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## MORPHOLOGICAL CHANGES IN PERIODONTAL TISSUES IN LABORATORY ANIMALS USING MESENCHYMAL STEM CELLS

**Abstract.** The article presents the analysis of the morphological changes of the periodontal tissues of laboratory animals using mesenchymal stem cells (MSCs).

The goal of the study is to create a model of experimental periodontitis and to identify the characteristics of morphological changes in the periodontal tissues using a biomedical cell product based on the allogeneic adipose tissue of MSCs (AT MSCs).

The application of a mixture of AT MSCs and osteoinduced AT MSCs (in the ratio of 1:1) allows reducing the time of bone defect regeneration in comparison to that of bone tissue regeneration when AT MSCs and osteoinduced AT MSCs are used separately, which is expressed in the filling of the bone defect with a fibroreticular osteogenic tissue, as well as with a muscle tissue one month after surgery.

In 2 months, in the defect area filled with a collagen membrane with a mixture of AT MSCs and osteoinduced AT MSCs, the initial signs of the formation of trabecula of bones were detected, which is evident of a more comprehensive osteosynthesis process compared to the blood clot use.

**Keywords:** mesenchymal stem cells, periodontitis, morphometry, fibroreticular tissue

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## МОРФОЛОГИЧЕСКИЕ ИЗМЕНЕНИЯ В ТКАНЯХ ПЕРИОДОНТА ЛАБОРАТОРНЫХ ЖИВОТНЫХ ПРИ ПРИМЕНЕНИИ МЕЗЕНХИМАЛЬНЫХ СТВОЛОВЫХ КЛЕТОК

**Аннотация.** В статье приведены результаты исследования морфологических изменений в тканях периодонта лабораторных животных при использовании мезенхимальных стволовых клеток (МСК).

Цель исследования – разработать модель экспериментального периодонтита и установить характер морфологических изменений в тканях периодонта при применении биомедицинского клеточного продукта на основе аллогенных МСК жировой ткани (МСК ЖТ).

Применение смеси МСК ЖТ и остеоиндуцированных МСК ЖТ в пропорции 1:1 позволяет сократить сроки регенерации костного дефекта по сравнению с длительностью восстановления костной ткани при применении МСК ЖТ, а также остеоиндуцированных МСК ЖТ, что выражается в заполнении дефекта костной ткани наряду с мышечной фиброретикулярной остеогенной тканью через 1 мес. после оперативного вмешательства. Через 2 мес. в зоне дефекта, заполненного коллагеновой мембраной со смесью МСК ЖТ и остеоиндуцированных МСК ЖТ, выявлены признаки начала формирования костных балок, что свидетельствует о более полноценном остеосинтезе, чем при заживлении под кровяным сгустком.

**Ключевые слова:** мезенхимальные стволовые клетки, периодонтит, морфометрия, фиброретикулярная ткань

**Для цитирования:** Морфологические изменения в тканях периодонта лабораторных животных при применении мезенхимальных стволовых клеток / С. П. Рубникович [и др.] // Вес. Нац. акад. навук Беларусі. Сер. мед. навук. – 2020. – Т. 17, № 1. – С. 444–451 (на англ.). <https://doi.org/10.29235/1814-6023-2020-17-1-444-451>

**Introduction.** Periodontal diseases are among the leading dental diseases across the world [1, 2]. The character of the inflammatory process during periodontal diseases, the disease progression and turning chronic lead to significant pathological changes in the periodontal tissues, the loss of teeth and dysfunctions of the dentition system as well as of various associated organs and systems of the human body [3]. Although measures have been developed for prevention and treatment of this pathology, there is no obvious trend towards the reduction of these diseases in patients of all age groups [2]. Therefore, the issues of efficacious treatment of periodontal diseases and prevention of the associated complications remain relevant.

The inflammatory process in the periodontal tissues is destructive and accompanied by the resorption of the bone tissues and the damage of the periodontium that supports and maintains the tooth [3]. Given a weak reparative capacity of these structures, the restoration of the bone deficiency by means of physiological regeneration takes a long time and can be complete in rare cases [4, 5]. Hence, one of the directions for comprehensive treatment is to improve surgery approaches for eliminating infectious destructive spots in the periodontium.

At present, the ability of periodontium tissues for regeneration by using different materials is actively investigated. It has been demonstrated that the method based on the use of membranes that limit the access to the regeneration space of the epithelial cells and connective tissue of the gum, not involved in the construction of periodontal tissues, is very efficient [6]. However, although the membranes slow down the migration of the epithelial cells, they do not facilitate repopulation of the periodontium space with progenitor cells [7, 8]. In order to enhance the osteoinductive capacity, it is expedient to combine the use of membranes with regeneration stimulating agents. The current investigations demonstrate a high ability of adipose tissue mesenchymal stem cells (AT MSCs) to initiate and accelerate the regenerative processes in the periodontal tissues, as well as to secrete the factors stimulating the resident progenitor cells [9], which enhances the treatment efficacy significantly [8–10].

At present, investigations are conducted to identify the efficiency of application of cell technologies in various fields of medicine, including periodontology [11–17]. The scientific sources include evidence on the reduced propagation and intensity of the gingival recession [16, 18, 19] and on improvement of periodontium microcirculation processes when MSCs are used [17, 20–35].

The above proves the expediency of experimental clinical studies to justify the use of MSCs in dentistry in order to regenerate bone tissue and, consequently, to increase the efficacy of treatment of patients with periodontal diseases.

The goal of this study was to identify the character of morphological changes using a biomedical cell product based on adipose tissue mesenchymal stem cells.

**Subjects and methods of study.** This study was approved by the independent ethics committee of the State health institution “Belarusian Medical Academy of Postgraduate Education” (BelMAPO). The experimental part of the study was performed by the pathophysiological group of the BelMAPO Research Laboratory in vivarium conditions complying with the requirements specified by Technical Code of Standard Practice 125-2008 “Good Laboratory Practice” and Sanitary Rules and Norms 2.1.2.12-18-2006 “Arrangement, Equipment and Maintenance of Experimental Biological Clinics (Vivaria)” [25].

Isolation and cultivation of allogenic AT MSCs from experimental animals (EAs), cell culture quality control, including the counting of the number and the assessing of the vitality of MSCs, determination of the cell phenotype using monoclonal antibodies, evaluation and control of contamination by microorganisms, as well as the induction of cell development in the osteogenic direction and evaluation of differentiation and immobilization of cells on the medium were performed in laboratory conditions in the state research institution “The Institute of Biophysics and Cell Engineering of the National Academy of Sciences of Belarus”. A porous membrane based on type I bone collagen “Osteoplast” (produced by the “Vitaform”, Russian Federation) was used as a biodegradable carrier for immobilization of MSCs.

Subjects of the study included 36 *Chinchilla* rabbits, both males and females, with the body weight 3.7 [3.5; 3.8] kg. The EAs were kept in conditions of vivarium according to the veterinary sanitary rules approved in the Republic of Belarus [25]. Before the experiment, the EAs had been observed in quarantine conditions in the vivarium for 2 weeks. The EAs were examined and weighed also on the day when the experiment commenced. Taking into consideration the chronobiological dependence of most biochemical processes in animals, the experiments were made at the same hours in the morning.

The EAs were anesthetized using one intramuscular injection of the Ketamine solution (50 mg/ml), 1 mg/kg body weight. Then anaesthesia was enhanced and maintained as needed during the surgery by means of a mixture of 0.05 Fentanyl solution and 0.25 % Droperidol solution (1:2). A cutter was used to produce in the anesthetized animals bone defects (BDs), 2 mm wide and 5 mm deep, in the area of the interradicular septum of the mandibular central incisors on the vestibular side.

The EAs were divided into 4 homogeneous groups: the control group (including 9 EAs) and 3 experimental groups (each including 9 EAs) according to the planned method of treatment. In the control group the artificial BD was filled with a blood clot; in group I with a porous membrane based on bone collagen with 50,000 allogenic AT MSCs immobilized on the membrane; in group II with a membrane with 50,000 immobilized allogenic osteoinduced AT MSCs; and in group III with a membrane including a mixture of 25,000 allogenic and 25,000 allogenic osteoinduced AT MSCs. After the BD was filled, the surgical wounds were closed (stitched).

During the postsurgical period, the EAs were observed to monitor the body weight, the condition of the visible mucous membranes, hair coat, motion activity, behaviour and the consumption of food and water.

After the observation was over, the control and experimental group animals were brought out from the experimental condition in compliance with the bioethical principles specified in the good laboratory practice standards (GLP).

Samples of periodontal bone were taken from mandible of the EAs, containing teeth and periodontal tissues.

The excised mandibular sections were fixed in 10 % neutral formalin for 48 hours. Decalcification was made using formic and hydrochloric acids, including mandatory control of decalcification by means of calcium oxalate. Then the mandibular sections were washed in a water stream for 24 hours and dewatered in alcohol of ascending concentrations (70, 80, 96 and absolute alcohol). This material was treated with alcohol-xylene, xylene, xylene-paraffin and poured into paraffin. The paraffin blocks were used to make cuts 3–5  $\mu\text{m}$  thick, which were stained with haematoxylin and eosin according to Masson's protocol.

Morphometric analysis of histologic specimens was made under  $\times 50$  magnification. Each microspecimen was investigated in 5 random fields of vision. The area of the field of vision was constant, making 4,561,048.00  $\mu\text{m}^2$ . The area of fibroreticular tissue was determined. The obtained results were presented as the ratio of the area of fibroreticular tissue to the total area of the field of vision, expressed in percentage. The microspecimens were investigated and micro pictures were made by means of the microscopes Axio Imager (Zeiss, Germany) and DMLS with the software (Leica, Germany).

The obtained data were statistically processed using Statistica and Excel. The type of quantitative characteristics distribution was determined using the Shapiro-Wilk test. In order to describe the quantitative characteristics with normal distribution, it was required to specify the mean value and the root-mean-square deviation. The quantitative characteristics with the distribution different from the normal were described using the median (Me), the lower 25<sup>th</sup> quintile (LQ) and the upper 75<sup>th</sup> quintile (UQ). The Mann–Whitney test was used for group comparison. The results were considered statistically significant at  $p < 0.05$ .

**Results and discussions. *The morphological pattern of bone tissue regeneration in the control group.*** On day 14, in animals from the control group healing under blood clot, a triangular BD was observed, separated from the maternal splenial bone by a thin strip of fibroreticular osteogenic tissue growing focally into the splenial bone (BD encapsulation). Loose oedema connective tissue (CT), muscular tissue (MT) and spots of granular tissue with local bleeding were observed in the BD lumen. Localized fibroreticular osteogenic tissue was observed in the BD bottom area. Hyperaemia of haversian canal vessels was observed in the splenial bone adjacent to the BD.

After 1 month, the BD retained the triangular shape with surface retraction and was separated from the maternal splenial bone by a thin strip of fibroreticular osteogenic tissue. The BD lumen included loose oedema CT with localized bleeding and cystic transformation. The surface section of the BD bottom area included necrotic CT, loose CT and dilated blood vessels. Localized formation of fibroreticular osteogenic tissue was observed. Hyperaemia of haversian canal vessels was seen in the splenial bone adjacent to the BD.

After 2 months, the BD had a circular wedge shape filled (two-thirds) with fibroreticular osteogenic tissue. Multiple spots of extensive bleeding were observed in the DB area. Necrotic tissue sports were seen

in the BD surface section. Focal aggregations of osteoblasts and fibroblasts were found in the BD bottom area. The adjacent area of the maternal bone included ectasia of vascular bone channels with fragmentation and elimination of their connective tissue component, and localized adipose transformation of the trabecular tissue.

**The morphological pattern of bone tissue regeneration in group I.** On day 14, a baggy BD with surface retraction filled with MT with dilated blood vessels and loose oedema CT were observed. Fragments of the stratified CT strip, separating the defect from the matrix bone, were observed in the CT surface section along the BD perimeter. Fragments collagen membrane, necrotic areas and a chondrification spot were found in the CT surface section. Islets of fibroreticular osteogenic tissue were found in the BD surface area. Constriction of the osteon channels and proliferation of the cell component were observed in the osseous tissue of the matrix bone.

After 1 month, a baggy CD was found, separated along the entire edge from the compact bone by an unevenly narrow strip of connective tissue. Muscular tissue with a small destruction spot and lymphoid cell infiltration were observed in the defect lumen. Segments of loose fibrous, granulated and compact fibroreticular tissue were observed in the bottom section. Localized hyperplasia of the trabecular CT was observed outside the BD area.

After 2 months, the BD was oval and surrounded unevenly by a narrow strip of connective tissue, delaminated from the compact bone (Fig. 1). Collagen membrane fragments as well as necrosis areas, as well as necrotic areas were identified in the BD surface section. Loose oedema CT and localized spots of fibroreticular osteogenic tissue were seen in the BD bottom area. Ectasia and hyperaemia of osteon channel vessels with loose perivascular tissue and hyperplasia of the cell component were observed in the perifocal splenial bone tissue.

**The morphological pattern of bone tissue regeneration in group II.** On day 14, the BD was oval and separated, along the edge, from the matrix bone by a thin strip of stratified loose fibrous or fibroreticular tissue. Loose fibrous immature CT with diapedesis bleeding was identified in the BD bottom area. The BD lumen was filled with MT and loose fibrous CT. Collagen membrane fragments were observed in the BD surface section. Ectasia and hyperaemia of osteon channel vessels were observed in the perifocal splenial bone tissue.

After 1 month, an oval BD, surrounded by a thin strip of stratified loose fibrous or fibroreticular tissue, was observed. Loose fibrous immature CT with diapedesis bleeding was found in the BD bottom area. The BD lumen was filled with MT and loose fibrous CT. Collagen membrane fragments were found in the BD surface section. Ectasia and hyperaemia of osteon channel vessels were observed in the perifocal splenial bone tissue.

After 2 months, the BS had an oval baggy shape and was separated from the matrix splenial bone by a narrow strip of loose CT in the surface section and fibroreticular in the bottom section (Fig. 2). The BD lumen was filled with oedema MT and had localized spots of bleeding. Fibroreticular osteogenic

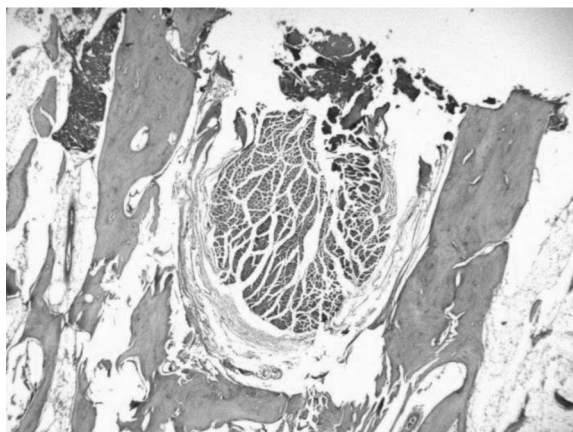


Fig. 1. The bone defect in 2 months. The collagen membrane with AT MSCs. Staining with haematoxylin and eosin,  $\times 20$

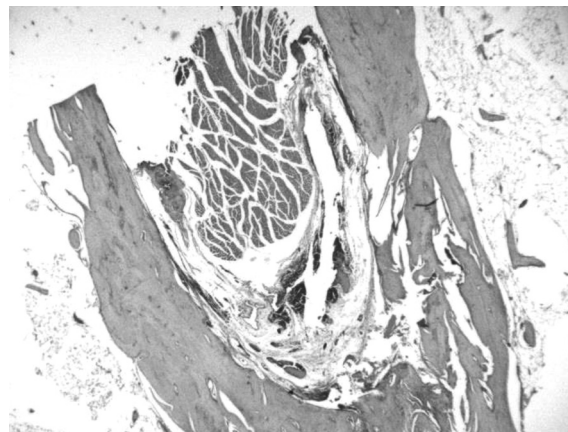


Fig. 2. The bone defect in 2 months. The collagen membrane with osteoinduced AT MSCs. Staining with haematoxylin and eosin,  $\times 20$



Fig. 3. The bone defect in 2 months. The collagen membrane with a mixture of AT MSCs and osteoinduced AT MSCs in the 1:1 ratio. Staining with haematoxylin and eosin,  $\times 20$ .

tissue and localized spots of bleeding were observed in the bottom section. Osteogenic islets with rare osteoblasts on their surface were observed in the area of fibroreticular tissue. Some haversian channels of the bone matrix were diluted, and the vessels were hyperaemic.

**The morphological pattern of bone tissue regeneration in group III.** On day 14, a bag-shaped BD was observed. The splenial bone tissue defect was separated along the edge by a narrow strand of connective tissue, segmentally stratified, *inter alia*, in the BD bottom area. Small fragments of necrotic tissue were seen in the CT surface section. The entire BD lumen was filled with dystrophic muscle tissue, including a focal necrosis. A crosswise narrow strand of connective tissue with a focal necrosis and diapedesis bleeding, separating the BD from the lower splenial

bone tissue, was found in the BD bottom area. The matrix bone included pyknotic osteocytes with spots of their necrosis.

After 1 month, a strip of fibroreticular osteogenic tissue was observed along the edge of the splenial bone tissue, separating the BD. The BD lumen included (in the surface section) fragments of small-cell or acellular homogeneous tissue with focal small-cell transformation, localized atrophic lymphoid tissue and, predominantly, areas of dystrophic muscular tissue with focal spots of necrosis. Spots of loose small-cell fibrous tissue and fibroreticular osteogenic tissue, chondrification focus, were seen in the BD bottom area. Extensive hyperaemia of the matrix bone haversian channels was observed.

After 2 months, a slightly slope and shallow BD was unevenly surrounded in some segments by a thin small-cell strip of fibroreticular osteogenic tissue (Fig. 3). Areas of fibroreticular osteogenic tissue with osteogenic islets and trabeculae of bone were observed in the BD lumen. Localized aggregations of osteoblasts and their high density were observed. The bone matrix of the matrix bone had a regular histologic structure, with wide hyperaemia of the matrix bone haversian channel vessels.

Morphometric investigation made it possible to identify that in experimental group III, where the collagen membrane mixture of AT MSCs and osteoinduced AT MSCs was used in the 1:1 ratio, the indicators of fibroreticular tissue area were definitely higher than in groups I and II ( $p < 0.05$ ) (see Table). The maximum values of the osteogenic tissue area were identified in experimental group III throughout the experiment ( $p < 0.05$ ) (Fig. 4).

#### Indices of fibroreticular tissue area (%) depending on the duration of the experiment

Group	Periods of observation		
	14 days	1 month	2 months
Control	1.65 (1.45; 1.82)	3.25 (3.25; 3.53)	5.19 (5.00; 5.33)
I	1.50 (1.35; 1.60)	2.28 (2.18; 2.36)	3.51 (3.30; 3.79)
II	2.24 (2.14; 2.34)	2.45 (2.23; 2.67)	1.70 (1.49; 1.92)
III	2.46 (2.30; 2.56)	6.10 (5.96; 6.30)	5.44 (5.12; 5.78)

Thus, the formation of the connective tissue capsule with areas of mature granulation tissue, fibroreticular tissue and small necrosis focal spots (BD encapsulation) were observed along the BD perimeter in the animals from the control and experimental groups. In the control group, the BD capsule comprised fibroreticular osteogenic tissue at every stage of observation. In the experimental groups, fibroreticular tissue capsules were observed in animals from groups II and III after 1 и 2 months of the experiment, and in animals from group I only in the bottom section after 2 months. After 2 months, the BD was filled with fibroreticular osteogenic tissue in animals from the control group and experimental group III; and osteogenic islets or emerging small trabeculae with a high density of osteoblasts on their surface were found in the fibroreticular

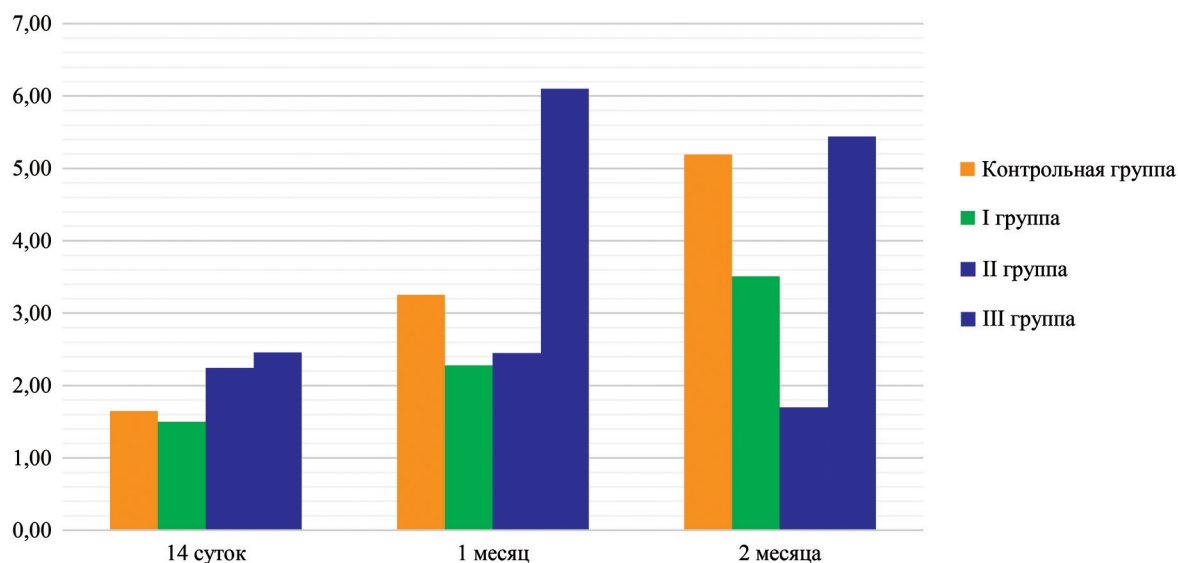


Fig. 4. The trends of the osteogenic tissue area indices, %. Left to right: control group, group I, group II, groups III; 14 days, 1 month, 2 months

tissue in animals from experimental group III in the BD bottom area. In animals from the control group, no trabeculae or osteogenic islets were identified in the BD bottom area two months later.

**Conclusion.** The developed model of experimental periodontitis reproduces signs of bone tissue pathology and shows that it can be used for evaluation of the regenerative processes.

The use of a collagen membrane with osteoinduced AT MSCs allows reducing the BD regeneration period as compared to the bone tissue regeneration period using AT MSCs, which is demonstrated by faster filling of the defect lumen, along with muscular tissue, by loose connective tissue with areas of fibroreticular osteogenic tissue, and by the formation of granulation spots and obvious vessel response of the bone defect tissue.

The use of a mixture of AT MSCs and osteoinduced AT MSCs in the 1:1 ratio reduces the time of bone defect regeneration compared to the time of bone tissue regeneration using osteoinduced AT MSCs, i. e., bone defect tissue was filled, along with muscular tissue, by fibroreticular osteogenic tissue one month after the surgery intervention. After 2 months, the area of the defect, filled by a collagen membrane with the mixture of AT MSCs and osteoinduced AT MSCs in the 1:1 ratio, demonstrated signs of generation of the trabecula of bone, indicating a more comprehensive osteosynthesis than in the case of healing under a blood clot.

**Conflict of interests.** The authors declare no conflict of interest.

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