Transplantologiya - 2016. - № 2. - P. 36-44.

Efficiency of allogenic platelet-rich plasma combined with collagen in rat's femur injury healing

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In experiment we studied the influence of collagen, filled with platelet-rich plasma (PRP), on rat bone regeneration in distal femur. The used dose of PRP contained 130–135 pg/ml of platelet-derived growth factor (PDGF). According to this level of PDGF, PRP-collagen treatment rapidly enhanced trabecular bone growth, stimulated angiogenesis and reduced intensity of inflammation. Allogenic PRP used in all experimental animals twice shortened the period of femoral defect healing.

Keywords: platelet-rich plasma, platelets with granules, PDGF, osteogenesis.

Introduction

With the development of hip replacement technique, the treatment of patients with femoral neck fractures ceased to be such a critical problem as it used to be. Meanwhile, osteosynthesis remained a preferred treatment of such fractures without displacement and of impacted fractures [1, 2]. Positive clinical effect of osteosynthesis has been demonstrated in patients of different age groups [1-5], including patients over 60 years old [2, 3] which

suggests the efficacy of this technique. However, the osteosynthesis still can not completely exclude the risk of nonunion or avascular necrosis of the femoral head [6-10]. Our data have shown that the complication rates in the treatment of femoral neck fractures in patients of all age groups can reach 29% [11]. In many ways, the degenerative processes developing in the bones are associated with malnutrition, inadequate angiogenesis in bone tissue at the injury site, and with the osteoblast migration and proliferation. Various biologically active grafts can be used to stimulate the processes of fracture healing and bone regeneration. One of the most well-known and available materials used for this purpose is human Type 1 collagen that attracts the connective tissue cells to the bone defect, stimulates their migration and proliferation, enhances the vascular sprouting, facilitates the fixation of prostheses and immobilizing structures. The research and clinical practice involve the use of gels, sponges, three-dimensional collagen-based matrices which composition often includes additional components: the hyaluronic acid, hydroxyapatite, demineralized bone [12-14]. Collagen-based grafts are considered to perform primarily an osteoconductive function, i.e. they serve scaffolds for further bone growth, cell migration, but do not possess osteoinductive properties themselves. Regenerative processes in bone defect/fracture can be directly stimulated by using various growth factors: the platelet derived growth factor (PDGF), fibroblast growth factor (FGF), transforming growth factor (TGF-b1), insulin-like growth factor (IGF-1), vascular endothelial growth factors (VEGF, VGF) [17, 18]. All of these factors are contained in the granules (secretory vesicles) of biologically high-grade platelets [15]. It has been shown so far that the human platelets have very high reparative and regenerative potentials that can be used to repair a damaged connective tissue, including the bone [16-19]. Thus, it is

reasonable to believe that collagen- and platelet-based biografts possess the potential to significantly speed up the regeneration of a bone defect.

The aim of this study was to evaluate the effect of allogenous platelets in combination with collagen on reparative osteogenesis processes in cases of damaged bone articular ends.

Material and Methods

An efficacy experimental study to investigate the collagen effect on the regeneration of bone defects was performed at the Laboratory of Experimental Pathology (headed by Professor T.S. Popova, Dr. Med. Sci.) of N.V. Sklifosovsky Research Institute for Emergency Medicine. The experiments were conducted in conformity with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes No.123 of 18.03.1986 and the USSR Healthcare Ministry Order No.755 of 08.12.1975 "On measures to further improve the organizational forms of work with the use of experimental animals."

The experiment was conducted on 64 outbred rats that were kept in vivarium on a regular diet. The animals were sacrificed by intraperitoneal hexenal injection in a lethal dose.

Preparation of collagen

Allogenous collagen type 1 was prepared from the rat tails using the standard technique [20]. Rat tail tendons were shredded in a homogenizer to mushy state, the resulted mass was placed in a 1 M NaCl solution on TrisHCl buffer (pH 7.2-7.5) in a volume ratio of 1:30-1:50. After 3-4 days, the deposited collagen was collected, washed thoroughly with distilled water to remove the salt residue and dissolved in 0.1-0.5 M acetic acid. That

resulted in a collagen gel formation. Then the collagen gel was washed off the acidic residue with distilled water several times. The resulting collagen gel had mild acidity (pH: 6.5-6.7).

Allogenous platelet-rich plasma preparation

Rat platelet-rich plasma (PRP) was procured up to Messora et al. method [21]. By using a disposable syringe containing sodium citrate, 5 ml of each animal blood was collected and centrifuged at 160 g for 20 minutes, the supernatant was collected and subjected to a new centrifugation at 400 G, for 15 minutes; after that, the bottom fraction (the ready rat PRP, average volume 0.5 ml) was collected. Platelet amount in the obtained rat PRP was 1100-1300 x 10^3 /mcL.

Bone defect model

Before surgery, the rats were anesthetized and the hair was shaved on the hind legs from both knees and hips. The incision of 1.0-1.5 cm length was made along the outer hip surface proximal to the knee joint so as to expose the outer distal femoral condyle, without arthrotomy. The bone defect was inflicted in the femoral condyle to the depth of cortical internal femoral condyle using a drill of 2.0 mm in diameter. The study drug was injected inside the defect using a syringe, and then the wound was sutured in layers. The surgery was performed on both hind limbs. The rat collagen used for the experiment had been produced in the Laboratory of Cell Transplantation and Immune Typing of the Sklifosovsky Research Institute for Emergency Medicine.

Postoperatively, the rats were kept in the vivarium on a regular diet. The experimental animals were allocated into three groups of 20 animals

each. The rats were withdrawn from the experiment on the 7^{th}, 14^{th}, 28^{th}, and 84^{th} day.

Characteristics of the experimental groups

After inflicting the bone defect in the animals of the 1^{st} group (controls), the surgical wound was sutured without filling it with a plastic material. In the 2^{nd} group, the bone defect was filled with rat collagen (in the amount of 150-200 mcL). In the 3^{rd} group, the bone defect was filled with the mixture of rat collagen/allogenous rat PRP in the ratio of 1: 1 (in the amount of 150-200 mcL).

Morphofunctional analysis

Recovery of bone defect over time was monitored using histology slides stained with hematoxylin and eosin, and with Van Guison staining solution. The biologically high-grade status of rat platelets was assessed using the original method of cell vital staining with fluorochrome trypaflavine and acridine orange stain with further fluorescence microscopy examination [22]. The following parameters were assessed: the total platelet amount (in thousand per mcL), the amount of biologically high-grade platelets (cells with granules, in %), the number of granules per a biologically high-grade platelet, a scored morphofunctional platelet activity (MFPA) indicating the cellular structure integrity; a scored platelet adhesive ability (PAA) indicating the functional activity of the cells.

Results and discussion

An efficient use of platelet-derived material as a biograft component essentially requires the quality assessment of original cells and the

evaluation of their saturation with growth factors and regeneration factors. This is necessary in order to eliminate the PRP with low content of normal platelets and also to select an adequate dose of the employed platelet-derived material. So, the morphofunctional assessment of rat platelets was absolutely necessary at the first stage of the study.

A total amount of granule-containing platelets in the studied rat PRP averaged 570±110 thousand/mcL that was comparable to the similar parameter in human PRP. Distinguishing features of rat platelets include their small diameter (2.7±0.2 mm) and the lack of the distinct heterogenicity by cell diameter typical for human platelet population (Fig. 1a). A great majority of rat PRP cells have similar linear dimensions; meanwhile, the platelet content varies from 25% to 60%, making mean 44.5±5.5%. This is significantly lower than in human PRP (55.6±7.8%); MFPA and PAA values of rat platelets are also lower, making 40.8±4.5 and 44.0±5.2, respectively. However, the mean number of visually distinguishable granules in a biologically high-grade rat platelet makes 8.0 ± 1.0 per cell and approximates that of human platelets. Thanks to the previously identified correlation between morphofunctional platelet parameters and their platelet-derived growth factor (PDGF) content [23], it was possible to indirectly assess the PDGF level in rat PRP that made mean 105 pg per 100 million biologically high-grade cells. The mean granule-containing platelet count in the mixture of rat collagen/allogenous PRP was 26 million cells, the total their PDGF content was about 27 pg. When the rat PRP was applied on collagen, the activation of biologically high-grade platelet activation occurred that was accompanied by an intensive degranulation and formation of multiple platelet aggregates (Fig. 1b), meanwhile, in the first 10 minutes, many activated cells could be clearly observed releasing granules on their

surface. After 10-20 minutes of contact with collagen, the granules lost their contact with the platelets and ceased to be identified in cytoplasm indicating a complete release of the granules and their components.

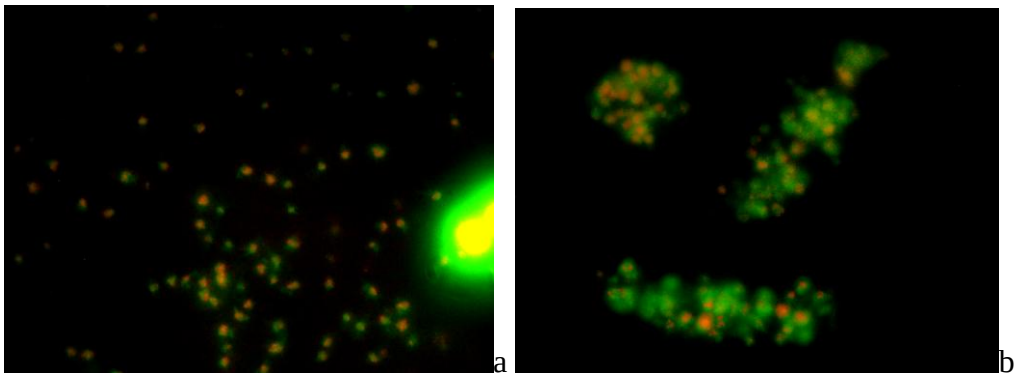

Fig. 1. Vitally stained rat platelets in the original PRP (a) and in PRP after the contact with collagen (b). Trypaflavine acridine orange stain. Magnification x1500

The postoperative period in animals of all experimental groups proceeded without apparent complications. Animals kept the activity and appetite. At day 7, 2 animals in the control group demonstrated signs of postoperative superficial wound suppuration. In the animals of the control group, the histological examination on day 7 demonstrated the bone defect being filled with a blood clot containing a large number of red blood cells and fibrin strands. In the group of rats treated with collagen, the defect was also filled with a blood clot and collagen gel; meanwhile, the collagen fibers in the gel had retained their structure (Fig. 2). An intensive infiltration with inflammatory cells (mainly macrophages) was observed in the clot and along the edges of the defect in the animals of the 1st and 2nd groups; occasional infiltration foci were also found in the surrounding tissues (Fig. 3a). A slight growth of bone trabeculae was seen along the edges of the defect, the newly

formed trabeculae displayed pronounced oxyphilic staining along the greater length, and slight basophilic staining at some sites indicating a slight accumulation of inorganic bone matrix in them (Fig. 3b.). Solitary thin-walled vessels could be seen formed along the edges of the defect. In the group treated with collagen and rat PRP, the extent of the bone defect infiltration with inflammatory cells was less pronounced than that in animals of the 1st and 2nd groups, no infiltration foci in the surrounding tissues were identified in 3 of 5 rats. An intensive growth of bone trabeculae was seen along the edges of the defect, the trabeculae displaying slight basophilic staining; on the other hand, their osteoblast content was 2-3 times higher than in the control group. Also, there was a rapid growth of blood vessels with their penetration into the entire depth of the defect (Fig. 4a). In the majority of animals, there was noted an active ingrowth of slightly pigmented trabeculae and vessels in the central part of the defect, and the migration of fibroblasts and osteoblasts (Fig. 4b).

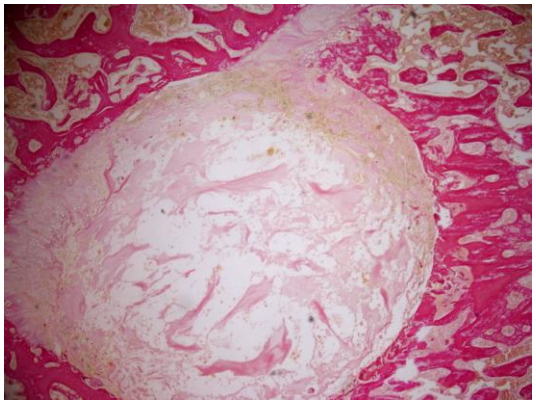

Fig. 2. Histology of the bone defect in rats treated with collagen, at day 7 after the start of the experiment. Van Gieson staining, magnification x 200.

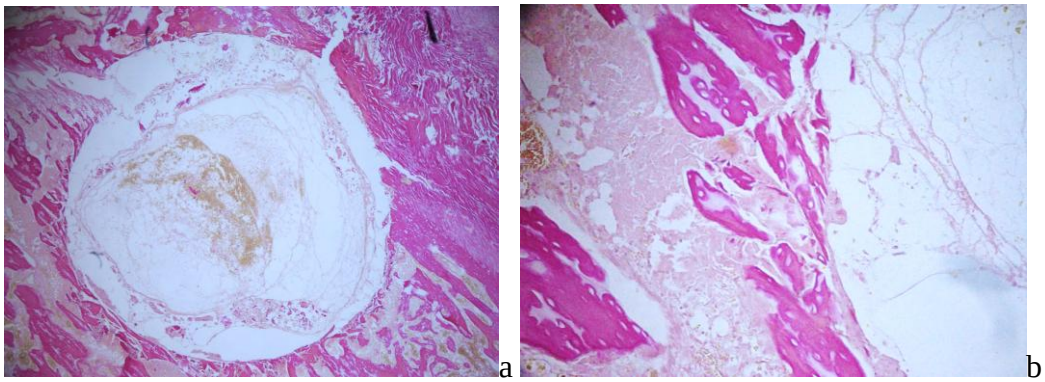

Fig. 3. Histology of the bone defect in rats of the control group (no treatment) at day 7 after the start of the experiment. Van Gieson staining (a: magnification x 200; b: magnification x 250)

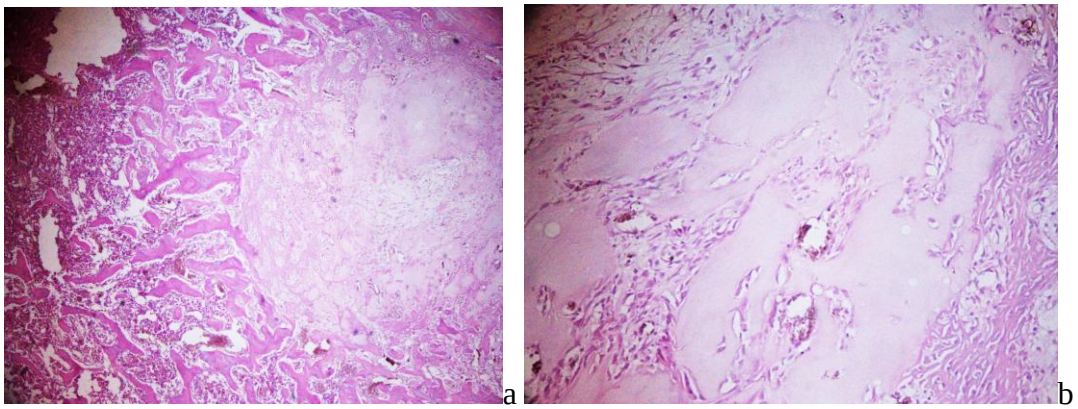

Fig. 4. Histology of the bone defect in the rats of the collagen and rat PRP treatment group at day 7 after the start of the experiment. Stained with hematoxylin and eosin (a: magnification x 100; b: magnification x 250)

In the control group, the bone defect area infiltration with inflammatory cells alleviated at day 14, the growth and thickening of bone trabeculae were observed (Fig. 5a), although the count of osteoblasts in their structure and the degree of basophilia were markedly lower than in the group of treatment with collagen and rat PRP at day 7. In the collagen treatment

group, a significant area of the defect (75%) was filled with slightly pigmented bone trabeculae, inflammatory cells were missing, and there was an intense migration of fibroblasts and osteoblasts (Fig. 5b). In all studied animals of the collagen and rat PRP treatment group, the defect area was virtually indistinguishable in histological sections: a full-fledged trabecular osseous tissue had been formed over the site of the defect with normally developing bone trabeculae (Figure 5c) where most of the present cells were fusiform osteocytes.

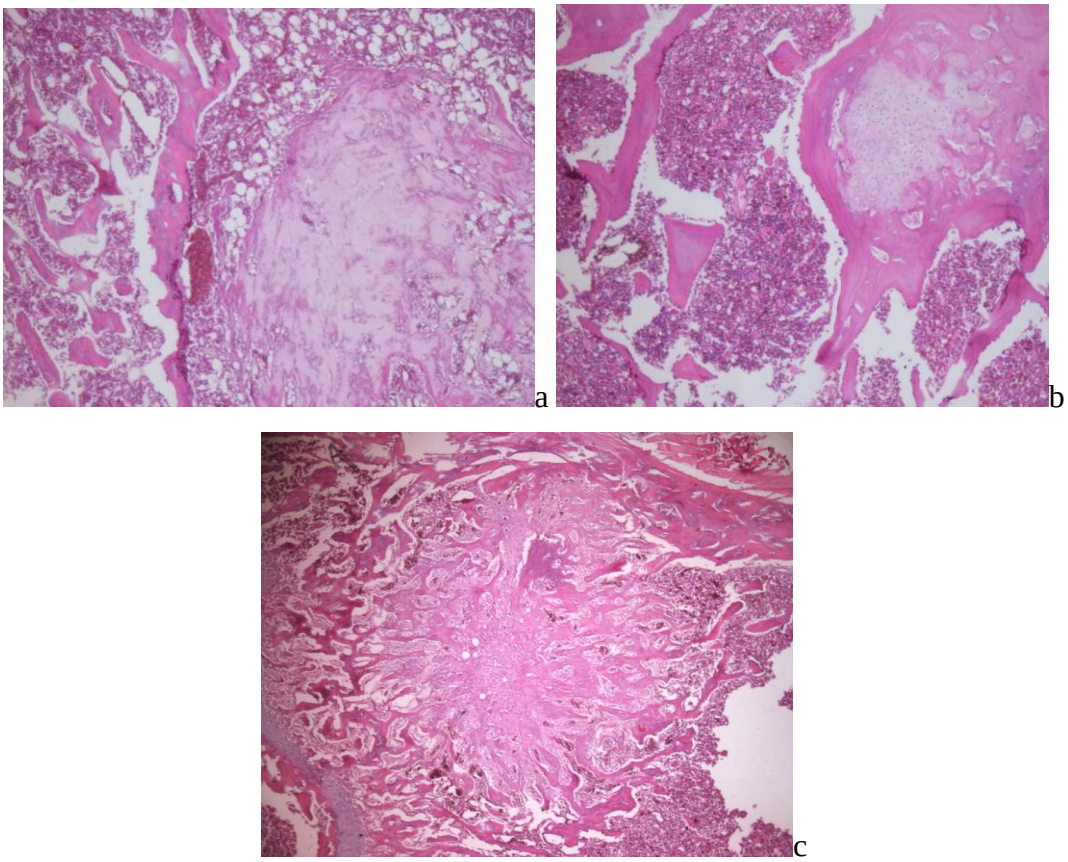

Fig. 5. Histology of the bone defect at day 14 from the start of treatment. H & E stain. Magnification x 100 (a: the control group [no treatment]; b: collagen treatment group; c: collagen and rat PRP treatment group).

After 28 days, the area with completely restored trabecular bone made 80% of the defect in the control group, and 100% in the collagen and rat PRP treatment groups. In all the cases, the bone defects were not detectable histologically (Fig. 6). After 84 days (12 weeks), a complete recovery of bone structural integrity was noted at the site of the defect in the experimental animals of all groups.

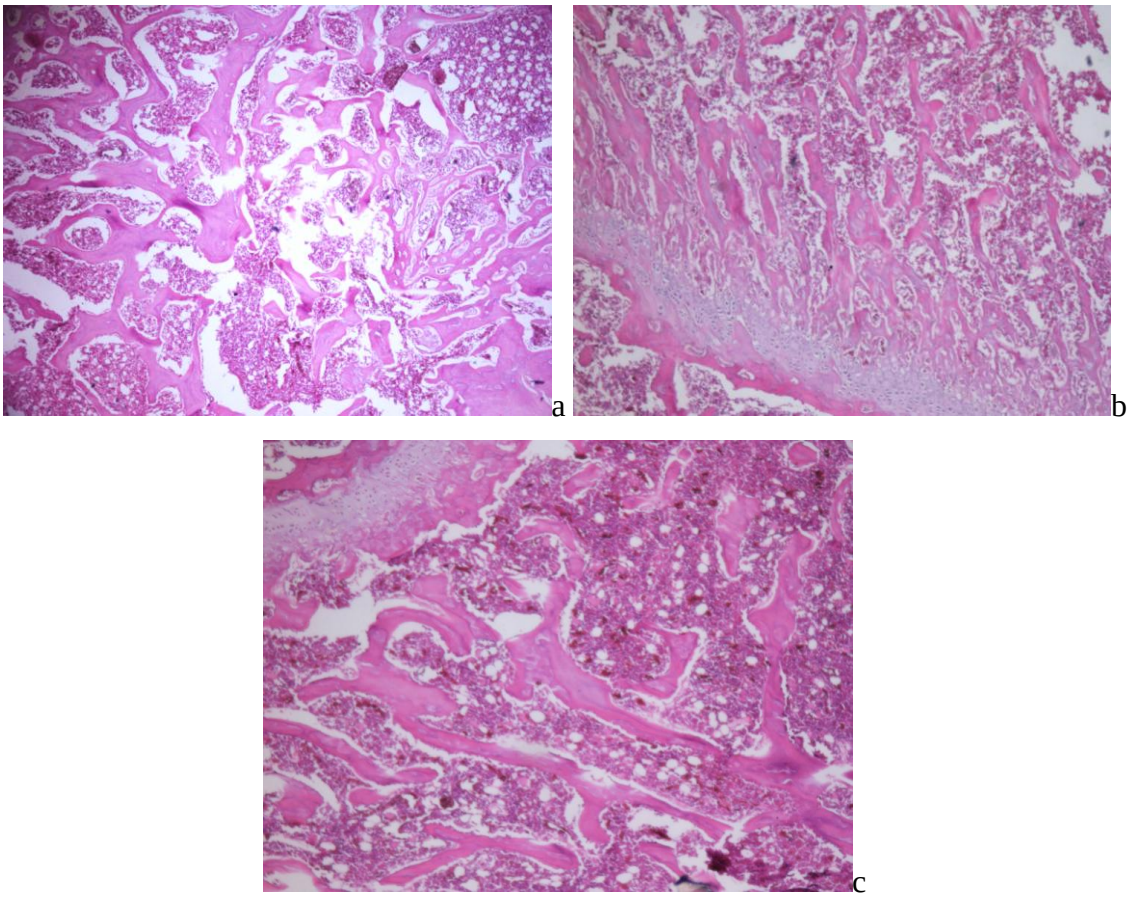

Fig. 6. Histology of the bone defect at day 28 from the start of treatment. H & E stain. Magnification x 100 (a: the control group [no treatment]; b: collagen treatment group; c: collagen and rat PRP treatment group)

Current treatment of hip fracture implies making the fixation of bone fragments through small incisions without the fracture site exposure, so the substances that stimulate osteogenesis should be preferably administered by injection. In this setting collagen and PRP provide very suitable components that may be used in both a dissolved and gel form. The use of platelet gel has already been widely described for the treatment of mechanical bone injury, specifically, the jaw bone fractures, diabetes-associated fractures, diabetic osteoarthropathy [16, 24-26]; ready-made gels are considered preferable for use than ordinary PRP. However, there is a large number of known cases where the use of products containing platelet components in the treatment of mechanical bone defects did not produce a desired clinical result. Moreover, PRP components can inhibit osteoblast proliferation and prevent the maturation of bone tissue. That is attributed to the fact that platelets contain a large spectrum of chemical factors, and many of those factors may produce pathophysiological effects in conditions of their excess or lacking balance with other factors [15]. Therefore, the success of the PRP application depends on an adequately chosen dose that can be determined both by the count of biologically high-grade platelets, and by the level of growth factors. We used the method of morphofunctional platelet analysis that implies the evaluation of the PDGF level based on the count of granule-containing cells [22, 23]. PDGF is a potent reparative agent; moreover, its concentration in platelets correlates with the concentration of many other factors [15], which means that by determining morphofunctional status of platelets, we could roughly estimate their saturation with growth factors, as a whole. A ready-made collagen+PRP dose used in our study contained an average of 130-135 pg of PDGF per 1 ml, and appeared very effective in the treatment of cancellous bone defect in rats. Rat PRP used in combination with collagen

allowed a 2-fold reduction in the period of bone defect healing, and the PRP produced a pronounced anti-inflammatory effect that is particularly important in clinical practice. The platelet degranulation is known to trigger the release of anti-inflammatory and pro-inflammatory factors (IL-1, IL-6, TNF-b) that, being in excess, may inhibit bone formation [27, 28]. Meanwhile, the growth factors and the growth-inhibiting factors are contained in the same platelet granules, and that emphasizes further the importance of assessing the platelet saturation with granules. We previously showed that the serum prepared from the PRP containing PDGF, 100-150 pg/mL, dramatically increases the proliferative activity of diploid cells in vitro without causing their damage or transformation [23]. Thus, the selected combination of collagen and PRP is certainly a lucky find and requires further research both on experimental animals and in clinical practice.

Conclusions

1. The PRP use decreases the intensity of inflammatory response in the area of the bone defect in rats. In 3 of 5 animals, the histology sections demonstrated no infiltration with inflammatory cells and an intensive growth of blood vessels at the site of injury with the migration of fibroblasts and osteoblasts.

2. The use of rat PRP in combination with collagen triggers the release of growth factors from platelet granules, thus allowing a 2-fold shortening the time of bone defect healing in a rat model.

3. The conducted experiment has yielded favorable results that give rise to using the combination of autologous PRP and allogenous collagen in clinical practice.

4. The selection of the optimal dose of PRP and collagen is required to get the best effect in clinical practice.

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