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The effect of osteosubstitution by platelet-rich autofibrin and hydroxyapatite ceramic with β-tricalcium phosphate on biochemical parameters of blood in rabbits

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Abstract: The paper presents the results of a rabbit blood biochemical study after the substitution of simulated defects in spongy and compact bone tissue by platelet-rich fibrin and its combination with hydroxyapatite granules with β-tricalcium phosphate. The aim of the work is to establish the biochemical features of reparative osteogenesis in the time of osteosubstitution by platelet-enriched fibrin and its combination with hydroxyapatite and β-tricalcium phosphate. Bone defects (d = 3 mm) were modeled in rabbits from control and experimental groups (n = 12) under general anaesthesia. In the control group, a replacement was performed with autologous fibrin enriched with platelets (PRF), and in the experimental group, it was combined with hydroxyapatite ceramics. Content of biochemical substances was determined: soluble fibrin, the activity of antithrombin-III in blood plasma, nitric oxide (NO), haptoglobin, and markers of bone metabolism (bone isoenzyme alkaline phosphatase (BAP) and tartrate-resistant acid phosphatase (TrAP) in serum. Measurements were performed with a spectrophotometer Stat Fax 4500. It is established that the dynamics of changes in biochemical parameters reflect certain features of osteosubstitution by different materials. In particular, the inflammatory-resorptive stage of reparative osteogenesis in the period up to the 7th day is characterized by an increase in blood concentrations of NO, haptoglobin, and soluble fibrin. However, the intensity of the acute phase in the case of combined osteosubstitution is significantly lower. The next peak of NO concentration on the 14th day indicates early angiogenesis with combined osteosubstitution. The two-phase degree of osteoresorption at the level of TrAP is significantly lower than combined osteosubstitution, and the intensity of osteogenic processes at the level of BAP, on the contrary, earlier. Also used biochemical parameters can be prognostic criteria for the analysis of fracture consolidation in conditions of osteosubstitution with different materials.

Keywords: Platelets, bone markers, tartrate-resistant acid phosphatase, bone alkaline phosphatase isoenzyme

1. Introduction

Reparative osteogenesis is a cascade-complex molecular-biological and histomorphological process [1]. It is quite long in time; therefore, it often makes it difficult to consolidate fractures, which prompts the search for new methods and means aimed at optimizing reparative osteogenesis [2–4]. This is topical for human medicine, veterinary traumatology, and orthopedics, especially in dogs, horses, and exotic animals.

Thus, among bone injuries in dogs, a fairly significant proportion of comminuted fractures is 60%. With this type of bone pathology, there is a high risk of dysregeneration or loss of reparative potential [5–7]. In any case, in the area of bone injury, hemorrhage and the formation of a hematoma occur, the structural and functional elements of which are the matrix for the deployment of regenerative processes [8]. At the same time, it is fibrin, which is a component of the hematoma, that ensures the physiological stability of its elements and functions as the primary scaffold [9], which is gradually infiltrated by fibroblasts and endothelial cells with subsequent collagen synthesis, vascular network ingrowth from the edges of the maternal bone to the center. This is how simple fractures heal by primary intention [7]. However, during the bone fragments' reposition and their osteosynthesis in case of fragmental fractures, the hematoma is destroyed and removed along with bone fragments, which leads to a decrease in osteoregenerative potential [10–12].

To optimize the healing of fractures with significant bone defects, a number of osteoreplacement materials with osteoconductive and osteoinductive properties are currently being proposed, including in companion animals [13]. Although bone autotransplants are still considered the most optimal material for bone defects' restoration, their use, especially in veterinary orthopedics is limited [2] mainly due to their insufficient volume and additional injury to the patient [14].

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The materials offered for osteoreplacement include bioactive ceramics, bioactive glass, and biological and synthetic polymers. Now the most promising in this case is calcium phosphate ceramics–hydroxyapatites, tricalcium phosphates, and their combinations, which have [14,15] biocompatibility, sufficient osteoconductivity, and gradual biodegradation [13,15–21].

Induction of reparative osteogenesis occurs through various growth factors: platelet growth factor (PDGF) [2], transforming growth factor-beta (TGF-b) [22], fibroblast growth factor (FGF) [23], insulin-like growth factor-1 (IGF-1) and 2 (IGF-2) [24], vascular endothelial growth factor (VEGF) [10], epidermal growth factor (EGF) and interleukin 8 (IL-8) [2], keratinocyte growth factor (KGF) and connective tissue growth factor (CTGF) [25–27]. It is these growth factors that provide the cellular type of bone tissue's regeneration characteristic [28].

Various materials for osteoreplacement, for the most part, have predominantly osteoconductive properties, which lead to their combination with components capable of inducing and regulating the molecular biological mechanisms of reparative osteogenesis [29,30].

At the same time, platelet-rich fibrin (PRF) is a biomaterial that has been widely used in regenerative medicine [31,32]. It provides both adhesive processes for osteogenic cell migration and is a strong source of growth factors. A significant amount of them is contained in platelet α-granules, which, when activated [33], provide attachment, migration, proliferation, and differentiation of cells involved in reparative osteogenesis. Studies [34] indicate that in the case of PRF, the release of growth factors occurs gradually over 10 days compared with the platelet-rich plasma (PRP)–first-generation PRP materials.

Previously, we [35–38] during histomorphological monitoring of technologies for obtaining platelet masses with fibrin established their optimal parameters to achieve the highest concentration of platelets. We also proved clinically, radiologically and histomorphologically osteoinductive properties of platelet-rich autofibrin under conditions of model fractures in rabbits.

The aim of the work is to establish the biochemical peculiar features of reparative osteogenesis in case of osteosubstitution by platelet-enriched fibrin and its combinations with hydroxyapatite and β-tricalcium phosphate.

2. Materials and methods

2.1. Experiment design

The experiment protocol was approved by the Ethics Committee of the Bila Tserkva National Agrarian University on the treatment of animals in scientific research and the educational process (protocol no. 1 dated January 23, 2019).

The studies were carried out on clinically healthy rabbits of the Californian breed at the age of 6 months and bodyweight of 2.5–3 kg, which were kept in the vivarium of the Bila Tserkva National Agrarian University, in individual cages with combined lighting and daily cleaning according to species needs. Rabbits had unlimited access to water, feeding was provided with a compound feed of the trademark "Selevana" (Kyiv region, Boryspil) at the rate of 200 g per head per day. For this purpose, control and experimental groups of animals were formed (n = 12).

2.2. Defect modeling

Perforated defects (d = 3 mm) were modeled with a drill in compact bone tissue–the middle of the diaphysis of the radius' dorsolateral surface, and in spongy–the tibial crest from the medial side. Anesthetic management included acepromazine-thiopental general anesthesia and local anesthesia with 0.5% lidocaine.

2.3. Osteoreplacement

Bone damage in the control group was filled with autologous PRF, and in the experimental group with a combination of autologous PRF and hydroxyapatite with β-tricalcium phosphate (HA/β-TCP–700). Interrupted sutures were applied to soft tissue wounds, which were removed on the 7th day. During the entire period of the study, rabbits were clinically observed.

2.4. Osteoreplacement materials

Implants are two-phase calcium phosphate granules HA/β-TCP–700, consisting of 70% hydroxyapatite and 30% β-tricalcium phosphate with a granule size of 700 μm, synthesized at the Institute of Materials Science of the National Academy of Sciences of Ukraine (Kyiv).

Preparation of autologous PRF (Figure 1): blood was collected from the jugular vein using a 5 mL syringe, which was transferred into a sterile plastic tube without anticoagulant and centrifuged at 3000 rpm for 10 min. A clot of fibrin enriched with platelets formed in the middle part, blood serum was separated above and around it, and erythrocytes were located in the lower part of the tube. Using sterile tweezers and scissors, the erythrocyte mass was separated from the autobio material. The required volume of PRF was taken to fill the bone defect from the lower part of the clot with the highest concentration of platelets (Figure 1.A), as we previously established [38].

2.5. Biochemical studies

For biochemical examination, blood sampling was performed from the external jugular vein of rabbits before anesthesia, on the 3rd, 7th, 14th, 21st, and 42nd day after modeling and replacement of defects in bone tissue.

In the blood serum, the level of NO was determined by the Green method in Golikov's modification [39,40]. Cadmium granules were used as a reducing agent.

In the blood plasma, the level of soluble fibrin was determined by phosphate buffer precipitation [41]. The

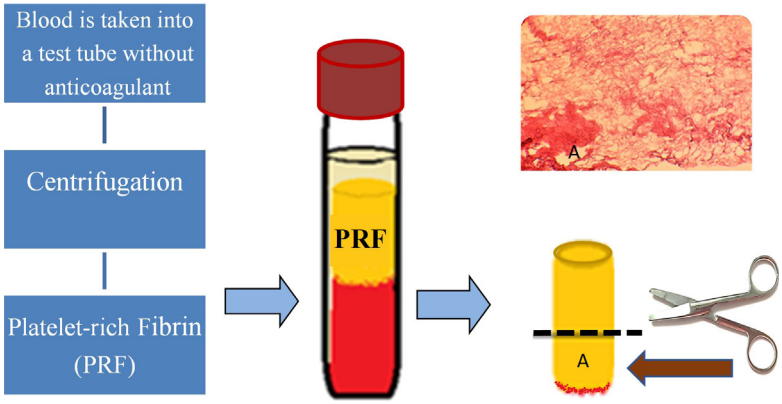

Figure 1. Scheme of obtaining fibrin enriched with platelets.

activity of antithrombin-III (AT-III) by using the kits of LLC "Laboratory Granum" (Ukraine), expressed as a percentage of normal.

The content of haptoglobin in the serum was determined by reaction with rivanol kits of PJSC "Reagent" (Ukraine).

BAP activity was determined in blood serum. The bone isoform's activity of alkaline phosphatase was calculated by the difference between the activities of total and thermostable alkaline phosphatases. The test principle is based on the cleavage of phenyl phosphate to form phenol. Alkaline phosphatase is able to break down the substrate –4-nitrophenyl phosphate with the formation of phosphate and 4-nitrophenol. The reaction was stopped with a 30 mM solution of Trilon B in 1 M NaOH. The released reaction product –4-nitrophenol gives a yellow color in an alkaline medium. Its intensity is determined spectrophotometrically at $\lambda = 410$ nm and is a measure of alkaline phosphatase activity, expressed in units/L, where units–the amount of μmol of 4-nitrophenol, which is formed under the action of alkaline phosphatase for 1 min at +37 °C and is contained in 1 L of serum. To determine the activity of thermostable alkaline phosphatase, serum was first aged at +56 °C for 15 min, after which the samples were transferred to an ice bath for 5 min [42].

The level of TrAP in blood serum was determined by sets of LLC "Laboratory Granum". The principle of the method is the cleavage of alpha-naphthyl phosphate into alpha-naphthol + phosphate using as a specific agent for the bone isoenzyme acid phosphatase tartrate. Measurements were performed with a spectrophotometer Stat Fax 4500.

Statistical processing of the results was performed using the program Statistica 10 (StatSoft Inc., USA, 2011). The data in the tables are presented as $x \pm SD$ ($x \pm$ deviation). Differences between groups were determined by ANOVA, $p < 0.05$ was considered significant (adjusted for Bonferroni correction).

3. Results

On the first day following surgery, a moderate inflammatory reaction was noted in the area of the formed defect, manifested by slight edema and an increase in local temperature. The functions of the limbs were not impaired. A small amount of serous exudate was released from the edges of the wound, which dried up and formed a crust. On the 7th day in the animals of the control and experimental groups, there was no swelling and soreness. The edges of the wound were connected by connective tissue adhesions. This was the basis for removing the sutures.

3.1. Biochemical research

Dynamic changes in the concentration of NO in the blood were established, which had certain patterns in groups of animals (Figure 2), which is a reflection of the intensity of the inflammatory process in the early posttraumatic period. During the experiment, except for the 42nd day, the difference between the groups in terms of NO level was significant. From days 7th to 14th, it decreased, and its next slight increase was noted from days 21st to 42nd, which was significantly higher ($p < 0.001$) than the indices of animals in the preoperative period. In the experimental group, a phase pattern of NO level dynamics was established with peaks on the 3rd, 14th, and 42nd days.

When determining the concentration in the blood plasma of soluble fibrin it was found to increase significantly during the first 7 days (Table 1) with a subsequent dynamic decrease. In experimental animals, on day 21st, it was 1.5 times ($p < 0.05$) less as evidence of dynamic normalization of the hemostasis response.

At the same time, the activity of AT-III in the plasma of animals of both groups did not change significantly (Table 1). Its decrease on the 3rd day of the posttraumatic period was not lower than 85%.

It was found that the content of haptoglobin in blood serum (Table 1) reached its peak value in two stages: on the 3rd and 14th days, which was significantly higher (p

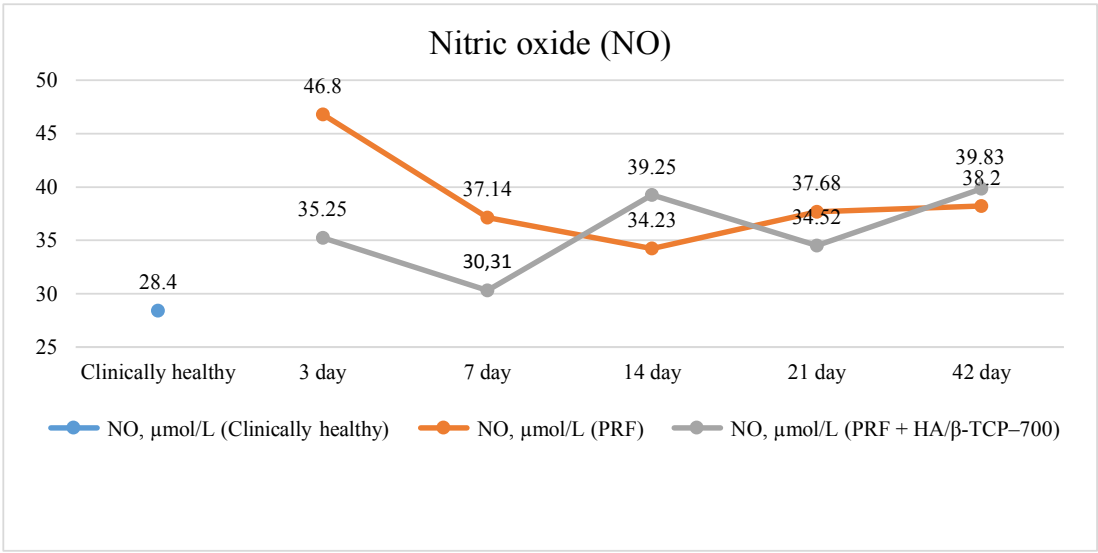

Figure 2. Nitric oxide's dynamics level in rabbit serum in reparative osteogenesis.

Table 1. Dynamics of biochemical parameters in the serum and plasma of rabbits.

	Indicators	**Soluble fibrin, mg%**	**Haptoglobin, g/L**	**Antithrombin -III, %**
	Clinically healthy rabbits (n = 12)	0.14 ± 0.01	1.59 ± 0.02	95.57 ± 0.84
3rd day	PRF Group (n = 4)	13.26 ± 0.74•••	2.06 ± 0.24	88.5 ± 1.70••
	Group PRF + HA/β-TCP–700 (n = 4)	12.12 ± 0.93•••	1.9 ± 0.01•••	88.13 ± 0.92•••
7th day	PRF Group (n = 4)	13.64 ± 1.35•••	1.51 ± 0.01••	96.14 ± 1.55
	Group PRF + HA/β-TCP–700 (n = 4)	14.39 ± 0.65•••	1.4 ± 0.02•••***	98.5 ± 2.07
14th day	PRF Group (n = 4)	4.67 ± 0.47•••	1.82 ± 0.01•••	96.33 ± 2.19
	Group PRF + HA/β-TCP–700 (n = 4)	5.76 ± 0.63•••	1.91 ± 0.04•••	97.3 ± 1.71
21st day	PRF Group (n = 4)	4.11 ± 0.33•••	1.4 ± 0.02•••	93.6 ± 0.81
	Group PRF + HA/β-TCP–700 (n = 4)	2.7 ± 0.44•••*	1.47 ± 0.03••	94.8 ± 1.50
42nd day	PRF Group (n = 4)	2.20 ± 0.13••	0.86 ± 0.10•••	95.33 ± 0.88
	Group PRF + HA/β-TCP–700 (n = 4)	1.89 ± 0.12•••	0.78 ± 0.09•••	92.65 ± 1.45

Note: control group–the defect was replaced by PRF; experimental – PRF + HA/β-TCP–700; * – $p < 0.05$; ** – $p < 0.01$; *** – $p < 0.001$, compared with the control group in this period of the study; • – $p < 0.05$; •• – $p < 0.01$; ••• – $p < 0.001$ compared to clinically healthy animals.

< 0.001) than in clinically healthy animals. A significant difference ($p < 0.001$) between the experimental and control groups in terms of the haptoglobin content was established only on the 7th day.

The serum level of the BAP (Figure 3) during all 42 days of the study was significantly higher in both groups than in clinically healthy animals. Meanwhile, in the experimental group, it turned out to be significantly higher on the 7th ($p < 0.001$) and 42nd ($p < 0.05$) days.

The level of TrAP (Figure 4) also throughout the study in both groups was significantly higher than in clinically healthy rabbits. At the same time, in both cases, two phases

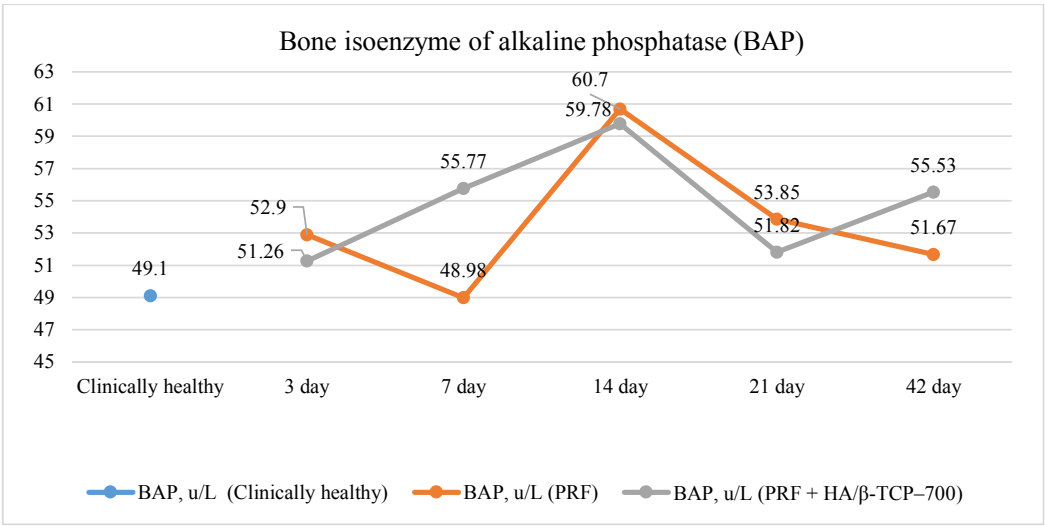

Figure 3. Dynamics of alkaline phosphatase's bone isoenzyme in reparative osteogenesis in rabbits.

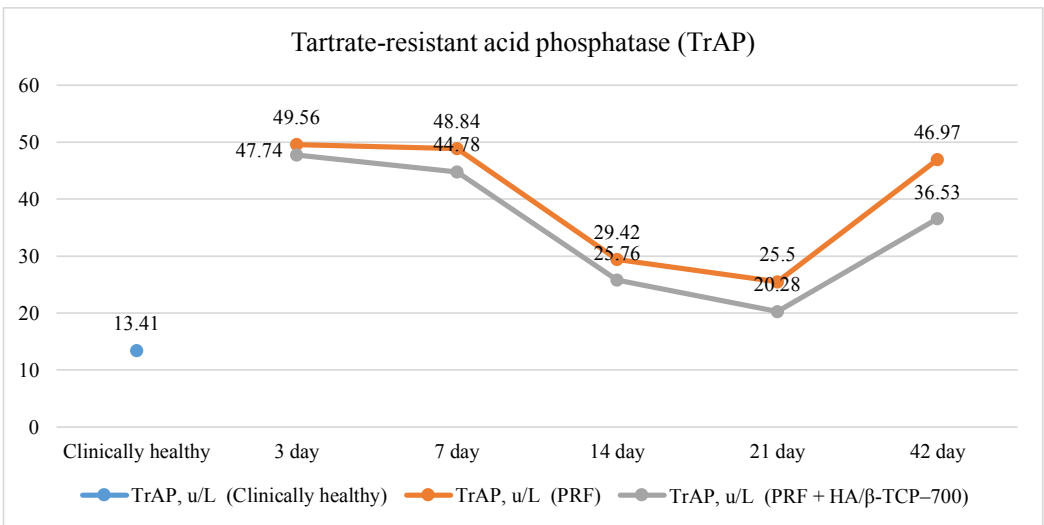

Figure 4. Dynamics of tartrate-resistant acid phosphatase in reparative osteogenesis in rabbits.

of the peak values of this bone resorption's biochemical marker were traced–in the period of 3–7 days and 42 days. The activity of TrAP on the 3rd, 7th, 21st, and 42nd days in animals of the experimental group was less than in animals of the control group ($p < 0.05$).

Considering the bone isoenzyme's dynamics (ratio) (Figure 5) of the BAP and TrAP coefficient, it follows that in the case of osteosubstitution with PRF + HA/β-TCP–700, starting from the 7th day, the osteogenic reaction is significantly more powerful than when using only PRF in bone defects.

In general, the dynamics of the studied biochemical parameters reflect certain features of osteosubstitution by different materials.

4. Discussion

One of the priority areas in solving the problem of restoring the reparative potential of bone tissue is generally recognized as osteoreplacement. The need for its use arises not only in cases of significant amounts of bone loss in comminuted fractures but also in arthroplasty or osteoplasty of meta-epiphyseal areas in arthrological pathology, in systemic osteopathies or neoplasia processes in bone tissue. Although a fairly large number of osteoreplacement materials have now been proposed, however, questions about the degree of their osteoconductive, osteoinductive, and osseointegrative properties, biodegradation, and nosological validity remain open. To a large extent, the ability of an osteoreplacement material to restore bone

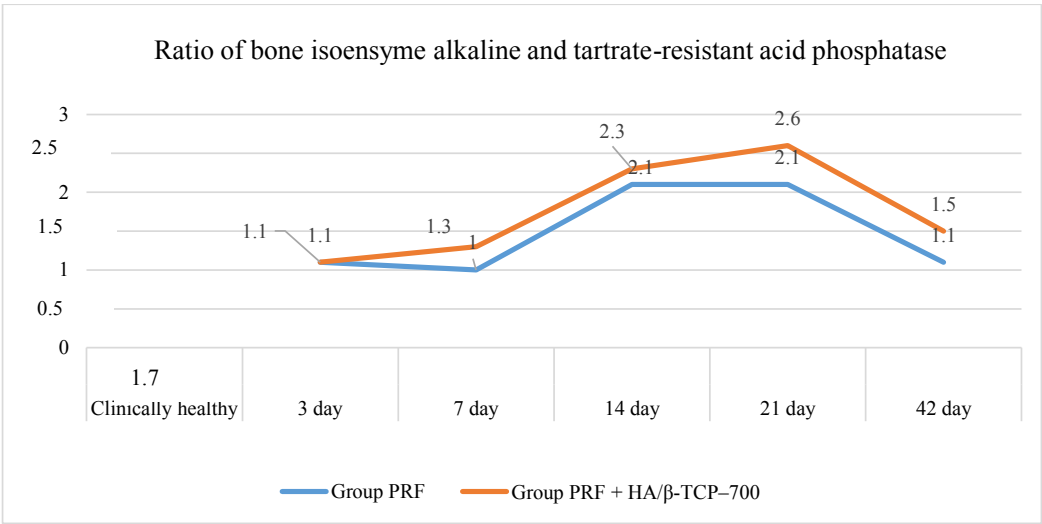

Figure 5. Dynamics of the ratio of bone isoenzyme alkaline and tartrate-resistant acid phosphatase in reparative osteogenesis in rabbits.

tissue depends on the presence of osteoinductive properties in it. They are trying to be achieved by including various compounds in the composition of composite materials or by combining them with other biomaterials [1–4].

The use of products containing an increased concentration of platelets in their composition can provide osteoinduction due to components that are concentrated in platelet α-granules: PDGF, TGF-b, FGF, IGF-1, IGF-2, VEGF, and others [33,34,43].

In particular, in the last decade, clinical and experimental data on the effect of PRF on the repair of various tissues have been accumulated. However, information on the use of calcium phosphate ceramics in combination with PRP-technologies is quite limited. It requires further in-depth study of PRP-technologies products' osteoinductivity realization in combination with calcium phosphate materials.

Previously obtained results of macro- and histomorphological research results [32,38,43,44] showed that hydroxyapatite ceramics with β-tricalcium phosphate when PRF is added, acquire pronounced osteoinductive properties, confirmed by early osteoblastic and endothelial reactions. The research results presented here are quite extrapolated from histomorphological ones.

The oxygenation degree affects significantly the production of type I collagen in the bone tissue and contributes to the induction of angiogenesis processes, which in turn ensures the dynamism and success of reparative osteogenesis. NO is a free radical, an angiogenesis inducer, produced in macrophages, neutrophils, bone marrow cells, eosinophils, and monocytes with an inducible form of NOS [45]. Its level in the early posttraumatic period increases, which is more pronounced in the control group. The subsequent rise peaks on the 14th day in the experimental group and on the 21st day in the control group due to its production and due to the endothelial NOS. In this case, the expression of interleukins and integrins by endothelial cells occurs, which causes cell migration and differentiation and promotes the formation of the vascular tubules' network in the repair zone [46,47]. Our previous work [48] and other studies [46,47] indicate a positive effect of NO donators on angiogenesis and microcirculation in the fracture zone due to a moderate increase in the level of nitric oxide in the blood and increased synthesis of tissue plasminogen activator.

The results of the presented study indicate an earlier increase in the NO level in the case of bone defect substitution by PRF + HA/β-TCP–700, compared with the independent use of PRF. This fact may indicate a faster development of the endothelial reaction, and hence the formation of the vasculature, reflecting an earlier initiation of angiogenesis in the experimental group in the bone injury area.

Haptoglobin is one of the major acute-phase proteins in rabbits. Its role is in the evolutionarily established antibacterial defense of the body. It may remain elevated throughout the fracture consolidation process [49–52]. In this study, in the early period, this is due to the body's response to injury. Moreover, its level in the experimental group on the 7th day was lower, which indicates a limitation of the inflammatory-resorptive phase. In the meantime, the next peak of the haptoglobin level on the 14th day indicates an increase in cytokine activity associated with an increase in endothelial reactivity.

One of the hyperactivation indicators of the blood coagulation system is the presence of soluble fibrin in

the circulating blood, which indicates thrombinemia. The emergence of thrombin in the blood is not only the activation of the cascade-coagulation process but also the stimulation of platelets with the release and binding of fibronectin and fibrinogen on the cell surface, which enhance the formation of fibrin and contribute to its organization. Along with this, the processes of thrombus formation in the body are constantly controlled by the system of natural anticoagulants, which includes AT-III [53,54] – serine proteinase, providing 75% of the antithrombin potential of blood plasma. The level of AT-III activity in the range of 86%–105% is considered to be normal [53–56]. The presented study established that its concentration did not change significantly, but on the 3rd day it decreased slightly. This fact indicates a shift in the hemocoagulation balance towards hypercoagulation, which, according to [57], is a typical reaction of the hemostasis system to surgical intervention.

Typical markers of bone metabolism are osteogenesis–bone isoenzyme of alkaline phosphatase and osteoresorption–tartrate-resistant acid phosphatase. They are used to assess reparative osteogenesis [58]. Osteoclasts provide the processes of osteoresorption, oxygen compounds lead to the destruction of bone's matrix components. Its degradation products, together with TrAp, are released into the blood and reflect the degree of bone resorption [21,59]. In this study, a two-phase increase in the concentration of this marker is due to the occurrence of inflammatory-resorptive processes in the first phase. They are more pronounced in the control group than in the experimental group and in the second phase–with the remodeling of bone regenerates (42nd day).

Osteogenic activity of osteoblasts in the area of the formed defect causes the elimination of the BAP into the bloodstream. Gradual resorption of composite material's granules in the experimental group is accompanied by the formation of trabeculae of bone tissue. This in turn causes an increase in BAP activity [21,60]. These data are consistent with the histomorphological picture, namely the detection of higher osteoblast density in the experimental group, as we reported earlier [38]. In particular, the inflammatory-resorptive stage of reparative osteogenesis in the period up to the 7th day is characterized by an increase in blood levels of NO, haptoglobin, and soluble fibrin. However, the intensity of the acute phase in the case of combined osteosubstitution is significantly lower. The next peak of NO concentration on the 14th day indicates earlier angiogenesis. The two-phase degree of osteoresorption, which reflects the level of TrAp, is significantly less than the combined osteosubstitution in rabbits of the experimental group. At the same time, the intensity of osteogenic processes, which reflects the activity of the bone isoenzyme alkaline phosphatase, in experimental rabbits is earlier.

From a clinical point of view, osteosubstitution by calcium-phosphate ceramics limits the inflammatory-resorptive phase due to osteoconductivity, and PRF gives it osteoinductive properties and improves its osteointegration characteristics.

5. Conclusion

Substitution of bone defects by autologous platelet-rich fibrin and hydroxyapatite granules with β-tricalcium phosphate has pronounced osteoinductive properties and greater opportunities for osteointegration. This is evidenced by the dynamics of biochemical markers such as nitric oxide, bone isoenzyme alkaline, and tartrate-resistant acid phosphatase. The studied biochemical parameters can be prognostic criteria and can be used to analyze the effectiveness of fracture healing in preclinical studies. The presented combination of hydroxyapatite ceramics with PRF may be promising for osteosubstitution in animals.

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Conflict of interest

The authors declare no conflict of interest.

Compliance with standards for working with animals

The studies were carried out in accordance with the Law of Ukraine "On the Protection of Animals from Cruelty" of March 28, 2006, the rules of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes of November 13, 1987, and the Order of the Ministry of Education and Science No. 416/2072 dated March 16, 2012 "On approval of the procedure for conducting research, experiments on animals by scientific institutions".

Informed consent

The experiment protocol was approved by the Ethics Committee of the Belotserkovsky National Agrarian University on the treatment of animals in scientific research and the educational process (protocol no. 1, dated January 23, 2019).

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