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GENERATION OF THREE-DIMENSIONAL SPHEROIDS FROM MOUSE MESENCHYMAL STEM CELL

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ABSTRACT

In comparison with two-dimensional (2D) culture, three-dimensional (3D) cultures of mesenchymal stem cells (MSCs) in spheroids (3D spheroids) offer a more physiologically realistic condition. Accordingly, 3D culture has been used to improve the immunomodulatory properties of MSCs and their survival after transplantation. Several methods have been developed to generate 3D spheroids of MSCs to date; however, these methods are time-consuming or expensive, thus limiting their wide application. In this study, we compared two inexpensive and simple culture methods for the generation of 3D spheroids from the MSCs of mouse compact bone: pellet culture and rotating vessels using low-binding 96-well plates. The effectiveness of the two methods was determined by the number of obtained spheroids, their diameter, and the expression of the MSC marker CD44 using morphometric and immunofluorescent methods. A higher number of homogeneous 3D spheroids was obtained from the MSCs of compact bone using a rotating vessel compared to that obtained with pellet culture. The immunofluorescence assay further showed that the developed 3D spheroids highly expressed CD44 on the cell surface. Thus, we suggest that the method of rotating vessels is effective for generating 3D spheroids from the MSCs of compact bone. This method can be used to promote research on the biology of stem cells and develop new therapeutic approaches in cell therapy and regenerative medicine.

Keywords: compact bone mesenchymal stem cells, 3D spheroids, pellet culture, rotating vessels.

INTRODUCTION

Mesenchymal stem cells are a heterogeneous fibroblast-like cell population that can be isolated from nearly all mammalian tissues and organs, such as bone marrow, adipose tissue, synovial membrane, skeletal muscle, umbilical cord, etc. MSCs have attracted the attention of scientists and clinicians due to their multilineage differentiation potential, low immunogenicity and active participation in tissue repair and regeneration after migration to the site of tissue injury [1-3]. When stimulated by appropriate signals, MSCs are capable of differentiating into several specialized cell types, such as adipocytes, chondrocytes, osteoblasts, endothelial cells and cardiomyocytes [4-6]. Moreover, recent studies have shown that MSCs possess strong immunosuppressive and immunomodulatory properties that are mediated both by cell-cell contacts and production of various signaling factors [7-9].

Traditionally, MSCs are expanded according to the standard 2D culture technique. However, this technique does not provide appropriate physiological conditions as some *in vivo* characteristics of MSCs are lost or compromised [10]. On the other hand, the 3D culture of MSCs represents more physiological conditions and has been used to improve the immunomodulatory properties of MSCs and

their survival after transplantation. For example, it has been shown that conditioned medium from spheroids of MSCs inhibited the production of pro-inflammatory cytokines such as TNF-α, IL-6, IL-12p40, IL-23, and CXCL2 from one hand, and increased the production of anti-inflammatory cytokines IL-1ra and IL-10 by LPS stimulated macrophages from another hand [11]. In addition to this, it was shown that to self-activation of TNF-induced protein 6 (TSG6) and COX-2 that increase PGE-2 production and conversion of pro-inflammatory M1 macrophages into anti-inflammatory M2 macrophages, as well as more effectively suppressing TNFα production by LPS stimulated macrophages [10-11].

Currently, several methods have been developed to generate 3D spheroids of MSCs, such as hanging drop technique [12], hydrogels [13], magnetic levitation [14] and nanoparticles [15, 16]. Despite being effective, some of these methods are time consuming or expensive. In this regard, we have been compared two inexpensive and simple culturing methods for generation of 3D spheroids from MSCs of compact bone that only requires a low-binding plate with U-shaped wells, orbital shaker or centrifuge.

In this study, we defined that the method of rotating vessels for the generation of the 3D spheroids of MSCs in comparison to pellet culture method is more efficient and reproducible, allows obtaining more mature spheroids in 2 days.

Materials and methods

Animals. Male C57BL/6 mice 2-4week old were purchased from SPF-vivarium of M. Aikimbayev≥s Kazakh Scientific Centre for Quarantine & Zoonotic Diseases (Almaty, Kazakhstan). The animals were housed in a temperature-controlled environment (23 °C) with 60% relative humidity applying a 12 h light/dark cycle. The animals had *ad libitum* access to food and water. Experimental procedures involving animals were in full compliance with current international laws and policies (Guide for the Care and Use of Laboratory Animals, National Academy Press, 1996) and were approved by the Local Ethics Committee for Animal Use in National Center for Biotechnology (Nur-Sultan, Kazakhstan).

Isolation and culture of MSCs from mouse compact bone. Isolation of MSCs from mouse compact bone was performed according to the method described by Zhu et al. [17]. Briefly, mice were humanely killed by cervical dislocation. Humeri, tibia, and femur were dissected from the hind limbs in a laminar flow hood using sterile instruments and washed thoroughly with cold α-MEM (Gibco, USA) containing 100U/ml Penicillin/100 μg/ml streptomycin (Gibco, USA). Muscle and connective tissues from bones were removed by a scissor and sterile gauze. After removing the epiphysis, the bones of the humeri, tibiae, and femurs were kept in a Petri dish with 5 ml α-MEM medium supplemented with 2% fetal bovine serum (FBS) (Gibco, USA). Next, a syringe needle was inserted into the bone marrow cavity and the marrow was flushed out with α-MEM medium. The bone cavities were thoroughly rinsed 3 times using a syringe until they were blanched. The diaphysis of humeri, tibiae and femurs was cut into 1ć3 mm^3 chips by a scalpel. The bone fragments were transferred with forceps into a Petri dish containing 3 ml of α-MEM media supplemented with 10% FBS and 1 mg/ml collagenase type II (Gibco, USA). The fragments were incubated in a shaker-incubator (Biosan, Latvia) for 2 hours, at 37°C and 200 rpm. After the enzymatic treatment, the media was removed and enzyme-treated bone fragments were washed 3 times with 5 ml α-MEM, transferred into a T25 culture flask and cultured in MSCs complete medium: α-MEM media supplemented with 10% FBS, 0,1% Pen/Strep and non-essential amino acids (Gibco) at 37°C and 5% CO_2 for 3-5 days. After 5 days of incubation, the culture flasks were washed with phosphate-buffered saline (PBS) to remove non-adherent cells and kept in MSCs complete medium. The culture medium was changed every 2 days. When the cells reached 70ć80% confluence, cells were harvested using Tryple Express (Gibco, USA), counted and split into new culture flasks [18].

Colony-forming units assay (CFU). The cells were harvested using Tryple Express, and the total cell number was determined by using an automatic cell counter BioRad TC20 (BioRad, Germany). Harvested cells were seeded into culture flasks at density 100 cells/cm2 and 1000 cells/ cm2 cells and cultured for 2 weeks. Generated colonies were washed with PBS and stained with 0.5% Crystal Violet (Sigma, USA) in methanol for 10 minutes at room temperature. After staining, flasks were washed with PBS, allowed to dry, and colonies were counted and an

alyzed by using a stereomicroscope SZ61 (Olympus, Germany).

Multilineage differentiation assay. For adipogenic differentiation, 1×10^4 cells/cm^2 were plated in a 6-well culture plate and cultivated in an adipogenic differentiation medium composed of high glucose DMEM (Gibco, USA), supplemented with 15% FBS, 0.2 mM L-glutamine (Gibco, USA), 100 µM L-ascorbic acid (Sigma, USA), 200 µM indomethacin (Sigma, USA), and 100 nM dexamethasone (Sigma, USA). The medium was changed twice a week. After 21 days, the cells were fixed with 4% paraformaldehyde solution (PFA) (Sigma, USA) and stained with Oil Red O (Sigma, USA).

For differentiation, the cells at 90% confluence were cultivated in an osteogenic differentiation medium composed of low glucose DMEM (Gibco, USA) supplemented with 15% FBS (Gibco, USA), 200 µM L-ascorbic acid (Sigma, USA), 10 mM b-glycerolphosphate (Sigma, USA), and 100 nM dexamethasone (Sigma, USA). The medium was changed twice a week for 3 weeks. Osteogenic differentiation was evaluated using Alizarin Red S (Sigma, USA) staining.

For chondrogenic differentiation, the cells were resuspended at 1.25×10^6 cells/ml in a chondrogenic differentiation medium composed of high-glucose DMEM supplemented with 1% ITS+Premix (Sigma, USA), 100 µmol/L ascorbate-2-phosphate (Sigma, USA), 0.1 µm dexamethasone, and 10 ng/ml TGF-β1 (Gibco, USA). To create chondrogenic micromass pellets, 2.5×10^5 cells from this cell solution was placed in a 96-well polypropylene tube, centrifuged at 500×*g*, and placed in an incubator at 37°C and 5% CO_2. The medium was changed twice a week. After 3 weeks, the cell pellets were harvested, fixed with 10% neutral-buffered formalin (Sigma, USA), paraffin-embedded, sectioned at 5 µm, and stained with Toluidine blue (Sigma, USA).

Flow cytometry analysis. The characterization of the cells was conducted by antibody panels which commonly used for basic characterization of MSCs preparations [19]. CD29, CD31, CD44, CD45, CD90, CD105, CD106, Sca-1 antibodies as well as mouse IgG2a and IgG2b isotypes (all from BD Biosciences, USA) were used to evaluate developed MSCs of compact bone. Analysis of cells, stained with antibodies was performed on Attune flow cytometer (Thermo Scientific, UK). Resulted data was counted using Flowjo program.

Generation of the 3D spheroids by the method of rotating vessels and their dissociation. For the generation of 3D spheroids was selected Achil 70-80% of monolayer [20]. Cells at were seed at 2×10^4 cells per well to U-shaped bottom 96-well non-adhesive plate (Nunc, USA) in 100 µL/well of MSCs complete medium. The plate was incubated in shaker-incubator at 250 rpm/min at 37 ° C and 5% CO2 for 36-48 hours. The size and number of 3D spheroids were observed using an inverted microscope (Axio Observer A1, Carl Zeiss, Germany) at a phase-contrast with 10x magnification. The number of 3D spheroids was counted by the next formula [21]:

Total number of spheroids=spheroid count (in 50µl)/50µl × Volume of spheroid suspension (µl)

To obtain spheroid derived cells, 3D spheroids were dissociated using 3-7 min (depending on the size of spheroids) incubation in Tryple Express and plated to adhesive culture dish [22].

Generation of 3D spheroids by pellet culture method. According to the literature, the method which commonly used for chondrogenic differentiation of MSCs [19] was implicated for 3D spheroids development [23]. In this method, 2×10^4 cells were placed in 96 well low-adhesive plates at 200 µL/well of MSCs complete medium and centrifuged at a speed of 450g for 10 min. Then, the cells were incubated at 37°C and 5% CO_2 incubator for 48 h.

Viability of cells. Spheroid derived cells were stained with trypan blue, counted and examined on the percentage of viable cells using automatic cell counter (Bio-rad, Germany).

Immunofluorescence analysis of 3D spheroids. 3D spheroids were analyzed through the standard immunostaining protocol [19]. 1×10^5 cells per well were seeded into a 4-well slide (BD Biosciences, USA) and incubated overnight in a CO_2 -incubator to form a monolayer. Then cell monolayer was fixed with 4% PFA in PBS (pH 7.2) for 20 min. After a 5 min treatment with Triton X-100 cells were washed three times with PBS and incubated in 1% solution of bovine serum albumin (BSA) for 30 min. In this assay, spheroids were stained with MSCs markers: CD44 FITC (1:100) (BD Biosciences, USA) antibody. For imaging by confocal microscopy, the cell nucleus was treated with propidium iodide dye (PI). Analysis of the stained samples was conducted confocal laser

scanning microscope LSM 780 (Carl Zeiss, Germany). Images processing were conducted using Zen 2011 software and ImageJ program.

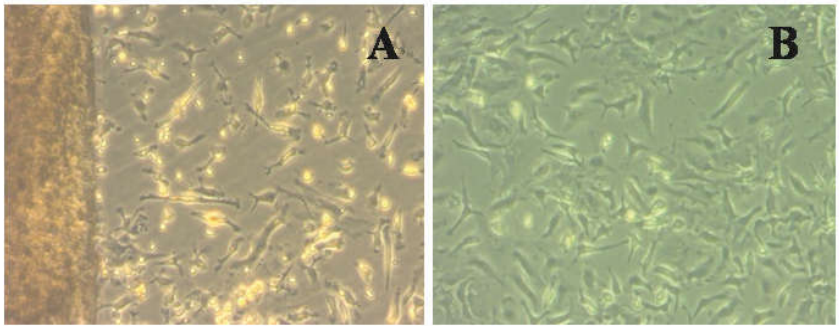
Statistical analysis. Data are presented as mean ± SD. The statistical significance was calculated using Student≥s test, P <0.05 was considered statistically significant. Statistical analysis was conducted with Statistica 6.0 (StatSoft,

RESULTS AND DISCUSSION

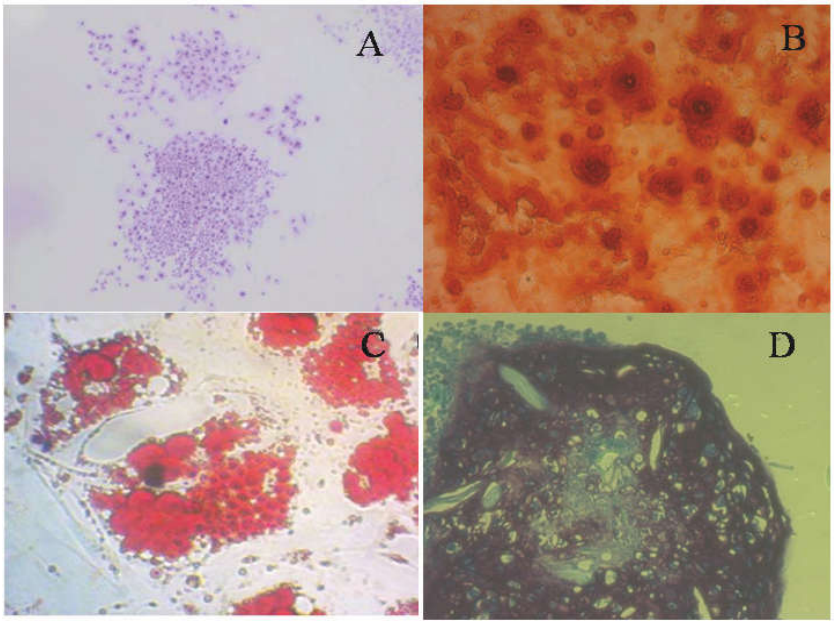
After isolation of MSCs from mouse compact bone, they were first analyzed and characterized based on morphology. Morphological analysis revealed that MSCs have a typical fibroblast-like morphology with an irregular shape, long processes, and oval nuclei containing two or three nucleoli (figure 1). Next, MSCs were evaluated based on their ability to form clonal fibroblastic colonies (figure 2A). CFU assay showed that MSCs of compact bone possesses the high proliferative activity and ability to form colonies which are characteristic for MSCs.

To evaluate the multilineage differentiation ability of the MSCs of compact bone, three different types of differentiation assays were performed. It was revealed that MSCs can differentiate into adipocytes, chondrocytes, and osteoblasts (figure 2B-2D).

To determine a phenotype of the MSCs of compact bone, flow cytometry analysis was performed. A comprehensive panel of positive and negative CD markers was used to characterize the expression of MSCs markers. It was observed that MSCs significantly expressed surface markers such as CD29, CD44, CD90, CD105, CD106, and Sca-1. In contrast, the expression of hematopoietic and endothelial markers CD45 and CD31 in the culture of the MSCs of compact bone was not detected (figure 3).



A) Migration of MSCs from mouse compact bone chip in 3 days.
B) Formation of the monolayer in 10 days of MSCs cultivation. Magnification, 100×
Fig. 1. Morphological features of the MSCs of the compact bone mouse.



A) MSCs formed fibroblastic colonies. Crystal violet staining.
B) Osteogenic differentiation. Alizarin red S staining.
C) Adipogenic differentiation. Oil Red O staining.
D) Chondrogenic differentiation. Histological section of pellet culture stained with toluidine blue.
Fig. 2. Characterization of the MSCs of compact bone

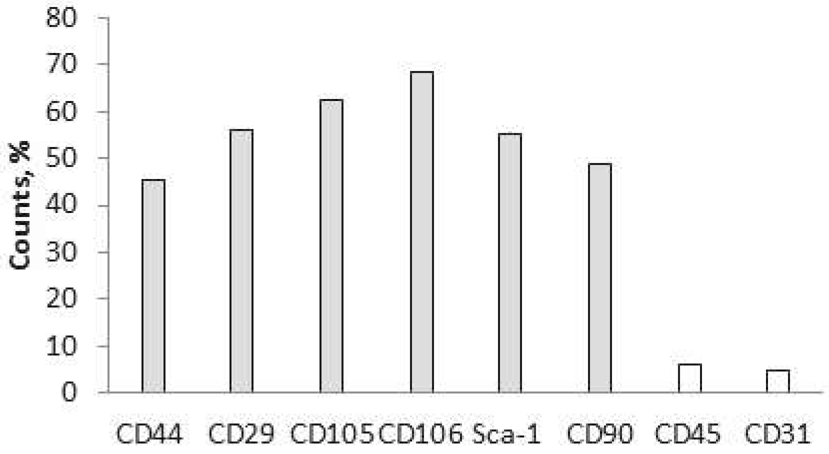


Fig 3. Flow cytometry analysis of the phenotype of mouse compact bone MSCs

To find a technically easy-to-use and productive method for generation of 3D spheroids we examined two methods: "pellet culture" and "rotating vessels". Schematic illustration of the methods of pellet culturing and rotating vessels are shown in figure 4.

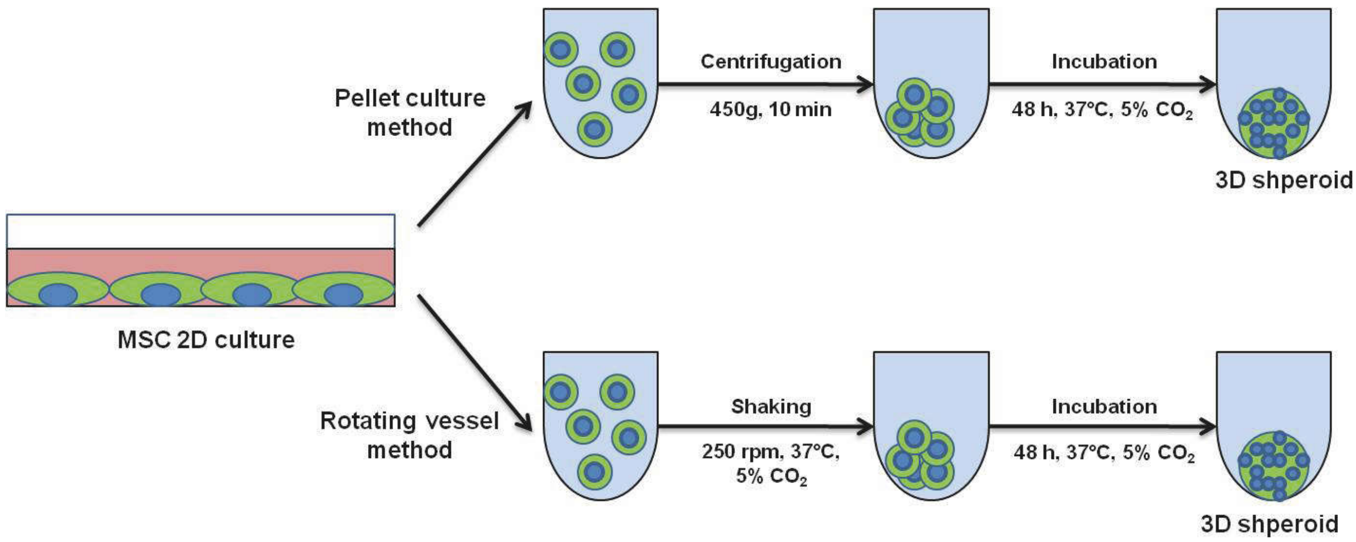


Fig 4. Schematic illustration of methods used for the generation of 3D spheroids

In the method of pellet culture after examining a 500g speed, which normally was used for chondrogenic beads assembling, and 450g speed, it was revealed that 450g speed was optimal for the generation of 3D spheroids.

Based on literature data, it is well-known that 3D spheroids aggregation maintains by the next two mechanisms: cluster-based self-assembly and collision-based self-assembly [20]. In this study, 3D spheroids of MSCs, generated by pellet culture, were aggregated with cluster-based self-assembly mechanism when centrifugal power forced cell-to-cell adhesion at the bottom of the conical well. However, it causes a poor effect on the viability of the cells. Therefore, the collision-based self-assembly mechanism of rotating vessels method, in comparison with the pellet culture method where generated 3D spheroids of the MSCs of compact bone had a large size and were minimal in number per well (16 spheres after 24 h and 24 spheres after 48 h of culturing), resulted in a high yield of homogeneous in size and shape spheres per well (34 spheres after 24 h and 41 spheres after 48 h of culturing) (figure 5). The diameter of 3D spheroids was 73 nm and 78 nm at pellet culture method and 68 nm and 71 nm at rotating vessels method after 24h and 48h respectively (figure 5 H).

Confocal microscopy imaging of stained 3D spheroids illustrated that rotating vessels obtained 3D spheroids of the MSCs of compact bone are highly expressed MSCs marker CD44 on the surface (Fig. 6) than pellet culture-derived 3D spheres. Thus, the lowest number of viable cells was generated by the method of pellet culture because centrifugal force damaged the cell structure. Moreover, on confocal microscopy, it was observed that 3D spheroids, obtained by the method of rotating vessels had round uniform shape with PI expression in the nucleus

(figure 6 H). Orthogonal views of 3D spheroids of the MSCs of compact bone indicated the evidence of three-dimensional structures in the xćz and yćz directions relative to the image plane (figure 6).

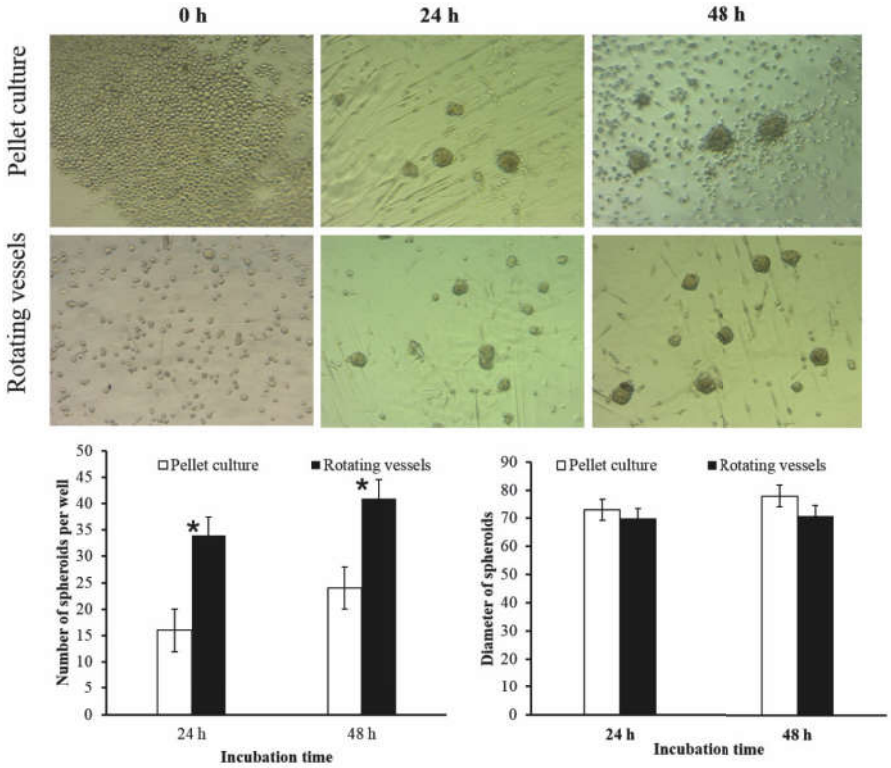


Fig 5. Generation of 3D spheroids of the MSCs of compact bone by the methods of pellet culture and rotating vessels. *p<0.05 means significant difference, mean±s.d. Magnification, 50×.

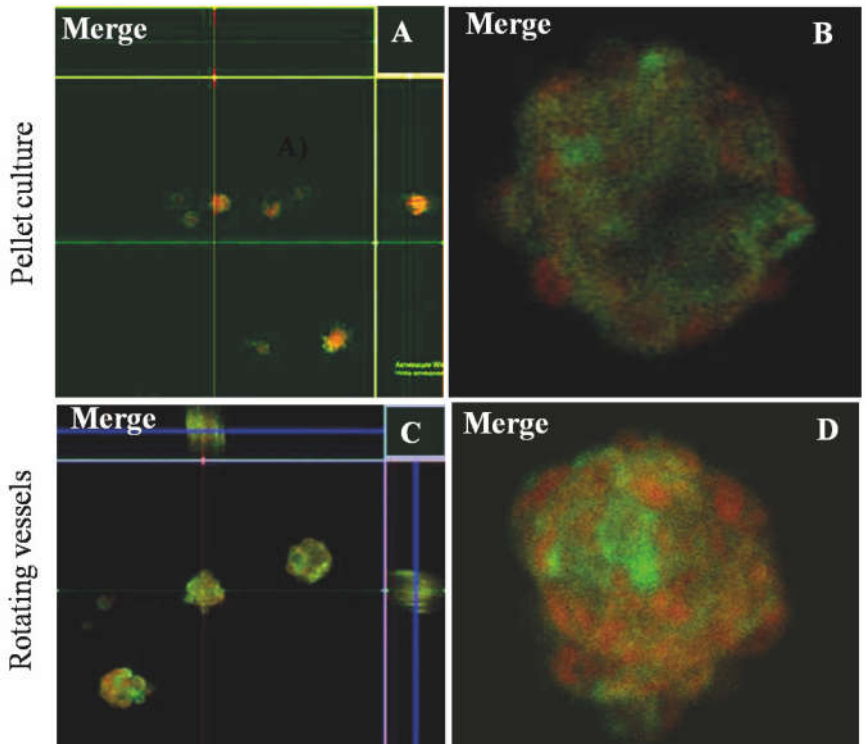


Fig 6. Confocal laser scanning microscopy imaging of the 3D spheroids of MSCs. Self-assembled MSCs was stained by CD90 -FITC antibodies (green). The cell nuclei were stained by PI (red). Magnification 100×

CONCLUSION

In this work were discussed the techniques of 3D spheroids formation: pellet culture and rotating vessels. Limitations of the method of pellet culture include the formation of heterogeneous spheroids as well as low viability of cells in the structure of 3D spheroid because centrifugation impacted to the cre-

ation of low oxygen in the center of the sphere.

Advantages of the generation of 3D spheroids from MSCs of compact bone by the method of rotating vessels: high yield of equal shaped spheroids, easy control of microenvironment of the cells and no need in specialized and expensive equipment. This method creates an internal microgravity condition in the wells with rotating cells supplying a low pressure of the shear environment. Thereby, self-assembling occurs by cell-to-cell adhesion and by the influence of soluble components in MSCs culture medium (nutrients, oxygen, growth factors). Furthermore, during our study, it was shown that the number of the MSCs of compact bone after 3D spheroid formation by the method of rotating vessels was increased and the cells after dissociation process had normal adhesive capabilities.

The number of studies suggested that aggregation of cells in 3D culture increase paracrine effects of the MSCs in inflammation conditions [22, 24] in comparison with 2D systems when was observed the decline of MSCs stemness. In this regard, the development of 3D spheroids from MSCs which enhance their anti-inflammatory, tissue reparative effects have therapeutic potential in regenerative medicine.

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ТЫШҚАННЫҢ ЫҚШАМ СҮЙЕГІНІҢ МЕЗЕНХИМАЛДЫ ДІҢГЕК ЖАСУШАЛАРЫНАН 3D СФЕРОИДТЕРІН АЛУ

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ТҮЙІН

Сфероидтердегі (3D сфероидтер) мезенхималды діңгек жасушалардың (МДЖ-лар) үш өлшемді (3D – three-dimensional) өсіруі екі өлшемді (2D - two-dimensional) өсіруге қарағанда физиологиялық жағдайларды әлдеқайда көп тудырады. МДЖ-лардың иммуномодулярлық қасиеттерін жақсарту және трансплантациядан кейін олардың аман қалуына үшін 3D өсіруі қолданылды. Қазіргі уақытта, МДЖ-лардың 3D сфероидтарын алу үшін бірнеше әдістер жасалды, алайда олардың көпшілігі уақытты қажет ететін немесе қымбат. Бұл зерттеуде біз түйіршікті өсіру және флакондарды айналдыру әдістерінде 96-ұңғымалы төмен ұштастыратын планшеттерді пайдаланып тышқанның ықшам сүйегінің МДЖ-ларынан 3D сфероидтерін алудың екі қолжетімді және қарапайым әдістерін салыстырдық. Әрбір әдістің тиімділігін зерттеу үшін алынған сфероидтердің санын есептеп, олардың диаметрін өлшедік, сондай-ақ морфометриялық және иммунофлуоресцентті әдістерді пайдаланып CD44 маркердің (МДЖ-лардың маркері) экспрессиясын анықтадық. Зерттеудің нәтижелері бойынша, флакондарды айналдыру әдісі түйіршікті өсіру әдісімен салыстырғанда ықшам сүйегінің МДЖ-ларынан үлкен сандағы біртекті 3D сфероидтерді алуға мүмкіндік береді. Осылайша, біздің деректерімізге сүйенсек, флакондарды айналдыру әдісі ықшам сүйегінің МДЖ-ларынан 3D сфероидтерді алуға тиімді. Бұл әдіс діңгек жасушаларының биологиясын зерттеу және жасушалық терапия мен регенеративті медицинада жаңа терапиялық тәсілдерін әзірлеу үшін қолданылуы мүмкін.

Негізгі сөздер: ықшам сүйегінің МДЖ-лар, 3D сфероидтер, түйіршікті өсіру әдісі, айналмалы флакондар әдісі.

ПОЛУЧЕНИЕ ТРЕХМЕРНЫХ СФЕРОИДОВ ИЗ МЕЗЕНХИМАЛЬНЫХ СТВОЛОВЫХ КЛЕТОК МЫШИ

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АБСТРАКТ

Трёхмерное культивирование (3D – three-dimensional) мезенхимальных стволовых клеток (МСК) в сфероидах (3D сфероиды) создает больше физиологических условий в сравнении с двухмерным культивированием (2D - two-dimensional). 3D культивирование используется для улучшения иммуномодулирующих свойств МСК и их выживаемости после трансплантации. В настоящее время, были разработаны несколько методов для получения 3D сфероидов МСК, однако большинство из них занимает много времени или дорогостоящие. В данном исследовании мы сравнили два доступных и простых метода культивирования для получения 3D сфероидов из МСК компактной кости мыши, используя 96-луночные планшеты с низким уровнем связывания в методах культивирования после центрифугирования и шейкирования клеток. Для изучения эффективности каждого метода, мы подсчитали количество полученных сфероидов, измерили их диаметр, а также определили экспрессию маркера CD44 (маркер МСК), используя морфометрические и иммунофлуоресцентные методы. Результаты нашего исследования показали, что метод шейкирования, позволяет получать большое количество однородных 3D сфероидов из МСК компактной кости по сравнению с методом культивирования после центрифугирования клеток. Кроме того, иммунофлуоресцентный анализ показал, что полученные 3D сфероиды демонстрируют высокую экспрессию CD44 маркера на поверхности клеток. Таким образом, основываясь на наших полученных данных, мы предполагаем, что метод шейкирования клеток наиболее эффективен для получения 3D сфероидов из МСК компактной кости. Этот метод может быть использован для изучения биологии стволовых клеток и разработки новых терапевтических подходов в клеточной терапии и регенеративной медицине.

Ключевые слова: МСК компактной кости, 3D сфероиды, метод центрифугирования, метод шейкирования.