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Application of β-1,3-glucan in production of ceramics-based elastic composite for bone repair

Research Article

Anna Belcarz[1,*], Grażyna Ginalska[1], Teodozja Pycka[1], Aneta Zima[2], Anna Ślósarczyk[2], Izabela Polkowska[3], Zofia Paszkiewicz[2], Wojciech Piekarczyk[2]

[1]*Chair and Department of Biochemistry and Biotechnology, Medical University of Lublin, 20-093 Lublin, Poland*

[2]*Faculty of Materials Science and Ceramics, AGH-University of Science and Technology in Cracow, 30-059 Cracow, Poland*

[3]*Faculty of Veterinary Medicine, University of Life Sciences in Lublin, 20-612 Lublin, Poland*

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Abstract: Background: Unsatisfactory surgical handiness is a commonly known disadvantage of implantable granular bioceramics. To overcome this problem, β-1,3-glucan, biotechnologically derived polysaccharide, has been proposed as a joining agent to combine granular ceramics into novel compact and elastic composite. Hydroxyapatite/glucan elastic material was processed and evaluated as a potential bone void filler Methodology: The procedure of composite formation was based on gelling properties of glucan. Its properties were studied using X-ray microtomography, SEM-EDS, FTIR spectroscopy, compression test and ultrasonic method. Sorption index was determined in phosphate buffered saline; bioactivity in simulated body fluid; sterility in growth broth and human blood plasma; implantation procedure in dog model. Results: HAp/glucan composite is sterilizable, flexible and self-adapting to defect shape. It exhibits bioactivity, good surgical handiness, high sorption index and profitable mechanical properties, resembling those of spongy bone. Results of pilot clinical experiment on animal (dog) patients of a local clinic of animal surgery suggested good healing properties of the composite and its transformation into new bone tissue within critical-size defect. Conclusions: The results obtained in this study confirm that flexible HAp/glucan composite has potential as a bone-substituting material. Promising results of pilot clinical experiment suggest that further *in vivo* experiments should be performed.

Keywords: *Hydroxyapatite ceramics • Bone filler • Sorption index • Sterility • Bioactivity*

1. Introduction

Hydroxyapatite (HAp), especially in a porous form, is appreciated as a bone filler due to its biocompatibility, bioactivity, osteoconductivity, minimal risk of appearance of allergic reactions, lack of carcinogenic properties and lack of sensitivity to sterilization processes [1-3]. There are numerous commercially available bone graft materials based on biologic and synthetic HAp, including ProOsteon® (Interpore Cross International, USA), Endobon (BIOMET Orthopaedics, Switzerland), Cerapatite (Ceraver Osteal, France), Synatite (SBM, France) and others. HAp may serve not only as a bone filler but also as a carrier of active substances: antibiotics, chemiotherapeutics, growth factors, *etc.* [4-9]; it can also be used in composites, as a factor increasing their cytocompatibility, bioactivity, osteoconductivity, adhesion of coatings and compression modulus [10-14]. On the other hand, HAp application is often limited due to its relatively poor resorption and slow replacement by the host bone after implantation, substantially high Young modulus, and low fracture toughness [15,16], although these properties may be improved by addition of elasticity-increasing polymers. It is considered that polymer-ceramic composites reveal superior properties (at least in some aspects) over polymer and ceramic

* *E-mail: anna.belcarz@umlub.pl*

alone [17]. However, most polymers used for bone filler fabrication meet only some of the criteria for implantable materials.

Bone replacing material such as ceramics or crushed bone offers an insufficient surgical handiness, especially when the orthopedic surgeon has to handle granules (in case of defects in maxillary bone) or scaffolds (rigid and non-adaptable to the implantation site). Some commercially available biomaterials contain the artificial or natural polymers serving as plasticizing agents and thus significantly reduce this inconvenience. Among them, EasyGraft™ (Degradable Solutions SA, Switzerland), Plexur M and P (Osteotech, USA) and Cerapatite-Collagen (Ceraver Osteal, France) can be listed. EasyGraft™ contains PLGA-coated β-TCP granules which hardens into a compact mass *via* partial and temporary PLGA dissolution by organic solvent; Plexur M and P are composed of cortical bone and resorbable artificial polymer; Cerapatite-Collagen, made from HAp granules and naturally-derived collagen fibers, becomes elastic after hydration with blood or saline. Bone or ceramic granules and powders may also be turned into a paste using collagen gels (e.g., Tecnoss® Gel O; Tecnoss, Italy) or fibrin glue [18,19]. However, the natural polymeric compounds used in commercially available composites (collagen, fibrin glue) originate from animal sources; this can be considered as a limiting factor because of the increased risk of transmissible contamination (e.g. viruses) and undesirable immune responses. Thus, the necessity of careful purification of these animal polymers exacts the high price of final product.

To solve the problems originating from low elasticity of HAp ceramics and the risk of negative effects of biologic factors mentioned above, β-glucan may be applied as polymeric phase in such materials, due to its specific gelling properties. β-glucan is a natural, relatively cheap, non-animal and nontoxic polymer biotechnologically produced by *Alcaligenes faecalis*, a bacterium commonly found in soil, water and human-associated environments. It was used in production of dietetic and diabetic food as thickener and stabilizer. In recent years, this polysaccharide attracted growing attention in biomedical and pharmaceutical applications [20]. Encapsulation of theophylline, salbutamol sulfate, prednisolone, indomethacin, doxorubicin and epirubicin with β-glucan has been shown to improve the pharmacokinetics of drugs [21-24]. Moreover, numerous studies reveal positive effect of β-glucans and its derivatives on health, particularly in the field of immunology: they improve wound healing and show antioxidant, antiviral, antibacterial, anti-inflammatory and DNA-protecting activity [25-28]. It also possesses anti-coagulant activity [29]. Morikawa *et al*. [30] found that β-glucan-injected mice produced a high level of macrophages and polimorphonuclear leukocytes, which were spontaneously cytotoxic to mammary carcinoma cells *in vitro*; macrophage stimulation has been also observed *in vitro* in contact with β-glucan-treated plates [31]. However, the use of glucan as a component of implantable bone filler has not yet been reported, although – on a base of available knowledge - it could show profitable healing properties.

The aim of this study was to prepare a biphasic HAp/glucan composite of elastic properties, which would allow for easy manipulation and good adaptation to the shape and dimensions of even large bone defects. Some of its physical and biological properties were examined and presented. Pilot clinical experiment concerning the repair of critical bone defects (oronasal fistula) in animal patients with HAp/glucan material was performed to initially evaluate its healing properties.

2. Experimental Procedures

2.1 Composite preparation and structure studies

HAp-glucan composite samples were prepared according to the procedure described in Patents [32,33]. Briefly, the granules and β-glucan in appropriate proportions were mixed carefully and baked at 100°C for 10 minutes. The samples tested in described experiments contained porous HAp granules and glucan at ratios within the ranges: 43.3-90.9 wt % for HAp and 9.1-56.7 wt % for β-glucan. Microporous HAp granules (0.2-0.6 mm; open porosity 68%; unimodal pore size distribution; surface area 24.94 m/g; average pore size of 0.1 μm) with high ability for water absorption were obtained according to the method described in Patent [34]. β-1,3-Glucan from *Alcaligenes faecalis* (DP 450) was supplied by Wako Chemicals, Japan. Composition of tested samples is presented in Table 1. Space distribution of HAp granules within the structure of composite (sample 3A) and volume of granules (in vol %) were evaluated by 2D X-ray microtomography using Skyscan 1174 apparatus with UDS 1.3Mp FW camera (50 kV; 800 μA; pixel size of 12 μm). Structure and chemical composition of cross-section of 3A sample was studied using SEM-EDS technique. FT-IR spectra of pure gelled β-glucan, HAp ceramics and composite (sample 3A) were obtained using IR spectrometer (Vertex 70, Bruker, USA) in ATR mode, 32 scans with 4 cm^{-1} resolution, at wavenumber range of 370-4000 nm.

2.2 Soaking capacity

For pilot determination of soaking kinetic curves, the samples of composites (ø=2 mm; h=1 cm) with maximum (90.9 wt %) and minimum (43.3 wt %) HAp content (Table 1) were incubated in phosphate buffered saline (PBS) pH 7.4. The measurements of sample weights were performed periodically during 48 h experiment.

Soaking capacity was estimated as changes of samples volume and weight after soaking in PBS pH 7.4. The composites (ø=2 mm; h=1 cm) with different HAp content (samples 1-7, Table 1) but the same granule size (0.2-0.3:0.5-0.6 in proportion 1:3) were compared. Samples were incubated in PBS pH 7.4 at 37°C for 24 h; then they were taken off from bath and placed on Whatman paper to remove the excess of liquid. Afterwards, their size and weight were measured.

Sorption index (S_w) for composite material was calculated from formula 1:

$$S_w=[(m_s-m_d).100]/m_d \quad [\%] \quad (1)$$

where:

m_s – mass of soaked sample [g]; m_d – mass of dry sample [g].

Volume index (S_v) for composite material was calculated from formula 2:

$$S_v=[(V_s-V_d).100]/V_d \quad [\%] \quad (2)$$

where:

V_s – volume of soaked sample [cm^3]; V_d – volume of dry sample [cm^3].

Structure of dry and completely swollen biomaterial (2 mm thin sample 3A, after 10 minutes in PBS) was studied by Differential Interference Contrast (DIC) technique in inverted Olympus IX81 microscope.

2.3 Mechanical parameters

Compressive strength was determined in uniaxial compression test using an Instron apparatus (Model: 3345). The crosshead speed was 2.0 mm/min.

Young modulus was measured by ultrasonic method (MT-541 ultrasonic apparatus, f=0.5 MHz). The measurements were performed along the diameter of each sample in two mutually perpendicular directions as well as along the height of samples. At least six independent measurements were done for each direction and average values of wave velocity and the standard deviations were calculated.

The composite samples (samples 1-3, with HAp content over 80 wt %) in the form of cylinders (ø=8 mm; h=15 mm) were used in compressive tests. Measurements were performed on dry and wet (after 24 h incubation in physiological solution) samples. In all mechanical studies, parameters of each sample were measured in 10 repeats.

2.4 Sterilization, storage and estimation of their effectiveness

For further experiments, only 3A composite sample (of medium HAp content) was chosen. Pieces were sterilized either by autoclaving in a glass bottle (121°C, effective sterilization time: 20 min., working pressure: 1.05 bar) or by ethylene oxide method in paper/plastic peel pouch (1 h at 55°C, followed by 20 h aeration). The sterilized samples were stored for 1 year at 25°C and afterwards their sterility was determined by 6-day incubation (37°C) in sterile Mueller-Hinton broth (Oxoid, USA) or human blood plasma (A Rh^+, Regional Blood Donation Center, Lublin, Poland). Presence of fungi and bacteria in the broth or plasma was estimated by macroscopic observations, quotidian measurement in PhoenixSpec nephelometer (Becton, Dickinson and Company) or by serial dilution method (incubation of 1 ml aliquots on Mueller-Hinton agar plates at 37°C, 20 h, followed by counting of colony forming units).

The same samples, sterilized and stored for 1 year at 25°C, were submitted for mechanical parameters estimation, as described in subchapter 2.3.

2.5 *In vitro* bioactivity test

Pieces (approx. 0.4 g) of composite (sample 3A) were dried (50°C, 24 h, followed by 20 h in exicator filled with anhydrous calcium chloride) and weighed. Then the pieces were sterilized by ethylene oxide method (1 h at 55°C, followed by 24 h degasation) and placed in sterile glass bottles containing 100 mL sterile SBF [35]. The bottles were then incubated for 1 month at 37°C, according to standard method ISO 23317 [36]. After this period, the samples were washed twice in 100 mL deionized water to remove traces of SBF, dried as previously and weighed. Investigation of surface topography changes and chemical composition was performed using SEM-EDS technique.

2.6 Clinical case

An 8-year-old male Dachsund was brought by his owner to The Department and Clinic of Animal Surgery, Faculty of Veterinary Medicine, University of Life Sciences in Lublin for pus discharge from the right nostril. An X-ray revealed purulent lesions around the right canine tip and a tooth extraction was prescribed. Long-term inflammatory lesions had caused an oronasal fistula. In order to heal the wound, the alveolus was curetted, washed with a solution of chlorhexidine, and composite (sample 3A, of dimensions similar to those of removed tooth) was

inserted. Stitches were applied on the mucous membrane with absorbable Mersilene 3-0 sutures (Ethicon, USA). During the 7-day postoperative period, the patient was treated with clindamycin, Upjohn, USA (600 mg, daily) and the stitches were removed after 9 days. Control check-ups were performed after 1 and 12 months, together with X-ray examination (Preva CD 04x04, Progeny Dental, USA or Planmeca Intra ProSensor, Planmeca, Finland). Experiments were conducted in accordance with Agreement of II Local Ethical Committee of University of Life Sciences in Lublin (15/2010).

3. Results and Discussion

3.1 General characteristics of composite

HAp/β-glucan composite was successfully prepared in different ceramic:polymer proportions, presented in Table 1. It should be noted that nonporous granules were useless for this purpose and did not allow the formation of compact material. Probably, the pores in HAp granules were required as attachment points for polymerized glucan chains. The composite can be formed into different shapes, both at the preparation step and afterwards (until wet), using lancet, scissors or any sharp device (Figure 1C). 2D X-ray microtomography of the composite (sample 3A) confirmed a homogenous distribution of granules (Figure 1B) and allowed us to estimate the volume of granules: 70 vol%. It is relatively brittle and pumice-like when dry, but after soaking in water, it shows flexibility, can be compressed or bent and adapts easily to appropriate shapes. Angles of the bending portability depends on size and dimensions of composite: thick cylindrical samples (length =3 cm, ø: 8 mm) can be bent to a 90 degree angle without damage while flat slices (δ=2 mm, ø: 13 mm) to a 180 degree angle (Figure 2). Although susceptible to bending and compression, wet samples of the material remained compact, relatively tough and retained its initial shape.

SEM/EDS studies of composite cross-sections (Figure 3A) brought some interesting observations. It was observed that a thin gap, visible in the center of the photo (magnified on Figure 3B), appeared between the granule and glucan layer during SEM analysis. Only the traces of P and Ca were detected on outer β-glucan layer (point 1), while significant quantities of these elements were found both on HAp granule surface (point 2) and on the inner side of β-glucan layer, which spontaneously separated from HAp granules during SEM microscopy of composite sample (point 3). This observation suggested that HAp granule and the surrounding polymer, forming a thin layer on the granules surface, must have been bound with high integrity during the process of composite preparation, probably due to high porosity of ceramic particles; thus, the structure of fabricated composite is significantly compact. Good integrity between two phases of composite is known to be necessary for functionality of osteochondral constructs (scaffold + growing osteoblasts cells) [37] and was observed for hydroxyapatite/chitosan porous scaffolds. In this context, its high integrity is a promising feature of HAp/β-glucan composite.

Figure 4 showed the FT-IR spectra of pure gelled β-glucan, HAp ceramics and composite. Characteristics bands due to PO_4^{3-} ions were visible in the spectrum

sample code		HAp granules		β-glucan
		wt % in composite	fraction size (mm)	wt % in composite
1		90.9	0.2-0.3: 0.5-0.6 (1:3)	9.1
2	A	88.6	0.2-0.3: 0.5-0.6 (1:3)	11.4
	B		0.2-0.3	
	C		0.3-0.4	
	D		0.5-0.6	
3	A	83.3	0.2-0.3: 0.5-0.6 (1:3)	16.7
	B		0.2-0.3	
	C		0.3-0.4	
	D		0.5-0.6	
4		76.9	0.2-0.3: 0.5-0.6 (1:3)	23.1
5		69.4	0.2-0.3: 0.5-0.6 (1:3)	30.6
6		61.8	0.2-0.3: 0.5-0.6 (1:3)	38.2
7		43.3	0.2-0.3: 0.5-0.6 (1:3)	56.7

Table 1. Composition of HAp-polymer composite samples (as percent of dry weight).

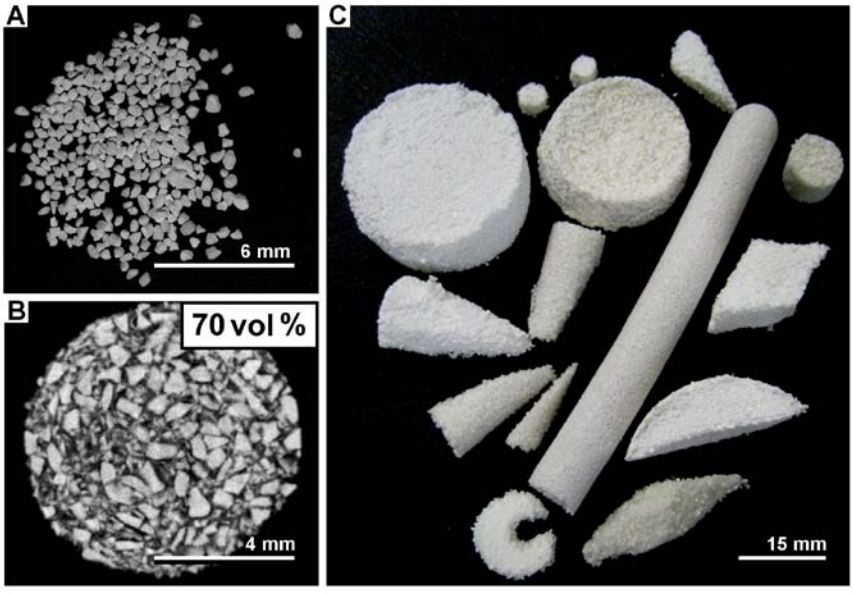

Figure 1. General appearance of composite. A - HAp granules (size: 0.2-0.6 mm) used as basic inorganic compound; B – 2D X-ray microtomogram of composite (3A) sample and volume of granules (in vol %) in composite (in inset); C - samples formed into different shapes.

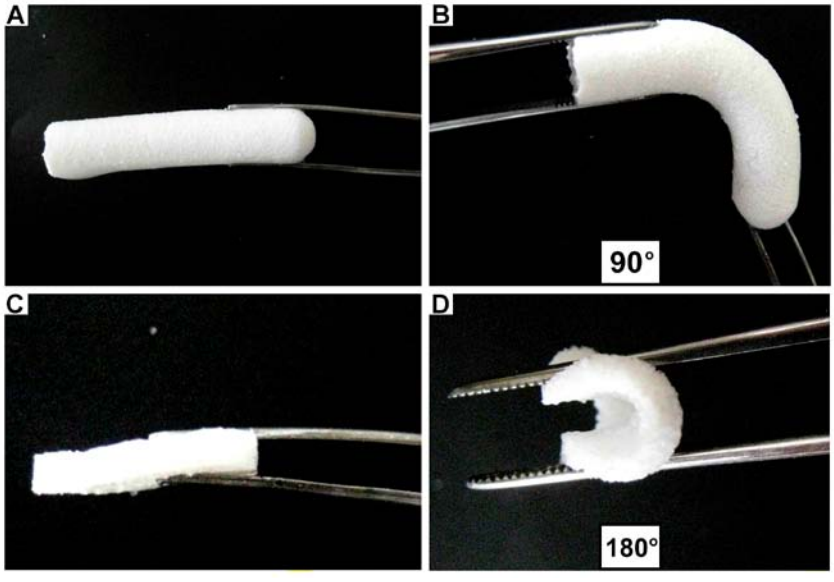

Figure 2. Elastic properties of composites (sample 3A) of different shapes; unbent (left; A,C) and bent (right; B,D). A,B – cylinder (length =3 cm, ø: 8 mm); C,D – slice (δ=2 mm, ø: 13 mm). Insets in Fig. B and Fig. D shows the angle of the bending portability for presented samples.

of HAp: v_1 at 963 cm^{-1}, v_3 at 1022 cm^{-1} and 1088 cm^{-1}, v_4 at 562 cm^{-1} and 599 cm^{-1}; bands 3572 cm^{-1} and 630 cm^{-1} appeared due to the presence of OH^- groups [38]. All these bands were also present in composite spectrum. The spectrum of composite showed also the presence of bands characteristic for β-glucan: broad bands in the region of about 3300 cm^{-1} (-OH vibrations), at 2920 cm^{-1} (C-H stretching vibration), at 1157 cm^{-1} and at 888 cm^{-1} (C_1-O-C_3 stretching vibration, characteristic for β configuration) [39,40]. A band at 1640 cm^{-1} suggested trace water in the β-glucan sample. No additional bands appeared in composite spectrum in comparison to those of pure β-glucan and HAp, thus confirming the lack of chemical interactions between β-glucan and HAp granules in the composite sample, which was expected because both components lack appropriate active groups capable of covalent bonds formation. Mechanism of composite synthesis seems therefore to be based on simple physical occlusion of HAp granules by β-glucan.

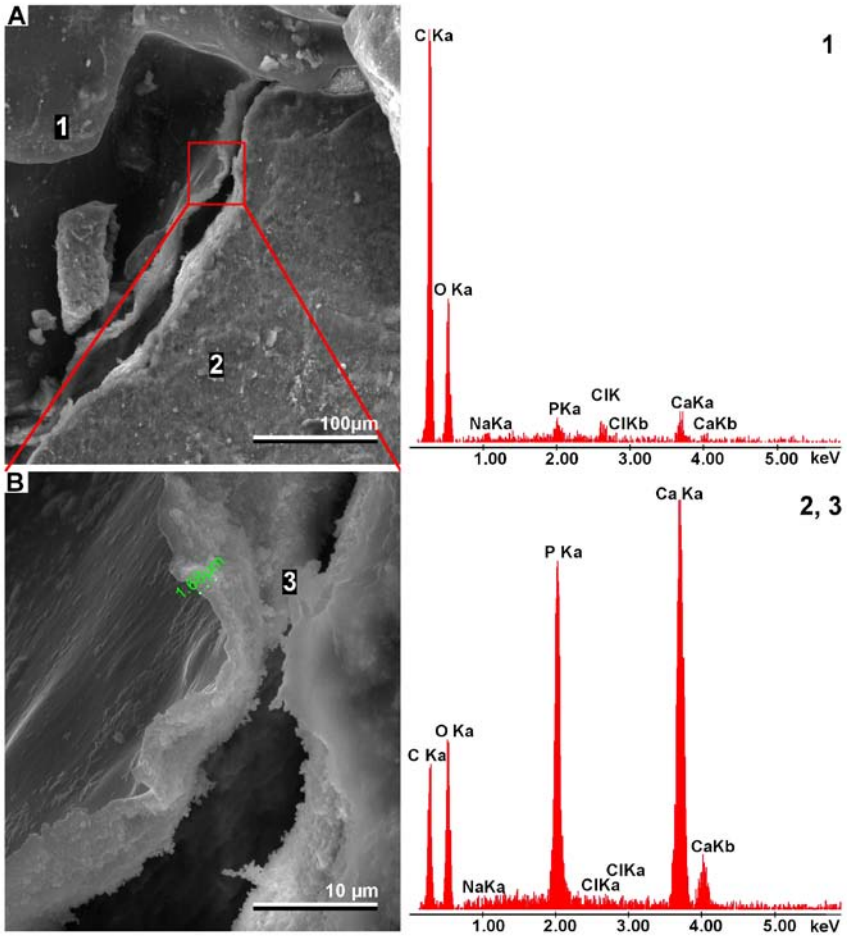

Figure 3. Analysis of composite cross-section (sample 3A). Left: SEM micrographs (A - Mag 1000x; B - Mag 8000x of selected area). Right: EDS spectra of particular points (1- polymerized β-glucan layer; 2 - HAp granule; 3 - inner side of β-glucan layer). EDS spectra for points 2 and 3 were almost identical, thus were presented as one spectrum.

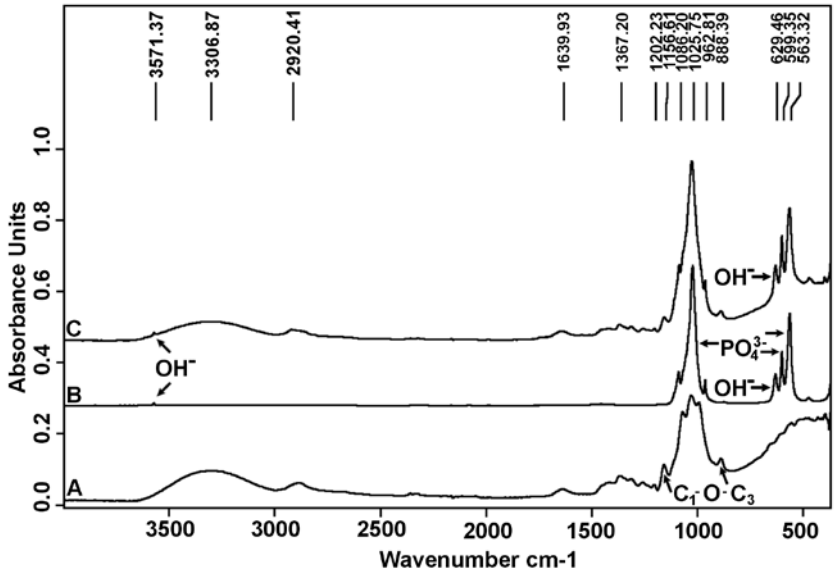

Figure 4. Comparison of FT-IR spectra of pure gelled β-glucan (A), HAp ceramics (B) and composite (C).

3.2 Soaking properties

The kinetics of soaking estimated in pilot experiment (Figure 5) showed that the composites with maximum (90.9 w t%; sample 1) and minimum (43.3 wt %; sample 7) HAp content reached the sorption equilibrium within 6 h and 24 h, respectively. Further estimation of sorption and volume indexes for all prepared samples was therefore limited to period of 24 h. A convenient feature of the investigated material is that it can be dried (0.016-24 h at 15-40°C) and soaked several times with no effect on mechanical properties. It should be noted that the soaking rate depended also on composite dimensions. In practice, advantageous for manual handling, the soaking process and recovery of elasticity for small pieces of composite material (e.g., of medium cyst dimensions) takes only 2-3 minutes, especially for samples with high HAp content. This was confirmed by DIC images of dry (Figure 6A,C) and soaked (Figure 6B,D) composite (sample 3) showing that the polymer phase was visibly swollen just after 10 min., with small air bubbles and irregularities suggesting the porosity of wet composite.

After a 24 h incubation in PBS pH 7.4, the volume index (S_v) and sorption index (S_w) of studied materials significantly varied in ranges: 5.5-190.3% (S_v) and 102.6-195.8% (S_w), depending on HAp granules content (Table 2). The general tendency, observed for sorption and volume indexes of tested samples, was as follows: their values decreased with an increase of HAp content in the composite. It seems that the greater the β-glucan content in composite, the more values of both tested indexes depend on properties of glucan itself.

The capability to uptake and preserve liquids within its structure is an important feature because high content of water (with oxygen and nutrients dissolved within) is requisite for osteochondral and cartilage defect regeneration. Moreover, due to short soaking time, the composite can be potentially used as a carrier for active substances (drugs, growth factors, *etc.*).

Sample code	Sorption index S_w [%]	Volume index S_v [%]
1	102.6 ± 2.1	5.5 ± 2.8
2A	101.1 ± 2.2	6.5 ± 2.1
3A	121.7 ± 3.2	19.5 ± 2.9
4	142.2 ± 1.1	31.2 ± 5.6
5	162.1 ± 2.8	46.5 ± 7.4
6	182.5 ± 4.1	68.8 ± 7.9
7	195.8 ± 4.0	190.3 ± 10.3

Table 2. Sorption (S_w) and Volume (S_v) index for HAp-polymer composite after 24 h of swelling in PBS pH 7.4.

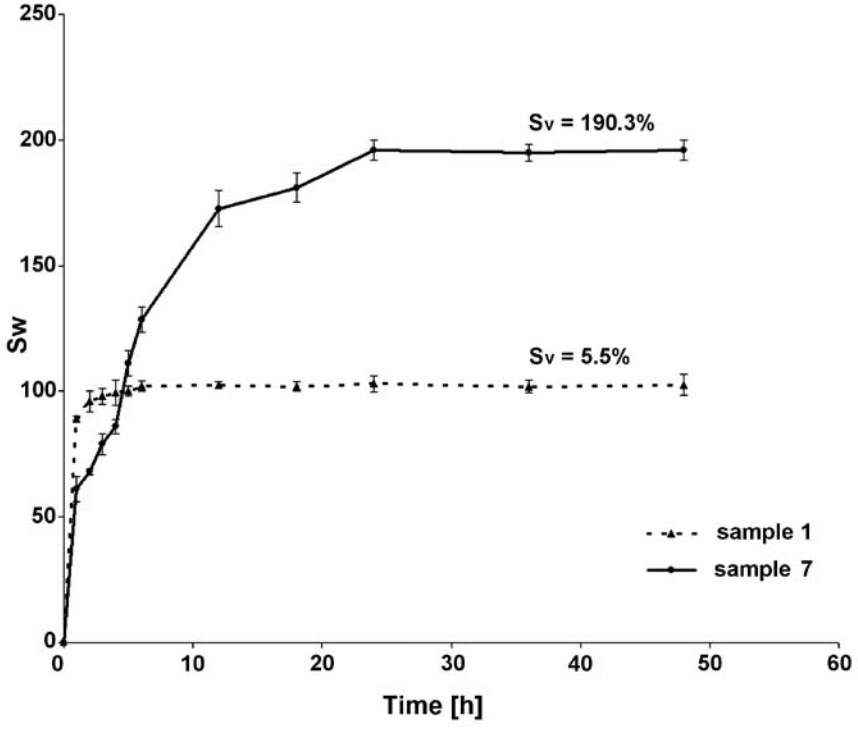

Figure 5. Kinetics of water absorption (S_w) of HAp-polymer composite with minimum (sample 1; dotted line) and maximum (sample 7; solid line) concentration of β-glucan. Volume indexes (S_v) for particular samples were inserted above the respective curves.

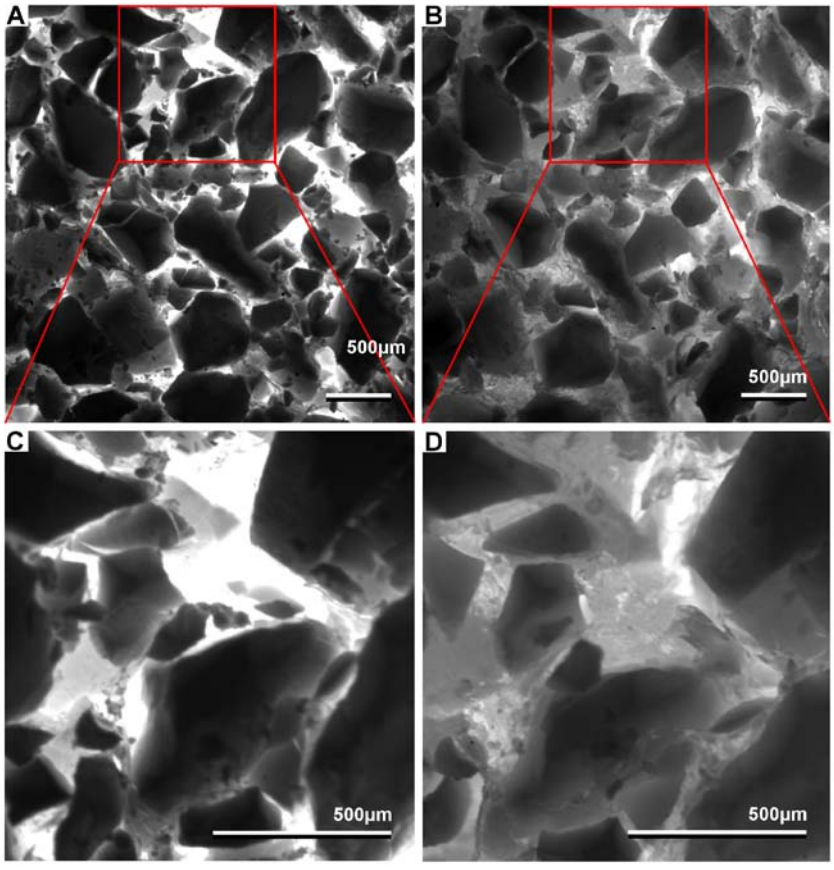

Figure 6. Differential Interference Contrast (DIC) images of composite (sample 3A; δ=2 mm), dry (A, C) and completely soaked (B, D). Mag: A, B - 40x; C; D - 100x.

This presumption is in agreement with the opinion that hybrid scaffolds are more profitable bone fillers and delivery systems than single component scaffolds [41]. However, the materials that reach the equilibrium of soaking several hours after the implantation may evoke undesired side effects because of the increased pressure within the implantation site, generated by swelling material. This, in turn, may result in local collagen fibers necrosis and degeneration of bone tissue [42]. Therefore, further studies on HAp/β-glucan composite were limited to samples 1-3, containing 83.3-90.9 wt% HAp and showing tolerable S_w and S_v values.

3.3 Mechanical parameters

The compressive strength and Young's modulus values for tested composite samples (1-3) were the highest for sample 3 (83.3 wt % HAp), although generally similar. Granule size did not seem to affect these parameters. However, significant difference has been observed between the values of both parameters for dried and completely soaked samples (Table 3). Our general conclusion is that the combination of tough and rigid HAp ceramic [43] with β-glucan reduces its compressive strength and Young's modulus and increases composite elasticity (Table 4). Interestingly, estimation of compressive strength and Young's modulus allowed us to state that composite of particular content (83.3 wt % HAp) revealed similar mechanical properties (compressive strength: 3.2-5.9 MPa and Young's modulus: 0.4-0.8 GPa) to those of spongy bone (Table 4) [44]. The above values are also in good agreement with those of normal human cartilage (1.9-14.4 MPa) [45,46]. However, mechanical parameters of tested composite are distinct from those of natural compact bone (compressive strength: 170-193 MPa; Young modulus: 17-19 GPa; Table 4) [47]. Thus, HAp/glucan composite differs from other flexible bone-replacing composite described in literature, based on particulate calcium phosphate and polyolefin as the polymeric phase. The latter was reported to show the Young's modulus within the range 2-40 GPa, characteristic for compact bone tissue (Table 4) [48].

Sample code		Compressive strength (MPa)		Young's modulus (GPa)	
		dry samples	wet samples	dry samples	wet samples
1		3.27 ± 0.22	0.18 ± 0.01	0.486 ± 0.025	0.271 ± 0.007
2	A	4.36 ± 0.82	0.21 ± 0.03	0.542 ± 0.036	0.178 ± 0.002
	B	3.23 ± 0.15	0.16 ± 0.03	0.417 ± 0.021	0.178 ± 0.012
	C	4.39 ± 0.46	0.27 ± 0.03	0.629 ± 0.075	0.191 ± 0.015
	D	3.97 ± 0.35	0.21 ± 0.04	0.621 ± 0.022	0.210 ± 0.021
3	A	5.92 ± 0.25	0.26 ± 0.02	0.777 ± 0.041	0.186 ± 0.006
	B	5.37 ± 0.31	0.24 ± 0.02	0.595 ± 0.011	0.182 ± 0.006
	C	5.29 ± 0.55	0.26 ± 0.02	0.620 ± 0.014	0.180 ± 0.004
	D	5.08 ± 0.10	0.21 ± 0.05	0.697 ± 0.027	0.180 ± 0.020

Table 3. Compressive strength (MPa) and Young's modulus (GPa) of composite samples.

	Composite (3A) dry / *wet*	HAp ceramics[43]	Compact bone[47]	Spongy bone[44]	Polyolefin-CaP ceramics composite[48]
Compressive strength (MPa)	5.3 / *0.26*	120-900	170-193	1.9-7	-
Young's modulus (GPa)	0.62 / *0.18*	35-120	17-19	0.18-0.33	2-40

Table 4. Comparison of compressive strength (MPa) and Young's modulus (GPa) of composite sample (3A) *versus* HAp ceramics, flexible polyolefin-CaP ceramics composite and spongy and compact bone.

[43, 44, 47, 48] – see: References
- – not available

Based on the above data, the potential application of HAp/glucan biomaterial should probably be limited to non-load bearing applications: for spongy bone or cartilage defect replacement. Moreover, the most promising parameters were observed for composite containing 83.8 wt % HAp (mixed-size granules) and 16.7 wt % glucan; therefore, all further experiments were performed for this selected sample (sample 3A).

3.4 Sterilization, storage and estimation of their effectiveness

Composite was sterilized by two methods: ethylene oxide (EO; one of the most frequently used for implantable materials) and autoclaving (widely available). These methods are considered to be less harmful for structure and stability of ceramic/organic composites than gamma irradiation, although the latter is thought to be the simplest and the most effective way of sterilization with no risk of intoxication by EO vapours. However, gamma irradiation causes the break of chemical bonds and thus affects the mechanical properties of organic polymers, as was shown for collagen sponges [49] and could damage the tested composite.

The ethylene oxide method allows for sterilization of dried samples because it is performed in paper/plastic peel pouch, which enables air exchange and forces the drying of sample. Autoclaving, using hot steam as a sterilizing factor, is a method more suitable for wet, ready-to-use samples and should be performed in glass bottles or other devices, which could reduce the loss of humidity during the process itself and further storage.

Microbiological testing showed that after 1 year of storage, HAp-composite remained sterile. After 6 days of incubation of composite samples (sterilized both by autoclaving and ethylene oxide method) with either rich growth medium or human blood plasma, bacteria and fungi were not detected. Human blood plasma – for its similarity to human tissue liquid, present in bone tissue – is an excellent medium for estimation of bone filler sterility. Absence of microorganisms growing in this liquid ensures the lack of biomaterial-originating bacterial contamination after its implantation. Comparison of mechanical properties of freshly prepared (a) and sterilized (EO and autoclaving) and stored (b) samples suggested that the sterilization and storage processes did not affect the mechanical parameters of the composite (Table 5). This is a promising feature for

potential application – commercial bone fillers must be not only sterile but also stable during long-term storage.

3.5 *In vitro* bioactivity

Testing for bioactivity revealed that after 1 month of incubation in SBF, the composite samples showed an approx. 2.5% increase in weight. Moreover, SEM/EDS results confirmed that after incubation in SBF, calcium phosphate deposits were detected on organic polymer layer (Figure 7). Therefore, the result of the experiment provided evidence for the bioactivity of obtained composite.

Some studies [50] show that β-glucan itself does not form apatite deposits after SBF treatment, even after presoaking in $Ca(OH)_2$ solution, probably due to lack of active groups responsible for apatite nucleation. Therefore, the appearance of calcium phosphate on HAp/β-glucan composite surface after treatment in SBF seems to result from HAp presence exclusively. High microporosity of ceramic granules used for composite synthesis probably enhanced this phenomenon. It was observed that increased microporosity of biomaterials and their specific surface areas affected ionic solubility in microenvironment and was essential for apatite

Time of storage (since the preparation)	Compressive strength (MPa)		Young's modulus (GPa)	
	dry samples	wet samples	dry samples	wet samples
a) directly after the preparation	5.92 ± 0.25	0.26 ± 0.02	0.777 ± 0.041	0.186 ± 0.006
b) after 1 year	6.38 ± 0.30	0.31 ± 0.05	0.777 ± 0.027	0.176 ± 0.007

Table 5. Compressive strength (MPa) and Young's modulus (GPa) of HAp-polymer composite (sample 3A) directly after the preparation and after 1 year of storage (sterilized by ethylene oxide method). The values for samples sterilized by autoclaving were identical.

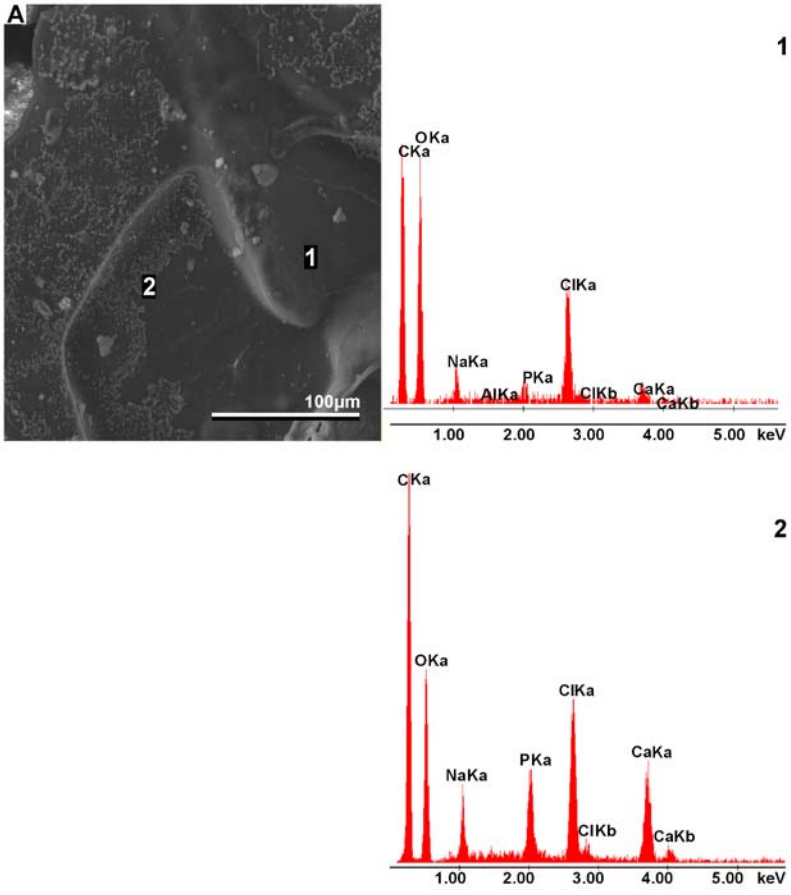

Figure 7. SEM-EDS analysis of surface of composite after incubation in SBF (1 month). Left (A): SEM micrograph (Mag 1000x) of material surface. Right: EDS spectra of particular points (1 – area of β-glucan layer with no visible deposits formation; 2 - area of β-glucan layer region with visible deposits formation).

layer formation [51,52]. Besides, microporosity of ceramics also induces differentiation of relevant cells into osteogenic lines [52,53]. This suggests that micro- and macroporous HAp/β-glucan composite may create an advantageous environment not only for bone-like apatite formation but also osteinduction in biomaterial.

3.6 Clinical case

A pilot *in vivo* experiment was performed to initially evaluate the possibility of practical medical application of the tested composite. The clinical case described here concerned the treatment of the dog's oronasal fistula – a critical size defect of maxillary bone and a serious complication in veterinary dentistry, often resulting in serious pathological processes, including infections and aspiration pneumonia [54]. The surgical procedure was performed quickly, as biomaterial soaked immediately with the dog's blood restored its flexibility. It filled precisely a fistula in the dog's oral cavity. After the operation, the patient showed normal activity, with only a mild depression several hours after implantation. Clinical examination revealed that the wound healed properly, with no signs of infection or other unexpected changes in the operation area during 1-year follow-up (Figure 8). RTG evaluation was performed directly after the composite implantation, as well as after 6 and 12 months (Figure 9). Radiographically, significant resorption of granules and mineralization within implanted composite were observed after 6 months. After 12 months, HAp granules were invisible in radiologically, which demonstrated the appearance of typical bone tissue in the implantation site. The results of the clinical case suggest that the composite is a promising material for repair of such bone defects.

Generally, HAp ceramics are considered to be poorly biodegradable which prevents the bone ingrowth for extended periods. However, porous HAp was found more osteoconductive and resorbable than dense HAp [16]. Moreover, Liu *et al*. [55] reported that *in vivo* biodegradation rate of HAp depended on surface/volume ratio, although they observed that – even in case of highly porous Interpore 200®, tested in their experiments – the scaffold itself remained in the implantation site during 6 months of implantation, despite the ingrowth of bone tissue within the biomaterial pores. The explanation of complete remodeling of HAp/β-glucan composite in the presented clinical case may lay in very high porosity and surface area of HAp granules used for composite fabrication. In this context, the complete bone remodeling and replacement of implanted HAp/glucan composite during the experimental period is a promising observation.

The positive healing effects of HAp/glucan composite presented in this article are not surprising because β-glucans have already been used for development of biomaterials. The porous gelatin/(1→3),(1→6)-β-glucan scaffolds showed promising results of skin defect repair; moreover, they could serve as fibroblast-containing artificial skin, strongly enhancing the healing process [56]. Also, β-glucan-treated polyurethane films reduced the inflammatory response in fibroblast culture and decreased *S. aureus* adhesion [57]; β-glucan-chitosan membranes revealed antibacterial properties [58] and β-glucan-collagen matrix showed the effectiveness during the treatment of partial-thickness burns in children [59]. Therefore, positive results of implantation of HAp/β-glucan bone filler in the presented case may support the opinion about the beneficial effect of β-glucans on health improvement.

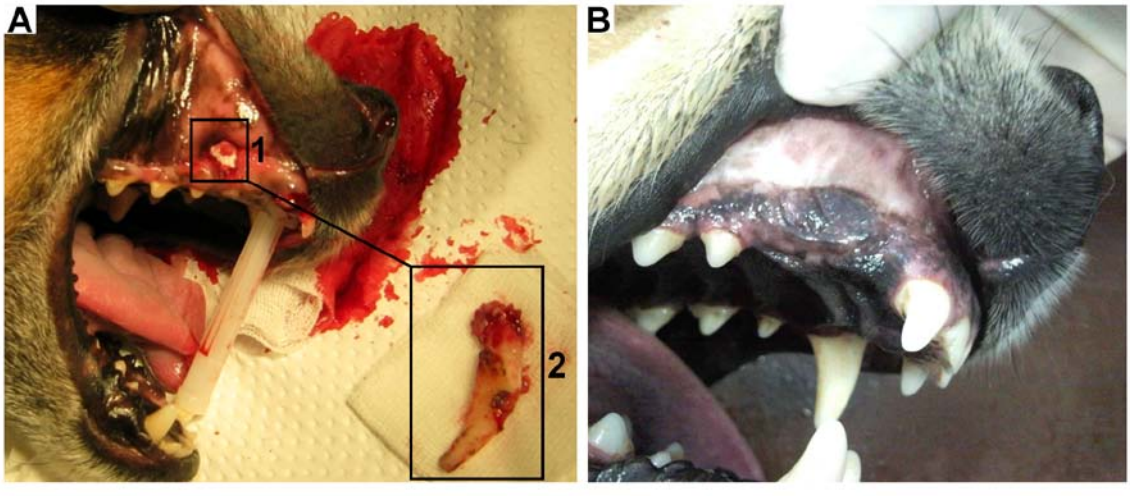

Figure 8. Treatment of dog's fistula. A - implantation procedure (frame 1 – place of tooth removal and implant insertion; frame 2 – removed tooth); B - control photo after 1 month.

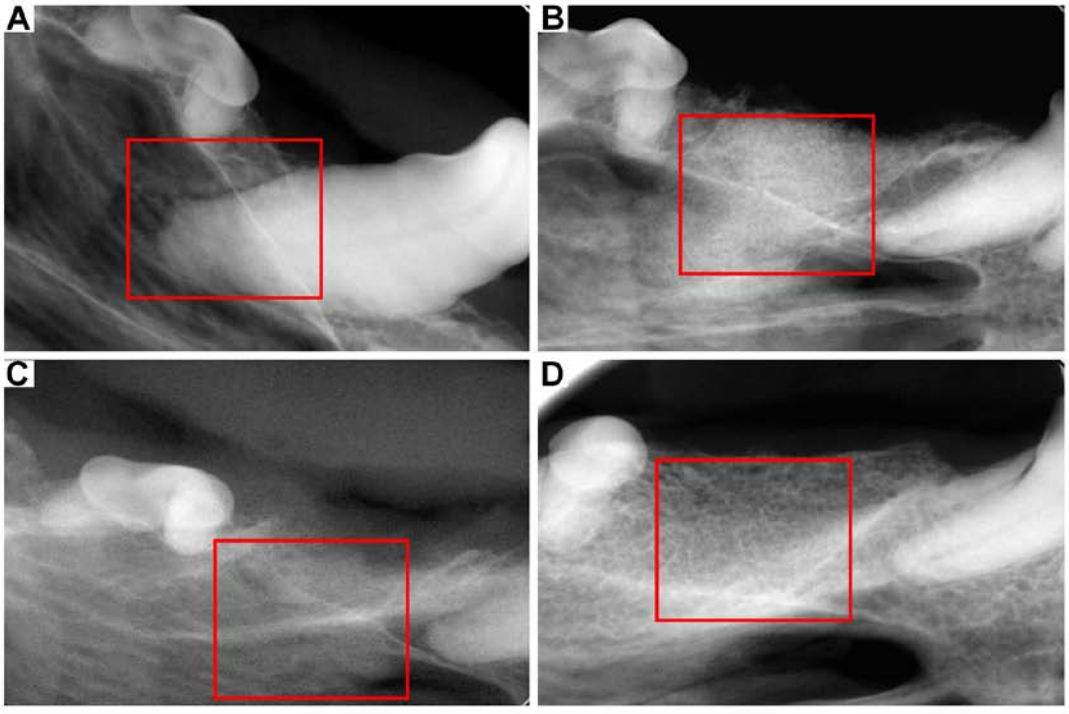

Figure 9. Radiographic evaluation. A – Class III perio-endo lesion with visible etopic inflammatory changes; B,C,D – composite implanted into a space after the tooth removal (B – directly after the implantation; C - after 6 months; D - after 12 months).

4. Conclusions

An innovative, easy-to-use and cost-effective HAp-based composite material was prepared using β-glucan as a polymeric phase. The new composite reveals sufficient mechanical strength for some applications, and that it may be sterilized and stored for at least 1 year without loss of its properties, it is bioactive, soaks and swells efficiently in contact with different liquids and shows good adaptation to the shape and dimensions of bone defects during implantation. Although further *in vivo* experiments are necessary, the results obtained thus far suggest the potential use of prepared biomaterial for repair of bone defects in various dental and orthopedic applications. Study on implantation of the material in animal patients should be performed in the near future.

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