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Early histological evaluation of bone defect healing with and without guided bone regeneration techniques: Experimental animal studies*

Wczesna histopatologiczna ocena procesu gojenia ubytków śródkostnych z i bez zastosowania metod sterowanej regeneracji kości – badania na materiale zwierzęcym

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Summary

Introductions: To obtain osseous tissue regeneration, treatment methods referred to as guided bone regeneration are used which utilize the mechanical, chemical, and biological properties of materials.

Material/Methods: Eighteen white rabbits were used. Under general anesthesia, a 5 mm in diameter defect was created transcutaneously in the femur trochanter major. The rabbits were divided into three groups depending on the type of the intraosseous defect (ID) treatment: in group I (C) the control IDs were left to heal spontaneously, in II (BOC+BG) the IDs were filled with Bio-Oss Collagen® and Bio Gide Perio® membrane, and in III (PRP) the IDs were filled with BOC® and platelet-rich plasma (Curasan Centrifuge®). The animals were sacrificed 1 and 3 months after the surgical procedure. The histological material was stained with hematoxylin and eosin and using the van Gieson method.

Results and Conclusions: In the earlier histological examinations (1 and 3 months after the procedures), resorption of the biomaterial and the formation of new bone trabeculas were observed in both groups II and III. At the first observation the extent of biomaterial resorption and the intensity of the osteogenic process were greater in group III, but after 3 months group II had a slight advantage. Fragmented remnants of the biomaterial in both groups were surrounded by newly formed bone and locally by fibrous connective tissue. At both observation times the number of bone trabeculas after implantation in groups II and III was greater than in the control group.

Key words: **intraosseus defects • healing process • guided bone regeneration • animal studies**

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Streszczenie

Cel pracy: Wczesna (1 i 3-miesięczna) ocena histopatologiczna procesu gojenia ubytków śródkostnych kości udowej u królików bez i z zastosowaniem wybranych technik sterowanej regeneracji tkanek.

Materiał/Metody: Badania przeprowadzono na 18 białych królikach o wadze 2,5–3 kg (zgoda I Lokalnej Komisji Etycznej ds. Doświadczeń na Zwierzętach we Wrocławiu nr 30/05). W znieczuleniu ogólnym, z dojścia przezskórnego, w krętarzu większym kości udowej, narzędziami ręcznymi wykonywano otwór o średnicy 5 mm. Ta sama procedura operacyjna była przeprowadzana na obu kończynach. W zależności od postępowania z ubytkiem śródkostnym zwierzęta zostały podzielone na trzy grupy, po 3 króliki w każdej z grup:
I Grupa kontrolna (C) – ubytki śródkostne pozostawiane były do samoistnego gojenia,
II Grupa (BOC+BG) – ubytki śródkostne zaopatrywano materiałem Bio-Oss Collagen® oraz błoną Bio-Gide Perio®,
III Grupa (PRP) – do wnętrza ubytku kostnego wprowadzono materiał Bio-Oss Collage® z polipeptydowymi czynnikami wzrostu (PRP). W grupie tej przed zabiegiem pobrano 4 ml krwi z żyły brzeżnej ucha w celu uzyskania osocza bogatopłytkowego PRP (MPW 223 prod. MPW Instruments + PRP-Kit).
Sekcje zwierząt przeprowadzano po 1 i 3 miesiącach. Materiał do badania histopatologicznego barwiono hematoksyliną i eozyną stosując metodę barwienia van Gieson.

Wyniki: Ocena histopatologicza po 1 miesiącu od zabiegu.
I Grupa (C) – ubytki śródkostne wypełnione szpikiem kostnym z dużą liczbą naczyń krwionośnych, widoczne nowo powstające pojedyncze beleczki kostne.
II Grupa (BG +BOC) – w ubytkach śródkostnych widoczne cząsteczki ulegającego resorpcji biomateriału. W centralnej części ubytku cząsteczki biomateriału otoczone pasmami tkanki łącznej, a na obrzeżach wszczepu, wokół cząsteczek widoczne nowo powstające beleczki kostne. Liczba nowo powstających beleczek kostnych znacznie większa niż w grupie C. Brak odczynu zapalnego wokół cząsteczek biomateriału oraz w ich najbliższym sąsiedztwie.
III Grupa (PRP) – liczba nowo tworzących się beleczek kostnych znacząco wyższa niż w grupie C i BG. Obecność beleczek wokół granul biomateriału widoczna nie tylko na obrzeżach ubytków śródkostnych, ale również w centralnych ich częściach. Miejscowo granule biomateriału otoczone tkanką łączną włóknistą. Wzdłuż większości nowo powstałych beleczek kostnych, linijne nagromadzenia osteoblastów, świadczące o intensywnym przebiegu procesów kościotwórczych.
Ocena histopatologiczna po 3 miesiącach od zabiegu.
I Grupa (C) – ubytki śródkostne wypełnione szpikiem kostnym, widoczne nieliczne beleczki nowo powstającej kości, mniejsza liczba naczyń krwionośnych w porównaniu do okresu 1 miesięcznego. Kanał wejścia pokryty zbitą tkanką kostną.
II Grupa (BG + BOC) – wzrost liczby nowo tworzących się beleczek kostnych, oraz większy stopień resorpcji biomateriału w porównaniu do okresu 1-miesięcznego. Obecność powstających beleczek kostnych nie tylko od obwodu ubytku, ale również w jego centrum. Na granicy faz biomateriał/kość widoczne bezpośrednie połączenie struktur, bez obecności komórek zapalnych, miejscowo nieznaczne ilości tkanki łącznej.
III Grupa (PRP) – wzrost liczby nowo tworzących się beleczek kostnych oraz większy stopień resorpcji biomateriału w porównaniu do okresu 1-miesięcznego. Proces kościotworzenia mniej nasilony w porównaniu do 1 miesiąca, mniejsza aktywność osteoblastyczna. Miejscowo między granulami biomateriału obecność tkanki łącznej.

Wnioski: W badaniach histologicznych wykonanych w okresie wczesnym, tj. 1 i 3 miesiące po zabiegu augmentacji ubytków śródkostnych zarówno w grupie PRP jak i BG stwierdzono obecność ulegających resorpcji biomateriałów i tworzenie nowych beleczek kostnych. W pierwszym okresie obserwacji stopień resorpcji cząsteczek biomateriału oraz nasilenie procesów kościotwórczych był intensywniejszy w grupie III, natomiast po 3 miesiącach zaobserwowano nieznaczną przewagę na korzyść grupy II. Pofragmentowane pozostałości biomateriałów w obu grupach otaczała młoda tkanka kostna, miejscowo tkanka łączna włóknista. W obu okresach obserwacji ilość beleczek kostnych po implantcji PRP i BG była większa w porównaniu z grupą kontrolną.

Słowa kluczowe: **ubytki śródkostne • proces gojenia • sterowana regeneracja tkanek • badania na zwierzętach**

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Introduction

Due to the limited regenerative capacity of bone tissue, the healing of intraosseous defects in the presence of particular local and/or general factors can occur through the reparative processes [18]. However, repair is not regarded as an optimal treatment method, as confirmed in many clinical cases [9]. To obtain osseous tissue regeneration, treatment methods referred to as guided bone regeneration (GBR) are used which utilize the mechanical, chemical, and biological properties of materials [2–4,6,7,10,12,13–16,21–25]. Despite the continuous and dynamic development of tissue engineering, it is the host bone which is considered the best regenerative material [25]. Nevertheless, this method is significantly limited by the frequent difficulty in obtaining an adequate amount of material. As a result, alternative solutions are being sought based mainly on xenogenic or alloplastic bone replacement materials, barrier membranes, as well as the application of morphogenetic proteins and polypeptide growth factors [25].

According to most authors, the most appropriate method of treating intraosseous defects is combined therapeutic management involving bone augmentation with a biomaterial and its simultaneous covering with a barrier membrane. This method is based on the cumulative regenerative potentials of both materials and their mechanical maintenance at the site of the defect. The biomaterial supports the barrier membrane, thereby preventing its collapse, while the barrier membrane stabilizes the material and ensures its protection at the entrance site [3,8,26]. The drawback of this method is the use of xenogenic materials. During biomaterial processing, proteins are eliminated, followed by growth factors locally activating osteogenesis. In cases of a poor blood supply to the operated region, their osteoconductive activity becomes impaired, triggered by the inflow of osteoinductive proteins in the blood [9].

Due to the above limitation, many authors recommend a treatment of choice in the form of combining xenogenic osteoconductors with natural sources of active growth factors, for example host bone or polypeptide growth factors [11]. The source of polypeptide growth factors is autogenic platelet-rich plasma (PRP) obtained by the isolation and centrifugation of full venous blood. When thrombin and calcium ions are added to the isolated plasma, a platelet-rich gel is obtained, which is an alternative to fibrin glue. Using polypeptide growth factors causes faster rebuilding of osseous transplants and an increase in the density of newly formed bone tissue, and the fibrin formed in the PRP acts as a tissue glue uniting the bone tissue and the transplant. This results in sealing and prevents displacement of the biomaterial in the defect. The use of platelet-rich gel also accelerates the healing of soft tissues in the operated area and has been reported to display barrier membrane characteristics, which is practically significant as it allows one to limit the amount of xenogenic material in the treatment [17].

The clinical and radiological assessment of the osseous rebuilding process in patients after intraosseous defect treatment, for example in cases of apectomy and cystectomy, is often subjective and tentative despite modern research technology possibilities (e.g. bone density scans of selected areas, mathematical Fourier analysis, and transsectoral CT). The most accurate test for reparative and regenerative process assessment is still histopathological examination. However, histopathological assessment in humans, where the operated site is exposed and the material is collected after apectomy or cystectomy, raises ethical concerns and would undoubtedly be rightly objected by the patient. A plausible option is therefore experimental animal studies.

The purpose of the study was early (1 and 3 months) histological assessment of the healing process of intraosseous defects in the femurs of rabbits with and without the use of selected guided tissue regeneration.

Material and Methods

Eighteen adult white rabbits weighing between 2.5–3 kg were used in this experimental study. Animal selection, management, and experimental protocol were approved by the Local Animal Experimentation Committee in Wroclaw (No. 30/05). The procedure was carried out under general anesthesia (Vetbutal, max. 25 mg/kg i.v., fentanyl 0.05 mg/ml in 10 cm NaCl i.v.) and the premedication given was atropinum sulfuricum (0.1 mg/kg s.c.) and ketaminum (max. 25 mg/kg). After inducing anesthesia, the shaved and disinfected incision site (femur trochanter major) was treated topically with 1% lingocaine solution. After administering local anesthetic, a 5-cm-long skin incision and blunt preparation of the femur muscles were performed. After the periosteoum had been reflected, a bony defect 5 mm in diameter was created using a hand steel bur. The same procedure was performed on both limbs.

The animals were divided into three groups according on the further management of the intraosseous defect. In group I (C) (six rabbits) the defect was left untreated and served as the control. In group II (BOC+BG) (six rabbits) the defects were filled with Bio-Oss Collagen® and covered by Bio-Gide Perio® membrane. In group III (PRP) (six rabbits) the defect was filled with Bio-Oss Collagen® combined with polypeptide growth factors. In this group a sam-

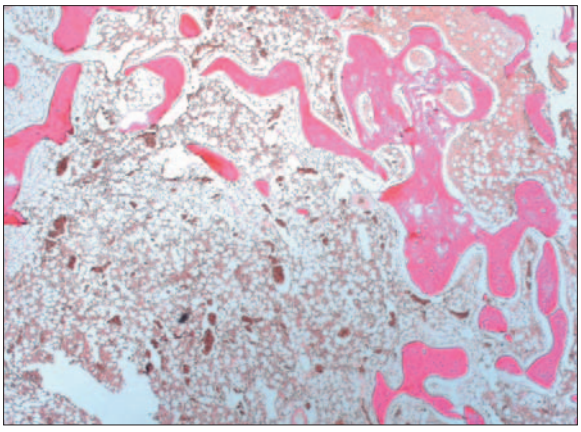

Figure 1. Histopathological specimen, HE staining showed newly formed single bone trabeculas; magnification ×40

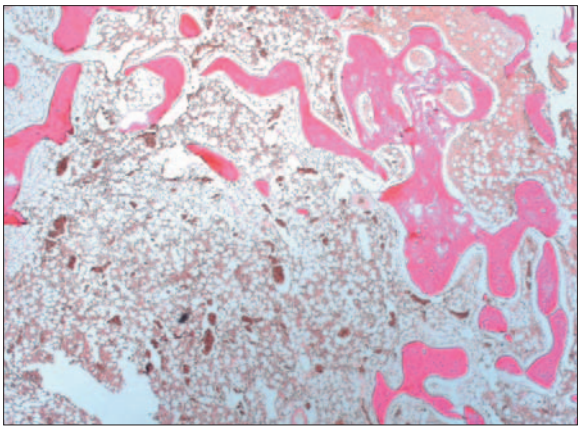

Figure 2. Histopathological specimen, HE staining showed no inflammatory reaction around the remnants of the biomaterial and in the immediate vicinity; magnification ×40

ple of blood (4 ml) was taken from the marginal ear vein in order to obtain platelet-rich plasma PRP (MPW 223, MPW Instruments + PRP-Kit).

After creating the defect and applying the biomaterial where necessary, the operated region was defined with two titanium pins of 3 mm length. Then the muscles and subcutaneous tissues were closed with a Dexon® 3-0 resorbable suture (Tyco, Puerto Rico, USA) and the skin incisions were closed with a AmifilM 3-0® non-resorbable suture (Sinpo, Poland). The postoperative wound was cleansed with hydrogen peroxide solution and disinfected with Prevacare (Johnson&Johnson, Warsaw). On the tenth postoperative day all the skin sutures were removed.

The rabbits were sacrificed 1 and 3 months later with an intravenous dose of pentobarbital (Morbital, Biowet, Pulawy). The maximum dose was 80 mg/kg administered in fractioned doses according to efficacy, i.e. the occurrence of respiratory and cardiac arrest.

The osseous specimens were fixed in a 10% solution of formic formaldehyde in buffered phosphate at room temperature for 72 hours. They were then decalcified in formic and hydrochloric acid solution and placed in 96% alcohol. Sections of the femur bones were cut transversely and longitudinally, dehydrated in acetone (56°C), examined in xylene at room temperature, and embedded in paraffin blocks. Histological 4-µm sections were prepared on a microtome (Leica 2025) and stained with hematoxylin and eosin (HE) as well as the Van Gieson method differentiating connective-tissue stroma. They were then placed in Canada balsam diluted in xylene. The histological specimens were assessed under an Axioscop light microscope (Zeiss) at varying magnifications. The characteristic histological picture was stored photographically using Axiovision (Zeiss) computer software for picture analysis and acquisition.

Quantitative and qualitative histological assessment was performed. Qualitative assessment included: 1) the tissue type inside the intraosseous defect, 2) the resorption process of the given biomaterial, 3) the integration of the xenogenic biomaterial with the surrounding tissues filling the intraosseous defect, and 4) the presence of cells indicative of an inflammatory process. Quantitative assessment was done at the smallest magnification of the operated region and involved comparison of the amount of newly formed trabeculas.

Results

Histological assessment after one month

In group I (C) the bone defects were filled with bone marrow and were highly vascularized. Newly formed single bone trabeculas were present (Figure 1).

In group II (BOC+BG), particles of resorbed biomaterial were observed in the bone defects. In the central part of the defects the remnants were surrounded by streaks of connective tissue. At the edge of the biomaterial, newly formed bone trabeculas were observed. The number of newly formed trabeculas was much greater than in group C. There was no inflammatory reaction around the remnants of the biomaterial and in the immediate vicinity (Figure 2).

Linear concentrations of osteoblasts were present alongside the newly formed trabeculas located at the entrance of the defect (under the barrier membrane). The barrier membrane was visible.

In group III (PRP) the quantity of newly formed trabeculas was significantly greater than in groups C and BG. Newly formed bone trabeculas around the biomaterial granules were observed not only at the edges of the defects, but also in their central parts. The biomaterial granules were surrounded locally by fibrous connective tissue (Figure 3).

Linear concentrations of osteoblasts were present alongside most of the newly formed bone trabeculas (Figure 4).

Histological assessment after three months

In group I (C) the bone defects were filled with bone marrow and there were very few trabeculas of newly formed bone and a smaller number of blood vessels in comparison with the assessment at one month. The entrance of the osseus defect was covered by compact bone tissue (Figure 5).

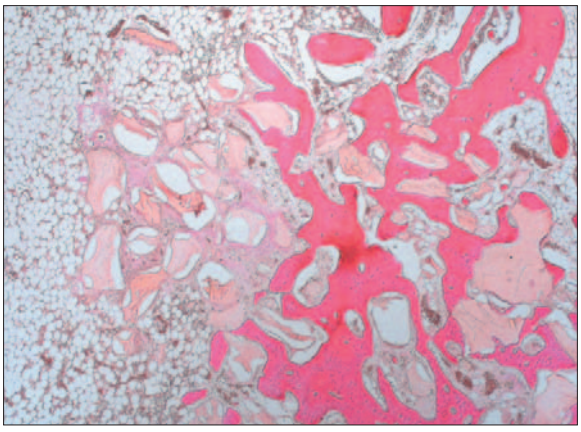

Figure 3. Histopathological specimen, HE staining; the biomaterial granules are surrounded by fibrous connective tissue; magnification ×40

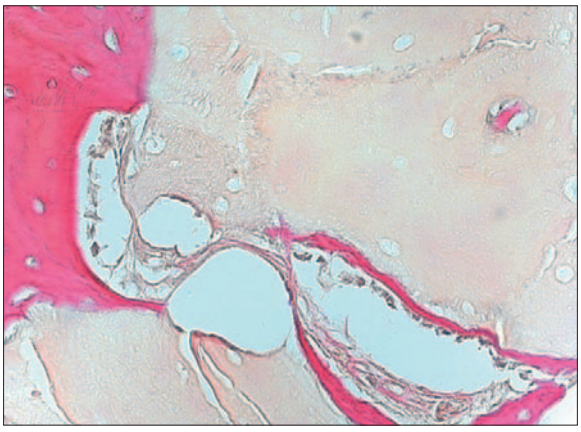

Figure 4. Histopathological specimen, HE staining; newly formed bone trabeculas, linear concentrations of osteoblasts; magnification ×40

In group II (BOC+BG) there was an increase in the number of bone trabeculas and a greater extent of biomaterial resorption than in the one month observation. Newly formed bone trabeculas were observed not only at the edge, but also in the central part of the bone defects. On the biomaterial/bone borderline, direct structure union was visible, with no inflammatory cells, and very little fibrous connective tissue present locally (Figure 6).

In group III (PRP) there was an increase in the number of bone trabeculas and a greater extent of biomaterial resorption than in the one month observation. The bone formation process was less intense than after one month, with less osteoblastic activity. Fibrous connective tissue was present locally between the biomaterial granules.

Discussion

In many clinical cases, for intraosseous defects to be healed with properly woven new bone tissue it is essential to use GBR methods utilizing materials affecting bone repair (BRMs, bone repair materials) [3–18]. In the control group the defects were left untreated and were later filled mainly with bone marrow, wherein single bone trabeculas were noted whose number was significantly lower than in the two GBR groups. After three months, progressive tissue rebuilding was observed in this group in the intraosseous defects, where the entrance was covered by compact bone. No significant increase in the number of bone trabeculas was found in the defect. Moreover, the bone marrow

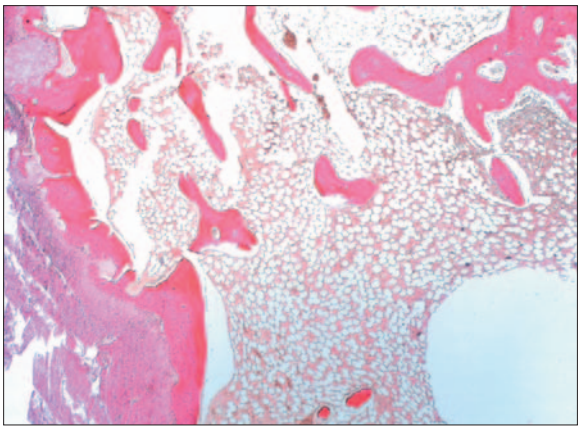

Figure 5. Histopathological specimen, HE staining showed the osseus defect entrance was covered by compact bone tissue; magnification ×40

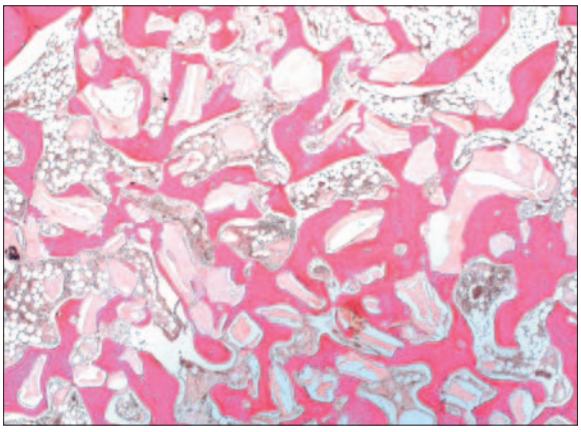

Figure 6. Histopathological specimen, HE staining showed no inflammatory cells on the biomaterial/bone borderline; locally visible, little fibrous connective tissue; magnification ×40

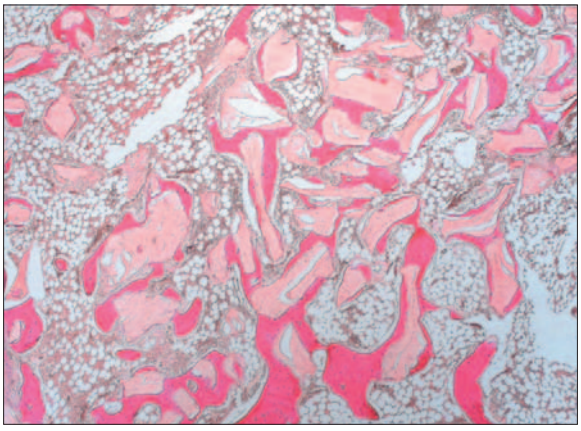

Figure 7. Histopathological specimen, HE staining; locally fibrous connective tissue was present between the biomaterial granules; magnification ×40

filling the defects was characterized by a decreased blood supply, which can be suggestive of slower dynamics of reparative processes.

Histological pictures of intraosseous defects in groups II and III confirm the beneficial influence of BRMs on osteogenetic processes and, accordingly, reparative processes. Newly formed trabeculas were noted in both groups. The trabeculas had formed in the immediate vicinity of the barrier membrane, around the biomaterial granules as well as inside them. As opposed to the control group, linear concentrations of osteoblasts alongside the bone trabeculas were noted after using BRMs in the defects. These concentrations are indicative of the intensity of the osteogenic process. The characteristic feature in the first observation was the regular distribution of osteoblast concentrations within the defect in the PRP group in contrast to the BG+BOC group, in which the concentrations are located mainly close to the barrier membrane. This, in turn, is reflected in the location of the bone trabeculas, which in the BG+BOC group were observed mainly at the edge of the defects, whereas in the PRP group both at the edge and in the center. One could suggest that in the first observation the dynamics of the reparative processes was higher in the case of Bio-Oss Collagen® with polypeptide growth factors than with the barrier membrane. This could be explained by the fact that in the BG+BOC group the active formation of new bone trabeculas occurred in areas with a good blood supply, i.e. mainly in the peripheral regions. In the central part the blood supply of the operated region was inadequate and consequently the inflow of osteoinductive proteins was restricted and, indirectly, so was the action of the osteoinductive biomaterial. This relationship does not apply to defects treated with platelet-rich plasma (PRP), which is a natural source of active growth factors essential for the bone regenerative process [7].

When comparing the ongoing processes in the PRP group, a definite slowing of the osteogenic processes was noted in the second observation, testified by smaller osteoblast activity characterized by the presence of very few linear concentrations of osteoblasts. Single osteogenic cells were found more often around biomaterial granules. The rationale for these processes can be found in the studies on the mechanism of polypeptide growth factors by Marx et al. [19,20]. They showed that the activity of the factors is maintained for 7–10 days. According to Arm et al. [1], the emission of polypeptide growth factors from the impregnated material can take place even after 40 days, peaking between days 10 and 20. Therefore, after three months the growth factors are inactive, with the regenerative function being taken over by the xenogenic osteoconductor itself [5,19,20]. In both BRM groups, connective tissue fibers were found locally around biomaterial granules. However, this does not result from non-osteogenic connective tissue penetrating into the defect and indicate reparative processes, but is an inherent feature of the biomaterial.

Conclusions

The histological evaluation performed during the early period, i.e. 1 and 3 months, after creating an intraosseous defect revealed the presence of resorbed biomaterials and the formation of new bone trabeculas in both the PRP (III) and BG (II) groups. At the first observation the extent of biomaterial resorption and the osteogenic process was more intense in group III, but after three months group II had a slight advantage. The fragmented remnants of the biomaterial in both groups were surrounded by new bone tissue, locally by connective fibrous tissue. At both times the barrier membrane or its fragments were present in group II. At both times the amount of bone trabeculas after PRP and BG implantation was higher than in the control group.

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