**ISSN:** 2310-6255
**Founder:** Academic Publishing House *Researcher*
**DOI:** 10.13187/issn.2310-6255
Has been issued since 2013.


**European Journal of Molecular Biotechnology**

UDC 616.72-018.3 : 577.3

# Recombinant Bone Morphogenetic Protein 2 Stimulates the Remodeling Chitosan-Based Porous Scaffold Into Hyaline-like Cartilage: Study in Heterotopic Implantation

[1] Nurshat M. Gaifullin
[2] Alexey V. Bachurin

[1] Lomonosov Moscow State University, Russian Federation
Lomovnosovskiy Avenue 31/5, Moscow, 119192
PhD (Medicine), Assistant-professor
E-mail: gaifulin@rambler.ru
[2] OXY-center, Russian Federation
Uritzkogo st. 186, Krasnodar, 350047
Senior embryologist

**Abstract.** To study the morphology of remodeling the chitosan-based three-dimensional porous scaffold, containing bone morphogenetic protein-2 (BMP-2) for chondroinduction, the experiments with heterotopic implantation using 28 Wistar rats were carried out. Scaffolds with growth factor (n=12) or without it (n=12), against intact control (n=4) were implanted subcutaneously. Classical methods of histology and morphometry as well as immune histochemical markers (CD-68, CD-31, MMP-9, TIMP-1, and osteonectin expression), one used to investigate zone of remodeling in euthanized animals at 4 and 8 weeks after implantation.

The BMP-2 application provides more intensive and rapid new cartilage formation from the scaffold matter. The additional chondroinductive effect proved more intensive settlement and proliferation of chondral cells in the regenerate, expression of chondral phenotype with the building the hyaline-like matrix, and the supporting necessary balance between the matrix metalloproteinases and their tissue inhibitors.

**Keywords:** cartilage tissue engineering; chitosan; MMP-9; TIMP-1; BMP-2; osteonectin; macrophages.

**Introduction.** The essence of tissue engineering is the development and application of biomaterials for transplantation to replace tissues and functions nave been partially or completely lost. Materials and framing constructs (scaffolds), created for reconstructive cartilage surgery, are no exception. In this regard, they should have complete biocompatibility, support the vitality of the settled cells, transform into natural autologous matrix, and managed to change the structure and properties in response to the action of environmental factors [1, 2, 3, 4].

Chitosan is deacylated form of chitin, which is widely distributed polymer of natural origin, it has a majority of the above properties, as was shown to be biomimetic to the osseous and cartilaginous tissues. Chitosan has gained popularity for various modifications of cartilage engineered scaffold due to availability of commodities for its production and ease enzymatic treatments to improve its physical and chemical properties [5, 6]. It easily forms copolymers with other materials (silk, hydroxyapatite, polyalginate, polylactic acids, etc.), forming the porous

composites with adequate mechanical properties and ability to adhesion and proliferation of cells [7, 8, 9].

Initially high viscosity of chitosan solutions allows the use of various methods to create three-dimensional porous scaffolds ranging from freezing-drying up to foaming gases, which bubbles form the stable pores of up to 500 μm in diameter. The necessity of creating porous structures is determined by the mechanical, biochemical and physiological needs of prosthetic tissue. Firmly-elastic properties of some chitosan-based scaffolds approach to values for the trabecular bone, they can carry compressive loads up to 75 MPa. It was proved the influence of pore sizes and thickness of chitosan membrane, forming a porous 3D matrix, on the quality of cell adhesion, intensity of subsequent cell adhesion, proliferation and neoangiogenesis [7, 10]. Porous surface of chitosan scaffolds ensure the efficiency of its use with options of surface spray and gel fillers for additional stimulation of chondrogenesis.

The alignment of different growth factors in the area of implantation used to be similar effective stimulant in cartilage tissue engineering. Many of growth factor (epidermal EGF, platelet derived growth factor, transforming growth factor TGF-β, and others), bone morphogenetic proteins (BMPs), matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs), as well the majority of known cytokines it was proven to impact on chondrogenesis [2, 3, 6]. For the needs of regenerative medicine, TGF-β and BMP-2 were most promising if they used as a temporary tissue deposits (for 5-7 days following implantation) had provided additional chondroinductive and chondroconductive properties of implants [11, 12].

Quite recently, Russian molecular biotechnologists have developed original method for obtaining recombinant BMP-2 by highly efficient producer strain based on *E. coli* BL21(DE3). New biomaterial have been created on its basis, and the activity of this factor have been shown both *in vitro*, and on the model of ectopic osteogenesis *in vivo* [12, 13].

We felt it necessary to study experimentally the chondroplastic properties of this BMP-2 in case of combination with chitosan-based scaffold, since it originally had precisely the ability to chondroinduction.

**Material and methods.** The work was performed using 28 white rats Wistar weight from 240 to 290 g. The experimental protocol corresponded to the ethical standards set out in the International Code of Medical Ethics (1994), the Good Laboratory Practice (GLP) principles, the Helsinki Declaration (2000) and the Directives of the European Community 86/609EEC.

Three-dinensional porous coin-like pellets were fabricated at the Bioengineering and Bioinformatics Department of Volgograd State University (Russia) using commercial chitosan product (Pharma Nutrients, USA). Fabrication included an additional cleaning and deacetylation of the primary material in an oxygen-free medium under reduced pressure, washing out and slow dehydration under conditions preventing cornification, resuspensing in the temporary medium, and the formation of porous structure with using freeze-drying method. As a result of this procedures the scaffolds of 5 mm in diameter, thick of 2 mm in the center, up to 1 mm edges, with regular pore size of 70-150 mm were obtained [14].

Scaffolds were implanted under the skin in the area of the withers in rats, anesthetized by intramuscular Zolethyl injection of 40 mg/kg of body weight. In the main group (12 cases) 2 mcL of BMP-2-containing matter «Gamalant™-pasta-FORTE Plus» developed in Gamaleya Research Institute of Epidemiology and Microbiology (Russia), were placed on the surface of each scaffold before implantation evenly with 24 point touches of capillary needles (12 on each side). Developers were previously shown good osteoinductive and osteoconductive effects of this stimulator during application on bioactive implant surfaces placed in the bone tissue [12]. The second group consisted of 12 animals which have similarly established implants without making a growth factor.

The dynamics of healing and scaffold remodeling traced through 4 and 8 weeks after implantation. All rats were output from the experiment by Zolethyl overdose (200 mg/kg of body weight), the pieces of tissue from the implantation area were taken for histological examination. Eight pieces of subcutaneous adipose tissue of four rats being in standard conditions of the same vivarium due to all experiment time, were studied as control samples.

The mobility *in situ*, the condition of surrounding tissues, the presence of supplying vessels, the severity of adhesions, and degree of scaffold biodegradation evaluated immediately after seizure of samples.

Complete cycle of histological slide production includes embedding into Paraffin with the STP 420 D dehydration/infiltration unit (Microm, Germany) and the EG 1160 modular embedding station (Leica, Germany), microtomy with rotary system Leica RM 2255 (Leica, Germany), staining with Link 48 and cover slipper (Dako, Denmark), microscopy and digital photomicrography with Leica DM 4000 (Leica, Germany). To stain slices classic hematoxylin-eosin and Masson's trichrome protocols were used, to reveal density of tissue matrix toluidine blue was applied [15]. Immunohistochemical study included the identification of cell numbers sach as vascular endothelium (CD31), macrophage/osteoclasts (CD-68), osteoblasts and osteocytes (Osteonectin), and chondral cells (MMP-9, TIMP-1). Commercial Ready-to-Use Kits of Dako (Denmark) and Leica Mycrosystems (Germany) were applied.

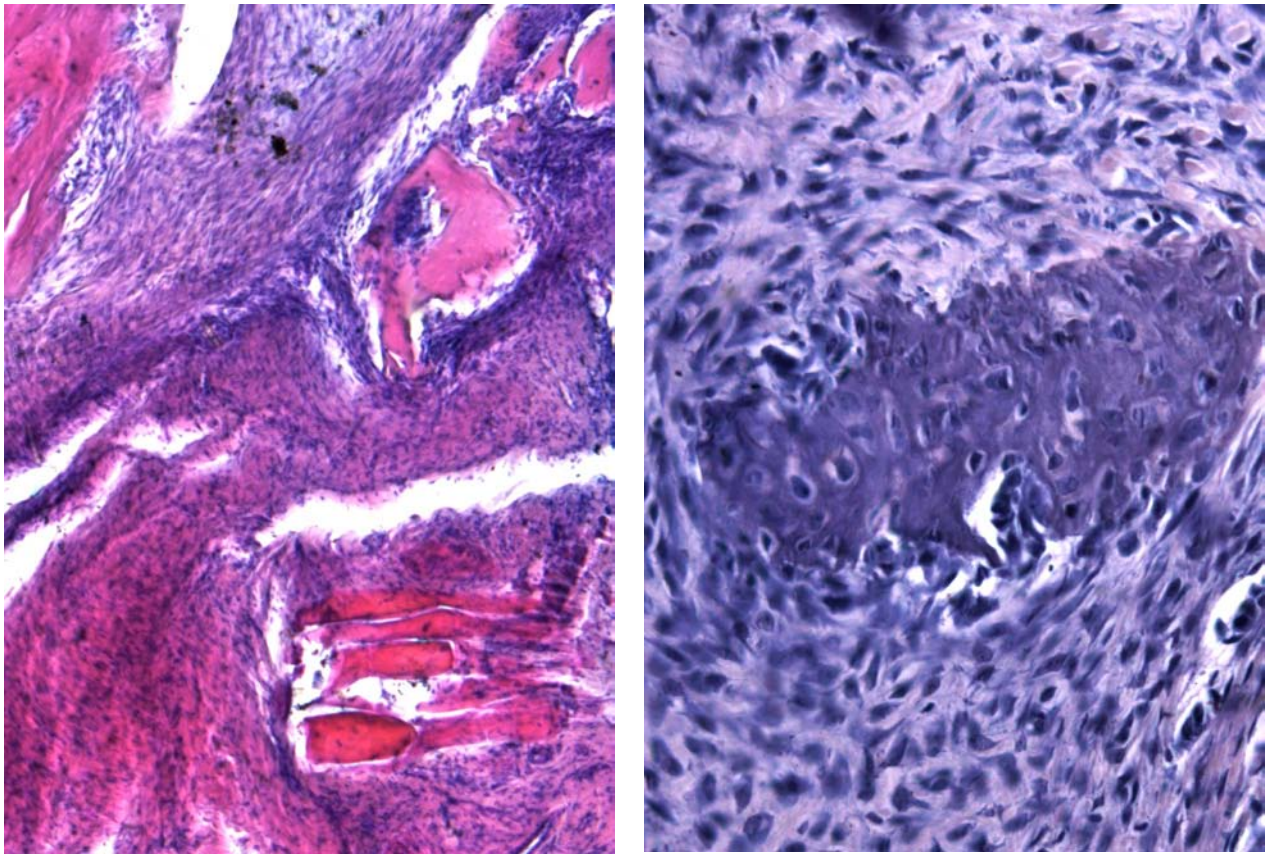
Morphometric analysis was performed using the software ImageJ (USA). Specific density of matrix (CU), the volume fractions of the tissue compartments in a regenerate (%), and numerical density of single cells (1/mm3) were determined quantitatively. Processing of these data have performed using Statistica 6.0 (StatSoft Inc., USA) software with common requirements for biomedical research. To analyze the differences between the samples Mann-Whitney criterion was used.

**The results of the study.** After 4 weeks the resorption of more than half of chitosan matter was noted in the group with BMP-2 application, and dense mixed regenerate have formed on this place. Morphology of the regenerate had bright structural variability and polymorphism of the cellular elements. Resorption of the surface layers of scaffold was accompanied by the growth of connective tissue elements with an abundance of extracellular chaotically oriented matrix and the presence of vessels. Deeper layers contained in the islets of hyaline-like cartilage, surrounded by connective tissue (Fig. 1A).

In the comparison group the scaffold bioresorption was no less intensive, but the number of cartilaginous elements in the regenerate was noticeably less.

At the 8$^{th}$ week the study of tissues regenerate confirmed almost complete bioresorption of scaffold matter. The layer of connective tissue remained on the surface of regenerate, it was slightly thicker, more dense, and with fewer vessels. Chitosan particles were diffusely distributed in the volume of the regenerate and surrounded by macrophages with morphological features of active phagocytosis. Focuses of previously formed cartilage became stronger and merged among themselves, as a result the main mass of regenerate was presented by newly hyaline-like cartilage with small areas of fibrous connective tissue. Small groups of chondrocytes resembling small clusters could be found (Fig. 1B).

In two cases out of five, we observed the presence of small osteogenic islets in the surface layers of regenerate, have been in contact with the vasculature of surface capsules.



**A** **B**

Figure 1. The place of remodeling porous chitosan scaffold with surface stimulation by BMP-2. A. 4 weeks. Mixed regenerate with a prevalence of fibrous cartilage and fragments of resorbing chitosan. Stain with hematoxylin and eosin. x 120. B. 8 weeks. Portion of hyaline-like cartilage in the stage of intensive remodeling. Stain with hematoxylin and eosin. x 480.

In the comparison group the scaffold bioresorption culminated in the formation of mixed regenerate with a prevalence of fibrous cartilage. Macrophages, often in groups of 3-5 cells, were detected on the surface of the non-resorbed polymer particles, captured chitosan fragments were frequently identified in their cytoplasm.

Data of classical morphometry are presented in table. 1.

Table 1

Quantitative morphological indicators of the regenerate after heterotopic implantation of chitosan scaffolds in rats (M ± m)

| Indices | Group | Controls | Timing of the experiment | |
|---|---|---|---|---|
| | | | 4 weeks | 8 weeks |
| Chitosan volume fraction, % | Main<br>Comparative | 0 | 37,2 ± 2,5<br>39,8 ± 2,7 | 6,9 ± 0,5<br>5,3 ± 0,4 * |
| Connective tissue volume fraction, % | Main<br>Comparative | 45,5 ± 2,9 | 28,1 ± 2,2<br>34,0 ± 2,6 | 15,1 ± 1,4<br>29,0 ± 2,3 * |
| Cartilage volume fraction, % | Main<br>Comparative | 0 | 27,3 ± 2,4<br>12,8 ± 1,0 * | 66,9 ± 4,9<br>52,7 ± 4,1 * |
| Specific density of matrix, CU | Main<br>Comparative | 0,25 ± 0,04 | 0,53 ± 0,06<br>0,38 ± 0,04 | 0,68 ± 0,07<br>0,45 ± 0,05 * |

* significant differences between groups.

As can be seen from the presented data, for 8 weeks after implantation the volume fraction of the cartilage in a regenerate on the place of chitosan scaffold increased to 52.7% without the use of BMP-2, and to 66.9% when growth factor had been used ($P<0.05$). This was accompanied by a more intensive and complete resorption of chitosan matter. The specific density of the cartilage matrix provided in the experimental group 1.51 times more than the rate value in the comparison group.

The data about the expression of the investigated immune histochemical markers are given in table. 2.

At 4th week numerical density of CD68+ cells mainly represented in regenerate by macrophages, increased in the main group in 14.8 times and in the comparison group in 16.5 times against the rate value in surrounding tissues. Later, the number of macrophages declined dramatically, but it still exceeded the values in the subcutaneous tissue in 5.9-6.8 times without significantly differinces between experimental and comparison groups. These data showed that the use of BMP-2 was almost not influence the intensity of macrophage reactions due to chitosan scaffold remodeling.

Table 2

The expression of different immunohistochemical markers in regenerates after heterotopic implantation of chitosan scaffolds in rats (M ± m)

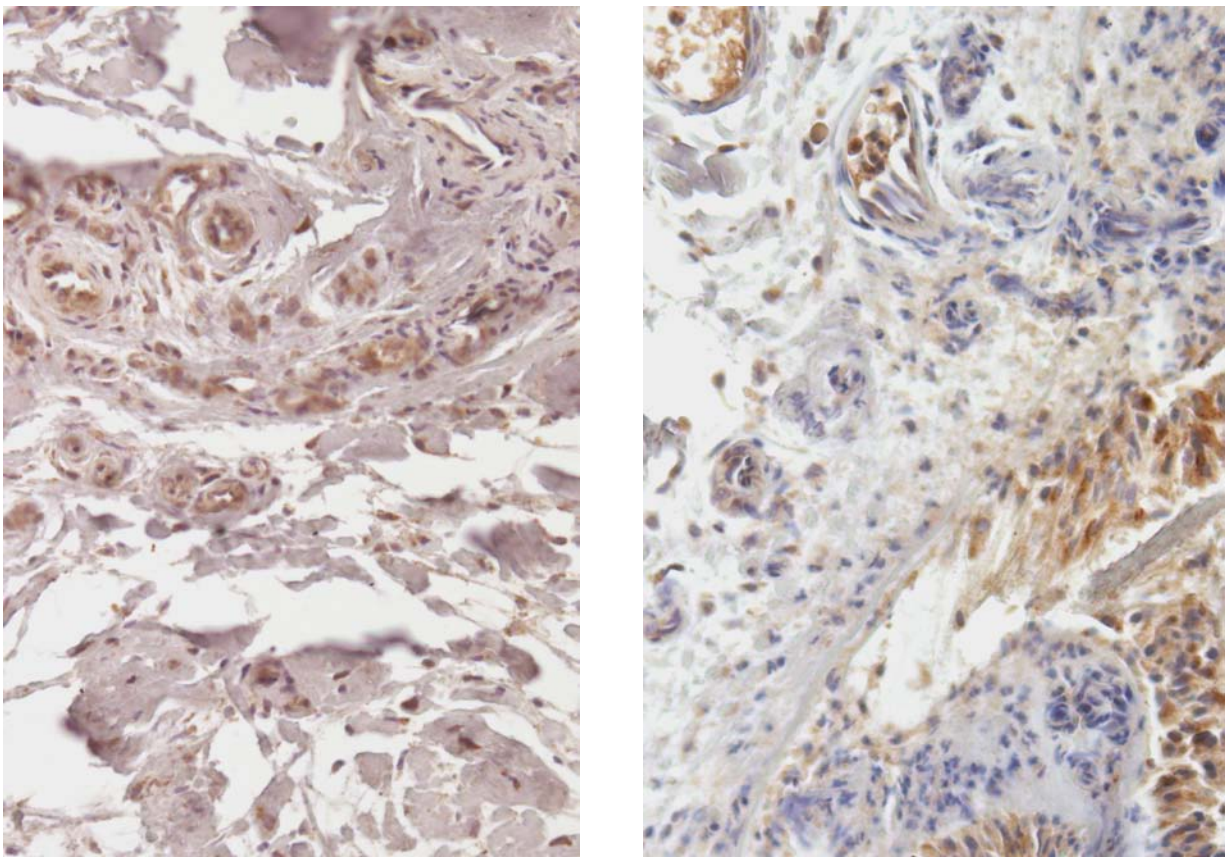
| Indices | Group | Controls | Timing of the experiment | |
|---|---|---|---|---|
| | | | 4 weeks | 8 weeks |
| CD-68+ cells, 1/mm³ | Main<br>Comparative | 49 ± 3,2 | 724 ± 43,2 *<br>807 ± 46,5 * | 290 ± 16,8 *<br>331 ± 23,4 * |
| CD31+ cells, CU | Main<br>Comparative | 0,17 ± 0,03 | 0,35 ± 0,05 *<br>0,64 ± 0,07 *# | 0,22 ± 0,04 *<br>0,47 ± 0,06 *# |
| MMP-9+ cells, 1/mm³ | Main<br>Comparative | 0 | 1152 ± 57,1<br>743 ± 40,8 # | 2739 ± 123,5<br>1794 ± 80,2 # |
| TIMP-1+ cells 1/mm³ | Main<br>Comparative | 0 | 118 ± 7,4<br>205 ± 12,6 # | 1634 ± 73,2<br>1029 ± 58,0 # |
| Osteonectin+ cells CU | Main<br>Comparative | 0 | 0<br>0 | 0,24 ± 0,03 *<br>0,11 ± 0,02 *# |

* significant differences between groups.

The degree of vascularization estimated from the total brightness of immune- positive material (CD31+ cells) also was the highest at 4th week of the experiment. It was higher in 2.1 times after using the BMP-2, comparing to 3.8 times in group without it ($P<0.01$ between groups). Similar but less drastic changes have been revealed at 8th week of experiment. So, the adding growth factor reduces the intensity of vascularization due to chitosan scaffold remodeling.

The number of chondrocytes synthesizing MMP-9, increased between the 4th to the 8th week of the experiment, and numerical density of cells was on those dates higher in the main group in 1.5 times higher than in the comparison one. The maximum expression of MMP-9 was revealed in chondrocytes, surrounded by newly hyaline-like matrix, which have been an evidence of intensive tissue remodeling (Fig. 2A).

At the 4th week of the experiment cells synthesizing the tissue inhibitor of metalloproteinase 1 (TIMP-1+ cells), were few in the regenerate, but by the 8th week their number had increased, so the ratio MMP-9+ : TIMP-1 had become of 1.7 : 1 in both groups. TIMP-1+ cells were localized in the thickness of the newly cartilaginous tissue (Fig. 2B).



**A** **B**

Figure 2. The place of remodeling porous chitosan scaffold with surface stimulation by BMP-2. A. 8 weeks. Numerous cells with high expression of MMP-9 placed in the zone of the newly formed cartilage. MMP-9-spesific monoclonal antibodies, immunoperoxidase assay. x 480. B. A smaller number of cells with high expression of TIMP-1 in the same zone of the regenerate. TIMP-1-spesific monoclonal antibodies, immunoperoxidase assay. x 480.

Co-localization of these markers in the regenerate evidenced on the integration of up-regulatory mechanisms of remodeling the neo-formed cartilage on the place of chitosan scaffold.

Osteonectin expression reflecting the presence of osteogenic elements was revealed in regenerates not earlier than 8$^{th}$ weeks after implantation, it was slightly higher in the main group. Osteonectin-positive substances were localized mainly around capsular vessels of regenerate, which was a consequence of osteoinductive activity of BMP-2. This fact is not a cause to concern if these scaffolds used for replacement of cartilage defects, because the conditions of tissue healing in the joint excluded osteogenesis, except osteochondal zone where this had a positive effect for tissue-engineering design.

**Discussion.** Since the chondroinductive effect of porous chitosan scaffolds no doubts and was repeatedly confirmed [5, 7, 10, 12], the main task was to find out main effects of small BMP-2 quantities after it's submit to the surface of the implant immediately before implantation. It is clear known, the initial processes in scaffold matter are inconceivable without migration and activation of tissue macrophages. These cells are able not only utilize a scaffold matter, but also develop a wide spectrum of cytokines and other signal molecules, causing stem cells and different immature cells to move from surrounding tissue in scaffold, proliferate, and transfer to chondral phenotype [3, 9, 16].

Bone morphogenetic proteins play a vital role in controlling the proliferation, differentiation, phenotypic expression (synthesis of collagen type 2, aggrecan, MMPs, TIMPs etc.), and life cycle duration through the classic Wnt/β-catenin signaling pathway activation and the expression of NF-κB factor [11, 17, 18]. In relation to explored model the BMP-2 adding led to significant growth of chondral cell number and results of their function in regenerates. One can see high expression of matrix metalloproteinases and their inhibitors in tissue on the place of scaffold implantation.

The sequence of MMP and TIMP activation in the tissues regenerate testifies about running active processes of tissue remodeling already after primary substitution of scaffold matter, which was a feather of restoring natural processes of cartilage matrix renewal.

**Conclusion.** Three-dimensional porous chitosan-based scaffold improved to have high capacity for chondroinduction, resulting in formation of fibrous cartilage with a moderate quarter of hyaline-like cartilage on the place of heterotopic implantation for 8 weeks. By surface adding nano-quantities of BMP-2 it can significantly improve chondroinductive properties of chitosan scaffold, as a result, the main volume of the regenerate after heterotopic implantation, judging by the cell representation, MMP-9 and TIMP-1 expression, and density of matrix is presented by hyaline-like cartilaginous tissue, close in composition and properties of natural hyaline cartilage.

## References:


1. Malanin D.A., Novochadov V.V., Samusev S.R., et al. (2009) Innovative technologies in restoration of damaged or diseased knee joint. *Herold Volgograd State Med. Univ. [Vestnik Volgogradskogo Gosudarstvennogo Meditzinskogo Universiteta].* (2), pp. 7-13. [in Rus.]

2. van Osch G.J., Brittberg M., Dennis J.E., et al. (2009) Cartilage repair: past and future – lessons for regenerative medicine. *J. Cell Mol. Med.* 13 (5), pp. 792–810.

3. Malanin D.A., Pisarev V.B., Novochadov V.V. (2010) Restoration of Cartilage Lesions in a Knee Joint: Monograph. *Volgograd: Volgograd Scientific Publishing*, 518 pp. [in Rus.]

4. Kock L., van Donkelaar C.C., Ito K. (2012) Tissue engineering of functional articular cartilage: the current status. *Cell Tissue Res.* 347(3), pp. 613-627.

5. Di Martino A. Sittinger M., Risbud M.V. (2005) Chitosan: a versatile biopolymer for orthopaedic tissue-engineering. *Biomaterials.* 26 (30), pp. 5983-5990.

6. Novochadov V.V. (2013) The control of the cell settlement and scaffold remodeling in cartilage tissue engineerng: a review. *Herold Volgograd State Univ. 11: Natural Sciences [Vestnik Volgogradskogo Gosudarstvennogo Universiteta. 11: Estestvennye Nauki].* (1), pp. 19-28. [in Rus.]

7. Correia C.R., Moreira-Teixeira L.S., Moroni L., et al. (2011) Chitosan scaffolds containing hyaluronic acid for cartilage tissue engineering. *Tissue Eng. Part C. Methods.*17 (7), pp. 717-730.

8. Chen J.P. Chen S.H., Lai G.J. (2012) Preparation and characterization of biomimetic silk fibroin/chitosan composite nanofibers by electrospinning for osteoblasts. *Nanoscale Res Lett.* 7 (1), pp. 170-178.

9. Lu T., Li Y., Chen T. (2013) Techniques for fabrication and construction of three-dimensional scaffolds for tissue engineering. *Int. J. Nanomedicine.* 8, pp. 337–350.

10. Muzzarelli R.A. (2011) Biomedical exploitation of chitin and chitosan via mechano-chemical disassembly, electrospinning, dissolution in imidazolium ionic liquids, and supercritical drying. *Mar. Drugs.*9 (9), pp. 1510-1533.

11. Bessa P.C., Casal M., and Reis R.L. (2008) Bone morphogenetic proteins in tissue engineering: the road from the laboratory to the clinic, part I (basic concepts). *J. Tissue Eng. Regen. Med.* 2, pp. 1-13.

12. Mironov S.P., Gintsburg A.L., Es'kin N.A., et al. (2010) Experimental evaluation of osteoinduction of recombinant bone morphogenic protein (rhBMP-2) of native production fixative on biocomposite or bone matrix. *Herold Traumatol. Ortop. (Moscow) [Vestnik Travmatologii i Ortopedii imeni N.N. Priorova].* (4), pp. 38-44. [in Rus.]

13. Sharapova N.E., Kotnova A.P., Galushkina Z.M., et al. (2010) Production of the recombinant human bone morphogenetic protein-2 in Escherichia coli and testing of its biological activity in vitro and in vivo. *Mol. Biol. (Moscow).* 44 (6), pp. 923-930.

14. Lyabin M.P., Semenov P.S. (2011) Improved technology obtaining chitosan. *Herold Volgograd State Univ. 11: Natural Sciences [Vestnik Volgogradskogo Gosudarstvennogo Universiteta. 11: Estestvennye Nauki].* (2), pp. 17-22. [in Rus.]

15. Handbook of histology methods for bone and cartilage. (2003) Ed. by Y.H. An and K.L. Martin. *N.-Y.: Humana Press,* 587 pp.

16. Goldring M.B. (2012) Chondrogenesis, chondrocyte differentiation, and articular cartilage metabolism in health and osteoarthritis. *Ther. Adv. Musculoskelet. Dis.* 4 (4), pp. 269–285.

17. Meszaros E., Malemud C.J. (2012) Prospects for treating osteoarthritis: enzyme–protein interactions regulating matrix metalloproteinase activity. *Ther. Adv. Chronic Dis.* (3), pp. 219-229.

18. Wang Y., de Li L., Zhang X.B., et al. (2013) Increase of TNFα-stimulated osteoarthritic chondrocytes apoptosis and decrease of matrix metalloproteinases 9 by NF-κB inhibition. *Biomedical and Environment Sciences*. (26). pp. 277-283.

УДК 616.72-018.3 : 577.3

# Рекомбинантный BMP-2 стимулирует ремоделирование пористой матрицы на основе хитозана в гиалиноподобный хрящ: модель гетеротопической имплантации

[1] Нуршат Минуллаевич Гайфуллин
[2] Алексей Владимирович Бачурин

[1] Московский государственный университет имени М.В. Ломоносова, Россия
119192, г. Москва, Ломоносовский пр., 31, корп. 5
Кандидат медицинских наук, доцент
E-mail: gaifulin@rambler.ru
[2] OXY-центр, Краснодар, Россия
350047, г. Краснодар, ул. Урицкого, 186
Старший эмбриолог

**Аннотация.** В экспериментах с использованием 28 белых крыс изучали дополнительные возможности хондроиндукции за счет внесения малых количеств костного морфогенетического белка BMP-2 на поверхность пористых скаффолдов на основе модифицированного хитозана. Скаффолды с фактором роста (n=12), без него (n=12) через 4 и 8 недель после гетеротопической имплантации и ткани подкожной клетчатки интактных крыс (n=4) были изучены с использованием классических гистопатологических и морфометрических методов, а также при иммуногистохимическом выявлении CD-68, CD-31, MMP-9, TIMP-1 и остеонектина. В результате исследования установлено, что предварительное внесение BMP-2 на поверхность скаффолдов сопровождается дополнительным хондроиндуктивным эффектом, который заключается в более интенсивном заселении и пролиферации в регенерате клеток хондрального ряда, экспрессии хондрального фенотипа с построением матрикса гиалиноподобного хряща и установлении необходимого баланса между матриксными металлопротеиназами и их тканевыми ингибиторами.

**Ключевые слова:** тканевая инженерия хряща; хитозан; MMP-9; TIMP-1; BMP-2; остеонектин; макрофаги.