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RESEARCH ARTICLE

PATHOMORPHOLOGICAL CHANGES IN THE MANDIBLE BONE WITH VARIOUS OSTEOREGENERATION METHODS IN THE EXPERIMENT

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ABSTRACT

The article presents a study of pathomorphological changes in the lower jaw bone in rats undergoing osteoplasty of a bone defect in an experiment. The study was conducted on male Wistar rats. Three groups of 20 animals were formed. In the first group, the defect was replaced with bone shavings, in the second - with Kollapan-L material, and in the third - with Kollapan-L with committed autologous mesenchymal stem cells. In the 3rd group of animals, the presence of young connective tissue rich in cells, vascular proliferation and maturation of connective tissue to coarse scar tissue with lymphohistiocytic infiltration were determined, especially at 30-60 days. The Ki67 proliferation index by day 30 of the experiment in the three groups was distributed as follows: 4.93%, 18.2 and 32.8%, indicating a stimulating effect of mesenchymal stem cells. The obtained data indicate acceleration of physiological regeneration under the influence of mesenchymal stem cells committed in the osteogenic direction. The immunomodulatory effect of stem cells allowed to significantly reduce the percentage of purulent infiltrates in the bone defect. Increased expression of proliferation markers Ki67 and angiogenesis markers CD 36 promotes accelerated bone tissue regeneration.

Keywords: bone defect, osteoplasty, artificial hydroxyapatite, biomatrix, mesenchymal stem cells, Ki67, CD38

1. INTRODUCTION

Osteoplasty has been used for many years in orthopedic surgery to restore bone tissue in case of defects or ruptures caused by trauma and tumors, as well as in case of periodontal defects¹⁻⁴.

Reconstructive surgery is important to restore

the aesthetic and functional role of these complex anatomical areas and can be challenging for both patients and surgeons^{5, 6}.

Bone regeneration requires that stem cells from both donor and host differentiate into osteoblasts, secrete collagen I and other organic components of the bone matrix, and then mineralize to form fully

functional bone. Previous studies in the literature have used autologous bone grafting, titanium or polyetheretherketone implants, and other modern materials to close bone defects ⁷.

However, each material has certain limitations, which requires further research to determine the best technique for bone reconstruction in dentistry.

General requirements for an ideal implant include biocompatibility, lack of allergy, radiopacity, cost-effectiveness, ease of use, and sufficient strength ^{8, 9}.

In recent decades, tissue engineering and regenerative medicine have attracted much attention with the expansion of various studies to evaluate biomaterial application strategies for better maxillofacial bone regeneration¹⁰. Biomaterial scaffolds provide support for cell growth, deliver cellular signals, and have a significant impact on the microenvironment of tissue formation. Various types of biomaterials such as bone grafts, bioceramics, natural and synthetic polymers, and dental derivatives have been clinically applied in the maxillofacial area. However, there are still shortcomings such as incomplete restoration and change in the structure of regenerated bone, complications, or lack of long-term stability ^{11, 12}.

Therefore, it is imperative to clarify the status quo of maxillofacial bone tissue engineering in order to develop more ideal biomaterial scaffolds for maxillofacial bone regeneration that will completely restore the normal physiological functions of the defect areas. Over the past threedecades, the use of stem cells delivered with various scaffolds or growth factors for bone tissue engineering has made enormous progress¹³.

Many types of stem cells have been isolated from various tissues for use in bone tissue engineering ^{14, 15}. Currently, the use of various postnatal stem cells, including bone marrow mesenchymal stem cells ¹⁶, muscle stem cells, adipose stem cells ¹⁸, dental pulp/periodontal ligament stem cells ^{19, 20}, periosteum stem cells ²¹, umbilical cord stem cells ²², peripheral blood stem cells ²³, urinary stem cells ²⁴, apical papilla stem cells, and induced pluripotent stem cells, for bone tissue engineering and repair has been widely applied in clinical practice ²⁵. Despite extensive preclinical studies using many different stem cells from diverse tissue resources, the clinical application of stem cells is still limited due to the fact that most

of them require culture and expansion. Ideally, stem cell-based strategies in bone tissue engineering require stem cells from both donor and host to differentiate into osteoblasts, secrete type I collagen and other organic components of the bone matrix, and then mineralize to form fully functional bone.

Therefore, the development of new methods or devices that allow the isolation of stem cells at the point of care for the repair of bone defects or the repair of non-union fractures is of critical importance.

The aim of the study was to study the pathomorphological changes in the mandibular bone of rats during osteoplasty of a bone defect in an experiment.

2. MATERIAL AND METHODS

The study was conducted on 60 male Wistar rats weighing 300±30 g. Osteodamage in animals was induced by drilling a defect with a dental bur in the area of attachment of the masticatory muscles in the angle of the lower jaw (2 mm in diameter). Then, for group 3, Kollapan-L granules were immersed in a MSC culture for 12 hours and the bone defect was filled.

We formed 3 groups of 20 animals each:

- group 1(control) - bone sawdust obtained during the application of the defect was placed into the bone defect;
- group 2 - the defect was filled only with Kollapan-L No. 1 mechanically crushed five times, thus obtaining fragments of 0.2 cm;
- group 3 - the bone defect was filled with Kollapan-L No. 1 with committed autologous mesenchymal stem cells.

In all groups, the mucosal defect was sutured with 4/0 Vicryl thread over the defect with plastic material. Bone marrow (BM) explantation was performed under aseptic conditions from the sternum or iliac crest (crista iliaca) in an amount of 20-40 ml with the addition of 625 units/ml heparin (Darnitsa, Ukraine). BM aspirate was layered on a Histopaque-1077 gradient, density 1.077 g/ml, (Sigma, USA) and centrifuged for 30 min. at 1500 rpm. The resulting mononuclear cells were collected with a pipette and successively washed 3-4 times in Hanks' solution (Biolot, Russia) by centrifugation at 1000 rpm for 14 min. The resulting mononuclear bone marrow cell suspension was seeded onto collagen-coated 75 cm² culture flasks (Corning-Costar, USA) at a concentration of 2–5×10⁶ cells per flask. MSCs were cultured in a mixture of DMEM/F12 nutrient medium,

1:1, (Sigma, USA) with the addition of 10% fetal calf serum (Biolog, Russia), 0.75 mg/ml glutamine (Institute of Poliomyelitis and Viral Encephalitis, Russia), 2 ng/ml basic fibroblast growth factor (Sigma, USA) and 100 U/ml penicillin and streptomycin (Darnitsa, Ukraine) in a CO₂ incubator (Jouan, France) at 37°C and 5% CO₂ atmosphere. The medium was changed every 3-4 days of cultivation. The cultures reached the primary monolayer on the 8th-11th day of cultivation, depending on the seeding density of the primary isolated cell suspension, individual characteristics of the donors and the level of proliferative activity of the cells. Passaging, or subculturing, was carried out using a mixture of trypsin/EDTA solutions (Biolog, Russia) in a ratio of 0.05%:0.02% in PBS, pH 7.4 (Sigma, USA).

The passaging ratio was 1:2 or 1:3. After that, the cells were cultured in a CO₂ incubator under the same conditions. As a result of these manipulations, an uncommitted cell culture of MSCs was obtained. After the uncommitted MSC cultures reached a confluent monolayer, they were incubated in an osteogenic growth medium containing DMEM/F12, 1:1, (Sigma, USA) supplemented with 10% fetal bovine serum (Biolog, Russia), 0.75 mg/ml glutamine (Institute of Poliomyelitis and Viral Encephalitis, Russia), and an osteogenic supplement - 50 µg/ml L-ascorbic acid (Sigma, USA), 10 mM β-glycerophosphate (Sigma, USA), 0.1 µM dexamethasone and 100 units/ml penicillin and streptomycin (Darnitsa, Ukraine) in a CO₂ incubator (Jouan, France) at 37°C and 5% CO₂ atmosphere. The growth medium was changed every 3-4 days of cultivation (Fig. 1).

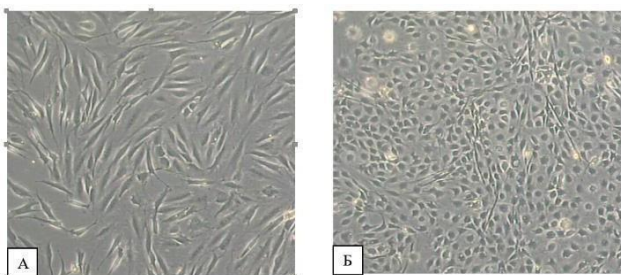


Figure 1. MSC culture (A) and after scaling (B) (FKM X200).

After 30 days, a standard histological examination of the tissues of the defect site was performed. At the 10th, 30th and 60th days, the proliferation marker KI-67 and the angiogenesis marker CD 34 were determined using immunohistochemical methods. Mouse antibodies from Cellmarque, USA, were used to study CD 34, and rabbit antibodies (BioVitrum, Russian Federation) were used for Ki-67.

Statistical methods. The Shapiro-Wilk test (W) was used to check the data distribution for normality, which made it possible to use it even with a small sample (n<30). To identify significant differentiations between the average values of different populations of comparable groups, variation statistics methods were using Student's t-test with Bonferroni correction for multiple comparisons with a type I error probability of p=0.05. The data were considered reliable at p<0.05. Results. The effect of osteoplastic materials modified with culture supernatant on the experimental bone wound was carried out by comparing the regeneration indices in relation to groups 1 and 2. Microscopy of histological preparations of animals on days 7-10, where traditional bone sawdust (group 1) and Kollapan-L (group 2) were used, the following patterns were revealed. In sections, in 35.6% of cases, in group 2, mild purulent inflammation was noted (Fig. 2).

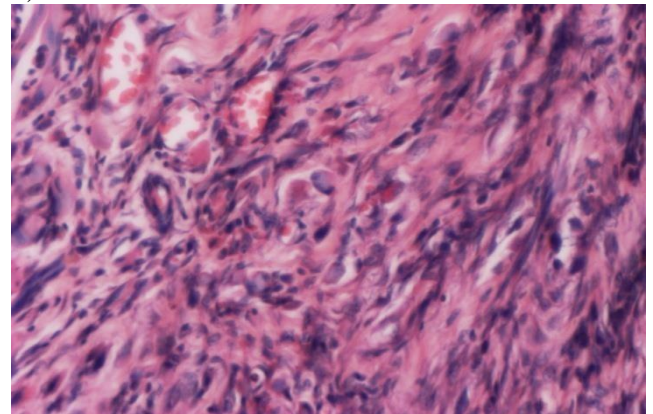


Figure 2. Osteoblastic granulation tissue with infiltration of lymphocytes, macrophages and neutrophils in group 1 on day 30 of the experiment

In comparison with the control group, where severe purulent exudate was detected in 52% of cases. In 78% of cases, osteotropic material was observed in the bone defect in group 3. In the area of the bone defect, the presence of both young connective and scar tissue was revealed, which indicated the onset of bone tissue regeneration (Fig. 3).

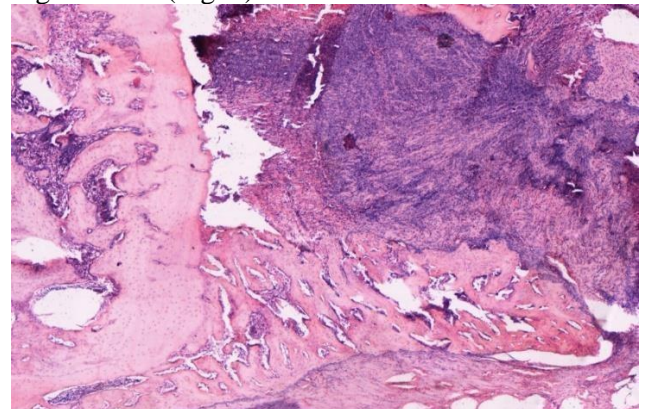


Figure 3. Active maturation of osteoblastic granulation tissue with the formation of spongy bone in group 2 on day 30 of the experiment.

Signs of bone regeneration were more pronounced in groups 2 and 3, compared to the control. Regeneration was manifested in the proliferation of numerous cells with round, oval nuclei, dispersed chromatin, most likely proliferating osteoblasts, pronounced proliferation of osteoblasts and the formation of small bone beams in 34.8% of cases (Fig. 4).

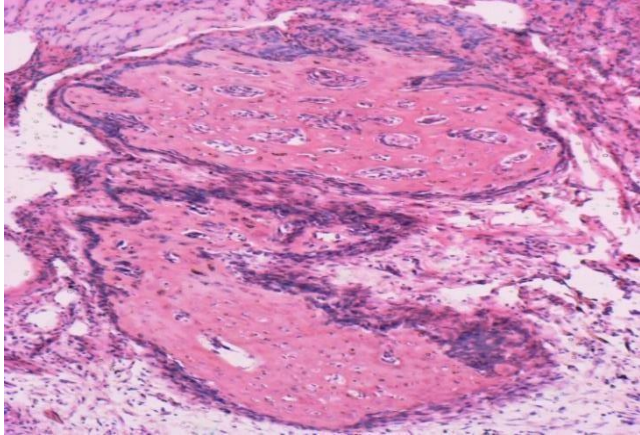


Figure 4. Active formation of bone trabeculae against the background of mild inflammatory infiltration on the 30th day after using Kollapan-L with committed mesenchymal stem cells. Hematoxylin-eosin, magnification 15. In the 1st group, insignificant proliferation of osteoblasts was noted, with the formation of osteoid in 18.2% of cases. Thus, when using Kollapan-L under experimental conditions, an osteoconductive effect was noted in the area of the bone wound and an osteostimulating effect manifested in the form of pronounced proliferation of mature forms of osteoblasts and the formation of small bone beams in 45% of cases.

In the 3rd group of animals, where Kollapan-L with committed stem cells was implanted, the wound showed the presence of young connective tissue rich in cells, proliferation of vessels and maturation of connective tissue to coarse scar tissue with

lymphohistiocytic infiltration, especially at 30-60 days (Fig. 3).

Bone regeneration was expressed in the presence of active (proliferating) bone marrow in the bone marrow empty spaces of the pre-existing bone and the proliferation of osteoblasts. In the 1st group, regenerative changes were noted, expressed in the formation of bone marrow cavities with the presence of young connective or osteogenic tissue.

The Ki67 proliferation index by the 30th day of the experiment in three groups was distributed as follows: 4.93%, 18.2 and 32.8%, respectively, which indicates the stimulating effect of mesenchymal stem cells in osteoplastic operations (Tables 1, 2). The reduction of the purulent process confirms the immunomodulatory effect of stem cells. The increase in CD34 content in the three groups was on day 30 in group 1 from 10.56±1.8 to 13.48±21, on day 60 to 14.22±12, in group 2 from 11.23±2.2 to 14.33±1.7 and 15.8±1.4, in group 3 from 12.23±1.3 to 15.1±2.2 and 16.4±1.1, respectively, which indicates the participation of angiogenic factors in the physiological bone regeneration of the bone defect of the lower jaw.

Table 1 Dynamics of Ki67 in groups at different times

Groups	10-day	30-day	60-day
1	7,96±1,89	9,11±2,18	10,11±2,41
2	9,26±3,47*	12,58±2,21*	14,22±1,37*
3	11,12±2,25#,@	18,46±2,11#,@	24,21±1,98#,@

Note: * - the difference between the indicators of groups 1 and 2 is reliable (p<0.05); # - the difference between the indicators of groups 2 and 3 is reliable (p<0.05), @ - the difference between the indicators of groups 1 and 3 is reliable (p<0.05).

Table 2 Dynamics of CD34 in groups at different times

Groups	10-day	30-day	60-day
1	10,56±1.8	13,48±21	14,22±12
2	11,23±2.2*	14,33±1,7*	15,8±1,4*
3	12,23±1,3#,@	15,1±2,2#,@	16,4±1,1#,@

Note: * - the difference between the indicators of groups 1 and 2 is reliable (p<0.05); # - the difference between the indicators of groups 2 and 3 is reliable (p<0.05), @ - the difference between the indicators of groups 1 and 3 is reliable (p<0.05).

The problem of stimulating physiological tissue regeneration raises a number of controversial aspects that require study. To eliminate these issues, bone tissue engineering is widely studied with the combination of three main components: osteogenic cell resources, biomaterials and signaling molecules²⁶. The authors analyze osteogenic tissue reparation using several types of plastic material, firstly, these are the patient's own bone chips, secondly, this is Collapan L and thirdly, Collapan L with its own autologous mesenchymal stem cells. The stem cells used in the experiment were committed, i.e. targeted to differentiate into bone tissue, this was done in order to accelerate the process of bone tissue regeneration and mesenchymal stem cells did not take the shortest path of differentiation into connective tissue. When studying the physiological regeneration characteristics of the lower jaw bone tissue in rats under the influence of mesenchymal stem cells on the jaw bone injury model, it was demonstrated that osteogenic differentiation of connective tissue cells and bone formation in Group 3 were more vigorous compared to other groups. This was confirmed by the concentration of Ki67 and CD 34, which demonstrated acceleration of angiogenesis and tissue proliferation. To study the question whether bone restoration stimulated by co-implantation of Kollapan-L with mesenchymal stem cells is mediated by increased proliferation in defective bone tissues, we determined the number of cells expressing Ki67, a proliferation marker, using immunostaining. In previously published works devoted to bone tissue regeneration, this marker was characterized as an objective criterion²⁷.

The number of Ki67-positive cells showed a slight increase after implantation in groups 1 and 2 compared to group 3. It turned out that combined implantation of Kollapan-L with mesenchymal stem cells increased the expression of Ki67-positive cells severalfold.

The integrity of bone, as a highly vascularized tissue, depends on angiogenesis and close communication of bone cells in time and space. Therefore, sufficient angiogenesis plays a key role in the development and restoration of bone. Local vascularization in the early stages after transplantation provides various necessary nutrients for the activity of osteogenesis, but also plays an irreplaceable role between the bone and adjacent tissues and organs²⁸.

An increase in CD34 expression in group 3 patients indicated the activation of angiogenic factors of bone tissue regeneration. In our previous studies, we confirmed that committed MSCs actively produce cytokines – IL-1 β , IL-2, IL-6, IL-8,

FNP- α and IL-4²⁹. During osteogenic differentiation, the production of IL-2, IL-6 and IL-8 significantly increases. Thus, it was proven that mesenchymal stem cells, being their own building material, stimulate regeneration, angiogenesis and enhance the synthesis of sulfated multipolysaccharides, which together lead to activation of bone tissue regeneration. In addition, arachidonic acid derivatives secreted by mesenchymal stem cells lead to both local and systemic immunomodulation, resulting in a decrease in the number of purulent complications and inflammation. The advent of bone tissue engineering technology has stimulated significant progress in the study of new materials and has significantly contributed to increasing the effectiveness of bone defect treatment. However, low-bionic materials, poor biocompatibility, cell migration, adhesion and proliferation still remain problems. Precise control of cell differentiation, gene and growth factor expression, and safety of clinical application need further study [30]. Through cross-integration and development of interdisciplinary concepts and technologies in the field of bone repair, such as cell biology, immunology, material science and manufacturing, bioactive bone-like tissues can be created by searching for and developing new substitutes for bone repair or directly in vitro. It is expected that regenerative restoration of bone defects will be widely used in clinical practice in the near future.

CONCLUSION

Thus, our data indicate the acceleration of physiological regeneration under the influence of mesenchymal stem cells committed in the osteogenic direction. The immunomodulatory effect of stem cells made it possible to significantly reduce the percentage of purulent infiltrates in the bone defect. An increase in the proliferation markers Ki67 and angiogenesis marker CD 36 leads to a favorable acceleration of bone tissue regeneration.

DECLARATIONS

Ethical approval and consent to participate
The work was carried out in compliance with all ethical standards for conducting an experiment and there is a positive decision of the Ethics Committee of the Order of the Red Banner of Labor Medical Institute named after S.I. Georgievsky (protocol No. 5 dated 05/18/2024).

Competing interest

The authors declare that there are no competing interest.

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Authors' contribution

I. G. Romanenko - study design, data collection and analysis, writing the text; V. Yu. Mikhailichenko - development of the research methodology and interpretation of the obtained data, preparation of the manuscript; N. L. Kulikova - protocol development, study implementation, analysis of the obtained data; D. S. Parshin - writing the text, scientific editing of the manuscript

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