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INDUCTION OF OSTEOGENESIS IN RABBIT MANDIBULAR BONE TISSUE USING AN ALBUMIN-BASED CRYOGENICALLY STRUCTURED POROUS 3D CARRIER LOADED WITH A BIOREGULATOR

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Objective: to study the induction of osteogenesis caused by introducing into the defect area broadly porous cryogenically structured 3D carriers, based on serum albumin and loaded with a bioregulator isolated from bovine serum on an experimental model of mandible defect in rabbits *in vivo*. **Materials and methods.** Cryogenically structured sponges in the form of cylindrical specimens, 5 mm in diameter and 5 mm in height, prepared from bovine serum albumin, were used as the bioregulator carrier. The experimental laboratory animals were male Chinchilla rabbits, weighing 2–2.5 kg. Bone tissue was skeletonized under anesthesia (intramuscular anesthetic Zoletil 100) with a 3-cm incision in the angle of the mandible and a 5-mm-diameter cutter was used to create a 2–3-mm deep defect to install an appropriate-size albumin sponge. A total of 24 animals participated in the experiment. X-ray control of the defect area was performed *in vivo* on day 14 using PanExam+ (Kavo) device (20 m X-ray). Histological examination of tissues was carried out at day 30 after the defect using a light microscope. **Results.** Experiments performed indicate an active restoration of bone tissue in the extensive defect area when using an albumin-based 3D carrier with the inclusion of a bioregulator as compared to the control experiments. There were osteointegrative and osteoinductive processes, almost complete decomposition (biodegradation) of albumin sponge with formation of islands of dense bone tissue with small foci of coarse fibrous tissue in the defect. This demonstrated good dynamics of recovery processes at this stage of healing. **Conclusion.** Under the action of a serum bioregulator contained in an albumin-based sponge, the repair process leads to restoration of normal bone tissue without formation of bone callus and altered bone tissue different from the native one.

Key words: osteoinduction, 3D technology, cryogels, bioregulators.

INTRODUCTION

In maxillofacial surgery, the problem of restoring bone defects with the use of special implant materials or compositions remains a hot topic. Moreover, it is important what materials and with what filling are used for replacement and restoration of bone tissue in various pathological processes. In particular, methods with replacement of bone defects using auto- and allografts as well as decalcified and decellularized artificial bones are used. However, our own bone material is not always available, especially for large defects, and the use of allografts and artificial materials can lead to complications, including rejection. Currently, there are still no implant materials and constructs that can fully meet all the requirements. In this regard, the development of new materials for implants, as well as means and methods of increasing their effectiveness should be considered very urgent [1]. Meanwhile, bone-made implants, subjected to the necessary pretreatment [2], as well as implants formed from biopolymer precursors suitable for this purpose are of interest [3, 4].

Membranous bones, such as cranial bones, provide better engraftment in the maxillofacial defect area [5]. Porous tissues such as cancellous bone can be used for faster sprouting of blood vessels in them and acceleration of ossification processes [6]. Grafts from decalcified bone matrix are also of interest since it has been shown that demineralized bone promotes osteoinduction processes [7, 8].

Previously, we investigated the effect of serum bioregulator in cryogenically structured sponge carriers (so-called cryogels and cryostructures [9]) on the repair of femoral defects in Wistar rats at different periods (day 7, 14, 30, 90). Moreover, it has been shown that during wound healing, there is an effective recovery of dense bone tissue, starting from early periods, leading to osteogenesis stimulation with complete recovery by late periods and with the formation of dense bone tissue in the experimental defect area [10, 11]. The best results in

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these experiments were achieved using albumin cryogels [12] as carriers of serum bioregulator [11]. The use of cryogels based on other proteins for the treatment of bone defects has also been reported [13]. For example, in the case of a cryogel formed from collagen filled with hydroxyapatite nanoparticles, safety, biodegradability and osseointegration induced by this material have been demonstrated over a long period (15 weeks) in vivo [14].

Given the positive results of the above experiments on the positive effect of serum bioregulator included in albumin cryogel on the regenerative process in the case of rat femur defect healing model, we planned to study the restoration of rabbit jaw bone, since there are significant differences between the tubular bones and the jaw bone. The jaw bone has a lamellar structure, the maxilla has a lot of spongy bone tissue and a thinner cortical plate of dense tissue, the mandible has a denser cortical plate [15]. Therefore, the jaw bones, as well as the skull bones, are less amenable to repair after damage. It was expected that the use of a bioregulator form adsorbed on a protein carrier as a transport conductor would accelerate osteogenesis stimulation, as a factor aimed at restoring the artificially created mandibular tissue defect.

MATERIALS AND METHODS

We used cryogenically structured sponge media in the form of cylindrical samples, 5 mm in diameter and 5 mm in height, prepared from bovine serum albumin according to a previously published technique [12]. Then, the resulting porous carriers were incubated in an aqueous solution of bioregulator, frozen, and lyophilically dried. The control samples were albumin carriers containing no bioregulator, also lyophilically dried.

The bioregulator was isolated from bovine blood serum (a commercial preparation used as a nutrient additive to culture media, BioLot) using a technique that included protein desalting with ammonium sulfate, dialysis, concentration, and then isoelectrophocusing in a sucrose density gradient at pH 3–10 [16]. The high degree of bioregulator purification was demonstrated by electrophoresis in polyacrylamide gel electrophoresis and reverse-phase HPLC. In this work, we used a commercial preparation Viorgon 1, manufactured by IPB.

The experimental laboratory animals were male Chinchilla rabbits, weighing 2–2.5 kg. Bone tissue was skeletonized under anesthesia (intramuscular anesthetic Zoletil 100) with a 3-cm incision in the angle of the mandible and a 5-mm-diameter cutter was used to create a 2–3-mm deep defect to install an appropriate-size albumin sponge. The study materials – 3D carriers containing and not containing the blood serum bioregulator – were placed into the formed defects. After filling the defects, soft tissues and skin were sutured. The animals were kept under standard vivarium conditions. The wound was sutured in layers after treatment with 3% hydrogen peroxide solution. The suturing was done with interrupted stitches with full coverage of the implant. Hemostasis was performed in the course of the operation. The skin wound was sutured with interrupted 4-0 polyglycolic acid sutures. Each rabbit was fitted with one test specimen of albumin sponge, either containing no serum bioregulator or containing serum bioregulator. In the negative control, nothing was inserted into the defect area and the wound was sutured. The following groups of 6 rabbits each were formed:

1. Native control (rabbits without defects).
2. Negative control (no albumin sponges were inserted in the defect area).
3. Control group (albumin sponges without serum bioregulator were inserted in the defect area).
4. Experimental group 1 (albumin sponges containing serum bioregulator at 10^{-10} mg/mL final concentration were inserted in the defect area).

A total of 24 animals participated in the experiment.

The defect area was subjected to X-ray imaging on day 14 in vivo on the PanExam+ (Kavo) device, (20 μR).

We divided the state of bone tissue on the radiographs into 3 points.

Score 1 – complete absence of osteoid tissue elements; the bone defect is determined radiographically as a shadow and defect zone, more filled with fibrous connective tissue.

Score 2 – defect area is 30–40% filled with islets of osteoid tissue and partially with coarse fibrous connective tissue, which presumably should restructure into young bone tissue with trabecular structure.

Score 3 – in the defect area, bone tissue density was almost equal to that of the parent tissue; small islets of connective tissue were observed against the background of active growth of normally structured bone tissue; but in general, identifying the defect area was difficult.

The state of bone defects was studied on day 14 via X-rays since the main regeneration processes, which further determine the quality of the bone tissue formed in the defect area, take place at this early stage. On day 30 after surgery, the animals were removed from the experiment, bone material was extracted from the defect area, fixed in formalin, decalcified, and embedded in paraffin to prepare 10 μm-thick histological sections. The histological sections were stained with hematoxylin and eosin and studied using light microscopy.

RESULTS AND DISCUSSION

As stated earlier, the main objective of this study was to test the possibility of osteogenesis induction caused by the action of serum bioregulator in the artificial bone defect area of a rabbit mandible, when such bioregulator was injected there in adsorbed manner on a cryogenically structured sponge cryogel prepared from denatured serum albumin. Formation of the above macroporous carrier is based on the previously discovered effect of cross-linking of the polypeptide chains of serum albumin

by disulfide bridges due to intermolecular thiol-disulfide exchange reactions resulting from the introduction of a denaturant (particularly urea or guanidine hydrochloride) and a small amount of a reducing agent (e.g., cysteine) into the initial protein solution, followed by freezing, freeze-drying, and further thawing [12, 17]. This sequence of operations leads to formation of a spongy protein cryogel (Fig. 1), which is easily degraded by proteases in case of a specific application [18]. It is this albumin carrier that was used in this work to introduce the serum bioregulator, adsorbed by it, into the body of laboratory animals.

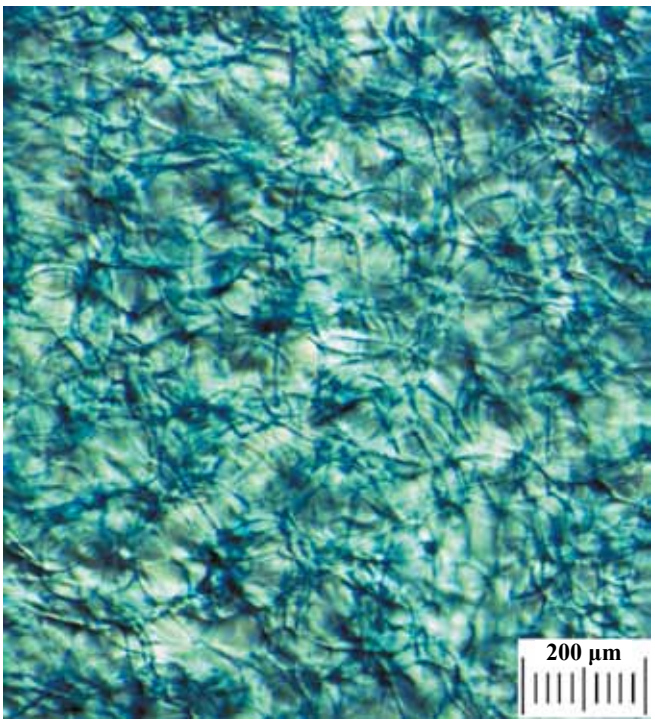

Fig. 1. Microstructure of serum-albumin-based cryogel contrasted with 0.125 mM aqueous methylene blue solution (optical stereomicroscope SMZ1000 (Nikon, Japan), equipped with digital system MMC-50C-M (MMCSoft, Russia) for image recording)

In the early stages after the defect (14 days), the state of the bone tissue was examined in vivo by X-ray.

The negative control on the mandible showed a defect zone without clear contours, there was a tendency to fill the formed defect. In the center, there are defined islands of dense substance, most likely zones of bone mass formation (score 1.5–2 on the scale described above). In the peripheral area of the defect, towards the center of the defect from the edge of the bone tissue, there were structurally more differentiated islands of bone substance. Radiographs show loose connective tissue located near the edge of the defect, which consists of intertwined bundles of collagen fibrils (Fig. 2).

In the control with a sponge, the defects were filled with connective tissue (coarse fibrous collagen tissue). In some areas closer to the edge of the maternal bone, islands of new bone substance deposition are detected (score 1 on the scale described above) (Fig. 3).

In the experimental group with the inclusion of serum bioregulator in the albumin sponge, exophytic growth of newly formed bone tissue with a trabecular structure interspersed with a fibrous matrix was determined in the defect area. Foci of osteogenesis in the form of osteoid deposition in the connective-tissue layer were observed (score 2.5–3 according to the above described scale) (Fig. 4).

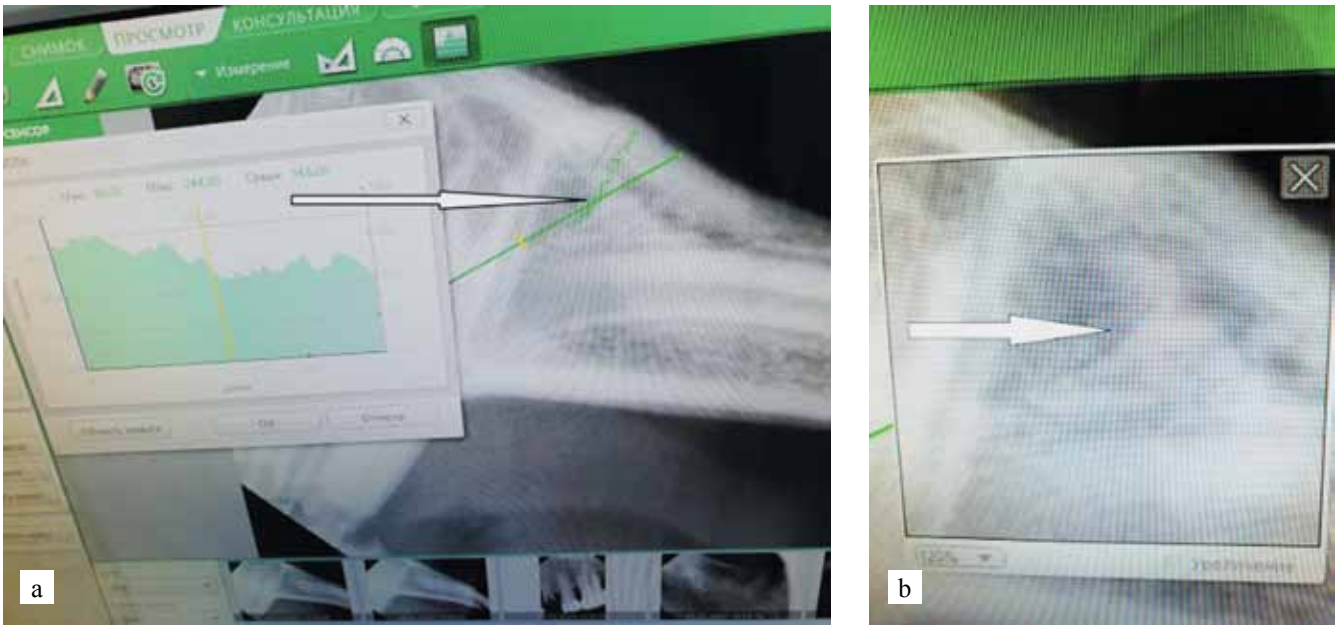

Fig. 2. X-ray of the rabbit mandible in the defect area (indicated by arrow) on day 14 in the control group: a, low magnification; b, high magnification

The histological description of the bone tissue state in the defect area on day 30 after its application in different groups gave the following picture.

Native control: Dense bone tissue, bone marrow (mostly yellow – fatty tissue, but red bone marrow is also presented) is visible inside cavities of bone beams. Elements of teeth with dentin, enamel and immature mesenchymal cells are well expressed. Dense bone tissue is well expressed, there are visible vessels. Osteons are well expressed, with small lacunae between the osteocytes. Nuclei of osteocytes are large, oval in shape. A mature lamellar osteoid bone is represented (Fig. 5, a).

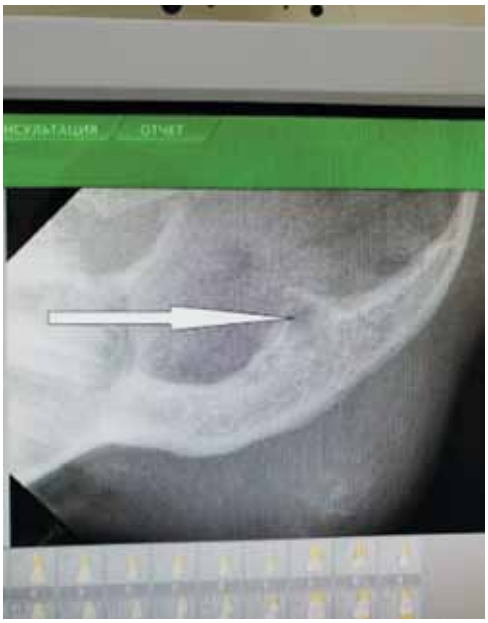

Fig. 3. X-ray of the rabbit mandible in the defect area (indicated by arrow) on day 14 in the albumin sponge group

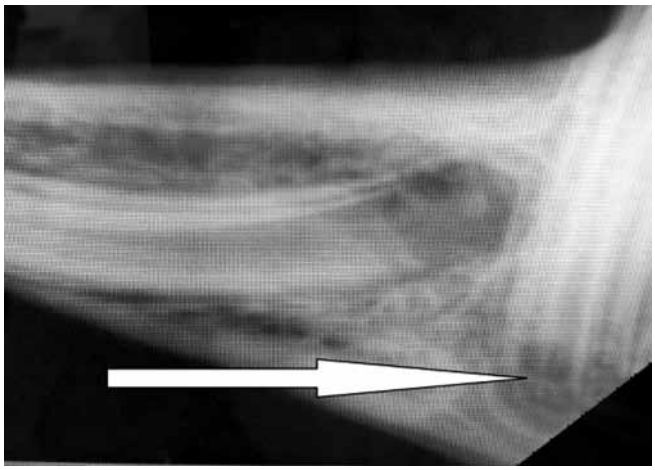

Fig. 4. X-ray of the rabbit mandible in the defect area (indicated by arrow) on day 14 in the group with albumin sponge, containing serum bioregulator

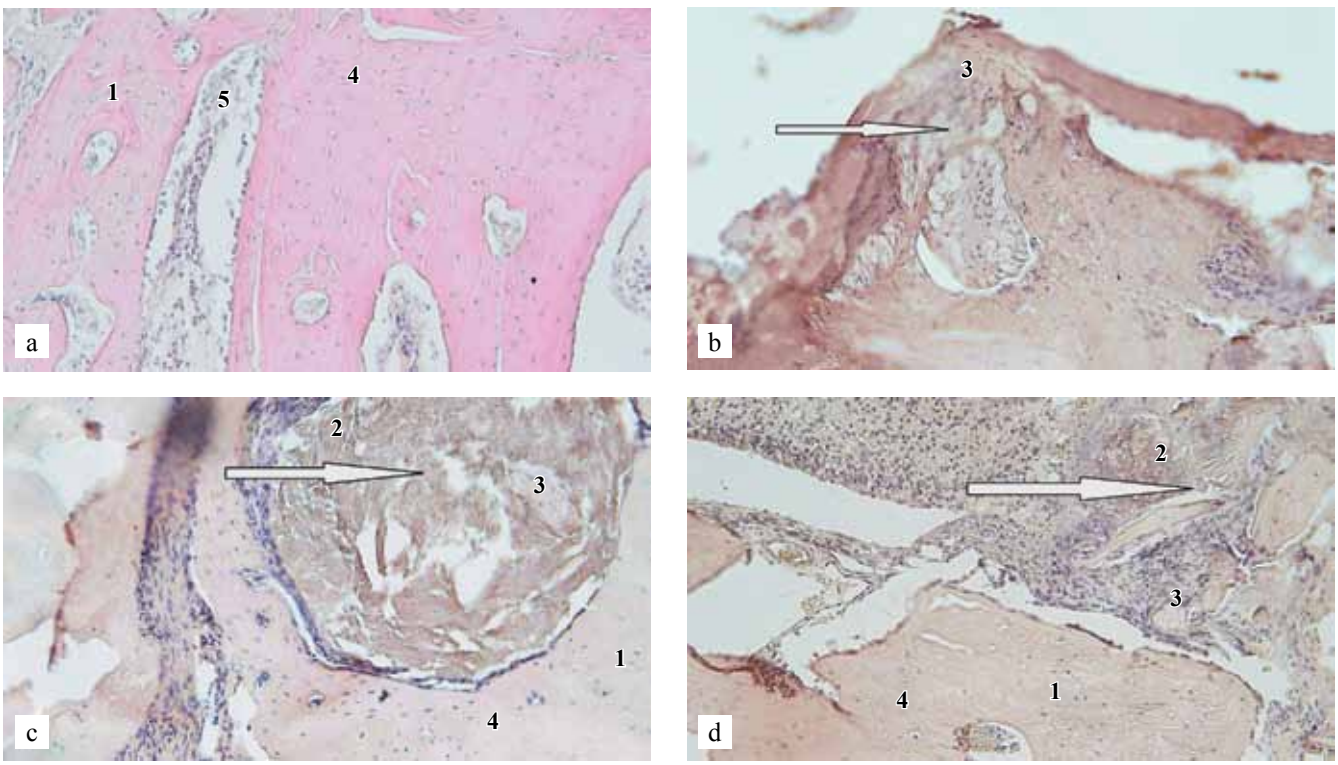

Fig. 5. Bone tissue of rabbit mandible: a, native; b, 30 days after application of defect (negative control, without introduction of any materials into the defect area; c, 30 days after application of defect (control, with introduction of albumin sponge, containing no serum bioregulator, into the defect area); d, 30 days after application of the defect (experimental group, with introduction of albumin sponge, containing serum bioregulator, into the defect area). 1, osteons; 2, remains of albumin sponge; 3, immature osteoid tissue; 4, dense bone tissue; 5, bone marrow. 200× magnification. The arrow indicates the defect area

In the negative control group (no filling of defect area with materials), there is no complete healing of defects. The wound cavity is filled with tissue detritus, between which a new bone is forming, bone marrow is not expressed. Immature osteoid tissue is observed, lacunae are not expressed. Fibrous bone tissue, vessels are poorly represented (Fig. 5, b).

In the control group (filling of defect with albumin sponge containing no serum bioregulator), the wound was healing without bone marrow formation. One can see active formation of bone tissue, osseointegration of albumin sponge into bone, formation of cavities in it and partial settlement by cells. And also on the border, there is active cell granulation with formation of immature osteoid tissue (Fig. 5, c).

In the experimental group (filling of defect with albumin sponge containing the serum bioregulator), the remains of decomposing albumin sponge are visible in the damage area, the newly formed bone is dense, with small lacunae. Inside the bone cavity, bone marrow is visible. Formation of dense mature osteoid tissue with formation of osteons and haversian canals and bone marrow is proceeding. The alveolar bone tissue and the lamellar dense bone are being rebuilt (Fig. 5, d).

Any connective tissue is a precursor of bone tissue. It is necessary for the growth of integration processes and filling of defects with bone tissue. In the negative control group with an albumin sponge containing no bioregulator, we observed the beginning of the bone repair process with the formation of immature bone, in contrast to the group in which albumin sponge containing a serum bioregulator was introduced into the defect area. Thus, the albumin sponge is a carrier, which is necessary for the settlement of newly formed cells in it as an osteoconductor, and the osteoinducer, which accelerates the process of mature bone tissue restoration, is a serum bioregulator included in it.

CONCLUSION

In the negative control, the picture of recovery of dense bone tissue of the rabbit's mandible is not pronounced, the restored tissue is mainly coarse-fibrous. In the control group with albumin sponge, one can see incomplete decomposition of this sponge and only the beginning of osseointegrative processes. Results obtained indicate active restoration of bone tissue in the extensive defect area when using a 3D carrier based on bovine serum albumin (albumin sponge) with inclusion of a bioregulator isolated from blood serum. We can see the processes of osteointegrative and osteoinductive activity, almost complete albumin sponge decomposition in the defect area, with the formation of islands of dense bone tissue with small foci of rough fibrous tissue in the defect site, which indicates good dynamics of restorative processes at the given stage of the defect healing. This may suggest that under the influence of serum bioregulator in the albumin sponge, the repair process leads to restoration of normal bone tissue without formation of bone callus and altered bone tissue different from the native one.

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The authors declare no conflict of interest.

REFERENCES

1. *Gunko VI.* Znachenie kostno-rekonstruktivnyh operacij pri medicinskoj reabilitacii s vrozhdennymi deformaciyami chelyustey. *Aktualnye voprosy stomatologii*. M., 2000: 117.
2. *Min'kov SA, Shkitov YuS, Sakovich GN, Kazimirskiy VA.* Klinicheskij opyt otsrochennoj implantacii nizhnej chelyusti vo vremya eyo rezekcii. *Aktualnye problemy stomatologii*. M., 2000: 126.
3. *Baker EJ, Onissema-Karimu S, Rivera-Galletti A, Fransis M, Wilkowski J, Salas-de-la-Cruz D, Hu X.* Protein-polysaccharide composite materials: fabrication and applications. *Polymers*. 2020; 12 (2): 464. doi: 10.3390/polym12020464.
4. *Mbundi L, Gonzalez-Perez M, Gonzalez-Perez F, Juanes-Gusano D, Rodriguez-Cabello JC.* Trends in the development of tailored elastin-like recombinamer-based porous biomaterials for soft and hard tissue applications. *Frontiers in Materials.* 2021; 7 (1): 601795. doi: 10.3389/fmats.2020.601795.
5. *Zins JE, Whitaker LA.* Membranous versus endochrondral bone: Implications for craniofacial surgery. *Plast reconstr Surg*. 1983; 72: 778.
6. *Gadzhiev AP.* Auto- i allotransplantaciya kompaktnoj i gubchatoj kostnoj tkani pri zameshhenii defekta nizhnej chelyusti [Dissertation]. M., 1986. 214.
7. *Polezhaev LV.* Regeneraciya putyom indukcii. *Zhurnal obschey biologii*. 1966; 27 (2): 223–233.
8. *Sampath TK, Reddi AH.* Dissociative extraction and reconstitution matrix components involved in local bone differentiation. *Cell Biology*. 1981; 78 (12): 7599–7603.
9. *Lozinsky VI.* Cryostructuring of polymer systems. 50. Cryogels and cryotropic gel-formation: terms and definitions. *Gels.* 2018: 4 (3): 77. doi: 10.3390/gels4030077.
10. *Krasnov MS, Shayhaliev AI, Korshakov EV, Efimenko MV, Soloshenkov PP, Davidova TR et al.* Induction of osteogenesis of rat bone tissue using cryogenically structured porous 3D materials containing a bioregulator. *Bulletin of Experimental Biology and Medicine.* 2019; 168 (7): 113–117. doi: 10.1007/s10517-019-04657-z.
11. *Krasnov MS, Shaikhaliev AI, Korshakov EV, Gasbanov GA, Korgoloev RS, Sinitskaya ES et al.* Changes in rat bone tissue at the site of the defect *in vivo* under the effect of a cryogenically structured albumin sponge containing a bioregulator. *Bulletin of Experimental Biology and Medicine.* 2021; 170 (12): 805–808. doi: 10.1007/s10517-021-05160-0.
12. *Rodionov IA, Grinberg NV, Burova TV, Grinberg VYa, Lozinsky VI.* Cryostructuring of polymeric systems. 40.

Proteinaceous wide-pore cryogels generated by the action of denaturant/reductant mixtures on bovine serum albumin in moderately-frozen aqueous media. *Soft Matter*. 2015; 11 (24): 4921–4931. doi: 10.1039/c4sm02814g.

13. *He Y, Wang C, Xiao Y, Lin W.* An overview on collagen and gelatin-based cryogels: fabrication, classification, properties and biomedical applications. *Polymers.* 2021; 13 (14): 2299. doi: 10.3390/polym13142299.
14. *Vilela MJC, Colaço BJA, Ventura J, Monteiro FJM, Salgado CL.* Translational research for orthopedic bone graft development. *Materials*. 2021; 14: 4130. doi: 10.3390/ma14154130.
15. *Pavlova IA, Vinogradova AV, Sergeeva ND, Spasich TA*. Anatomiya, fiziologiya chelyustno-licevoj oblasti v vozrastnom aspekte: metodicheskoe posobie. Irkutsk: N CRVH SO RAMN, 2014. 59.
16. *Yamskova VP, Krasnov MS, Yamskov IA*. Mehanizm deystviya membranotropnyh gomeostaticheskih tkane-specificheskih bioregulyatorov. Saarbrucken: Lambert Academic Publishing, 2012. 136.
17. *Lozinsky VI, Konstantinova NR, Solov'eva NI.* Method for the preparation of porous protein gel. Russ. Pat. No. 2,058,083 (1994).
18. *Lozinsky VI, Shchekoltsova AO, Sinitskaya ES, Vernaya OI, Nuzhdina AV, Bakeeva IV et al.* Influence of succinylation of a wide-pore albumin cryogels on their properties, structure, biodegradability, and release dynamics of dioxidine loaded in such spongy carriers. *Int J Biol Macromol*. 2020; 160 (1): 583–592. doi: 10.1016/j.ijbiomac.2020.05.251.

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