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Original article

Autologous activated platelet-rich plasma (PRP) in bone tissue healing – does it work? Assessment of PRP effect on bone defect healing in animal models

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Abstract

Introduction: Platelet-rich plasma (PRP) preparations can be used in bone tissue healing but there are numerous doubts among clinical orthopedists about effectiveness of this method.

Materials and methods: The studies were carried out in 12 rabbits of *white termond* breed. In operating room we operationally generated cylindrical, unicortical defects of the diameter of 4 mm in the middle of the shafts of both femurs. The defects in the left bones were left without filling and served as controls, and 0.7 ml of the ready-to-use PRP was administered to the defects in the right bones (experimental group). We evaluated the usefulness of the diagnostic methods applied: biomechanical tests, micro-CT tests, densitometry, typical radiology, macroscopic measurements, histopathological examinations.

Results: The macroscopic measurements showed a statistically significant increase in the dimension in the area of the right defect filled with PRP in relation to the control group. In experimented group, the assessment of the X-ray images showed the formation of a callus cuff around the defects. Densitometric examinations showed no statistically significant differences between defects in the experimental and control group. The analysis of the micro-CT examinations showed an increase in the total volume of the tissue examined (Vb) and the low density tissue fraction (Vb_2) in the experimental group. The biomechanical examinations revealed significant decrease in the maximum breaking force (F max) necessary to break the bone in the experimental group in relation to the control group.

Conclusions: Platelet-rich plasma (PRP) stimulates bone formation in the area of bone defects and may accelerate bone regeneration.

Key words: platelet-rich plasma, bone regeneration, bone defects, rabbit model

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Introduction

Platelet-Rich Plasma (PRP) is a commonly used method in a wide range of indications in veterinary and human medicine. Platelet-rich plasma preparations are used not only in bone and joint surgery, but they are also recognized and widely described therapeutic tools mainly in soft tissue pathologies, for example, in entezopathies, in healing of traumatic and non-traumatic soft tissue injuries and ulcerations (Memeo et al. 2013, Albanese et al. 2013, Mi et al. 2017). Having potentially beneficial effects on preservation of autografts in ligament reconstructions, PRP is also used with controversial effects in cruciate ligament reconstructions in humans and animals (Andriolo et al. 2015, Komzák et al. 2015). The beneficial effect of PRP on joint cartilage regeneration has not been confirmed credibly in the available literature. In the Clinical Practice Guidelines, the American Association of Orthopedic Surgeons (AAOS) points out that on the grounds of the available studies they are not able to express definitely whether they are for or against using PRP in osteoarthritis (Hsu et al. 2013). Similarly, the fact of beneficial effect of PRP on bone tissue healing is still raising numerous doubts among clinical orthopedists. The available studies show a large discrepancy in the descriptions of the studies results, which can be associated with a large number of variables related to the method applied, for example, due to the absence of standards for PRP production, difficulties in objective monitoring of its effectiveness or lack of a uniform experimental model (Malhotra et al. 2013).

The purpose of this work was to objectively assess the effectiveness of the autologous activated PRP in the early phase of bone tissue regeneration based on the repeatable animal model. Another purpose was to evaluate the usefulness of the diagnostic methods applied (biomechanical tests, micro-CT tests, densitometry, typical radiology, macroscopic measurements, histopathological examinations) in assessing the progress of bone defect healing.

Materials and Methods

The studies were carried out in 12 rabbits of *white termond* breed, males that were 4 months old. Before the studies started, the consents required by legal regulations had been obtained, including those of the First Lublin Local Ethics Committee for Experiments on Animals (Resolution No. 6/2013 of 18.01.2013). The animals for experiments were purchased at the Experimental Station of the National Research Institute of Animal Production in Chorzelów (Poland). The rabbits were vaccinated against myxomatosis and viral rabbit hemorrhagic disease (RHD). The animals were subject to a routine two-week quarantine during which there were disclosed no signs of diseases that could affect the course of the experiment. Afterwards, the rabbits were marked, weighed and the homogeneity of the group in terms of their weight (3.04 kg ± 0.1) was confirmed.

Preparation of PRP. Immediately before the procedure, the whole blood was collected from each subject: 2 ml in order to do blood count and assess the level of platelet compaction in the PRP preparation in relation to their level in the whole blood and 4 ml to BD Vacutainer® CPT™ (*GE Healthcare*) test tubes, in which, after a standard centrifugation and activation with the thrombin previously obtained from the serum of one of the rabbits, the ready-to-use PRP was obtained. The level of the platelet concentration in relation to the whole blood is presented in Table 1.

Surgery. Before the surgery, the animals were premedicated and they underwent general anesthesia by intramuscular administration of Ketamine (1.5 mg/kg) and Diazepam (30 mg/kg). In operating room conditions, sticking to aseptic principles, there were operationally generated cylindrical, unicortical defects of the diameter of 4 mm in the middle of the shafts of both femurs. The defects in the left bones were left without filling and served as controls, and 0.7 ml of the ready-to-use PRP was administered to the defects in the right bones (experimental group) with a needle.

Assesment. Immediately following the procedures and before the animal woke up, a control X-ray had been performed in two projections in order to assess whether the surgical procedures had been performed correctly and in order to exclude inter-operative complications. Every 7 days a control X-ray was performed in two projections so as to assess the healing process. The study was completed 8 weeks after the surgical procedure. After the premedication, the animals were anesthetized by intravenous administration of Morbital (1ml/kg m.c. i.v.). Then, both femoral bones were collected for the tests, marking the preparations of the left femurs (served as controls) with the symbol "L", whereas the right femurs (experimental group) with the symbol "P". Next, the femurs were measured in the defect area setting out two dimensions X and Y perpendicular to each other. A DEXA densitometry was performed using the Norland XR-46 machine. The bone mineral density (BMD) and bone mass value (BMC) were determined for the 4-cm fragment of the femoral shaft tested, in the centre of which the defect was generated (marked as: BMD-FR and BMC-FR respectively). At the subsequent stage, using the Skyscan 1174 x-ray microtograph (Bruker-SkyScan)

Table 1. Average platelet count (PLT) in whole blood and in the preparation of autologous activated platelet-rich plasma (PRP) in rabbits.

	PLT [$10^3/\mu l$] Day 0	PLT [$10^3/\mu l$] PRP	Level of concentration
Test group	487.2±94,1	2117.5±393.0	4.36±0.15

Table 2. Average value and standard deviation of X and Y dimensions and their differences.

	Experimental group	Control group	Delta
X dimension [mm]	10.84 ± 1.62*	10.11 ± 1.00	0.74 ± 0.76
Y dimension [mm]	7.52 ± 0.84	7.53 ± 0.51	-0.02 ± 0.77

* $p<0.05$

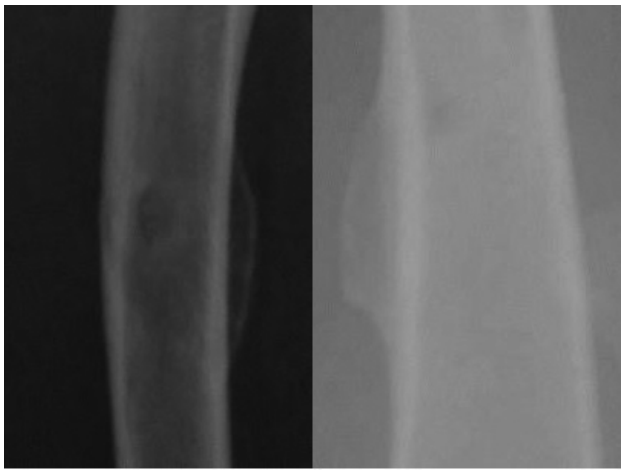

Fig. 1. Rabbit B5, right femur on the 56th day; callus in the area of the generated defect.

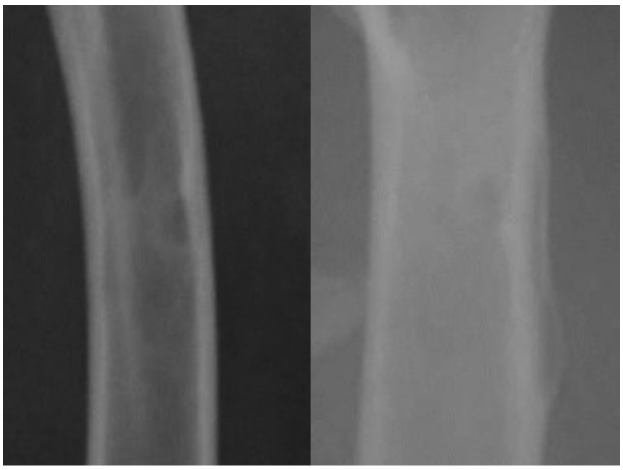

Fig. 2. Rabbit B5, left femur on the 56th day; healing area of the defect.

in the area covering 1-cm fragment of the shaft, in the centre of which the defect was generated, the following parameters were analyzed: the total tissue volume (Vb [mm^3]), the volume of high density tissue fraction (Vb_1 [mm^3]), the volume of low density tissue fraction (Vb_2 [mm^3]). Using the TA.XT Plus texture analyzer, a three-point preparation bending test was performed with the application of a breaking force at the place where the defect was generated. The maximum breaking force necessary to brake the bone at the area of the defect F max [N] was analyzed and the work W was calculated according to the formula: $W = \Delta F * m$ [J]. Then, the tissue material for the histopathological examination was collected from the fracture area. The bone scraps obtained were stained with hematoxylin and eosin (H+E) and by the van Gieson's stain method and then they were microscopically evaluated.

Results

Out of 12 animals, 10 subjects reached the study endpoint. In the course of the experiment, there were general and local complications: on the third day of the surgery, with no external infection signs, the rabbit marked with symbol B6 died, on the 22nd day after the surgery the rabbit marked with symbol B8 developed a generalized infection and the right femur was fractured in the area of the generated defect.

The macroscopic measurements showed a statistically significant increase in the X dimension in the area of the right defect filled with PRP in relation to the control group (Table 2)

The assessment of the X-ray images showed the formation of a callus cuff around the defects filled with the PRP preparation (the test group) (Fig. 1). In the area of the defects left without the filling (the control group) there was observed a gradual increase in the density of the tissue filling the defect without altering the bone contour in its area (Fig. 2).

The densitometric examinations of the fragments of the bone preparations showed no statistically significant differences between the experimental group and control group with respect to BMD and BMC in the area of the bone fragment examined (Table 3).

The analysis of the micro-CT examinations showed

Table 3. Average level and standard deviation of bone mineral density (BMD) and bone mineral content (BMC) in the preparations tested.

	Experimental group	Control group
BMD-FR [g/cm2]	0.2839 ± 0.0230	0.2903 ± 0.0262
Delta BMD-FR [g/cm2]	-0.0064 ± 0.0091	
BMC-FR [g]	1.553 ± 0.304	1.477 ± 0.230
Delta BMC-FR [g]	0.076 ± 0.353	

Table 4. Average level and standard deviation of total volume of the tissue tested (Vb), volume of the high density tissue fraction (Vb_1), volume of the low density tissue fraction (Vb_2).

	Experimental group	Control group	Delta [mm^3]
Vb [mm^3]	88.05 ± 6.91**	67.87 ± 9.67	20.18 ± 8.77
Vb_1 [mm^3]	23.65 ± 16.71	34.35 ± 10.37	-10.70 ± 14.41
Vb_2 [mm^3]	64.39 ± 13.45**	33.51 ± 10.37	30.88 ± 18.16

** $p<0.01$

Table 5. Average level and standard deviation of maximum breaking force (F max) and work W necessary to break the preparations tested.

	Experimental group	Control group
F max [N]	247.1 ± 73.1*	282.3 ± 63.6
Work W [J]	0.33 ± 0.15	0.36 ± 0.15

* $p<0.05$

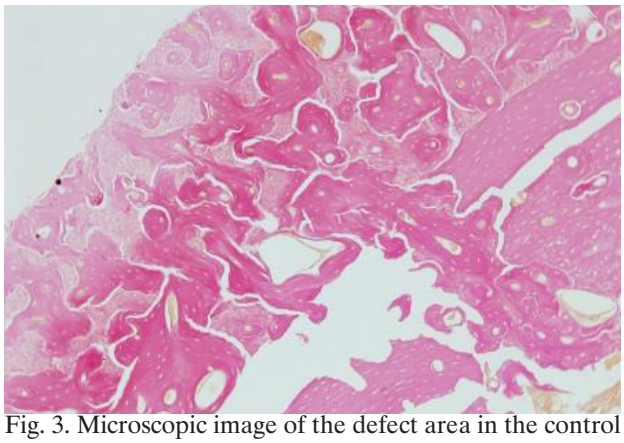

Fig. 3. Microscopic image of the defect area in the control group. x200

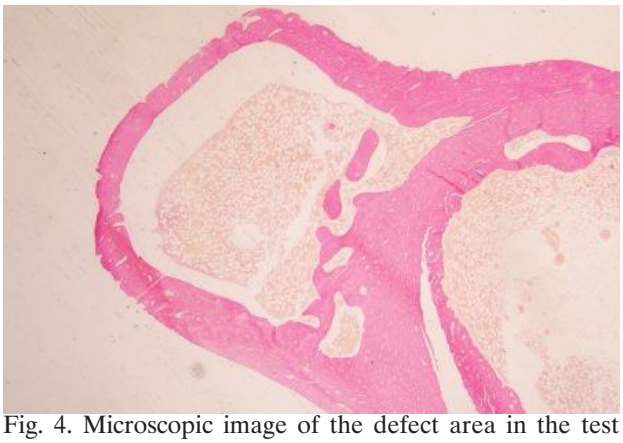

Fig. 4. Microscopic image of the defect area in the test group. x200

a statistically significant impact of PRP used in the experimental group in relation to the control group on the increase in the total volume of the tissue examined (Vb) and the low density tissue fraction (Vb_2), however, they showed no significant impact on the volume of the high density tissue fraction (Vb_1) (Table 4).

The biomechanical examinations revealed a statistically significant decrease in the maximum breaking force (F max) necessary to break the bone in the area of the defect in the experimental group in relation to the control group (Table 5). In the control group, in the defect area, the histopathological evaluation demonstrated an irregular osteoid tissue (Fig. 3). In the test group, in the defect area, there was observed an exogenous osteogenesis with „secondary" medullary cavity and irregular trabecular bone. In the primary zone of the cortical layer, thickening of trabecular bone from the side of the medular cavity with more irregular system of bone beams was observed (Fig. 4).

Discussion

In recent years, both in veterinary and human medicine, the PRP preparations have been widely used both

in soft tissue and bone pathologies as well. Due to their autologous origin, using the PRP preparations is very safe, and the systematic overview available in the literature and related to the safety of the platelet preparations revealed single local adverse effects (Martínez-Zapata et al. 2009). In our experiment two animals died. In one case the death was associated with the fracture of the limb operated (which indicates a biomechanically significant size of the defects generated), whereas in the other case, the cause was not determined.

In the clinical practice, an objective assessment of the effectiveness of PRP is difficult. Due to a wide range of indications, lack of commonly accepted classifications of platelet-rich plasma, lack of systematized methods of PRP production and specific administration protocols, the therapeutic effects vary a lot (Zhao et al. 2010, Iqbal et al. 2011, Mazzocca et al. 2012, Malhotra et al. 2013, Roffi et al. 2013, Russell et al. 2013).

In the available literature, various types of PRP preparations are used in experimental and clinical studies and they mainly differ in: platelet concentration in the PRP preparation as compared to peripheral blood, a method of activation (or its absence) or leukocyte content.

Dohan et al. (2012) developed classifications introducing a division of platelet-derived preparations in terms of leukocyte content, activation, fibrin concentration and architecture. In our experiment, we used a liquid form of P-PRP (pure PRP), most commonly used in clinical practice.

The density of platelets in PRP in a given indication seems to be crucial in the effectiveness of the method. Contrary to the generally accepted opinion, it is not true that the greater concentration, the more effective the preparation is (Mazzocca et al. 2012). Some reports show that too high concentration of platelets in the preparation may inhibit bone tissue healing (Malhotra et al. 2013). In our study, we obtained platelet density in the preparation of 4.36 ± 0.15. Based on the data obtained from the literature, in the absence of evidence proving an advantage of multiple PRP applications in healing of bone defects in animal models, we administered the preparation we had obtained only once (Özdemir et al. 2012).

An objective assessment of PRP effectiveness is also difficult because of very differentiated research models in the assessment of their effect on bone tissues. In the experimental studies of the PRP impact on a bone tissue, the animal models differed significantly primarily in terms of the location and area of the bone tissue that the tested preparation is supposed to affect, which makes it really difficult to interpret and compare the results (Grageda 2004).

In our study, we generated the defects in the femurs. These were the defects that did not meet the Critical Size Defect (CSD) criterion, which, on the one hand, eliminates the need to use a surgical stabilization of the defect area, but on the other hand, it allows only to assess the effect of PRP on the natural healing processes because the defects in the control group left without filling also healed. In turn, in case of CSD in load-bearing long bones, using a stabilization makes the study technically very difficult and introduces another factor that might affect healing of the fracture itself (a necessity to use the same stabilization in mechanical and biological terms). Only in a few studies, bone defects in load-bearing bones that met the CSD criteria, were used as a research model, but they always required an additional surgical stabilization (Kanthan et al. 2011). Some experimental studies used defects meeting the CSD criteria, however, they were mostly limited to non-load-bearing bones, for example, temporal bones (Denicolo et al. 2013, Lim et al. 2013).

A macroscopic evaluation of anatomical preparations obtained by a section, in correlation with the results of radiological examinations, allows to conclude that PRP causes an increase in bone formation in the area of the defect. In the analyzed literature, a similar image of a radiological increase in bone formation after application of the rich-platelet preparations as an independent factor affecting regeneration of a bone tissue was obtained in a number of works (Markou et al. 2009, Souza et al. 2012). However, in most studies, PRP is used (with varying effects) as an osteoinductive "supplement" to the osteoconductive bone substitute material of bone grafts (Faratzis et al. 2012, Kamoda et al. 2012, Metzler et al. 2012).

The use of the densitometry showed no differences between the test group and the control group and it showed no qualitative differences of the bone tissue in the area of the defect. Analyzing the literature, a rarely used two-dimensional densitometry seems to be gradually replaced with more accurate research tools allowing for a three-dimensional qualitative and quantitative assessment (e.g. microCT) (Kim et al. 2010, Souza et al. 2012). In our experiment, the micro-CT examination turned out to be a valuable research tool that allowed for both qualitative and quantitative assessment of the bone healing process. In the area of the defect filled with PRP, compared to the control group, the total volume of the bone tissue and the low-density bone fraction increased, which proves the effect of the applied platelet-derived preparation stimulating the repairing bone formation processes. In the micro-CT examinations mostly used to assess the effect of PRP on bone substitute healing, many authors demonstrate similar bone-formative stimula-

ting properties of PRP (Plachokova et al. 2007, Rai et al. 2007, Kim et al. 2010, El Backly et al. 2013).

From a clinical point of view, a very essential aspect of PRP-assisted bone healing was shown in strength tests. It was observed that the individual administration of PRP in the early phase of bone healing, on the one hand increases the bone repair rate, but on the other hand, it can weaken the strength of the bone tissue in this phase. In the analyzed literature, we found a few works which studied the impact of the PRP itself on the biomechanical properties of the bone regenerate in the early phase of healing, particularly in case of fractures (Guzel et al. 2013). In such cases where PRP was used with a bone substitute, the areas of defects demonstrate the same or greater mechanical strength compared to other groups (Rai et al. 2007, Kasten et al. 2008). Thus, it seems reasonable, in terms of biomechanics, to combine platelet-derived preparations with bone substitutes or grafts wherever it is possible.

Assessing the newly-formed tissue in terms of histology, it seems that this new bone tissue is formed in a larger quantity and it is better organized in the area where PRP was used. Souza et al. (2012) indicates a beneficial effect of PRP on the histological image of bone healing in the experimental osteotomy of the radical bone in dogs while using PRP exclusively, compared to the control group where the defect was left without filling. Similar observations, but with the use of PRP together with bone substitutes, was presented in a number of works (Oryan et al. 2012, Guzel et al. 2013, Kurikchy et al. 2013, Messora et al. 2013).

According to the results obtained, platelet-rich plasma, as a source of growth factors, can add some benefits to bone healing by stimulating bone formation in the area of bone defects and may accelerate bone regeneration. It increases the total volume of the newly formed tissue in the defect area. However during the period considered in this study it can weaken the bone mechanical properties.

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