ORIGINAL ARTICLE

Lectin histochemistry of bone regeneration dynamics in the rabbit mandible following transplantation of natural mineral and organic osteoplastic materials

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ABSTRACT

Aim: To reveal the applicability of lectin histochemistry methods for the evaluation of bone tissue injury recovery.

Materials and Methods: The research was accomplished on 35 mature rabbits aged 6-7 months weighting 2.5-3 kg subdivided into three groups. Control group included 10 rabbits with post traumatic mandibular bone injury site healed under the blood cloth. First experimental group consisted of animals with damaged bone recovery supplemented with an osteotropic material based on native octacalcium phosphate (OCP-N). Second experimental group consisted of rabbits with bone defect filled with natural collagen cone (Col-C). Monitoring of the post-traumatic processing. was carried out for 84 days using panel of three lectins (WGA, LABA and CNFA) with different carbohydrate specificities.

Results: The lectins demonstrated high variability of binding with constituents of posttraumatic bone tissue. In particular, it was revealed rather selective WGA reactivity with microvascular endothelium, LABA reactivity with macrophages and CNFA with fibroblaststs and osteogenic cells. Injured bone in control and both experimental groups demonstrated distinct rearrangement of lectin receptor sites with drift towards posttraumatic recovery being more prominent after aplication of OCP-N osteotropic material. Traumatic bone injury induced accumulation of macrophages in the site of inflammation, as well as redistribution of lectin receptors in microvascular endothelium.

Conclusions: Lectins of different carbohydrate specificities can be recommended for monitoring bone tissue regeneration after posttraumatic injuries.

KEY WORDS: bone tissue, lectin histochemistry, osteoregeneration, octacalcium phosphate, natural collagen

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INTRODUCTION

The relevance of present research is due to the growing number of patients and victims of war who need adequate augmentation of bone defects, a significant number of relevant surgical interventions, and is confirmed by a huge number of specialized studies and publications in this area. However, the vast majority of studies are aimed on finding the so-called «perfect» material or justifying the optimal way to use it, in other words, at determining a certain rating of osteoplasty methods. Therefore, it is justified to study the dynamics of histoarchitectural remodeling during osteoregeneration depending on the osteoconductive and osteoinductive properties of osteotropic materials that differ in their physicochemical composition. The urgency of finding effective methods for stimulating bone tissue regeneration after injuries is due to the fact that extensive and infected post-traumatic defects, despite the natural ability of the bone to self-heal, significantly complicate the healing process and cause a number of complications [1, 2].

It should be noted that this is not the first study devoted to solving numerous issues related to ways to increase the effectiveness of osteoplasty using various osteotropic materials. However, when it comes to morphological transformations of bone and soft tissue components of the dento-maxillary apparatus when using certain osteoplastic materials, we are faced with a complete lack of ideas in this direction. Those fragmentary information and individual publications that try to cover it sometimes turn out to be contradictory or even antithetical in their general conclusions. In this aspect, the problem of our research is clearly distinguished precisely by the fact that it is aimed on studying specific morphogenetic mechanisms that ensure the osteoregenerative process

Table 1. Lectins used and their carbohydrate specificities

Lectins full names and abbreviated forms	Carbohydrate specificities	Complementary olygosacharide
Clitocybe nebularis fungus agglutinin, CNFA	DGalNAc	DGalNAc(β14)GlcNAc
Wheat germ agglutinin, WGA	DGlcNAc > NeuNAc	Man(β1-4)GlcNAc(β1-4)GlcNAc > NeuNAc(α2-6)Gal(β1-4)GlcNAc
Laburnum anagyroides bark agglutinin, LABA	LFuc	Gal(β1-4)Fuc(β1-3)Glc

Source: compiled by the authors of this study

at the tissue, cellular and subcellular levels when it is initiated using osteotropic materials with different properties, thereby providing the opportunity to choose the optimal material depending on the specific clinical situation. Extensive and infected post-traumatic bone defects significantly slow down the rate of remodeling, often requiring surgical intervention using osteoplastic materials [3].

In large bone defects, autologous bone remains the «golden standard», but it also has a number of disadvantages [4-6]. In the treatment of small and medium bone defects, alternative osteoplastic materials are increasingly used, which minimizes the risk of complications associated with autotransplantation. Effective integration of these materials with the bone bed without the formation of fibrous tissue is a determining condition for their successful use. Xenogenic materials, in particular, based on octacalcium phosphate, demonstrate good osteoconductive properties due to their structural similarity to human bone tissue and the optimal ratio of calcium to phosphorus. Biodegradation of octacalcium phosphate with subsequent replacement by new bone makes it a valuable material in dental regenerative surgery [7-10].

Also in our study, a comprehensive analysis of the effectiveness of a modified natural collagen matrix was conducted. This substance is widely used for post-extraction preservation of the alveolar process and promotes the formation of a blood clot with its subsequent reorganization into bone tissue. Collagen-based materials have high biocompatibility and a predictable result of preserving the height of the alveolar process. The main characteristics of natural collagen implants include: high ability to resorption processes, ensuring blood clot stabilization and effective local hemostasis. Collagen cones have found wide application in various fields of dentistry, in particular, in implantology, periodontology and maxillofacial surgery. They are used to control bleeding and hemostasis after tooth extraction, during biopsy and when performing closed sinus lifting [11]. Collagen-based materials in various forms, including membranes, sponges or matrices, hydrogels, and composite scaffolds, are also widely used in vivo to support bone regeneration in various clinical applications [12].

AIM

To reveal the applicability of lectin histochemistry methods for the evaluation of bone tissue injury recovery.

MATERIALS AND METHODS

The study was conducted on 35 mature male rabbits aged 6-7 months, weighing 2.5-3 kg. The animals were divided into three groups: a control and two experimental groups (10 animals each). Another 5 intact animals were used to study the normal structure of the bone tissue of the studied area of the lower jaw (LJ). The control group included animals with a bone tissue defect healed under a blood clot. The first experimental group consisted of rabbits in which the bone defect was filled with the osteotropic material Compact BoneB («Dentegris», Germany), the main crystalline element of which is native octacalcium phosphate (OCP-N). The second experimental group consisted of rabbits in which bone defect was filled with the material Collacone («Botiss dental», Germany), which is a natural collagen cone (Col-C). Animals of the control and experimental groups were given general anesthesia by intraperitoneal injection of Thiopenate (Bropharma, Ukraine) at a rate of 25 mg/kg of animal body weight at the level of the edentulous area of the alveolar part of the lower jaw using a dental bur, causing a bone-destroying injury in the form of a shaft 4 mm deep and 3 mm wide. Post-traumatic condition was monitored 1, 7, 14, 21, 28, 35, 56 and 84 days after the injury.

The dynamics of histoarchitectural rearrangements of bone tissue in the area of an experimental defect of the lower jaw were studied using the lectin histochemical method, which occupies a significant place among modern methods of morphological analysis [13,14]. To identify carbohydrate determinants of bone tissue structures, a panel of lectins conjugated with horseradish peroxidase was used (Table 1). All used lectins were purified and conjugated with peroxidase by Professor V.O. Antonyuk, a Faculty member of Lviv Medical University Pharmacy Department.

Serial sections of 5-7 μm thick were made from the obtained paraffin blocks of decalcified bone fragments on a sled microtome. Processing of sections with lectins was carried out according to the standard scheme de-

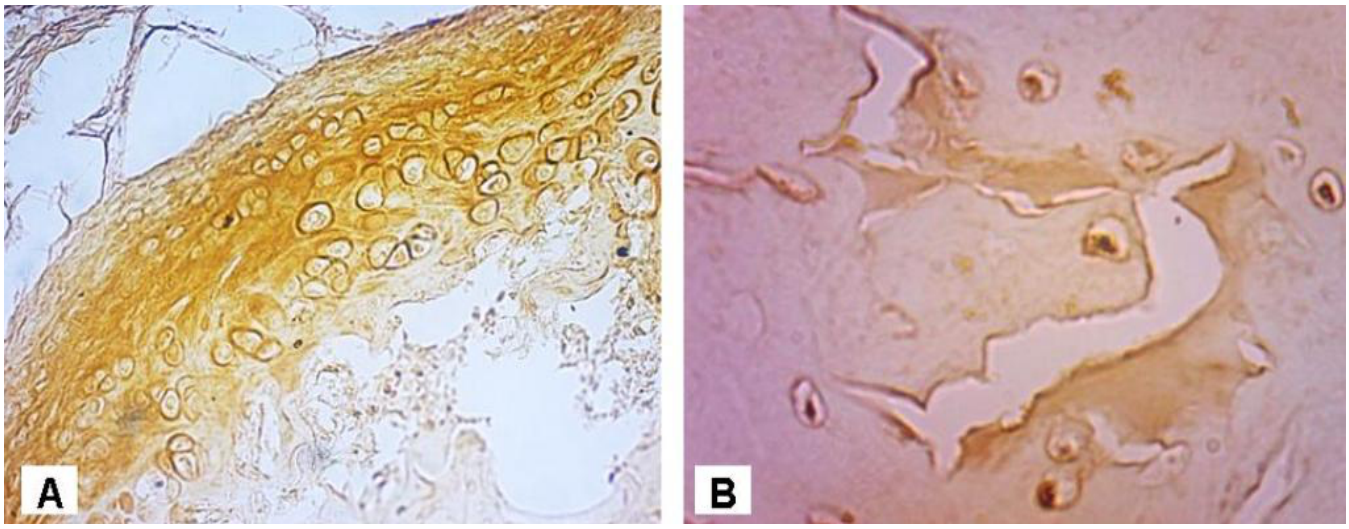

Fig. 1. Regenerate deep zone on the 3rd week post surgical treatment of control group rabbit as visualized by WGA-HRP. A). Destruction of chondroid during cartilage osteogenesis. ×400. B) Intense WGA labeling of osteoclasts and surface lining of the Howship`s lacunae. ×600
Picture taken by the authors

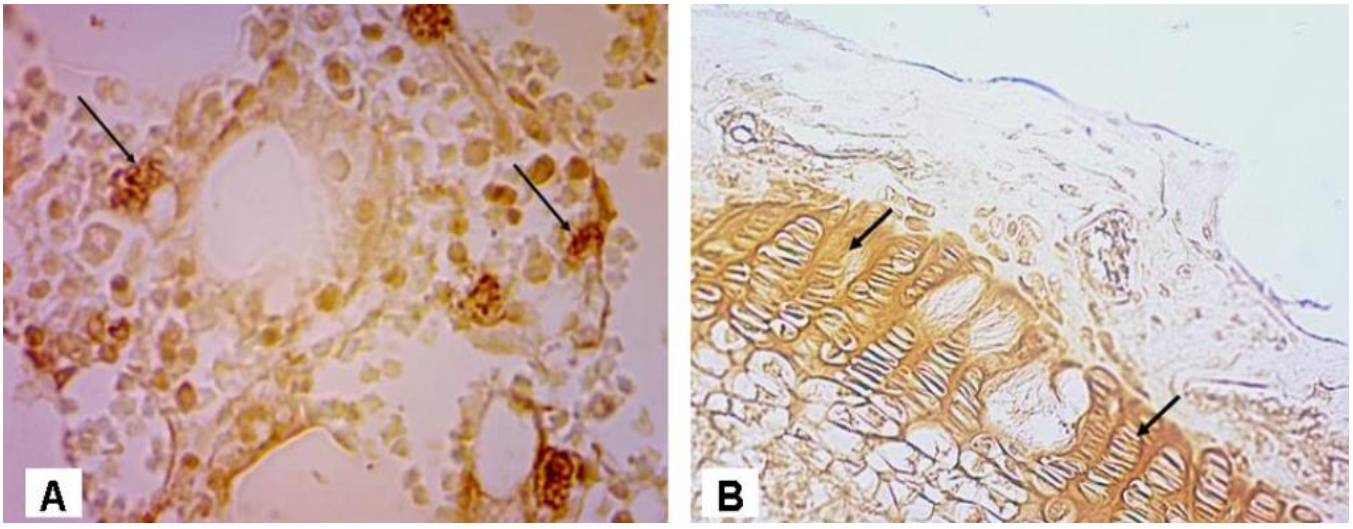

Fig. 2. Deep zone of the regenerative island: 2nd week post surgical treatment; control group rabbits; carbohydrate visualization by LABA-HRP conjugate. A). Accumulation of macrophages in the inflammatory zone (arrows). ×600. B). Islet of chondrohistogenesis: columns of chondrocytes subjected for resorption are highlited by arrows. ×400
Picture taken by the authors

scribed elsewhere [15]. The lectin panel is presented in Table 1. Stained histological slides were studied using a UlabXSP–137TLED photomicroscope (PRC). Pictures were taken with an XCAM-1080 P camera (PRC).

All animals were kept in standard vivarium conditions and procedures related to housing, care, marking and all other manipulations were carried out in compliance with the provisions of the «European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes» (Strasbourg, 1985);, the «General Ethical Principles of Animal Experiments» adopted by the First National Congress on Bioethics (Kyiv, 2001), the Law of Ukraine No. 3447-IV «On the Protection of Animals from Cruelty to Animals» in accordance with the EU Council Directive 2010/63/EU on the implementation of the regulations, laws and administrative provisions of the EU Member States concerning the protection of animals used for scientific purposes. The Bioethics Commission of Danylo Halytsky Lviv National Medical University approved the design and methodology of research project with final conclusion that conducted research meets ethical requirements in accordance with the Order of Ministry of Health of Ukraine № 231 of November 1, 2000 (protocol № 1 of January 20, 2025).

RESULTS

A study conducted using peroxidase-labeled lectins in the control group showed significant variability in the intensity

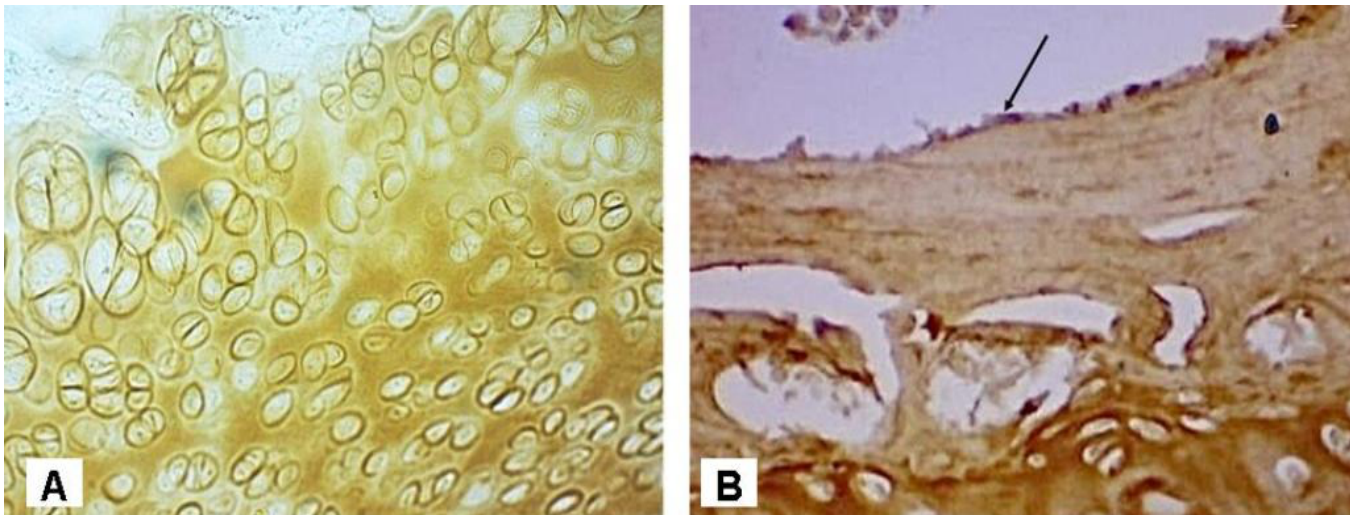

Fig. 3. Regenerate deep zone on the 3rd (A) and 7th (B) weeks post surgical treatment: control group rabbits subjected to CNFA-HRP conjugate. A). Islet of chondrohistogenesis in progres. ×400. B). Intense reactivity of osteoblasts covering bony trabecule (arrow); osteocytes and osteomucoid less reactive. ×400
Picture taken by the authors

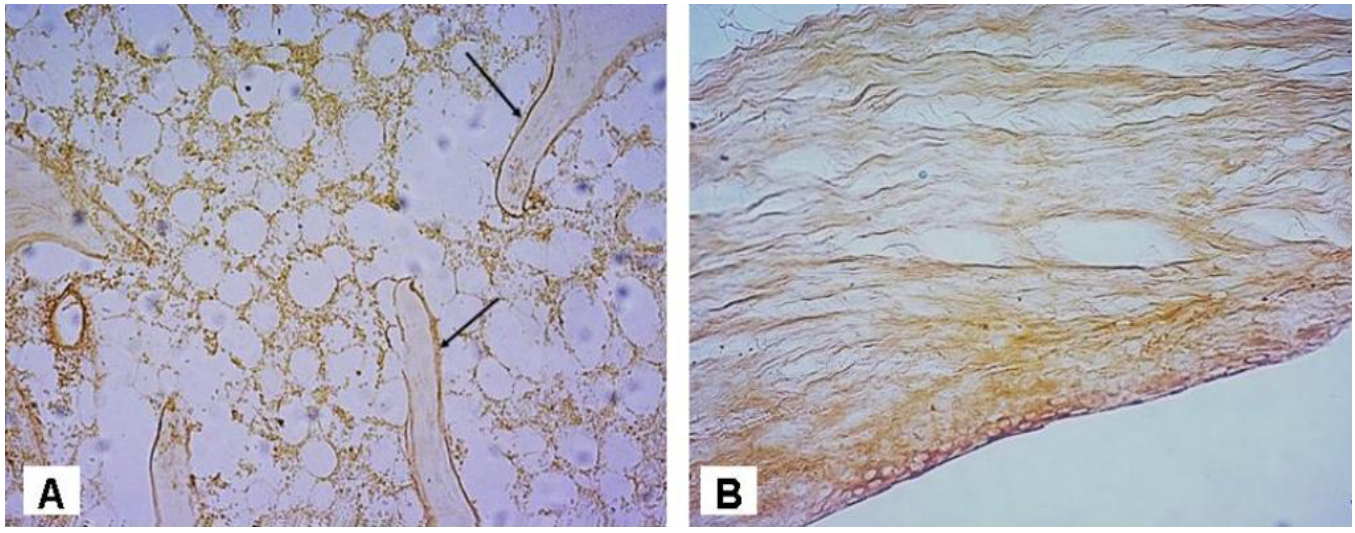

Fig. 4. Deep zone of osteoregenerate after 8 (A) and 5 (B) weeks of traumatic damage with subsequent tissue heeling under octacalcium phosphate. A). Bony trabecules covered by WGA reactive glycoconjugates (arrows). ×100. B). LABA reactivity of enosteal cover of bony trabecule, collagen fibers of newly formed bone are faint positive. ×200
Picture taken by the authors

of binding to carbohydrate determinants of structural elements of bone tissue of the alveolar processes of the mandible, periodontium, gums and blood clot structures. In particular, when using WGA (DGlcNAc > NeuNAc), high reactivity was detected at the early stages of the study in the microcirculatory bed endothelium, individual cellular elements of the periodontal ligament and in the fibroblasts of the gingival lamina propria. Strong WGA labeling was also characteristic for the surfaces of blood cells. Within 1-3 weeks after injury, the highest intensity of WGA binding was observed on the periphery of cartilage osteogenic islets, in the deep zone in the regenerate, close to the areas of chondroid destruction (Fig. 1A). From the 6th to the 12th week of the experiment, a slightly different activity of WGA lectin binding to the structures under investigation was observed. The vascular endothelium was mostly unresponsive, but individual areas of WGA binding were detected in the basement membranes of small blood vessels (Fig. 1B). The reactivity of cellular elements during this period did not show significant changes.

LABA reactivity (LFuc specific) was detected on individual cells in the studied samples. At the end of the day 1st of experiment, on the background of osteolytic injury, strong LABA reactive cells were identified in the

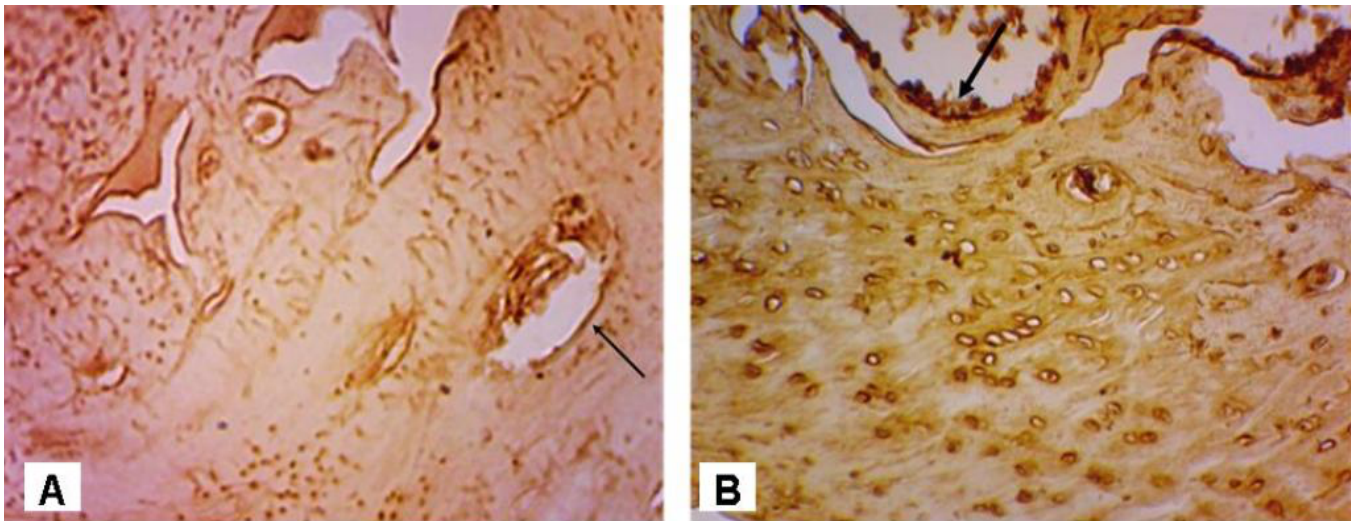

Fig. 5. CNFA lectin labeling of osteoregenerate peripheral trabecule on week 8th post traumatic damage and subsequent heeling under octacalcium phosphate (A) and natural collagen treatment (B). In both cases CNFA strongly labeled osteoblastic cover of bony trabecules (arrows), cell bodies of early osteocytes, surface of their hausing lacunas, surface of Howship`s lacunas with detryt deposits inside. A×400; B. ×200
Picture taken by the authors

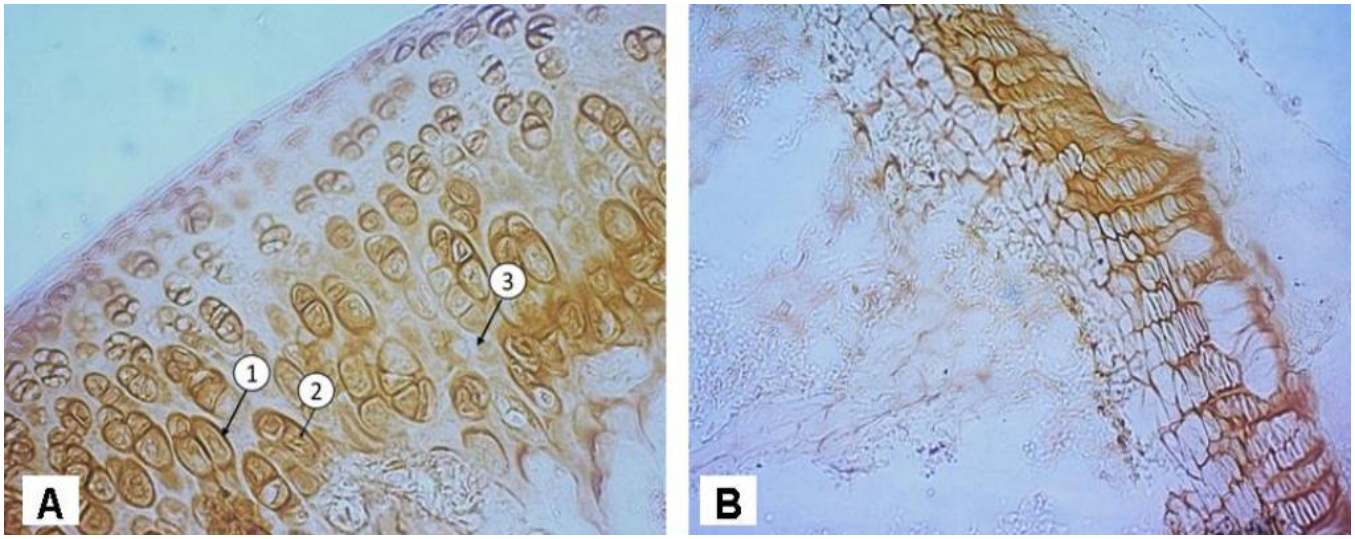

Fig. 6. Lectin reactivity with deep zone of osteoregenerate of the lower jaw of a rabbit. A. Two weeks after injury and subsequent filling of the defect with octocalcium phosphate: intense WGA reactivity with surface of chondrocytes and of their residence lacunae (1); cytoplasm of chondrocytes (2) and adjusting chondromucoid (3) are far too less reactive. ×200. B. Three weeks afer injury and filling of the defect with natural collagen: staining with LABA-HRP conjugate. ×200
Picture taken by the authors

damaged tissues, apparently representing macrophages accumulating in inflammatory affected post treaumatic tissues (Fig. 2A). Other cell populations, in particular, osteogenic cells, numerous fibroblasts and migratory cells, did not show LABA reactivity throughout the study. In the deep zone of the regenerate, from the 1st to the 4th week after injury, there was an intensive exposure of fucose-specific determinants in the matrix of hyaline cartilage and on the surface of chondrocytes between the islands of cartilagenous osteogenesis (Fig. 2B).

Beginning from the 4th week of experiment and until its completion, LABA lectin did not expose noticeable reactivity, though endothelium and subendothelial layer of small arteries, as well as the basement membrane of the gingival epithelium were faintly positive.

Treatment of preparations with lectin CNFA (specificity DGalNAc) revealed heterogeneity of binding to carbohydrate determinants of individual structures. In the period from 1st to 5th weeks after injury, considerable CNFA binding was identified between chondroblasts and isogenic groups in the foci of cartilage osteogenesis in the deep zone of the regenerate (Fig. 3A). Also lectin CNFA demonstrated rather selective binding with cellular elements of

periodontal ligament (2nd-3rd weeks), as well as with hemocapillary bed (5th-8th weeks). Additionally CNFA labeled cells of the fibroblastic series located in the periodontal ligament and gums. It is noteworthy that CNFA, unlike other used lectins, selectively labelled carbohydrate determinqants of bone trabeculae endosteum, this notion becoming especially evident in the latest stages of experiment (8-12 weeks) (Fig. 3B).

While studying lectin reactivity of lower jaw decalcified sections with injury in the form of bone defect and its subsequent replacement with osteoplastic material OCP-N, a number of changes associated with both the development of injury consequences the appearance of signs of reparative regeneration were found. In particular, while using WGA it was documented variable intensity of binding to carbohydrate determinants of the structural components of bone tissue in the area of surgical intervention. The affected sites included alveolar processes, periodontium and gums. WGA demonstrated strong reactivity with glycoconjugates of the microcirculatory bed endothelium, cellular elements of the dental ligament close to the apex of the tooth root. Such picture was typical for the 14th and 21st days of observation, while later (56th and 84th days), WGA reactivity decreased. At the same time, a slightly positive reaction was detected with the individual fragments of microvascular bed basement membranes of small blood vessels. Lectin reactivity of cellular elements of the studied structures did not expose significant changes. In the composition of the bone regenerate 56th days post OCP-N material implantation, a rather uniform distribution of WGA reactive glycoconjugates between newly formed trabeculae was observed (Fig. 4A).

While using LABA lectin after osteoplasty with OCP-N material, in contrast to the control group and intact animals, noticeable reactivity with cellular elements was documented mainly in the early stages of experiment (from 1st day to the 4th week). Since it is known that LABA lectin is a specific marker of rat macrophages, we relay this observation to the presence of large number of macrophages in the focus of the inflammatory process, which was naturally caused by bone-destructive trauma. When analyzing the newly formed trabeculae of the bone regenerate in the period from 4th till the 8th week of the experiment, uniform distribution of fucosoglycans was detected both on the periphery of the osteoregenerate and in its deep zone (Fig. 4B).

While labeling histological sections of the first experimenthal group animals with CNFA, this lectin binding unexpectedly was observed with carbohydrate determinants of damaged structures already on the first day of experiment. Similar pattern was observed in animals of control group. Regarding further terms of observation in animals whose bone defect was filled with OCP-N, on the 5th-8th weeks CNFA lectin selectively labeled surface of single cells in the periodontal ligament and within the walls of hemocapillaries. Similar pattern was inherent at the end of the experiment; however, the reactivity of the endothelium of the microcirculatory bed was comparatively higher in comparison with animals of control group (Fig. 5A). This observation can be related to enhanced vasculogenesis intensity in the area of defeect filled with OCP-N material. It is noteworthy that CNFA lectin exposed rather selective binding to carbohydrate residues of the structural components of the endosteum enveloping bone trabeculae, which was most clearly manifested at the end of the observation. At the same time, we did not observe similar CNFA reactivity in the animals of the control group and in intact rabbits.

Results of lectin hitochemistry investigation of Col-C treated animal series revealed significant differences in the intensity of lectins binding to carbohydrate determinants of structural components of the alveolar processes of lower jaw, periodontium, and gums. Namely, intensive exposure of WGA receptor sites was observed in the early (2-3 weeks) stages of experiment by the microcirculatory bed endothelium, single cells of the periodontal ligament, and cells of the fibroblastic origin of the gingival plate. Within 1-3 weeks after implantation in the regenerate, the highest intensity of WGA binding was observed on the periphery of the islands of cartilage osteogenesis (Fig. 5B).

In later stages of the experiment (5-12 weeks), WGA lectin showed certain differences of binding. In particular, vascular endothelium was predominantly areactive, though single loci of WGA-specific carbohydrate determinants were still observed in the small blood vessels basement membranes. This lectin reactivity of cellular elements did not tend to change significantly in both the experimental and control groups of animals.

While studying LABA lectin reactivity after osteoplasty using the Col-C material, mostly single cellular elements were detected in the slides under investigation. At the end of the first day of experiment on the background of bone-destroying trauma, clusters of LABA-positive cells were observed in the closely adjacent tissue. To our current thining these were macrophages, involved by an acute inflammatory process. At the same time, other cell populations, such as osteogenic cells and fibroblast were LABA-areactive throughout the entire investigation. The total countnt of macrophages in the dynamics of the experiment tended to decrease, which apparently can be due to the slowing down of macrophage migration

into the focus of inflammation, or due to masking or loss of the corresponding carbohydrate determinants. In the composition of bone regenerate in the first three weeks of the experiment, the highest content of LABA receptor sites were observed on the periphery of the islands of cartilage osteogenesis (Fig. 6A). Starting from the 4th week of experiment and until its completion, the LABA lectin did not show noticeable reactivity and the results obtained did not differ from the studied objects of the control group. However, it should be noted that LABA lectin exposed faint reactivity with endothelium and subendothelial layer of small arteries, as well as with the basement membrane of gingival epithelium.

Treatment of tissue sections of rabbits from Col-C series with CNFA lectin demonstrated certain mosaicism of its binding to carbohydrate determinants of individual structures. Namely this lectin unexpectedly bound to the destroyed material (detritus) of tissues one day after the application of the Col-C material. In the dynamics of the experiment, CNFA exposed certain selectivity of binding towards cellular elements of the periodontal ligament (2-3 weeks), hemocapillary wall (5-8 weeks). Other CNFA-positive cellular elements included primarely cells of the fibroblastic series in the periodontal ligament and gums in both the experimental and control groups of the study. It should also be noted that the CNFA lectin, unlike the others studied by us, selectively conjugated with carbohydrate residues of the endosteum of bone trabeculae, which was most clearly manifested in the late stages of the experiment (8-12 weeks) (Fig. 6B), while the endosteum of bone trabeculae in intact animals was completely unreactive.

DISCUSSION

Bone grafting is an integral part of modern dental practice, used in periodontal surgery, implantology, sinus lifting and alveolar augmentation [16, 17]. As a promising alternative to auto- and allotransplantation, in recent years, xenogenic materials have attracted increasing attention, which, due to their properties, promote bone tissue regeneration and are advantageously distinguished by a less invasive procedure for material collection [18, 19]. Wide availability, provided by the possibility of mass production, is a significant advantage of xenogenic materials, which meets the growing demand for bone reconstruction. Due to this, experimental and clinical studies aimed at the development and implementation of various types of xenogenic materials, especially those based on calcium phosphate, have been intensified [20].

According to the obtained data, these materials, promoting the penetration and growth of bone tissue into the framework, are effective in the treatment of small and medium bone defects, sinus lifting and alveolar appendages augmentation [21-23]. However, their use in large defects is limited by the lack of osteoinductive properties [24]. It should be noted that the use of collagen in bone regeneration has made a remarkable step forward [25]. Improving methods of production and purification of modern collagen preparations ensures the preservation of their natural structure and minimizes the risk of immune reactions. Further modification of collagen, which increases its strength and resistance to cleavage, opens up new prospects for its use for medical purposes [11,12].

Natural collagen preparations are a resorbable material that is a natural matrix for the formation of new osteogenic cells. This substance is widely used for post-extraction preservation of the alveolar process and promotes the formation of a blood clot with its subsequent reorganization into bone tissue [25,26].

To clarify a number of morphogenetic events that accompany the osteoregenerative process under the conditions of osteotropic materials, our work used the lectin histochemical method, which occupies an important place among modern methods of morphological analysis, since the terminal carbohydrate residues of glycopolymers, which are lectin receptors, form the so-called «glycocode» of a living organism, provide mutual recognition and various forms of interaction of cells with their microenvironment [27].

Results of de Sousa GF et al. [28], who were studying the role of plant lectins in cellular and molecular processes of skin wound repair, have demonstrated that lectins are good candidates for the role of healing agents. Cell surfaces are rich in glycoproteins (glycosidic receptors) that can potentially interact with lectins. This lectin-cell interaction is the molecular basis for triggering various changes in biological organisms, including healing mechanisms. A review of relevant studies indicates the presence of promising potential of plant lectins for wound healing and tissue regeneration processes. Also, when studying bone tissue contacts with a titanium implant using the lectin histochemistry method, similar results of lectin binding to structural components of bone tissue were obtained by Violin K et al. [29]. Lectin-histochemical studies of the structural components of bone and periodontal tissues allowed us to establish the features of the distribution of their carbohydrate determinants, both in normal conditions and under the experimental use of osteoplastic materials replacement.

Regarding the WGA lectin, the most indicative was the fact of its binding to carbohydrate determinants of histological structures directly adjacent to the

site of injection of OCP-N plastic material. This concerned, first of all, the endothelium of microvessels. The detected enhanced expression of vascular endothelium glycoconjugates may be evidence of active transendothelial transport, which, as is known, is one of the links in the activation of regenerative processes. In the case of using the Col-C plastic material, the expression of endothelial glycoconjugates was less intensive, which may indicate a lower level of regenerative processes. Regarding the lectin reactivity of individual fragments of microvascular basement membrane, which was detected by WGA, we found a rather noticeable expression of carbohydrate residues in experimental groups of animals. This fact may also indicate intensive transcytosis, and, therefore, an increased level of tissue metabolism [30].

While using lectin LABA, it was documented intense labeling of macrophages in experimental group animals post OCP-N plastic material administration. Such expression indicates an increase in the signaling function of macrophages and activation of their phagocytic activity. In addition, it can be argued that such macrophages create a cleared field for the implementation of future regenerative processes on it. Therefore, it can be assumed that the used plastic material has, at the same time, a stimulating effect on the positive course of restoration of the histoarchitectonics of damaged structures. In addition, confirmation of the acceleration of tissue restoration is also a decrease in the expression of macrophage receptors and a decrease in their content at the final stages of the study. The same lectin in the conditions of natural collagen application was less informative, its reactivity was barely noticeable and sporadic and concerned only the endothelium and subendothelial layer of small arteries, the basement membrane of the gingival epithelium. Regarding the mentioned cells of macrophage nature, the dynamics of the experiment showed that the content of such cells tended to decrease, which is probably due to the slowing down of the migration of macrophages into the focus of inflammation, primarily, or the masking or loss of the corresponding carbohydrate determinants.

In our opinion, the information we obtained when using the lectin CNFA as a carbohydrate-specific marker is valuable. Although, to date, its affinity to various carbohydrate determinants has not been fully elucidated, we have established a certain informativeness. This, first of all, concerns the active binding of CNFA to destroyed structures in the area of bone-destroying injury at the end of the first day of the experiment. In the group of animals where the material OCP-N was used, the most indicative was the fact of enhanced expression of glycoconjugates of the endosteum of the bone trabeculae of the jaw in the late stages of the experiment, starting from the 8th week until its completion. The obtained result can obviously be interpreted as the restoration and activation of the stromal component of the red bone marrow and, as a consequence, the enhancement of hematopoietic function. In the remaining groups of animals studied, the informativeness of CNFA was insignificant or absent, which does not allow us to state the above.

Another interesting fact that was established by the lectin CNFA is its conjugation with the determinants of fibroblast cells in the periodontal ligament. We registered this phenomenon in all groups of animals. Obviously, the obtained data indicate the activation of the synthetic function of fibroblasts aimed at enhanced synthesis of intercellular substance, and therefore, the restoration of the periodontal structure of the teeth in the area of bone replacement surgery. Thus, the use of these lectins allows us to assert that they are an auxiliary marker in the study of the course of regenerative processes after bone-destroying trauma and the use of osteoplastic materials to eliminate it. The results obtained by us give reason to recommend the used lectins in such studies.

CONCLUSIONS

The peroxidase-labeled lectins we used demonstrated a sufficiently high variability in the intensity of binding to carbohydrate determinants of the structural components of the bone tissue of the alveolar processes of the mandible and are an auxiliary marker in the study of the course of regenerative processes after osteodestructive trauma and the use of osteoplastic materials to eliminate it.

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CONFLICT OF INTEREST
The Authors declare no conflict of interest

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