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Biogenic calcium phosphate materials implanted into canine bone defects and their biocompatibility

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Purpose To study the biocompatibility of implantation calcium phosphate (CP) materials from bovine bone tissue and its dependence on their composition **Materials and methods** The authors studied the biocompatibility of three implantation calcium phosphate materials obtained by an original technology on experimental animals (dogs). The materials obtained from bovine bone tissue by its demineralization and sedimentation out of the CP salts solutions. Serum proteins (SP) of the molecular mass of 20-30 kDa that were isolated from blood serum of the dogs with active osteogenesis were added to CP composition in order to improve the characteristics of biocompatibility. The isolation was performed using the methods of salting-out, dialysis and gel permeation chromatography. CP materials and their composites were implanted into the defects of long bone metaphyses. The study was performed using the methods of infrared spectroscopy, X-ray electron probe microanalysis, and light and scanning electron microscopy. Chemical composition of CP materials, reparative osteogenesis character and its intensity were determined quantitatively and qualitatively. The diameter of the implant particles of the tissues that filled the cancellous defect area were evaluated histomorphometrically. **Results** It was found that the implanted materials differed in biodegradation, osteoinduction and osteoconduction properties. CP materials that were the closest to bone tissue by their composition had a higher degree of biocompatibility **Conclusion** The proposed CP materials can be used in clinical practice for filling posttraumatic defects and correction of the pathological conditions accompanied by osteoporosis or bone loss.
Keywords Calcium phosphate (CP) materials, serum proteins (SP), biocompatibility, osteoinduction, osteoconduction, biodegradation, biointegration

INTRODUCTION

Biocompatible implantation materials for recovery of human bone tissue have long been a necessity in practical orthopaedics and traumatology. Due to certain limitations of autologous cancellous bone tissue, the golden standard of bone grafting, the search for novel implantation materials for bone healing is based on using both biological and synthetic substances [1, 2]. Those substances should be osteoinductive and allow for differentiation of non-committed and committed cells into the osteogenic lineage as well as be osteoconductive and provide a good interfacial contact with the newly formed bone tissue [3-6]. The above properties result in biointegration or the capability of the material to form a mechanically strong structure jointly with the newly formed bone [7]. Biodegradation, the implanted material ability to be eliminated alongside with provisory bone trabeculae during physiological reorganization, is also a mandatory property [8]. The complex of the mentioned qualities should be considered as biocompatibility or the ability of a biomaterial to perform its desired function with respect to a bone problem and an appropriate host response in order to obtain clinical success [9]. Calcium phosphate (CP) compounds, including those that are produced from the natural bone matrix, correspond to that complex of the requirements to a considerable degree [10, 11]. In vivo trials of using them for cancellous bone defect filling in dogs and sheep confirmed their low degree of immunogenicity and high biocompatibility [12, 13]. The chemical content, particle size and shapes of any CP material have an effect on its behavior when implanted [5, 14]. Therefore, the desired result of its application will directly depend on the production technology [15]. This study used three technologies for obtaining CP materials from the bovine bone tissue and was aimed at studying their properties and biocompatibility when implanted into canine metaphyseal defects, and namely the influence of their composition on biodegradation, osteoinduction and osteoconduction. We also hypothesized that blood serum proteins (SP) obtained during an active phase of osteogenesis would have a stimulating effect on bone formation and biocompatibility as they contain the utmost concentration of osteogenic factors [16, 17].

MATERIALS AND METHODS

Production of CP materials

CP compounds were obtained from adult bovine long bone diaphyses according to the following three technologies:

1) CP1: bone demineralization with a 0.5 N HCl solution and CP sedimentation using a 52.2 % NaOH water solution;

2) CP2: bone demineralization with a 0.5 N HCl solution and CP sedimentation using a 52.2 % NaOH water solution, and additionally treated with an 8M water solution of carbamide;

3) CP3: bone demineralization with a 6 N HCl solution and CP sedimentation using a 0.12 % CaO water solution.

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The three CP materials were sterilized by 20 kGy dozed β-radiation using a linear resonance accelerator of electrons LUE-8-5V (NIIEFA, Russia).

Composition of CP materials

Qualitative content of the CP materials obtained was studied using an IR Fourier spectrometer (InfraLUM® FT-02, Lumex, Russia). EPMA was applied for quantitative assessment of calcium (Ca), phosphorus (P), magnesium (Mg) and sulfur(S) in the materials of interest and dogs' compact bone tissue using the Oxford INCA Energy 200 Dispersive X-Ray Analyzer (Oxford Instruments, UK) adjusted on the JSM-840 scanning electron microscope (JEOL, Japan) by an accelerating voltage of 20 kV. The quantitative findings were presented as mean values and standard deviations (M ± SD).

Separation of blood serum proteins

Homologous SPs were obtained from blood serum of the dogs of another series that were undergoing an active phase of distraction osteogenesis in the tibia using the Ilizarov apparatus at the same time [18]. Their blood serum was diluted twice in 0.15 M NaCl solution, saturated with ammonium sulfate up to 30 %, cooled and centrifuged for removal of the precipitation with the aids of the Optima LE-80K (Beckman Coulter, USA) (40000 g, 15 min). The supernatant was saturated up to 50 % with ammonium sulfate for further centrifugation. Purification of the proteins was performed using the gel filtration (GF) chromatography system LKB (Pharmacia LKB Biotechnology AB, Sweden). The precipitate that was formed after the second phase of salting out was dissolved in 8M urea solution and fractioned according to the molecular mass by GF chromatography using the TSK gel carrier TOYOPEARL HW65S (ToyoSoda, Japan). The fractions with the output volume corresponding to the relative molecular mass ranging from twenty to 30 kDa were dialyzed against distilled water and lyophilized. Further on, 0.02 to 0.03 g of SP were dissolved in 1 ml of saline and mixed with 5.0 to 5.2 g of each of the three CPs. The saline was added again to form a paste. Then, the three CP+SP biocomposites obtained were placed into glass vials, closed with rubber caps and aluminum cups and sterilized by 20 kGy dozed β-radiation using a linear resonance accelerator of electrons LUE-8-5V (NIIEFA, Russia).

Implantation of CP materials into canine cancellous bone defects

TThe implantation was carried out on 24 adult mongrel dogs of both sexes, aged from one to three years and an average body weight of 8.8 ± 3.2 kg. Conical non-through defects (five to 7 mm in diameter, n =1 20) were produced by drilling in the proximal metaphyses of both humeral and tibial bones. The defects were filled with pasted CP materials or CP + SP biocomposites: CP I (n = 16), CP2 (n = 16), CP3 (n = 16), CP1 + SP (n = 16), CP2 + SP (n = 16), and CP3 + SP (n = 16). Six dogs were a control group, and no CP materials were used in their bone defects (n = 24). The animals were euthanized after 21 or 42 days postoperatively with an intravenous lethal dose of 5 % sodium thiopental. The surgical procedures, animal care and euthanasia followed the requirements of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986). The study was approved by the ethic board decision #3 from 12.03.2001, and the institution review board permission for publication under # 3-28 from 27.09.2013.

Histological study of biocompatibility

Tissue samples harvested from the canine metaphyseal defect area on experimental days 21 and 42 were treated according to general histological techniques [19]. Reparative bone formation in the defect gaps and CP materials biocompatibility were studied with the method of light microscopy. Celloidin sections stained with hematoxylin and eosin or by Masson techniques were studied with a light photomicroscope (OPTON Feintechnik GmbH, Germany). Digital images of the visual fields were obtained with the aids of DiaMorph complex (Diamorph, Russia) adjusted to the photomicroscope. Digital images of whole histotopographic celloidin preparations were taken using the scanning system HP ScanJet 7400C (Hewlett-Packard, USA). VideoTesT-Morfologia softwear (VideoTesT, Russia) was applied for histomorphometric study. The volume of cancellous bone in the defect (Vcb, %), volumetric density of trabeculae in the cancellous regenerated tissue (Vtr, %), and implanted material granule diameter (Dg, μm) were calculated. The measurements were taken in no less than 30 visual fields. The granules of 100 μm were graded as small, the ones sized 100-250 μm were considered average, and granules larger than 250 μm were referred to big ones. The methods of non-parametric statistics were used to process the findings, and they were presented as medians (Me) with confidence intervals (95 % CI). As far as the findings could not be referred to the normal distribution, the Mann-Whitney U-criteria were used for group difference [20]. The difference was considered significant by $p<0.05$. The data were processed with the AtteStat software, version 10.8.8 (an add-in software product to Microsoft Excel, certificate 2002611109, dated 28.06.2002).

RESULTS

IR spectrometry of CP materials

IIR spectrometry showed that alongside with Ca and P the CP materials contained the following. CP1 had small quantities of carbonate groups and a sufficiently large amount of protein compounds as its IR spectrometry revealed hydroxyl groups, carbonate ions, carboxyl groups, and amides (**Fig. 1a**). The IR spectrum of CP2 defined an extremely low content of protein substances, and the absorption intensity for carbonate ions was not high (**Fig. 1b**). CP3 contained carbonate ions as well as small amounts of protein compounds (**Fig. 1c**).

SEM and EPMA findings of CP materials morphology and composition

SEM and EPMA revealed that the CP materials were mixtures of granules and powder. Their granules were of irregular shape and of various sizes (**Fig. 2**). The quantity analysis of chemical elements is presented in **Table 1**. The quantities of Ca, P or Mg in CP1 were higher than in the natural bone tissue due to the elimination of the organic matrix. The ratio of Ca to P approximated to the ratio found in the regular bone tissue. In our opinion, the presence of S can be explained by partial sedimentation of the matrix protein components. CP3 was the most different from the natural bone tissue.

Histological and histomorphometric study of CP materials biocompatibility in the implantation area

Experimental day 21

All groups with implanted materials showed a layer of newly formed trabecular bone tissue with gelatinous bone marrow covering the bottom and the walls of the defect gaps. The gaps were mostly filled in with loose connective tissue that was abundantly vascularized by sinusoid capillaries full of blood. The CP3 group connective tissue had big amounts of mononuclear phagocytes as well as extensive cystic cavities. The granules of CP1 and CP2 were solitary in the loose connective and bone tissues (**Figs. 3a, 3b**). The attached polynuclear phagocytes were seen on their surfaces. In the biocomposite groups, the interfacial contact of trabecular and granular surfaces was close, and the capillaries of the connective and bone tissues adjoined to the particles of the materials (**Figs. 3d, 3e**). As for CP3, solitary or accumulated friable granules were seen alongside with shapeless non-structured masses surrounded by loose connective tissue (**Fig. 3c**). It seemed that CP3+SP group had an increased rate of material resorption as far as the granules in the connective tissue were rare. In both CP3 groups, the surfaces of bone trabeculae and material granules were resorbed by polynuclear phagocytes (**Fig. 3f**). Osteoinduction of the CP materials was assessed by measuring Vcb and Vtr. CP2 and CP2 + SP groups had the highest Vcb and Vtr values, and the defect filling corresponded to the one in the controls but with the higher Vtr than in the control group. Implantation of CP1 and CP3 statistically decreased the formation of cancellous bone in the regenerated area, and their Vtr did not differ from the control group. Application of CP + SP biocomposites enhanced defect filling with cancellous bone and influenced on the Vtr (**Table 2**).

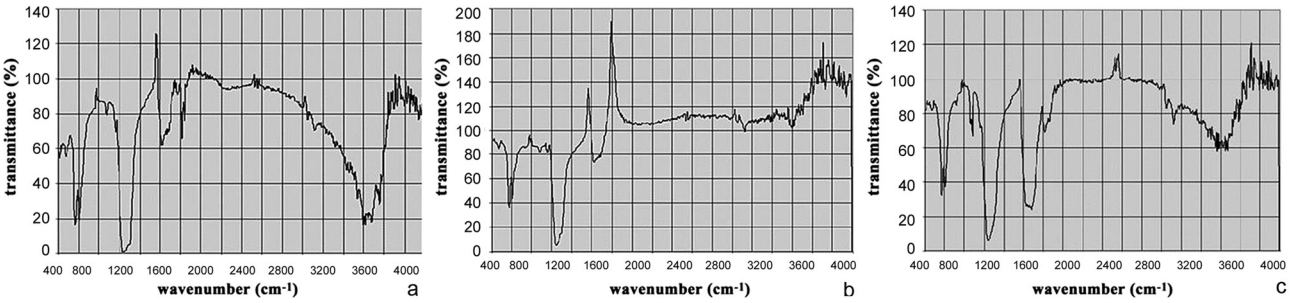

Fig. 1 IR spectrum: (**a**) CP1, (**b**) CP2, (**c**) CP3

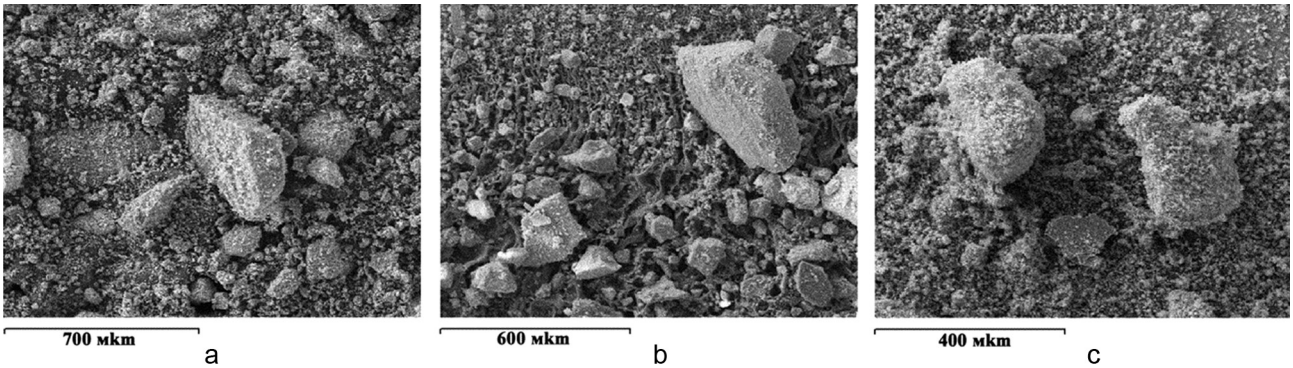

Fig. 2 CP materials structures by electron scanning: CP1 (**a**), magnification 70×, CP2 (**b**), magnification 80×, CP3 (**c**), magnification 120×

Table 1

Mean content of mineral components, M±SD (%)

	Ca	P	Mg	S	Ca/P
Bone tissue	22.8 ± 0.12	10.5 ± 0.09	0.27 ± 0.040	0.10 ± 0.004	2.17
CP1	33.18 ± 0.88	15.71 ± 0.52	0.43 ± 0.07	0.07 ± 0.03	2.11
CP2	30.20 ± 0.92	14.72 ± 0.82	0.59 ± 0.11	0	2.05
CP3	30.07 ± 0.87	10.25 ± 1.14	0.11 ± 0.03	0	2.93

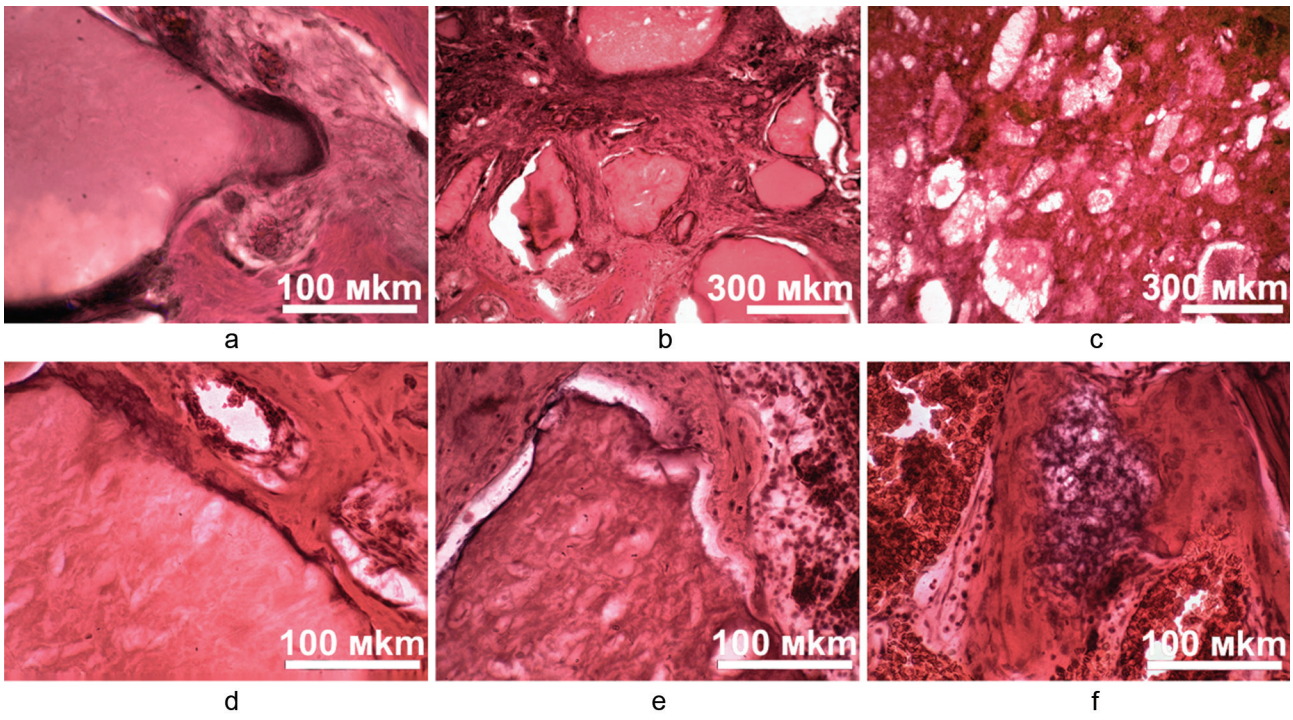

Fig. 3 CP granules in the defect tissues on day 21 of the experiment: CP1 (**a**), CP2 (**b**), CP3 (**c**), CP1+SP (**d**), CP2+SP (**e**), CP3+SP (**f**). Hematoxylin and eosin staining: Lens 40× (**a, d-f**), eye-piece 10×. Lens 10×, eye-piece 10× (**b, c**)

Table 2

Morphometric parameters of new bone tissue in the defect (21 days of experiment)

Parameter	**Control group**					
	Me		*95 % CI*			
Vcb	50.3		44.2–54.0			
Vtr	29.6		23.7–34.6			
	CP1		**CP2**		**CP3**	
	Me	95 % CI	Me	95 % CI	Me	95 % CI
Vcb	31.5*	27.6–33.8	45.3	38.3–53.0	25.4*	20.6–31.6
Vtr	32.7	29.3–37.6	44.9**	39.3–52.5	32.5	27.6–41.8
	CP1+SP		**CP2+SP**		**CP3+SP**	
	Me	*95 % CI*	*Me*	*95 % CI*	*Me*	*95 % CI*
Vcb	52.7	40.1–61.5	56.6	51.3–65.9	50.5	37.6–55.8
Vtr	43.9**	40.8–46.8	30.3	27.5–38.6	29.1	27.6–35.0

* – significant decrease relative to controls; ** – significant increase relative to controls

Experimental day 42

Cancellous bone tissue prevailed in the defects of all experimental groups (**Fig. 4**). Bone formation was higher in CP1 groups, especially in CP1+SP. The mean Vcb exceeded the values of the controls, and the Vtr was close to the values in the controls. The trabeculae were formed of lamellar membranous bone with red bone marrow in between. In CP2 and CP3 groups, the newly formed cancellous bone tissue was undergoing an active reorganization. The Vcb in their regenerated area was lower while the Vtr was higher as compared with the controls. The SP supplement in CP2 did not have any significant effect on bone formation parameters while their mean values in the CP3 + SP combination were reduced (**Table 3**). CP1 granules were seen in bone substance or had a close interface with lamellar trabeculae (**Fig. 5a**). CP2 granules were dispersed in the bone substance, intratrabecular spaces and fibrous connective tissue (**Fig. 5b**), and solitary granules were resorbed by polynuclear phagocytes. CP3 particles were identified as large flock-like accumulations in the fibrous connective tissue that was infiltrated with monocytes and macrophages (**Fig. 5c**). The SP supplement enhanced the osteoconductivity of CP1 and CP2. Also, their granules contacted bone marrow capillaries and connective tissue (**Figs. 5d, 5e**). The implanted material completely resorbed in the CP3+SP group (**Fig. 5f**).

CP material particles in the implantation gap

Experimental day 21

The study of the samples harvested detected the granules with the biggest averaged diameter of 400 μm in CP1 implantation gaps. The mean diameter of CP2 and CP3 particles averaged 260 and 140 μm, respectively. The SP supplement had a considerable effect on their biodegradation, as the mean value of CP1 particles decreased approximately down to 220 μm, and on the contrary the mean diameter of CP2 and CP3 particles grew up to 340 and 215 μm, respectively (**Table 4**). CP1 and CP2 materials showed prevalence of large and median granules while CP3 had mostly median and small ones. The SP stimulated mostly resorption of large granules in CP1 and CP2. However, small and median particles resorbed in the CP3 + SP group resulting in the reduction of their portion (**Fig. 6a**).

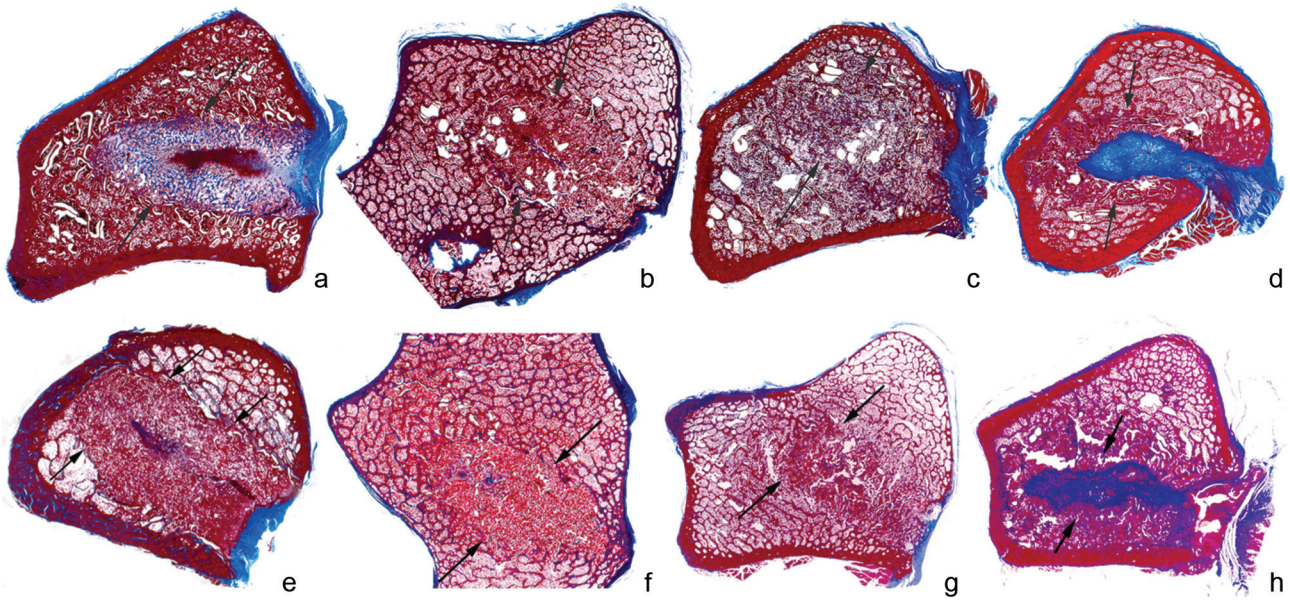

Fig. 4 Filling in the cone-shaped defect in the proximal tibia: control group (**a, e**), CP1 (**b**), CP2 (**c**), CP3 (**d**), CP1+SP (**f**), CP2+SP (**g**), CP3+SP (**h**) on day 42 of the experiment. Scans of histotopographic celloidin sections. Masson staining

Table 3

Morphometric parameters of new bone tissue in the defect (42 days of experiment)

Parameter	**Control group**					
	Me			*95 % CI*		
Vcb	71.3			62.7-79.9		
Vtr	13.5			7.6-15.9		
	CP1		**CP2**		**CP3**	
	Me	95 % CI	Me	95 % CI	Me	95 % CI
Vcb	85.8	74.5–90.4	67.7	63.4–72.0	58.3	49.8–70.4
Vtr	12.6	9.6–15.5	29.4**	26.0-32.2	32.8**	24.1–39.6
	CP1+SP		**CP2+SP**		**CP3+SP**	
	Me	95 % CI	Me	95 % CI	Me	95 % CI
Vcb	88.1**	84.9-91.7	78.7	74.3–85.0	43.5*	36.1–49.7
Vtr	12.5	10.8–18.3	23.9**	20.7–26.1	17.6	15.9–18.9

** – significant decrease relative to controls; ** – significant increase relative to controls

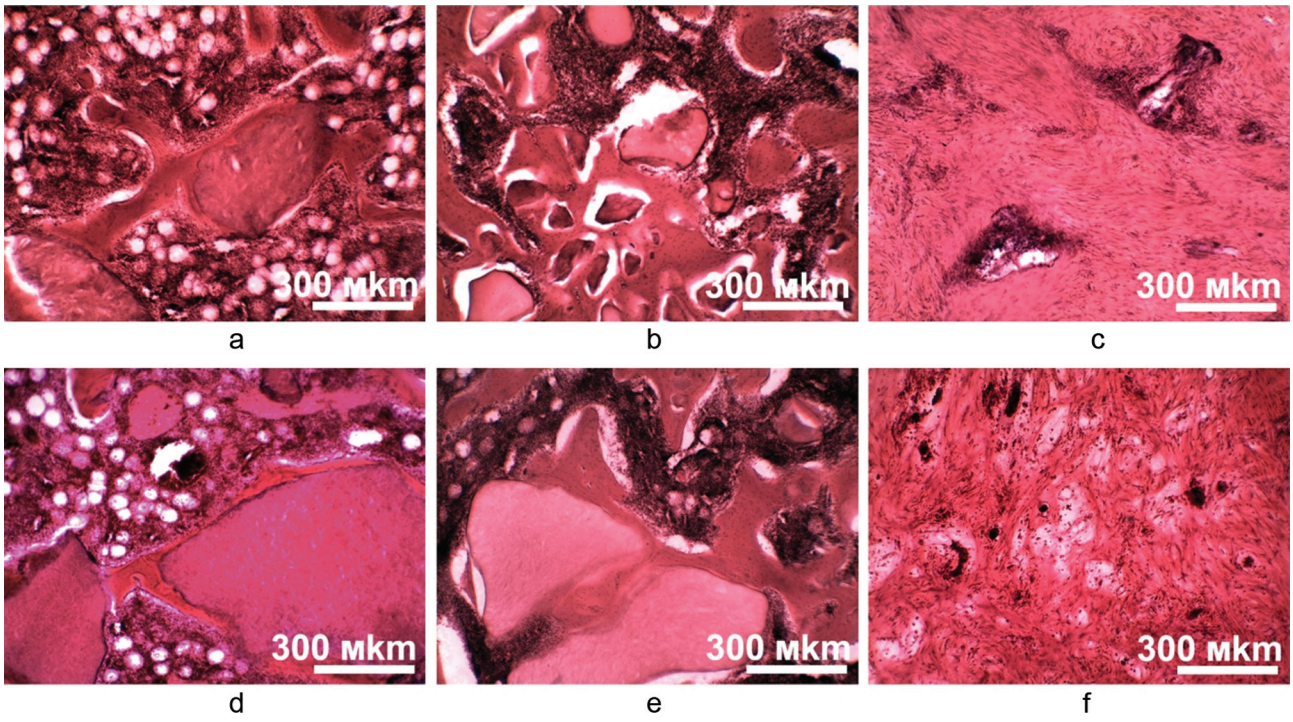

Fig. 5 CP granules in the defect tissues on day 42 of the experiment: CP1 (**a**), CP2 (**b**),CP3 (**c**), CP1+SP (**d**), CP2+SP (**e**), CP3+SP (**f**). Hematoxylin and eosin staining. Lens 10×, eye-piece 10×

Table 4

CP granules diameter in cancellous bone defects (μm)

CP materials	**21 days of experiment**		**42 days of experiment**	
	Me	*95 % CI*	*Me*	*95 % CI*
CP1	448	410–492	372	337–415
CP1+SP	224	202–252	302	278–338
CP2	262	231–287	270	246–301
CP2+SP	341	318–371	223	199–253
CP3	141	129–158	147	128–159
CP3+SP	215	204–249	0	0

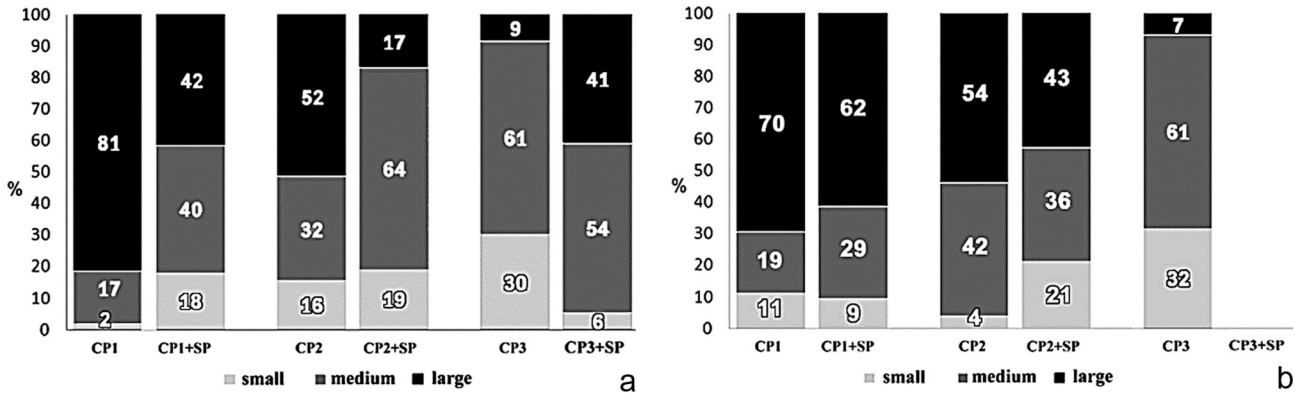

Fig. 6 CP particles fractions in the defect tissues according to their size (%): 21 days of the experiment (**a**), 42 days of the experiment (**b**)

Experimental day 42

The mean diameter of particles did not change considerably as compared to the previous experimental period, if CP materials were implanted alone. However, the mean diameter of CP1+SP granules increased considerably while the effect was the contrary in CP2 + SP group. In CP3 + SP group, the particles were resorbed completely (**Table 4**). The change in the particle sizes when compared

with the previous study period reflected the balance between the processes of biodegradation and biointegration. When the CP materials were implanted without the SP supplement, a slight difference from the values of the previous experimental period proved that resorption went down. It was conditioned by osteointegration in cases of CP1 and CP2, and by incapsulation of dense connective tissue in the CP3 group. Introduction of SP into the materials accelerated biodegradation that was manifested by the decrease in the portion of small particles and increase in the fraction of large integrated into bone tissue granules by CP1 + SP and CP2 +S P application while the CP3 + SP group showed a complete degradation, being substituted by loose connective tissue (**Fig. 6b**).

DISCUSSION

Our analysis showed that CP1 and CP2 were close to the natural bone tissue in their mineral content. The CP degradation products (calcium and phosphate ions, polypeptides) were natural metabolites and induced biological reactions similar to those by natural bone remodeling. The presence of considerable amounts of protein compounds in CP1 could stimulate adhesion and osteoblastic differentiation of insufficiently differentiated cells, and thus determined its osteoconduction and osteoinduction properties [21]. Small amounts of Mg could improve the mechanical features of CP1, its osteoinduction and, possibly, reduce its resorption rates or biodegradation [22]. However, the CP1 osteoinductivity seems to be not optimal as the bone formation rate was delayed when compared with the control group. Most likely, this phenomenon was associated with the size of CP1 particles that averaged 400 μm during the experiment. Theoretical calculations determined that the most preferable Dg range was from one hundred to 200 μm as it could provide the combination of both highly specific surface for resorption and the space between the granules for ingrowths of vessels and new bone deposits [23].

The CP2 contained less Mg, proteins, and was not osteoconductive. However, during the first three weeks of its implantation the defect filling with new cancellous bone was higher than in the control group and proved its high osteoinduction. The mean Dg reduced to 260 μm during that period of time and approximated to the optimal values for providing the ingrowth of the newly formed tissues. However, bone formation went down after 21 days as compared to the control group. It was most likely associated with the decrease of biodegradation rates of the material as far as the ratios of small, median and large particles remained unchanged up to 42 days of the experiment.

The CP3 material was the most different from the biological bone tissue as compared to CP1 or CP2. It did not contain protein compounds, and the Ca/P ratio of 2.93 exceeded the upper limit of the optimal range of 2.0 to 2.5 [24]. The mean Dg was 140 μm during the whole experiment that was optimal for biodegradation. The lack of bone matrix macromolecules and Mg could reduce the mechanical strength of its granules. It resulted in their destruction and dense unstructured accumulations of the particles of a smaller diameter. In general, CP3 implantation reduced the rate of bone tissue defect filling as those large accumulations of the material could be perceived as foreign bodies. The results obtained correspond to the findings that revealed that the CP particles of the diameter less than 100 μm depressed osteoblast function [25]. Another possible reason of low osteoinduction could lie in the structural features of the CP3 material. It was established that CP materials that have the ratio of C/P > 2 and contain CO to which CP3 is referred would resorb and would not have osteoinduction or osteoconduction properties [24, 26].

The SP added to the CP materials improved osteoinduction, osteoconduction and accelerated biodegradation. CP1 + SP and CP2 + SP biocomposites showed the highest biocompatibility while the CP3 + SP combination accelerated biodegradation and improved osteoinductivity but had no effect on osteoconductivity. Those effects could be explained by the high capacity of CP materials to adsorb bioactive compounds, in particular, serum polypeptides having the molecular mass within the range of 20 to 30 kDa obtained from the animals that were undergoing an active distraction osteogenesis. The serum fraction contained osteogenic growth factors due to active osteogenesis [17, 27, 28]. Their effect on mineralization, resorption and biointegration when added to CPs has been lately much discussed, and general acceleration of bone regeneration was noted [29]. Several types of CP materials have been allowed for use and are currently available on the market but their clinical trials were not numerous [30]. A better understanding of the mechanism of their interaction with the biological tissues could offer novel potential rational strategies for bone repair [31].

CONCLUSION

Our study on a canine model shows that osteoconduction, osteoinduction and biodegradation of the biogenic calcium phosphate materials developed depend on the production technology. The CP materials that are close to the natural bone can improve bone formation and can be used alongside with homologous serum proteins for better osteogenesis and

biocompatibility. We opine that the materials developed by us may find their place in the management of bone injuries including the ones associated with marked osteoporosis as well as for posttraumatic bone defects filling, bone cyst management or compensation of bone stock during bone grafting procedures.

Declaration of interest

The authors declare no conflict of interest.

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