REGENERATIVE EFFECTS OF MOUSE ADIPOSE-DERIVED MULTIPOTENT STROMAL CELLS IN A MICROMASS GRAFT FOR THE TREATMENT OF BONE INJURY MODEL

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Abstract

Background. Adipose-derived stem cells (ADSCs) are a promising source for the regeneration of bone tissue injuries. At the same time, three-dimensional cultures provide spatial organization of stem cells for optimal intercellular signaling, contact interaction and increase the efficiency of directed osteogenic differentiation prior to further transplantation.

The aim of the study was to establish the regenerative potential of mouse adipose-derived stem cells in micromass grafts differentiated into the osteogenic direction to restore the bone injury in mice.

Methods. Three-dimensional micromass cultures of murine ADSCs with further differentiation into osteogenic direction were obtained. The migration potential of cells from micromass in vitro and the effectiveness of differentiation by staining for alkaline phosphatase were evaluated. Mice with the model of femoral bone injury were transplanted with ADSCs micromass grafts and 21 days later the lesion site was examined by histological and morphometric methods.

Results. The protocols for the cultivation and directed osteogenic differentiation of ADSCs in the three-dimensional micromass culture have been developed. Alkaline phosphatase production was demonstrated in cells that migrated from micromass, confirming the effectiveness of differentiation. In macroscopic examination 21 days after graft transplantation, the defect sites in femur were filled with dense tissue, while in control bones without the use of transplants, the size of the defect by 80 ± 6 % corresponded to the initial diameter and depth of injury. Histological examination of femoral bone lesions in the area of transplantation of micromass grafts revealed the formation of granulation tissue followed by the replacement of defects with newly formed bone tissue with thickening of peristeme and compact bone substance, similar to callus in fracture regeneration. In animals that underwent transplantation of micromass without prior osteogenic differentiation, the diameter of the zone of active regeneration of the diaphysis at the site of injury was 1.3 ± 0.2 mm while in the group with transplantation of directed differentiated graft it was significantly lower (0.37 ± 0.12 mm, p ≤ 0.05).

Conclusions. Three-dimensional grafts of adipose-derived multipotent mesenchymal stromal cells cultured in micromass are able to improve bone tissue regeneration in a model of bone injury in mice. In this case, the grafts differentiated into osteogenic direction, provide better morphological indicators of bone recovery, compared with the micromass without prior differentiation.

Keywords: Adipose-derived stem cells; Micromass culture; Osteogenic differentiation; Bone injury; Cell therapy; Regeneration

Introduction

In modern regenerative medicine, cell and tissue technologies are advanced approaches to the treatment of various diseases. The prospects of cell therapy are drawing attention of researchers and clinicians around the world. Stem cells are given the role of a tool that can be used to repair
damaged tissues and correct the dysfunction of many organs [1, 2, 3]. For example, acute and chronic musculoskeletal injuries are one of the leading causes of disability, and in recent years they have increasingly affected young people [4]. At the same time, cell therapy is now becoming a new and promising strategy for the treatment of severe damage to bones and joints [5, 6].

Priority areas in regenerative medicine are the search for available and safe sources of autologous stem cells, the development of new methods for their cultivation, the use of different types of matrices as scaffolds for cells transplantation. Based on this, among the variety of types of stem cells that are offered in the clinic, of huge potential are the adipose-derived stem cells (ADSCs) [7]. These cells are most similar in their properties and regenerative potential to multipotent mesenchymal stromal cells (MMSCs) from bone marrow, but they have certain advantages, the main of which are relative availability, simplicity and safety compared to stem cell isolation methods from other sources. [8, 9]. Equally important is the ability to obtain the required number of autologous cells at any age, preventing graft rejection reactions, the risk of transmissible infections, and avoiding many legal and ethical limitations of cell therapy [10].

One of the main difficulties in tissue regeneration is to ensure the survival of transplanted cells at the site of their application and functioning. To this end, different types of three-dimensional matrices for cells are being developed capable of filling tissue defects. Thus, demineralized bone, polyethylene glycol, alginate, collagen, carbomer, chitosan, agarose, gelatin, ceramic and combined matrices [11, 12, 13] have been proposed for bone regeneration. They can be in the form of permanent grafts, which perform mainly mechanical function under load, or biodegradable. In addition to the shaping and supporting function, scaffolds are able to continuously release the necessary signal factors over time, providing not only growth but also directed cell differentiation in vivo [14]. For example, exposure to adipose tissue stem cells by bone marrow microenvironment factors or TGF-β1 change the secretion profile of cytokines, affects their survival in the recipient and the realization of regenerative effects [15].

The development of technologies for the engraftment of three-dimensional scaffolds will allow modeling the spatial self-organization of cells by mechanical support for more efficient repair of damaged tissues and organs, as this method of cultivation partially simulates niche conditions for intercellular interaction in vitro and improves their survival and specialization in vivo [11, 16].

An important point is to assess the ability of multipotent stromal cells to direct differentiation under three-dimensional culture condition and their possible use for the regeneration of tissue defects, in particular bone ones [12, 17, 18]. Animal experiments have demonstrated the possibility of using cultured and directed differentiated ADSCs to repair bone tissue in flat and tubular bone damage models [14, 19].

One of the methods for obtaining 3-D organoids is a micromass culture. Without an anchoring contact like the surface of a culture flask, cells aggregate in order to form a microsphere, which displays an in vitro tissue-specific model and a three-dimensional graft for tissue regeneration [20].

Many studies showed that cells being a part of micromasses have an upregulated cell activity. Especially in micromasses of human ADSCs, a significant increase of angiogenic growth factors, as Hepatocyte Growth Factor (HGF), Vascular Endothelial Growth Factor (VEFG) and Fibroblast Growth Factor 2 (FGF2), was found in comparison to a monolayer culture. The expression of specific angiogenic and antiapoptotic factors in ADSCs spheres under inducing cultivation medium was at a 20-times higher level as in monolayer cultures [21]. ADSCs within the spheroid are viable, and the cells produced more extracellular molecules, like laminin and fibronectin. Moreover, spheroid-derived ADSCs exhibited higher expansion efficiency and colony-forming activity [22].

The induction of MMSCs toward the osteoblastic lineage using osteogenic supplements prior to implantation is one approach to enhance their bone-forming potential. MMSCs rapidly lose their induced phenotype upon removal of the soluble stimuli; however, their bone-forming potential can be sustained when provided with continued instruction via extracellular matrix (ECM) cues [23]. Spheroid culture promoted the preservation of ECM components, such as laminin and fibronectin, in a culture time- and spheroid size-dependent manner [24].
The aim of the study was to establish the regenerative potential of mouse adipose-derived stem cells in micromass grafts differentiated into the osteogenic direction to restore the bone injury in mice.

Materials and methods

The study was performed using 12 wild-type FVB/N and four FVB-Cg-Tg(GFPU)5Nagy/J (transgenic for GFP) male mice aged 4-5 months, kindly provided by European Molecular Biology Laboratory (Monterotondo, Italy). Animals were kept under standard conditions of temperature and lighting with free access to water and food ad libitum in the vivarium of the State Institute of Genetic and Regenerative Medicine of the National Academy of Medical Sciences of Ukraine. All experiments with animals were carried out in compliance with the Law of Ukraine "On Protection of Animals from Cruelty", "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes", as well as principles of bioethics and biosafety standards [25].

Obtaining of murine ADSCs cultures. The males of FVB-Cg-Tg(GFPU)5Nagy/J mice were euthanized by cervical dislocation under intraperitoneal anesthesia with 0.25 % avertin solution at a dose of 400 mg/kg. Under sterile conditions, fragments of subcutaneous adipose tissue were dissected from the inguinal areas and washed in a solution of phosphate-buffered saline (PBS) containing 1 % of a mixture of antibiotics PenStrep (Sigma, USA). The primary cell suspension was prepared by mincing adipose tissue in 2 mL of DMEM-F12 medium (Gibco, USA) containing 0.1 % collagenase type 1A (Sigma, USA) and incubated for 90 minutes at 37 °C with constant stirring on a shaker. After fermentation, 10 mL of DMEM-F12 medium was added to the suspension, resuspended, filtered through a cell strainer with a pore diameter of 100 μm and centrifuged for 7 minutes at 400 xg. The supernatant containing mature adipocytes was discarded, the cell pellet was resuspended in complete DMEM-F12 medium (Gibco, USA) supplemented with 15 % fetal bovine serum (FBS) (Gibco, USA) and seeded into 75 cm² culture flasks (SPL Life Science, Korea).

Directed differentiation of ADSCs in the monolayer culture. Osteogenic cell differentiation was induced by standard methods with modifications [26, 27]. For osteogenic differentiation, cells were cultured for subconfluent monolayer at second passage and the complete culture medium was replaced with osteoinductive medium consisting of DMEM-F12 (Gibco, USA) supplemented with 10 % FBS, L-ascorbic acid-2-phosphate (0.05 mM), dexamethasone (100 nM) and β-glycerophosphate (10 mM). Replacement of this medium was performed every three to four days. In general, the cells were exposed to the osteogenic differentiation supplements for 21 days under standard culture conditions. At the end of induction, the monolayer of cells was washed with media.

Immunophenotyping of cell cultures by flow cytometry. Phenotyping of the obtained cell cultures for CD34, CD44, CD45, CD73, CD90, CD117 markers was performed using fluorochrome-conjugated rat anti-mouse monoclonal antibodies, according to the recommendations of the manufacturer (BD Bioscience, USA). 2•10⁶ cells in 50 μL of DMEM medium were transferred to 5 mL polystyrene tubes and antibodies were added at a 0.5 μg/10⁶cells. The samples were incubated for 20 minutes at +4 °C protected from light, then added 1 mL of wash buffer CellWash (BD Bioscience, USA), centrifuged at 350 xg for 5 minutes at +4 °C. After re-washing, the supernatant was discarded, and 300 μL of CellWash buffer containing 1 % FBS was added to the tubes. Immediately before the analysis, the suspension was passed through cell strainers with a pore diameter of 70 μm. The percentage of dead and viable cells was determined by the staining with 5 μL of 7-Aminoactinomycin D (7-AAD) for 10 min (BD Bioscience, USA). Measurements were performed on a cell sorter BD FACSria (Becton Dickinson, USA) using BD FACSDiva 6.1.2 software (Becton Dickinson, USA). At least 20 000 events were recorded to analysis. To adjust the overlap compensation of fluorochrome emission spectra in multiparameter analysis, control samples of cells without the antibodies (unstaining control), samples with each of the antibodies alone (single staining control) and samples with a combination of several antibodies without one (fluorescence minus one – FMO control) were used.

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PBS, fixed with 2 % formaldehyde solution for 30 minutes, re-washed with PBS. The fixed monolayer of cells was stained with Alizarin Red S to detect deposits of calcium salts in the extracellular matrix, which is one of the signs of osteogenic differentiation of culture. Cells were also stained for alkaline phosphatase using the BCIP/NBT kit.

Adipogenic differentiation was performed using DMEM High Glucose medium (Gibco, USA) supplemented with 10 % FBS, dexamethasone (1 μM), indomethacin (200 μM), isobutylmethylxanthine (500 μM) and insulin (5 μg/mL). The medium was replaced after 3 days and the cells were cultured for 14 days. Visualization of lipid granules in the cytoplasm of cells was performed by Oil Red O (Sigma, USA) staining after pre-fixation with 4% solution of formaldehyde.

Obtaining ADSCs micromass culture. The culture of ADSCs micromass was formed after the third passage. A cell monolayer was detached using 0.25% trypsin solution, washed in DMEM medium and 2×10⁶ cells in standard complete culture medium transferred in a 5 mL polystyrene tube (Falcon, USA). After micromass formation (after 24 hours), the cells were incubated under standard conditions for 10-14 days, periodically gently shaking the tube to prevent adhesion of cells to its surface. Replacement of the nutrient medium was performed every 2-3 days.

Directed osteogenic differentiation of ADSCs micromasses. On the 5th day of cultivation, the standard culture medium was replaced with osteogenic induction medium, which consisted of nutrient medium DMEM-F12 supplemented with 10 % FBS, L-ascorbic acid-2-phosphate (0.05 mM), dexamethasone (100 nM) and β-glycerophosphate (10 mM). Replacement of this medium was performed every three to four days. In general, the cells were exposed to the osteogenic supplements for 21 days.

To assess the differentiation effectiveness, after culturing with factors of osteogenic induction for 14 days, micromass were transferred to the 35 mm Petri dishes (SPL Life Science, Korea) and cell migration was observed. Migrating cells were stained for alkaline phosphatase using the BCIP/NBT kit.

Model of bone injury in mice and transplantation of ADSCs micromass grafts. Bone injury in FVB/N mice was performed according to our method (UA Pat. No. 60512). Under the anesthesia with 0.25 % avertin solution in a dose 400 mg/kg, surgery areas were treated with 70 % ethanol solution. In sterile condition, the skin was incised in the middle third of the thigh and the front and medial muscle groups were separated with surgical forceps to expose the femur. In the lower third of the femur perpendicular to the surface using dental drill with a diameter of 1 mm a defect of bone to the endosteum was performed. The defect was covered with muscle and the skin incision was sutured with retention suture for further transplantation of graft into the damaged area at an early stage.

After 24 hours, the wounds were reopened and in the defect of the right femur the micromass graft with a diameter of 1 mm was placed, while the injury on the left femur was used as the control of the natural regeneration of bone tissue. The bones were covered with muscles and the skin wound was sutured. Mice were divided into two groups of 6 animals who underwent transplantation of normal micromass or ADSCs micromass after previous osteogenic differentiation.

Three weeks after the transplantation, the animals were euthanized by cervical dislocation under anesthesia with 0.25 % avertin solution at a dose of 400 mg/kg. Femurs were excised and histological and morphometric examination of the lesion area was performed.

Histological examination of the murine femurs. The femurs for histological examination were fixed in 4 % paraformaldehyde solution in PBS (pH = 7.4) and decalcified. For decalcification, the bones were placed for 24 hours at 20-24 °C in a Richman-Gelfand-Hill solution containing 8 % hydrochloric acid and 10 % formic acid and washed in water. After decalcification, the bones were dehydrated twice according to standard methods in ethanol solutions of increasing concentration and in benzol. The samples were kept in a mixture of benzol and paraffin in a ratio of 1:1 for 30 minutes at a temperature of 37 °C, three times transferred to melted at 57 °C paraffin type 6 (Richard-Allan Scientific, USA).

Using a rotary microtome HM 325 (Microm, Germany), the 12-μm-thick histological sections were performed, placed onto slides and dried in a thermostat at a temperature of 37 °C. The sections were dewaxed twice for 5 minutes in benzol and kept for 5 minutes in 96 % ethanol and 5 minutes
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in 70 % ethanol solution. The sections were stained with Ehrlich's hematoxylin solution for 10 minutes, washed and stained with 0.1 % eosin solution for 2 minutes. The preparations were dehydrated in 96 % ethanol solution for 3 minutes, washed twice in benzol, mounted in Canada balsam and covered with coverslips. The sections were examined using a microscope BX 51 (Olympus, Japan). Morphometric analysis was performed using "Image J" software.

**Statistical data processing.** Statistical analysis was performed using the nonparametric Wilcoxon-Mann-Whitney U-test using Origin Pro 8.5 software (OriginLab Corp, USA) [28]. Sampling of data in *in vitro* experiments included the results obtained from 3 samples, and in *in vivo* study – from groups of 6 animals. The results are presented as the mean in each experimental group ± standard error of the mean (M ± SEM). Differences were considered statistically significant at p < 0.05.

**Results**

To confirm the proper characteristics of the adipose-derived stromal cells used in the studies, the obtained cultures were analyzed by morphology, immunophenotype and potential for directed differentiation into osteogenic and adipogenic directions.
According to morphological characteristics, the vast majority of ADSCs had an elongated or polygonal shape with several processes, the nuclei were clearly visualized (Fig. 1, A). Diffusion growth of the monolayer was characteristic of ADSCs culture, and in some cases, growth was observed by increasing the size of the cell colony. In the following passages, the culture was represented by elongated adhesive cells that formed a confluent monolayer (Fig. 1, B).

For the first passages, cell cultures from adipose tissue were characterized by low expression of hematopoietic markers CD45 and CD117 (< 5%), but by the 2-3rd passage there was a population of cells expressing CD34 (12 ± 4%). In the first passage, the expression level of CD73 was lower (58 ± 12%) than CD44 and CD90 (over 90 %) (Fig. 2). Immunophenotyping of adipose tissue cells at the 3rd passage showed a high level of expression of mesenchymal markers CD44, CD90 and CD73 with a low level of expression of hematopoietic markers CD45 and CD117 (Table 1). Intensive mineralization of the intercellular matrix with calcium salts, which was detected by staining with Alizarin red S, and the production of alkaline phosphatase, which was detected by BCIP/NBT staining, was observed during the third week of cultivation with osteogenic supplements (Fig. 3, A-B). The lipid droplets inclusion in the cytoplasm of cells was demonstrated by directed adipogenic differentiation of ADSCs (Fig. 3, C). Thus, in terms of morphological characteristics, immunophenotype and potential for directed differentiation, the obtained ADSCs cultures used in experiments met the minimal criteria of MMSCs [29].

**Table 1.** Expression of surface markers in the murine ADSCs culture according to flow cytometry, 3rd passage, % (n = 3).

| Surface marker, % cells | CD44          | CD90          | CD73          | CD45          | CD117         |
|-------------------------|---------------|---------------|---------------|---------------|---------------|
| CD44                    | 96,4 ± 3,5    | 96,2 ± 2,1    | 70,1 ± 8,3%   | 2,1 ± 0,8     | 2,2 ± 0,7     |

**Figure 3.** Micrographs of murine ADSCs cultures, differentiated into osteogenic (A, B) and adipogenic (C) direction; staining with alizarin red (A), BCIP-NBT (B) and Oil Red O (C). Light microscopy, phase contrast, scale – 50 microns.

To assess the effectiveness of directed differentiation, after culturing with factors of osteogenic induction for 14 days, micromass were transferred to the adhesive surface and intensive cell migration was observed. When staining these cells with alkaline phosphatase the samples demonstrated almost 100 % positive staining of varying intensity (Fig. 4).

A three-dimensional culture of ADSCs was transplanted into the area of bone injury model to assess the regenerative potential of three-dimensional grafts of this type of cells. In macroscopic examination 21 days after graft transplantation, the defect sites in femurs were filled with dense tissue, while in control bones without the use of micromass, the size of the defect by 80 ± 6% corresponded to the initial diameter and depth of injury (Fig. 5).
After 21 days, histological examination of femoral lesions in the area of transplantation of micromasses revealed the formation of granulation tissue followed by replacement of defects with newly formed bone tissue. The periosteum was thickened, and the compact bone substance was similar to callus during fracture regeneration (Fig. 6). In a comparative study of histological sections of the femurs of mice, which after bone injury model were transplanted with normal ADSCs micromasses, it was found that the edges of the damaged area of the diaphysis formed a cancellous bone containing trabeculae and cavities filled. In the central area of the damaged diaphysis, the trabeculae are thinned, the gaps between them are enlarged and formed channels into the medullary cavity. In the surrounding areas of the diaphysis, the periosteum and the compact substance of the bone bounded the cavities that did not contain cells (Fig. 6, A). The diameter of the zone of active regeneration of the diaphysis at the site of injury was $1.3 \pm 0.2 \text{ mm}$.

![Figure 4](image1)

**Figure 4.** Micrograph of migrating cells from murine ADSCs micromass, differentiated into osteogenic direction for 14 days; staining with BCIP/NBT to detect alkaline phosphatase production (dark). Light microscopy, scale – 50 microns.

![Figure 5](image2)

**Figure 5.** Photo of mouse femurs 21 days after bone injury modeling: A – natural regeneration; B – the local application of ADSCs micromass graft; oc. x10.
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At the same time, in the bone sections of mice, which after bone injury model were transplanted with ADSCs micromasses, differentiated into osteogenic direction, in the damaged diaphysis spongy bone tissue with cavities containing trabeculae was also formed. Some of the gaps are connected to the medullary cavity but did not form through channels. In the areas of the diaphyses adjacent to the regenerating zone of bone, periosteum and compact substance had a typical morphology for normal bone. (Fig. 6, B). The diameter of the regeneration zone of the diaphysis at the site of injury was $0.37 \pm 0.12$ mm, which is significantly lower compared to the group of animals that underwent transplantation of micromass without prior osteogenic differentiation ($p \leq 0.05$).

Thus, three-dimensional grafts of ADSCs micromass, differentiated into osteogenic direction, have better rates of regeneration of injured bone tissue, compared with micromass grafts without differentiation.

Discussion.

Bone regeneration is a complex process requiring highly orchestrated interactions between different cells and signals to form new mineralized tissue. During bone repair and remodeling, osteogenesis is coupled with angiogenesis [30, 31]. Blood vessels serve as a structural template, around which bone development takes place, and also bring together the key elements for bone homeostasis into the osteogenic microenvironment, including minerals, growth factors and osteogenic progenitor cells. VEGF is the master regulator of vascular growth, and it is required for effective coupling of angiogenesis and osteogenesis during both skeletal development and postnatal bone repair [32, 33]. Due to pronounced proangiogenic properties and multipotent potential 3-D cultures of ADSCs provide a favorable source for the induction of different morphogenesis processes, including vasculo-, angio- and osteogenesis.

In our experiment, the relatively high level of CD34 marker expression at early passages is typical for ADSCs and may indicate the potential of these cells to differentiate into endothelial direction. This feature can promote the activation of neoangiogenesis and better vascularization of grafts to ensure their survival.

The conventional monolayer technique used to culture MMSCs inadequately overcomes their low differentiation capacity. The culture of MMSCs in multicellular spheroids more accurately mimics the in-vivo microenvironment; thus, resolving this problem [34]. 3D culture systems have the ability to mimic the natural microenvironment by allowing better cell-cell interactions.

In our ADSCs micromass culture, examining the morphology of cells that migrated to the adhesive surface from osteogenically differentiated cells, it can be seen that it differs from the morphology of cells cultured as monolayers or cells that migrated to the culture surface from micromass without osteoinduction. Interestingly, a higher intensity of staining to detect alkaline phosphatase activity was observed in cells located near the micromass, while in cells that migrated a considerable

**Figure 6.** Micrograph of histological sections of the murine femurs 21 days after modeling the bone injury: A – after transplantation of a normal ADSCs micromass; B – after transplantation of the ADSCs micromass, differentiated into osteogenic direction. Hematoxylin-eosin staining; oc. x10, ob. x20.
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distance, almost no activity of alcaline phosphatase.

It is known that osteoblasts have reduced migratory activity, and with maturation generally lose it, forming osteons and integrated in the extracellular matrix. The test for migratory activity of cells differentiated in the osteogenic direction showed that ADSCs acquired morphological features of osteoblasts: morphology changed, migratory activity decreased, alkaline phosphatase was expressed. At the same time, with increasing time of directed osteogenic differentiation of ADSCs in micromass culture, the migration potential of cells decreases.

Fitzgerald S. et al. compared three culture methods, namely, elastin-like polypeptide-polyethylenimine coated surfaces, ultra-low attachment static culture, and suspension culture for their ability to form and retain productive spheroids from human ADSCs. DNA content and morphometric analysis revealed merging of spheroids to form larger spheroids in the ultra-low attachment static culture and suspension culture methods [35].

Fabrication of three-dimensional stem cell spheroids have been studied to improve stem cell function, but the hypoxic core and limited penetration of nutrients and signaling cues to the interior of the spheroid were challenges [36]. Spheroid cultures were more effective in preconditioning ADSCs to a hypoxic environment, upregulating hypoxia-adaptive signals (i.e., stromal cell-derived factor-1α and hypoxia-inducible factor-1α), inhibiting apoptosis, and enhancing secretion of both angiogenic and anti-apoptotic factors (i.e., hepatocyte growth factor, vascular endothelial growth factor, and fibroblast growth factor 2) compared to monolayer cultures [21].

In our experiment, intensive migration of cells from the micromass to the adhesive surface proves the ability of cells to survive in the 3-D culture. Under the stereomicroscope it was possible to find that the surface of undifferentiated micromass looks noncoherent. This is probably due to the proliferation and self-organization of cells in the micromass. However, the surfaces of micromass, which was cultured in the medium for osteogenic differentiation, look smooth and solid. Probably, in such conditions, the morphology of cells on the surface of micromass under the influence of differentiation factors changes and proliferative activity decreases.

Cheng N. et al. demonstrated that spheroid formation by human ADSCs on chitosan films induced significant upregulation of pluripotency marker genes (Sox-2, Oct-4 and Nanog). Furthermore, a strong influence on their differentiation capacity was observed, represented by the increase on the expression of specific markers for osteogenic (RUNX2), neurogenic (nestin), and hepatic differentiation (albumin) [22].

Murphy K. et al. hypothesized that entrapment of MMSCs spheroids formed from osteogenic induced cells would exhibit better preservation of their bone-forming potential than would dissociated cells from monolayer culture. Spheroids exhibited comparable osteogenic potential and increased proangiogenic potential with or without osteogenic preconditioning versus monolayer-cultured MMSCs [23].

Yamaguchi Y. et al. using real-time reverse transcription-polymerase chain reaction and immunocytochemical analysis indicated that osteogenic properties were accelerated in MMSCs spheroids compared with monolayer rat MMSCs when treated with an osteoblast-inducer reagents. In a rat calvarial defect model, computed tomography and histological analysis showed that spheroid-engrafted defects experienced enhanced bone regeneration [34]. Thus, boosted osteogenesis in 3D spheroid stem cell culture may serve as an alternative to 2D culture by providing a better microenvironment for the enhanced cellular functions and interactions in bone tissue engineering [37].

The mechanisms of cell differentiation are complex and, despite the advances in the knowledge of the processes, the pathways regulating them are not still fully understood. High-throughput analysis, e.g., transcriptome and translome, are strategies helping to shed light on the molecular events driving osteogenesis, chondrogenesis and adipogenesis in two- and three-dimensional ADSCs culture [38]. Yang S. et al. used microarray assays to analyze the expression profiles of exosomal miRNAs derived from undifferentiated as well as osteogenic differentiated ADSCs; 201 miRNAs were upregulated and 33 miRNAs were downregulated between the two types of exosomes. These differentially expressed exosomal miRNAs connect osteogenic
differentiation to processes such as axon guidance, MAPK signaling, and Wnt signaling [39]. The Wnt/β-catenin pathway is involved in the osteogenic differentiation of human ADSCs under cyclic strain [40].

Jung S. et al. have shown that human ADSCs derived from subcutaneous, visceral and omental fat tissue share immunohistochemical characteristics but differ in morphological aspects. The most stable results were observed for the cells of subcutaneous origin. An osteogenic differentiation from adipose-derived cells from all analyzed fatty tissues can be achieved easily and reproducibly. [41]. Micromasses obtained from subcutaneous adipose tissue also turned out to be more homogeneous and compact in shape than visceral and omental tissues, which was shown in a study by Kleineidam B. et al. [20]. Thus, in therapeutic clinical application, adipose-derived cells from subcutaneous tissue provide the optimal cellular source.

In general, our results confirm the experimental findings of other researchers and allow us to consider three-dimensional transplants of adipose-derived stem cells, differentiated into osteogenic direction, as a promising and effective tool for bone regeneration.

Conclusions

Three-dimensional grafts of adipose-derived multipotent mesenchymal stromal cells cultured in micromass are able to improve bone tissue regeneration in a model of bone injury in mice. In this case, the grafts differentiated into osteogenic direction provide better morphological indicators of bone recovery, compared with the micromass without prior differentiation.

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None

CONFLICTS OF INTERESTS

Author has completed the ICMJE Disclosure Form (http://www.icmje.org/disclosure-of-interest/) available on request from the corresponding author). Author declares that there are no potential conflicts of interest.

DISCLAIMER

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Регенеративні ефекти трансплантатів мікромаси мультипотентних стромальних клітин жирової тканини мишей у лікування змодельованого пошкодження кістки

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Резюме
Мультипотентні стовбурові клітини, отримані з жирової тканини (adipose-derived stem cells – ADSCs), є перспективним джерелом для регенерації пошкоджень кісткової тканини. При цьому їх тривимірні культури можуть забезпечувати просторову організацію стовбурових клітин для оптимальної передачі міжклітинних сигналів, контактної взаємодії та підвищення ефективності спрямованого остеогенного диференціювання перед подальшою трансплантацією.

Метою дослідження було встановити регенеративний потенціал трансплантатів ADSCs мишей у вигляді мікромас, диференційованих в остеогенному напрямку, для відновлення пошкодження кісткової тканини у мишей.

Методи. Отримано тривимірні культури мікромаси ADSCs мишей з подальшим їх диференціюванням в остеогенному напрямку. Оцінювали міграційний потенціал клітин із мікромаси в vitro та ефективність диференціювання шляхом фарбування на лужну фосфатазу. Мишам з моделлю пошкодження стегнової кістки трансплантували мікромаси ADSCs і через 21 добу досліджували зону ураження гістологічними та морфометричними методами.

Результати. Розроблено протоколи культивування та спрямованого остеогенного диференціювання ADSCs у тривимірній культурі мікромаси. У клітинах, які мігрували з мікромаси, було продемонстровано продукцію лужної фосфатази, що підтверджувало ефективність диференціювання. При макроскопічному дослідженні через 21 добу після трансплантації мікромас ділянки дефекту були заповнені шлифами тканиною, а в контрольних зонах без використання трансплантатів розмір дефекту досяг 80 ± 6 % відповідно, але в контROLEних зон з кістковою тканиною з потовщенням окістя та компактною речовиною, схожою на кістковий мозоль при регенерації перелому. У тварин, яким була проведена трансплантація мікромаси без попереднього остеогенного диференціювання, діаметр зони активної регенерації діафізі в місці пошкодження становив 1,3 ± 0,2 мм, тоді як у групі з трансплантацією мікромас, диференційованих в остеогенному напрямку, він був достовірно меншим (0,37 ± 0,12 мм, р ≤ 0,05).

Висновки. Тривимірні трансплантати мультипотентних мезенхімальних стромальних клітин жирової тканини, культуронів у мікромасі, здатні покращувати регенерацію кісткової тканини на моделі пошкодження кісток у мишей. при цьому трансплантації, диференційовані в остеогенному напрямку, забезпечують кращі морфологічні показники відновлення кістки, порівняно з мікромасами без попереднього диференціювання.

Ключові слова: стовбурові клітини жирової тканини; культура мікромаси; остеогенне диференціювання; пошкодження кістки; клітинна терапія; регенерація