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# New biocomposite [biphasic calcium phosphate/ poly-DL-lactide-co-glycolide/biostimulative agent] filler for reconstruction of bone tissue changed by osteoporosis

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Biphasic calcium phosphate-poly-DL-lactide-co-glycolide composite biomaterial with and without biostimulative agents (protein-rich plasma or fibrin) was synthesised in the form suitable for reconstruction of bone defects. The composite used as filler was obtained by precipitation in solvent-non-solvent systems. The material, calcium phosphate granules covered by polymer, was characterised by wide-angle X-ray structural analysis, scanning electron microscopy, infrared spectroscopy and differential scanning calorimetry. Reparation of bone tissue damaged by osteoporosis was investigated *in vivo* on rats. The method applied enabled production of granules of calcium phosphate-poly-DL-lactide-co-glycolide composite biomaterial of average diameter 150–200 $\mu$m. Histological analysis confirmed recuperation of the alveolar bone, which osteoporosis-induced defects were repaired using composite biomaterial. By addition of biostimulative agents, intensity of osteogenesis increases accompanied by the formation of regular, new bone structure.


## 1. Introduction

Composite biomaterials have enormous potential for natural bone tissue reparation, filling and augmentation [1]. Ceramics/polymer composites play a significantly role in these reparations, as their properties are very close to the natural bone tissue [2]. Calcium hydroxyapatite/poly-l-lactide (HAp/PLLA) composite biomaterials belong to this group of composites and due to their osteoconductive and biocompatible properties they can be successfully implemented in bone tissue reparation [3–5]. The structure and properties of this kind of composites depend on the polymer molecular weight, crystalline/amorphous ratio, porosity, etc. [6, 7]. Poly-DL-lactide-co-glicolide (DLPLG) polymer is also a biodegradable material that unites biocompatible properties of poly-l-lactide and polyglicol, whereas its resorption time is shorter compared to that of poly-l-lactide [8], which is preferable in some applications. Thus, DLPLG polymer as highly porous biodegradable foams has been used until now in reparation of bone tissue, cartilage and meniscus [9]. Reparation processes in organism depend on interactions of organism cells with implanted biomaterial. Human osteoblast adhesion is better on DLPLG polymer surfaces than on polylactide ones (PLA); therefore, it is to be expected that the formation of human osteoblasts is also more intensive on the surface of DLPLG [10]. It is also important to emphasize that adhesion and osteoblast production phenomena in this case depend on the structure and type of the polymer but not on time, provided that the advantage is given to DLPLG polymer over PLA.

Mixed with calcium phosphates, DLPLG polymer realizes more intensive activity of alkaline phosphatase, which is important for differentiation of osteoblasts that dictate regeneration processes within the organism [11]. A composite with calcium-deficient hydroxyapatite (CDHAp) and DLPLG polymer was produced by hydrolysis and hot pressing at a temperature of approx 70 °C. Different mechanical properties of this composite were obtained by varying the time and temperature of hydrolysis [12]. A similar composite was produced by extrusion, but instead of synthetic, natural hydroxyapatite was used. Cow's hydroxyapatite (osseine) mixed with DLPLG was extruded and the obtained composite showed greater potential for osteoblast protein synthesis than pure DLPLG [13]. Addition of biphasic calcium phosphate (BCP) to polymers can largely increase the polymer bioactivity [14], while BCP itself can be an exceptional carrier of growth factors, which facilitates its wider application in medicine and stomatology [15].

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Osteoporosis is a system disease that seizes all parts of the skeleton and whose main characteristic is reduction of bone mass per volume unit. Problems of bone tissue decrease due to osteoporosis occupy a significant place in the field of medical and stomatological sciences [16, 17]. All kinds of dental implants and suprastructures have bone as their basic support, securing their stability. Bone tissue deficiency entails impossibility of adequate healing.

In this paper we report on a new biphasic calciumphosphate/poly-DL-lactide-co-glicolide (BCP/DLPLG) composite biomaterial synthesized in the form of granules of desired shape and dimensions. In order to solve the problem of bone tissue deficiency due to progressive bone resorption within system osteoporosis, possible applications of this biomaterial were examined involving experimental animals with induced osteoporosis. To intensify osteogenetic processes, production and application of biomaterial with and without biostimulative agents as blood plasma and fibrin were studied.

## 2. Materials and methods

### 2.1. Synthesis and characterisation

A calcium phosphate gel was produced by precipitation of calcium nitrate and ammonium phosphate in an alkaline medium, according to the reaction:

$$5Ca(NO_3)_2 + 3(NH_4)_3PO_4 + NH_4OH = Ca_5(PO_4)_3(OH) + 10NH_4NO_3$$

The gel was dried at room temperature and calcined at 1100 °C for 6 h [3, 5]. Poly-DL-lactide-co-glicolide (DLPLG) (50:50) (Sigma Chemical Company, USA) was used as a polymer component. Granules of calcium phosphate were added into completely dissolved polymer, in amount of 80 mass%. The solution was mixed at a speed of 30 rev/min, and then methanol was added. After solvent evaporation, the particles were dried at the room temperature for 24 h. The granules of calcium phosphate/DLPLG composite biomaterial, sizes 0.15–0.20 mm, sterilized by $\gamma$ rays (25 kGy) before use, were separated by sieves. Wide angle X-ray structural analysis (WAXS) was made on a Philips PW 1710 diffractometer with Ni-filtrated Cu $K_{\alpha_1}$, by $\alpha_2$ radiation. Microstructure characterisation was done by scanning electron microscopy (JSM 5300) and phase analysis was made by infrared spectroscopy (IR) (KBr pastille). Samples were analysed by a dispersive Perkin—Elmer 983G spectrophotometer, in the IR spectrum range of 4000–400 $cm^{-1}$. Differential scanning calorimetry (DSC) tests were done by a Perkin Elmer DSC-2 differential scanning calorimeter in the 305–480 K temperature interval, at 20 K/min heating rate, in nitrogen.

### 2.2. *In vivo* research

The research was carried out on white female rats of Sprague Dolly syngenic type, age 6–8 weeks. Animals were divided into two groups:

1. Experimental group (A) (24 animals)
2. Control group (B) (8 animals)

Osteoporosis was induced by cortico medicaments to animals of the group A. Cortico medicaments were intramuscularly administered to animals during 12 weeks, alternately methylprednisolone succinate sodium (Lemod-Solu, Hemofarm, Serbia and Montenegro) and dexamethasone- sodium-phosphate (Dexason, ICN Galenika, Serbia and Montenegro). During the period of research shown in this paper, induced osteoporosive changes were irreversible. Corticosteroids used in our research induced irreversible processes whose final aim was a distinctive and irreversible thinning of alveolar bone [18–20]. After 12 weeks of cortico-treatment, animals were prepared for intervention applying Diazepam (Bensedin, ICN Galenika, Serbia and Montenegro) and anaesthetized with Ketamin hydrochloride USP (Ketalar, Rotexmedica Gmbh, Trittau, Germany). Defects 1.4 mm in diameter and 1.6 mm deep were made in animals in the region between medial line and foramen mental on the left side of the osteoporosive mandible and in thusly made defect BCP/DLPLG was implanted.

Group A was divided into three subgroups:

- A1—BCP/DLPLG was implanted in artificially made defect of osteoporosive mandible;
- A2—BCP/DLPLG mixed with fibrin glue, produced by mixing fibrin and thrombin of rats with calcium chloride was implanted in artificially made defect of osteoporosive mandible;
- A3—BCP/DLPLG was implanted in artificially made defect of osteoporosive mandible, mixed with autologous plasma, after centrifugal separation of $1dm^3$of blood of the animal on which intervention was executed.

Group B was a control group, without therapy.

Animals of both groups were sacrificed, six and twenty four weeks after implantation. Bone samples of mandible, from the medial line to the foramen mental (region where artificial defect was made and implantation executed), were taken. They were cut in the vestibule-oral direction. Samples were washed in physiological solution, fixed in 10% formaldehyde and then decalcified chemically and by electrolysis. Chemical decalcification was done in 15% solution of nitric acid. After decalcification, bone tissue was dehydrated in alcohol and then moulded into paraplast, dried and dyed. Thus obtained histological sections, 2–4 $\mu$m thick, were dyed routinely by hematoxylineosin (HE) and PAS methods, and pathohistologically analysed. Obtained histological preparations were histomorphologically analysed by a Lucia 3.2 G system for image analysis (Laboratory Imaging, Prague, Czech Republic), on a NU-2 microscope (Carl Zeiss, Jena, Germany).

## 3. Results and discussion

### 3.1. Properties

After production of powder and granules of calcium phosphate according to the previously given procedure,

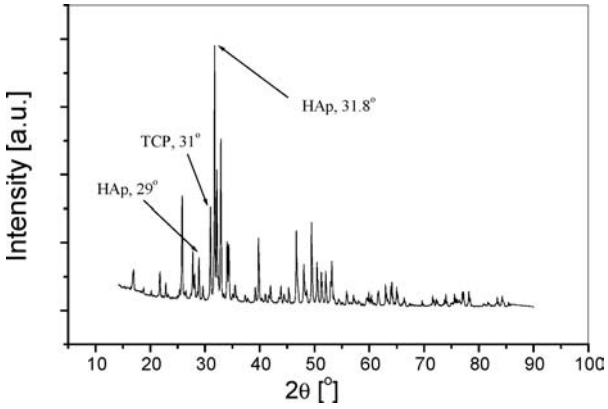



*Figure 1* WAXS of calcium phosphate powder.

phosphate phase was analysed by WAXS. Fig. 1 shows a diffractogram of the obtained powder.

As evident from Fig. 1, the obtained calcium phosphate powder is a highly crystalline. The most intense peaks at $2\theta = 29^\circ$ (2 1 0) and $31.8^\circ$ (2 1 1) originate from calcium hydroxyapatite (HAp) and that at $2\theta = 31^\circ$ (0 2 10) from 3-calciumphosphate ($\beta$-TCP). Based on earlier described methodology [21], mass content of HAp and $\beta$-TCP, 80 and 20%, respectively, were calculated. Thereby, this calcium phosphate is also called biphasic calcium phosphate (BCP) and is used for the BCP/ DLPLG composite biomaterial production.

Composite production procedure via solvent—non-solvent system realizes covering of BCP particles with DLPLG polymer. Using sieves, a fraction of granules, sized 0.15–0.20 mm, was separated. Fig. 2 shows SEM images of BCP/DLPLG composite structures.

Spherical forms are discernable in Fig. 2(a). BCP granules are coated with the polymer and the average diameter of 0.15–0.20 mm. The shape of composite biomaterial enables its application as filler, which can be optimal in some cases. A detail of spherical granule surface is shown in Fig. 2(b), where a part of BCP granule coated with DLPLG polymer can clearly be seen, as well as a weak adhesion between the phases at BCP-DLPLG interface. Connection between HAp and the polymer based on lactide is of weak ion nature and was most probably established between the $Ca^{2+}$ ion and oxygen from the polymer [14].

IR spectrum of BCP/DLPLG composite biomaterial is shown in Fig. 3.

Bands originating from BCP and DLPLG can be seen in the spectrum. BCP is identified within the spectrum by a doublet with maxima at 1052 and 1087 $cm^{-1}$, which are the most intense and originate from phosphate groups, and by a triplet with maxima at somewhat lower frequencies of 571 and 602, arising from the $PO_4^{3-}$ group vibrations, and at 632 $cm^{-1}$, assigned to the hydroxyl group vibrations appearing also at 3567 $cm^{-1}$ [7, 22]. DLPLG is characterized by an absorption band at 1756 $cm^{-1}$ corresponding to the C=O group vibrations and two smaller maxima at 2996 and 2944 $cm^{-1}$ ascribed to C–H group vibrations. Absorption maximum at 1449 $cm^{-1}$ originates from the $CH_3$ group [22].

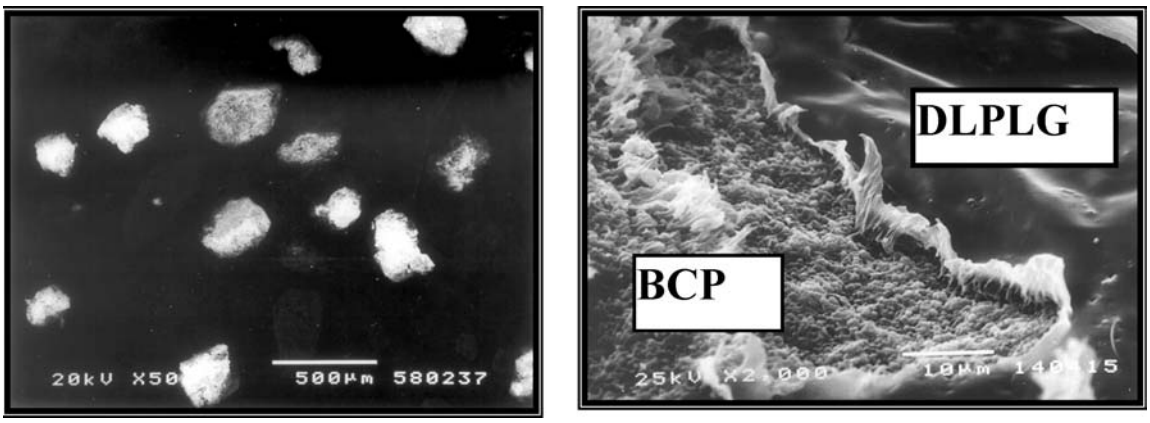
Composite biomaterial was analysed by DSC and thus obtained results are shown in Fig. 4.

According to DSC analysis, all transformations occurring in the temperature range from 305 to 480 K are connected with the polymer, while BCP is stabile. The only noticeable transformation, Fig. 4, is characterized by a glass transition peak at the glass transition temperature ($T_g$) 324.4 K. Non-existence of phase transition, characteristic of melting, indicates amorphous of the polymer. The obtained DSC curve of DLPLG is characteristic of this polymer [23]. Amorphous of the polymer is an important parameter in its short-term bioresorption compared to other biodegradable polymers, which is generally 4–8 weeks [8].

### 3.2. *In vivo* research

Osteoporosis is a disease of changed bone architecture and mass that due to its great frequency and spreading is becoming one of the leading health problems. It is of exceptional importance for stomatology since it grips alveolar bones. System osteoporosis directly attacks all segments of mandible bones, as indicated by the results of many examinations [24].

Fig. 5 shows histological preparation of the control group mandible, in which the number and size of trabeculae are usual, as well as the size of marrow spaces in spongious bone. Well-mineralised compact bone is present with well organized Havers' system and dense




(a) (b)

*Figure 2* SEM images of BCP/DLPLG composite biomaterial: (a) granules and (b) part of granule surface.

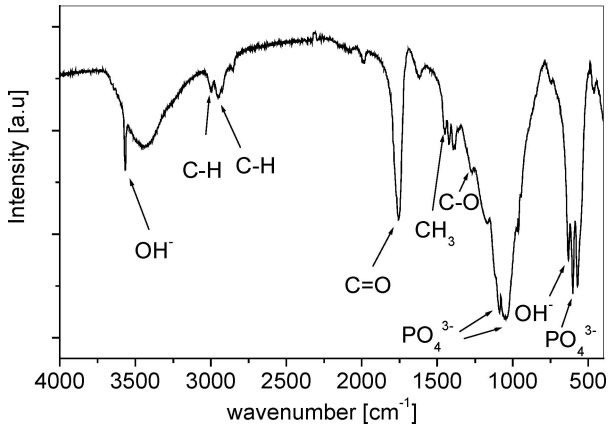



*Figure 3* IR spectrum of BCP/DLPLG composite biomaterial.

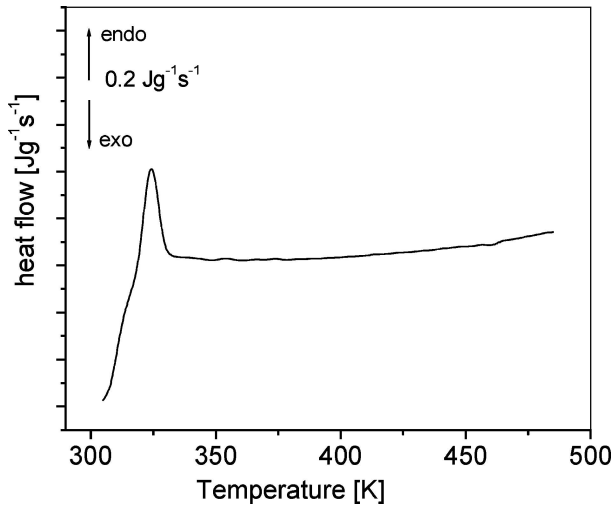



*Figure 4* DSC curves of BCP/DLPLG composite biomaterial.

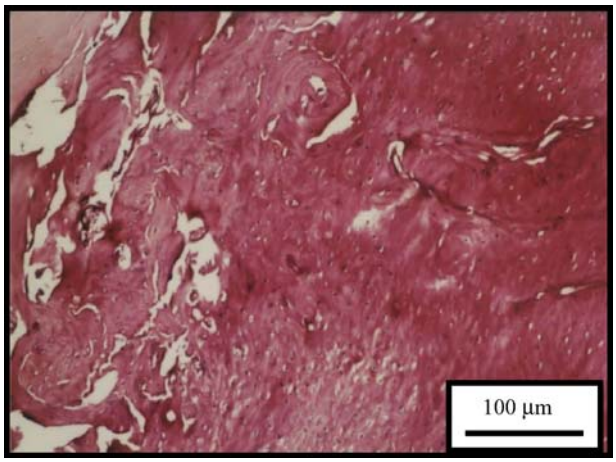



*Figure 5* Normal bone of the control group (HE, obj.x10).

cement lines, which speaks in favour of normal osteogenesis.

Cortico-medicaments, administered to experimental animals during intensive growth, development and mineralisation of bones, induced a pronounced alveolar bone decrease. Histopatological results of bone tissue with induced osteoporosis are shown in Fig. 6. At pronounced osteoporosis, physiological osteoporosive functions are weaken, so hypofunction of osteoblast oc-

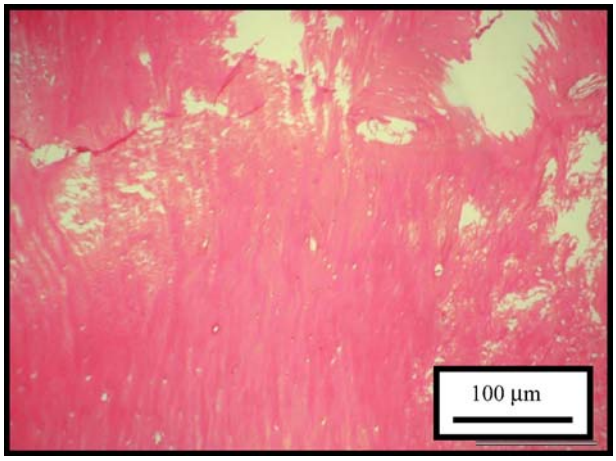
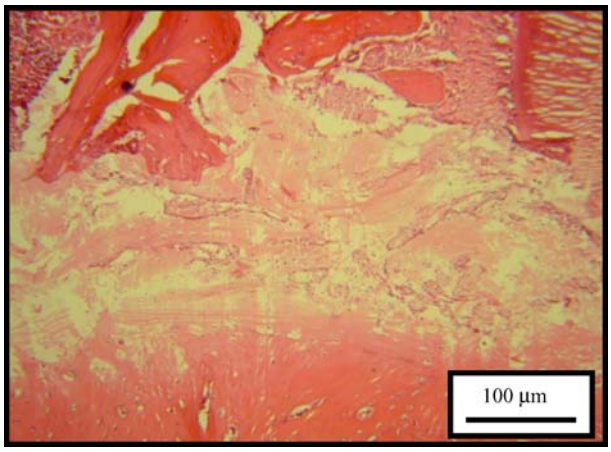
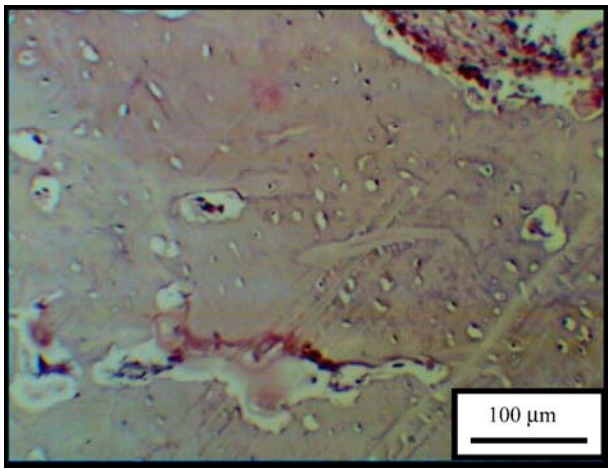



*Figure 6* Osteoporosis of alveolar bone of rat's mandible (HE, obj.x10).

curs [25]. Spongiosis is reduced and cortex extremely thinner. Histological analysis shows bone thinning and a decrease in the number of trabeculae with increasing marrow spaces in spongious bone. Compact bone is thinner and slightly mineralised. Osteomucoid increases instead of the mineral component. The Havers system is poorly organized with rare cement lines. The mentioned changes indicate inhibition of bone tissue




(a)




(b)

*Figure 7* Histological preparation after BCP/DLPLG implantation: (a) after 6 weeks and (b) after 24 weeks.

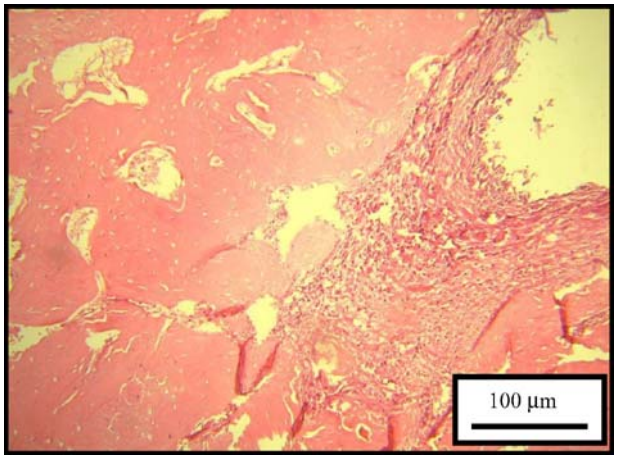
formation and prevalence of catabolism over bone anabolism (Fig. 6).

On histological preparations of alveolar bone where BCP/DLPLG was implanted, shown in Fig. 7, newformed bone tissue is noticeable, as well as spaces filled with mature bone. After 6 weeks, Fig. 7(a), there are spaces with insignificant amount of BCP/DLPLG residue imbued with fibroblasts. Polymer matrix on its surface provides good adhesion of osteoprogenitive cells, so that they induce intensive development of ostegenesis [8]. Results obtained 24 weeks after implantation are shown in Fig. 7(b). In the 24th week, formation of new bone tissue is evident, which intensely overgrows the compact as well as the spongious part of mandible that directly and positively influences remodelling of alveolar bone damaged by osteoporosis [26, 27].

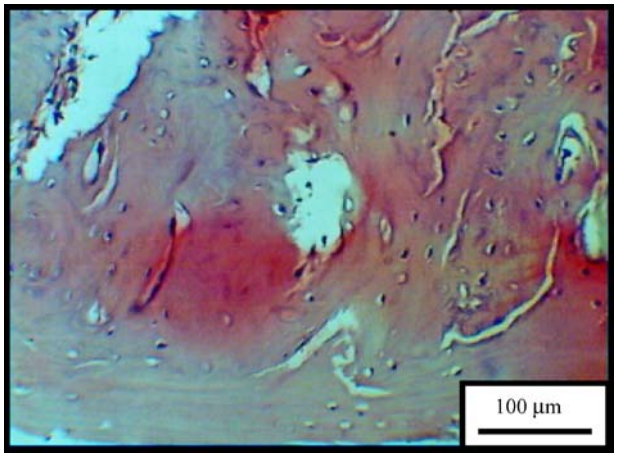
Resorption of BCP/DLPLG composite biomaterials compared to that of BCP/PLLA is faster [28], which is in agreement with the results on the polymer amorphous presented in Fig. 4. An increase in resorption rate may indicate an increase in osteogenesis rate. BCP/LPLA composite biomaterial with BCP and polymer of the same mass fractions as those in the studied BCP/DLPLG showed however different activity 6 weeks after implantation [28]. The presence of pro-inflammatory reactions with a visible bondary between the bone and the implant, as well as the presence of LPLA characterises the behaviour of this type of material.

When BCP/DLPLG composite covered with fibrin glue was implanted in osteoporosive alveolar bone of mandible, experimental group of animals, fibroblast reaction became pronounced, which reminds us of the matrix of a new bone formed already 6 weeks after intervention (Fig. 8(a)). Reparatory osteogenesis is intensive 24 weeks after implantation (Fig. 8(b)). Integration of the new with the existing bone tissue is complete in many places, so that the bondary between the tissues is barely visible. The given facts confirm intensive reparatory osteogenesis.

The best results of the process of regeneration, reparation and recuperation of alveolar bone diminished by osteoporosis were achieved after implantation of BCP/DLPLG mixed with autologous plasma. Overgrowth of implanted material with young bone tissue and creation of the Havers system is more intensive compared to other subgroups. Focal points with connective tissue residue that transfuse implanted material are increasingly rare 6 weeks after implantation (Fig. 9(a)). After 24 weeks the above-mentioned processes, characteristic of this biomaterial, are the most intensive. Cement lines are denser and represent the signs of pronounced reparatory process. Young bone

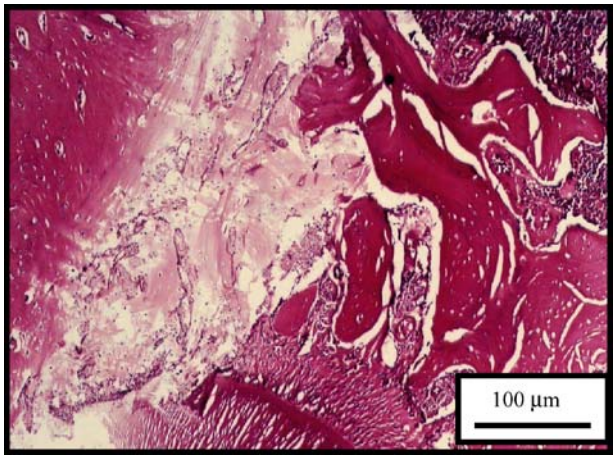



(a)

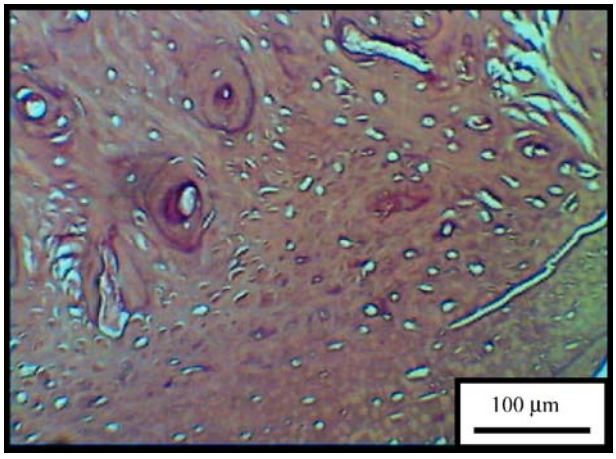



(b)

*Figure 8* Histological preparation after BCP/DLPLG/fibrin implantation: (a) after 6 weeks and (b) after 24 weeks.




(a)




(b)

*Figure 9* Histological preparation after BCP/DLPLG/plasma implantation: (a) after 6 weeks and (b) after 24 weeks.

tissue integration is complete in many places. On histological preparations of this subgroup, significantly higher degree of osteogenesis and more regular bone structure are evident (Fig. 9(b)), as well as creation of mature connective collagen tissue, which means that the reparation is entering its final phase.

Significantly higher intensity of osteogenesis in rats of this subgroup, where BCP/DLPLG mixed with autologous plasma was implanted, compared to previous subgroups, is probably a consequence of the joint action of BCP/DLPLG and growth factors [29, 30] that are present in autologous plasma and can significantly improve cell proliferation, differentiation and new bone creation.

## 4. Conclusion

BCP/DLPLG composite biomaterial was synthesized in the shape of spherical granules, 150–200 $\mu$m in diameter; each BCP particle is coated with amorphous DLPLG polymer. The composite is suitable for application as filler in reparation of osteoporosive bone tissue. Calcium phosphate present in the composite is in the form of biphasic calcium phosphate consisting of 80% calcium hydroxyapatite and 20% tricalciumphosphate.

Histological analysis of the experimental group of animals, with implanted composite biomaterial into osteoporosive bone tissue, revealed recuperation of the alveolar bone diminished by osteoporosis, starting from the sixth week of reparation. Application of BCP/DLPLG facilitated overgrowth of new-formed vascular tissue, fibroblasts and intensified the activity and adherence of osteoblasts.

Mixing of biostimulative agents (autologous plasma or fibrin) with BCP/DLPLG yielded composite biomaterial BCP/DLPLG/biostimulative agent, with whom the reparation was made. Composite with autologous plasma showed the highest reparatory results and the highest intensity of osteogenesis followed by regular bone structure formation compared to the same composite without biostimulative agents.

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