultured in a laboratory, where they are used to obtain cell products and as experimental models [6-9]. Recently, evidence has accumulated supporting the effectiveness of the MSC-conditioned medium (CM) in studies aimed at assessing therapeutic potential [10,11]. The positive clinical effect of MSCs depends mainly on their paracrine action, and not on engraftment in the tissue of the recipient with subsequent differentiation, which opens the way to acellular therapeutic strategies in regenerative medicine. Several study conclude that they are a favorable prospect for cell-based treatment. The therapeutic potential of MSCs, for wound healing tissue and repair, is basically established by their paracrine results[12-14]. Numerous pre-clinical and clinical investigations of MSCs have yielded promising outcomes. Additionally, those cells are somewhat safe for clinical applications. MSCs harvested from multiple anatomical locations, such as the adipose tissue, bone marrow, Wharton's jelly of the umbilical cord, show identical immunophenotypic shapes[15,16]. Nevertheless, a considerable body of proof exhibits that MSCs secrete various biologically active molecules, including chemokines, growth factors, and cytokines[17,18]. Given the statements above and the increasing significance of new biologically active substances of peptide nature and their therapeutic effects and due to the fact that this subject have not been completely analyzed so far, the current study intended to assess the osteoinductive potential of the conditioned medium concentrate on native mesenchymal stem cells. In addition, this study attempts to answer if the conditioned medium concentrate can be further utilized for the development of therapeutic drugs with osteoinductive and regenerative potential.

2. MATERIAL AND METHODS

The work was carried out on the culture of MSCs, which were obtained from the cell bank of the Research Laboratory "Cellular, assisted reproductive and DNA technologies" NRU "BelGU". biomass was grown in complete culture medium (PCS) based on DMEM with 10% fetal bovine serum (FBS) in a CO_2 incubator at 37 ° C, 100% humidity, and 5% CO_2. After receiving the required amount of MSCs to perform the work, the supernatant was removed from the vial, 5 ml of 0.05% trypsin solution with ethylenediaminetetraacetic acid was added, under an inverted light microscope for 5 min, the detachment of cells from the bottom of the culture flask was observed to block the action of trypsin in the vial 5 ml of DMEM medium with FBS was added. Then, the cells were separated from the enzyme by centrifugation at 1000 rpm for 5 min and resuspended in the PKS for two or two times. The resulting culture of rat MSCs was resuspended and planted in 225 cm^2 flasks in an amount of 6.3×10^6 cells. After obtaining a sufficient number of cells, the culture medium was removed, the cell monolayer was washed with phosphate-buffered saline (PBS). To stimulate the differentiation of MSCs in the osteogenic direction, we used a complete nutrient medium containing dexamethasone (0.1 μM), ascorbic acid (50 μM), and sodium β -glycerophosphate (10 μM) at the concentrations recommended in the guidelines [12-14]. Upon reaching the specified cultivation time, the conditioned medium was collected, then the cell monolayer was washed with PBS, and the same amount of serum-free growth medium without additives was added in the same amount in which the cultivation was carried out before. After completion of the cultivation, all of the conditioned medium was collected and centrifuged at 3000 rpm for 10 minutes. The resulting supernatant was passed through a 0.22 μm filter, ultrafiltration and dialysis of the conditioned medium were carried out. The conditioned medium was concentrated by tangential flow ultrafiltration using a Vivaflow® 200 system from Sartorius (Germany) using a membrane module with a cutoff of molecules with a molecular weight of 10 kDa[4,8,17]. The resulting concentrate is dialyzed using the Vivaflow® 200 system from Sartorius (Germany) by adding deionized water to the sample / diafiltration vessel[17,19,22]. Then, the conditioned medium concentrate purified from salts was dried in a Rotavapor® R-210 vacuum rotary evaporator from Buchi (Switzerland). The dried concentrate was collected aseptically from the round-bottom reaction flask using a spatula into a screw-capped microcentrifuge tube. The resulting fraction of a complex of small protein molecules was stored in a freezer at a temperature (-20 ° C), for a longer storage temperature (-80 ° C) was used. HPLC-MS / MS spectrometric analysis at the V.N.Orekhovich Moscow. Dry concentrate in an amount of 0.5 gram was dissolved in 4 ml of 50 mM ammonium bicorbanate, after which the mixture was transferred to a concentration filter Amicon Ultra 15 Centrifugal Filters Ultracel 10K for subsequent washing and concentration to a volume of 2 ml. To isolate the protein from the concentrate, the method of precipitation with a mixture of methanol-chloroform and water was used[18,21]. The total volume of 18 ml was centrifuged at 13000 rpm at room temperature for 2 minutes The supernatant was removed, and another 6 ml of methanol for the final washing of the precipitated protein, followed by centrifugation at 13000 rpm for 2 minutes The supernatant was taken, and the pure protein was dried in a centrifuge concentrator at 30 °C until the precipitating components completely evaporated. Dried protein dissolved in 5 M urea for further protein measurement by the method using bicinchoninic acid (BCA), washing on the filter according to the FASP protocol with reduction and alkylation, etc. by riptic cleavage of protein. To measure the concentration of total protein, an aqueous solution of bovine serum albumin (BSA) was used as a standard[14,21]. To 30 μ l of standard BSA solutions and experimental samples, 1 ml of a reagent containing 1% aqueous solution of sodium salt of bicinchoninic acid, 2% Na_2CO_3, 0.16% sodium tartrate, 0.4% NaOH and 0.95% $NaHCO_3$, pH = 11.25, 20 μ l of a 4% aqueous solution of $CuSO_4$. Then the solutions were mixed and incubated at 56 ° C for 20 min. on a thermal shaker Termomixer comfort (Eppendorf, Germany). Construction of a calibration curve for standard albumin solutions and measurement of the

concentration of total protein in experimental samples was carried out using a NanoDrop ND-1000 cuvette spectrophotometer (Thermo Fisher Scientific, USA) at a wavelength of 562 nm in 3 replicates. Obtained after washing and extraction, the protein mixture containing 250 μ g of protein was transferred to a Microcondevices YM-10 filter. Samples were washed by adding 200 μ l of buffer containing 50 mM ammonium bicorbanate. The washing procedure was repeated 4 times. After the last wash, the sample volume was 50 μl, and another 50 μl of working buffer (50 mM ammonium bicorbanate) was added to it for reduction and alkylation. To 100 μ l of the protein mixture was added 2 μ l (TCEP) + 4 ml (400 mM chloroacetamide), the mixture was incubated at 80 ° C for 30 min. After recovery and alkylation, the samples were washed twice in 200 μ L of buffer containing 50 mM ammonium bicorbanate, followed by centrifugation at 12000 rpm. within 15 minutes. c at a temperature of 20 ° C. For the hydrolytic digestion of the protein, trypsin was used in an enzyme / total protein weight ratio of 1/50 and incubated overnight at 37 °C. The resulting mixture of peptides was dried in a centrifugal concentrator at 45 ° C for 30 min, the peptides were redissolved in 20 μ L of 0.1% formic acid and subjected to further mass spectrometric analysis[7,8]. Proteomic analysis of peptides was carried out using an Ultimate 3000 RSLCnano chromatographic HPLC system (Thermo Scientific, USA) connected to a Q-exactive HFX mass spectrometer (Thermo Scientific, USA)[13-17]. One microgram of the peptide mixture was loaded onto an Acclaim μ-Precolumn (0.5 mm x 3 mm, 5 μm particle size, Thermo Scientific) enrichment column at a flow of 10 μl / min. within 4 minutes. in isographic mode using buffer "C" as a mobile phase (2% acetonitrile, 0.1% formic acid in deionized water). Then the peptides were separated on an Acclaim Pepmap® C18 HPLC column (75 μm × 150 mm, 2 μm particle size) (Thermo Scientific, USA) in a gradient elution mode. The total duration of the analysis was 90 min. Mass spectrometric analysis was performed on a Q-Exactive HFX mass spectrometer in the positive ionization mode using an NESI source (Thermo Scientific, USA)[9,10]. For mass spectrometric analysis, the following settings were set: emitter voltage 2.1 kV, capillary temperature 240 ° C. Panoramic scanning was performed in the mass range from 300 m / z to 1500 m / z, at a resolution of 120,000. In tandem scanning, the resolution was set to 15000 in the mass range from 100 m / z to the upper limit, which is determined automatically based on the precursor mass, but not more than 2000 m / z. Proteins were identified using the SearchGUI v.3.3.15 program with the simultaneous use of three search algorithms X! Tandem, OMSSA, MS-GF +. To identify proteins, we used the Uniprot sequence database with restrictions on the species of the organism of the studied samples - "Human". PeptideShaker v.1.16.40 was used to visualize and obtain search results and generate a report in the form of tables in .xls format. For validation of comparisons (pairing) of spectra and PSM peptides (Peptide-Spectrum Matches), identification of peptides and identification of proteins, an FDR (False Discovery Rate) value of no more than 1.0% was used. Proteins were considered as reliably identified if at least two validated peptides were found for them. Proteins were annotated in terms of gene ontology (GO) categories using the GeneXplain platform (www.genexplain.com). The analysis used the default settings[5,19]. During the annotation, a functional analysis algorithm was used to identify statistically significant representation of certain groups of proteins among all genes / proteins in the sample under study. The statistical significance was assessed using the probability p-value, as well as the Adjusted P-value, which was calculated taking into account the multiplicity of comparison[7-13]. The groups for which the Adjusted P-value was less than 0.001 were considered significant protein groups in the classifications. A part of the previously obtained concentrate of the conditioned medium was used to assess the proliferation and differentiation of rat MSCs in the osteogenic direction by staining for alkaline phosphatase [13-15]. The previously obtained culture of rat MSCs was resuspended and planted on the bottom of 35 mm Petri dishes with a coverslip (manufacturer: SPL Lifesciences, Korea), at a cell concentration of $0.2x10^6$ cells / ml. After incubation of MSCs for 30 minutes in a CO_2 incubator at 37 °C, 100% humidity, and 5% CO_2, 2 ml of complete culture medium based on DMEM with 10% fetal calf serum (FBS) containing a concentrate in the ratio 1:50. Then the cells were cultured in a CO_2 incubator at 37 °C, 100% humidity, and 5% CO_2. Rat MSCs without the addition of conditioned medium concentrate were used as a negative control. After 7 days of cultivation, cells were counted in a 35 mm Petri dish using specialized software Nikon EZ-C1 FreeViewer in 10 fields of view (the area of a 35 mm Petri dish is 8.8 cm2). Then MSC was washed with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde for 2 min. Fixed cells were washed with 1 ml of wash buffer (0.05% Tween-20 in PBS) and incubated with BCIP® / NBT substrate (Sigma Aldrich, USA) at room temperature for 10 min. After washing off MSCs using an Eclipse Ti-U microscope (NIKON, Japan) equipped with a camera, staining for alkaline phosphatase was detected. The resulting photographic color images were converted to black and white and negative. Then, using specialized software Nikon EZ-C1 FreeViewer (Fig. 1), the intensity of staining (in cu - arbitrary units) of MSC for the expression of alkaline phosphatase in 6 areas of 8 wells of the plate for the experimental and control groups was determined.



Statistical processing of the results obtained is carried out using the specialized software Statistica 6.0.

Fig 1: Assessment of the intensity of alkaline phosphatase staining with using Nikon EZ-C1 FreeViewer software.

3. RESULT AND DISCUSSION

Analysis of the conditioned medium concentrate sample by HPLC-MS / MS mass spectrometric analysis showed that the total protein content was 1.9 μ g / μ L. The spectra for the sample were obtained in the number of 3 technical replicates.

As a result of LC-MS / MS analysis and subsequent identification using the SearchGui program, 91 proteins were found in the sample, of which 45 were reliably identified. The list of proteins identified in the sample with the highest concentration is presented in Table 1.

Table 1. List of proteins found in the sample

Main connection	Description	MW [kDa]	Possible coverage [%]
A0A096P6L8	Fibronectin (A0A096P6L8_RAT)	272.2757	54.54
P60711	Actin, cytoplasmic 1 (ACTB_RAT)	41.70973	64.53
1LTJ5	Protein Hspg2 (F1LTJ5_RAT)	262.94755	50.96
Q68FP1	Gelsolin (GELS_RAT)	86.014062	60.26
P02454	Collagen alpha-1(I) chain (CO1A1_RAT)	137.86897	62.35
Q5M7T5	Protein Serpinc1 (Q5M7T5_RAT)	52.20084	77.42
M0RBF1	Complement C3 (M0RBF1_RAT)	186.20633	77.99
F1M6Z1	Apolipoprotein B-100 (F1M6Z1_RAT)	509.37581	73.26
P35444	Cartilage oligomeric matrix protein (COMP_RAT)	82.610699	63.58
P68136	Actin, alpha skeletal muscle (ACTS_RAT)	42.023853	64.72
Q6MG90	Complement component 4, gene 2 (Q6MG90_RAT)	192.0063	64.54
A0A0G2K625	Protein LOC297568 (A0A0G2K625_RAT)	160.9003	54.8
G3V852	Protein Tln1 (G3V852_RAT)	269.50409	72.33
M0R9G2	Alpha-2-macroglobulin (M0R9G2_RAT)	163.68158	67.8
D3ZBS2	Inter-alpha-trypsin inhibitor heavy chain H3 (D3ZBS2_RAT)	99.005684	64.49
A0A0G2JV24	Protein Thbs1 (A0A0G2JV24_RAT)	127.78878	69.44
A0A0G2K5E8	Protein NEWGENE_621351 (A0A0G2K5E8_RAT)	119.97178	68.84
P47853	Biglycan (PGS1_RAT)	41.679709	72.63
A0A0G2K8V2	Vinculin (A0A0G2K8V2_RAT)	123.51375	82.98
G3V843	Prothrombin (G3V843_RAT)	70.340627	70.34
A0A097BW25	Periostin isoform R1 (A0A097BW25_RAT)	87.098399	74.71
A0A0G2K151	Apolipoprotein E (A0A0G2K151_RAT)	41.173779	71.03
P14630	Apolipoprotein M (APOM_RAT)	21.498517	58.42
D3ZQ25	Fibulin-1 (D3ZQ25_RAT)	78.019457	63.12
A0A0G2K2V6	Keratin, type I cytoskeletal 10 (A0A0G2K2V6_RAT)	56.778705	60.82
Q63041	Alpha-1-macroglobulin (A1M_RAT)	167.01941	58.4
B2RYM3	Inter-alpha trypsin inhibitor, heavy chain 1 (Predicted), isoform CRA_a (B2RYM3_RAT)	100.5256	62.94
P68370	Tubulin alpha-1A chain (TBA1A_RAT)	50.10361	68.51
P62804	Histone H4 (H4_RAT)	11.360382	100

P13941	Collagen alpha-1(III) chain (CO3A1_RAT)	138.85052	57.96
A0A0G2K014	Protein Lcp1 (A0A0G2K014_RAT)	71.934642	78.45
A0A0G2K7X7	Oxidation resistance protein 1 (A0A0G2K7X7_RAT)	93.603519	74.82
D4A8G5	Protein Tgfbi (D4A8G5_RAT)	74.705787	63.65
F1LRE2	Insulin-like growth factor binding protein, acid labile subunit, isoform CRA_b (F1LRE2_RAT)	66.856694	53.57
Q4V8P9	Protein Tfap2c (Q4V8P9_RAT)	49.06785	61.33
P00762	Anionic trypsin-1 (TRY1_RAT)	25.942676	29.27
A0A0G2K3C8	Nidogen-2 (A0A0G2K3C8_RAT)	152.7235	50.61
Q80ZA3	Alpha-2 antiplasmin (Q80ZA3_RAT)	46.436186	71.77
Q7TP84	Ab1-346 (Q7TP84_RAT)	86.000916	64.03
D3ZAB1	Lactotransferrin (Predicted) (D3ZAB1_RAT)	79.765759	84.34
A0A096P6L9	Complement C5 (A0A096P6L9_RAT)	188.96131	60.8
P49001	Bone morphogenetic protein 2 (BMP2_RAT)	44.354758	60.81

Source: compiled by the authors based on the results of the current study

Table 1 illustrates the List of proteins found in the sample. It reveals that Histone H4 (H4_RAT) and Lactotransferrin (D3ZAB1_RAT) has the greatest shares with 100 and 84.34 percent in the sample, similar to Vinculin (A0A0G2K8V2_RAT) with nearly 83 percent. In contrast, it can be seen that Anionic trypsin-1 (TRY1_RAT) has the least share in the sample with merely 29.27 percent. Plus, It can be observed that most of the identified proteins belong to the group of proteins that regulate cellular processes, and groups of proteins have also been found that relate to the organization of the extracellular structure, the processes of cell development, or respond to the effect of organic substances on the cell. Next, we will consider in more detail a brief description of proteins that provide osteogenesis. Fibronectin is an extracellular matrix glycoprotein that binds to membrane receptor proteins called integrins. It has been experimentally established that fibronectin stimulates osteogenic differentiation of stem cells [14-16]. actin, cytoplasmic 1 - plays an important role in key cellular processes such as adhesion, migration; in addition to their role in the cytoplasmic cytoskeleton, actins are also localized in the nucleus and regulate gene transcription, motility, and repair of damaged DNA [17]. It was shown that intra-nuclear actin induces the expression of osteogenic genes of osterix and osteocalcin in a Runx2-dependent manner, which leads to the acquisition of an osteogenic phenotype [18-20]. Protein Hspg2 is a basic heparan sulfate proteoglycan protein specific to the basement membrane, modulating heparin binding growth factor availability during cartilage development. Deficiency of this protein affects bone formation and calcification [21, 22]. Gelsolin, an actin-binding protein, controls the length of actin filaments in vitro, and cell shape and motility in vivo. It was revealed that gelsolin participates in the assembly of podosomes and contributes to an increase in bone mass and strength [23]. Collagen alpha-1 (I) is an extracellular matrix protein that serves as a scaffold that determines the shape and mechanical properties of many tissues [24]. Complement C3 and C5 are proteins that are part of the complement system. Studies confirm that C3 can promote bone formation, where C3a and C5a influence bone cell migration, osteoblast-osteoclast interaction, and osteoblast-mediated modulation of the inflammatory response [25]. Cartilage oligomeric matrix protein is an extracellular matrix protein that interacts with fibronectin, participating in the assembly of the extracellular matrix and enhances osteogenesis by direct binding and activation of bone morphogenetic protein-2 [7]. Alpha-2-macroglobulin protein is able to suppress all four classes of proteinases using a unique trapping mechanism and bind osteogenic growth peptide [24]. Biglycan, a member of a small family of leucine-rich proteoglycans, is one of the major proteoglycans found in bones and is involved in osteoblast differentiation and bone formation [9-13]. Vinculin is a cytoskeleton protein involved in the formation of cell contacts with other cells and the extracellular matrix through integrin receptors. It was found that it is responsible for the relationship between attachment, the spread of osteoblasts and the formation of focal contacts on the underlying surfaces [12]. Along with this, vinculin regulates MSC differentiation by stimulating the nuclear localization of YAP / TAZ. [13]. Fibulin-1 is an ECM glycoprotein protein that is expressed in bone marrow and bone-derived osteoblasts [4, 5], while Fibulin-1 is required for bone formation and Bmp-2-mediated induction of Osterix [16, 27]. Histone H4 is a low molecular weight protein that is rich in positively charged basic amino acids (arginine and lysine) and interacts with negatively charged phosphate groups in DNA, ensuring the precision of osteoblast differentiation [8, 9]. Collagen alpha-1 (III) chain is an extracellular matrix protein synthesized by cells as pre-procollagen, found in bone tissue [10]. Protein Tgfbi is an extracellular matrix protein expressed in a wide variety of tissues including bone. TGFBI binds to collagen types I, II, and IV, as well as biglycan and decorin, and plays an important role in interactions between cells, cells and collagen and between cells and matrix [21]. Lactotransferrin belongs to the transferrin family. Has anabolic, differentiating, and anti-apoptotic effects on osteoblasts and can also inhibit osteoclastogenesis, possibly playing a role in the regulation of bone growth [2]. Bone morphogenetic protein 2 is a growth factor of the TGF-beta superfamily, an important regulator of osteogenesis. BMP-2 is a polypeptide growth factor containing 396 amino acids, the function of which is to induce the differentiation of mesenchymal stem cells into cartilage and bone tissue [13, 24].

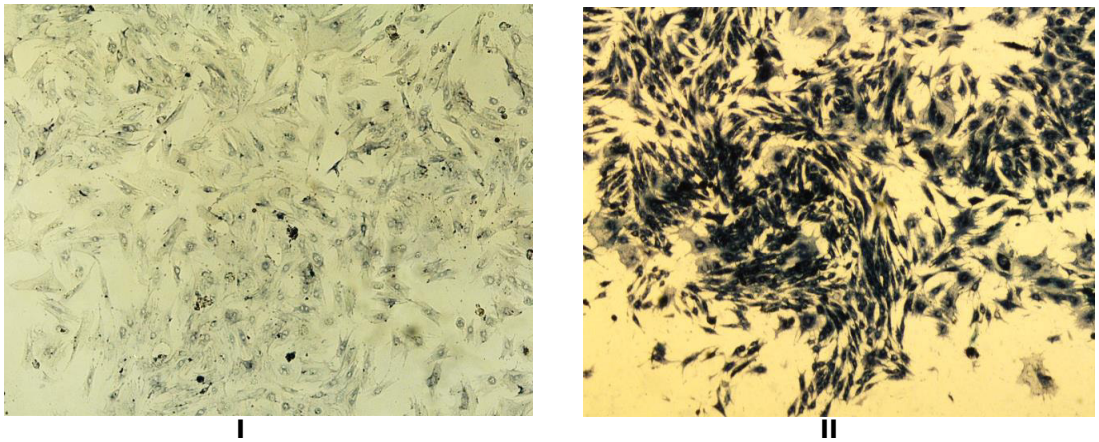


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Fig 2: MSCs from the control (I) and experimental (II) groups with the addition of the conditioned medium concentrate to the culture medium; X100.

In the course of this study, it was found that the number of MSCs in the experimental and control groups does not have significant differences and is 1.2 × 106 cells / ml. MSCs actively proliferate in both the experimental and control groups. When a conditioned medium concentrate is added to the nutrient medium in the experimental group, the intensity of staining for alkaline phosphatase in MSCs is greater than in the control group and amounts to 101.50 ± 2.3 a.u., while in the control group it is 39.15 ± 1.6 a.u. at p≤0.01 (Fig. 2).

4. CONCLUSION

In the course of the study, it was found that when the conditioned medium concentrate is added to the complete nutrient medium, differentiation of MSCs in the osteogenic direction is noted, in the absence of a proliferative effect in comparison with the control group. The intensity of staining for alkaline phosphatase - a marker of osteogenic differentiation in the experimental group of MSCs is higher than in the control group (cells without the addition of conditioned medium). Proteomic analysis of the conditioned medium concentrate showed that the studied concentrate contains peptides that play an important role in the differentiation of MSCs into osteoblasts and ensuring effective osteogenesis: fibronectin; actin cyto-plasmic 1; protein Hspg2; gelsolin; complement C3 end C5; cartilage oligo-meric matrix protein; biglycan; vinculin; fibulin-1; histone H4; protein Tgfbi; BMP-2. Thus, most of the identified proteins of the conditioned medium concentrate belong to the group of proteins regulating cellular processes; groups of proteins have also been found that relate to the organization of the extracellular structure, the processes of cell development, or are directly responsible for the differentiation of MSCs in the osteogenic direction. Along with this, the concentrate of the conditioned medium does not affect the proliferation rate of MSCs. In connection with the above, the conditioned medium concentrate can be further used for the development of therapeutic drugs with osteoinductive and regenerative potential.

5. AUTHORS CONTRIBUTION STATEMENT

N.S.V., B.V.S., N.N.A., P.L.A., S.V.I., V.S.V., M.D.V., and P.M.V. carried out the experiment. N.N.A., P.L.A., and S.V.I. conceived the original idea. N.S.V., and B.V.S. supervised the project. All authors provided critical feedback and helped shape the research, analysis and manuscript.

6. CONFLICT OF INTEREST

Conflict of interest declared none.

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