BONE TISSUE ENGINEERING BASED ON BONE MARROW IN BLOOD CLOT LOADED ON MINERAL MATRIX CARRIER: EXPERIMENTAL STUDY IN SUBCUTANEOUS MICE MODEL

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Repair of bone defects can be supported by appropriate biomaterials, cells and regulatory signals, which is the basic principle of bone tissue engineering. The components of bone marrow and blood clot come in close contact with bone defects after injury. Since these structures represent the source of different cell types and regulatory signals, we wanted to examine their effect on osteoreparatory process in ectopic implants. Subcutaneous implantation was performed on male Balb/c mice. Bio-Oss® biomaterial in the function of mineral matrix carrier was loaded with blood diluted with bone marrow cell suspension (MMB-type implants) or only with blood diluted with saline (MB-type implants, control). Implant extraction was performed 2, 8 and 12 weeks after implantation. MMB-type implants were characterized with more pronounced beginning of resorption in comparison with MB-type implants. High cell density of connective tissue was also preserved during the 12-weeks observation period in MMB-type implants, as well as good vascularity, which was most pronounced 8 weeks after implantation. Osteoblast-like cells and osteon-like structures could be observed. These results suggest that bone marrow in synergy with blood clot might have a stimulating effect on osteoreparation process. *Acta Medica Medianae 2017;56(3):5-11.*

Key words: bone marrow, blood clot, mineral matrix carrier, implant, osteoreparation process

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Introduction

Undisturbed remodeling of bone tissue and repair of small bone defects involve a balanced activity of osteoblasts and osteoclasts (1). However, despite this established mechanism, large bone defects cannot completely heal and require the application of biomaterials, when those on the hydroxyapatite base in the form of granules and blocks are often used (2, 3). Besides biomaterials, studies are directed toward their combinations with cells and/or regulatory signals (4, 5). This approach has proven to be highly suitable in bone tissue engineering, because it can favorably affect the speed and end-result of the osteoreparation process (6-12).

Investigation of the effect of bone marrow (BM) and blood clot (BC) on the osteoreparation process is important from several aspects. Bone is the natural environment of BM, so BM can come in contact with biomaterials that are used to fill large bone defects. In addition, BM is an excellent source of different cell types, including mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs), vascular progenitors and different regulatory signals (13-15), all of which play an important role in bone tissue reparation and regeneration processes. Tissue injury, including injury of the bone tissue, is often accompanied with bleeding and consequent BC formation, which contains different cells and regulatory signals. As such, BC has an important role in tissue reparation. During the application of biomaterials, BC can permeate them and thus participate in the healing of bone defects as well (11, 16, 17).

In the present study, osteogenic potential of bone marrow cells (BMCs) combined with BC and

implanted on mineral matrix carrier (MMC) was examined in a subcutaneous mice model.

Material and methods

Male Balb/c mice aged 8 to 10 weeks and weighing between 22 and 24 g were used in this study (with the approval of the institutional Ethics Committee, University of Niš Faculty of Medicine). Mice were kept under standard housing conditions with ad libitum access to food and water.

As a MMC in our experiment, we used the Bio-Oss® biomaterial (Geistlich-Pharma, Wolhusen, Switzerland), derived from the bovine bone by the process of deproteinization.

BMCs were obtained from femurs of five mice, as described earlier (18). In brief, femurs were cleaned of the surrounding tissue in a sterile chamber. Epiphyses were cut and BM was flush out into 10 ml tubes by inserting a 26-gauge needle attached to the syringe filled with cold, sterile RPMI 1640 medium (PAA Laboratories, Austria). Centrifugation of the cells at 1200 rpm and 4°C, for 10 min was followed by adjustment of their density.

Blood was collected from the retro-orbital plexuses of five mice and immediately diluted with prepared BMCs suspension or saline in 1:4 ratio.

Two types of implants were made:
1. MMB-type implants: MMC + BMCs + BC
2. MB-type implants: MMC + BC

Implants, lump-shaped, contained each 10 mg of MMC mixed with 30 µl of diluted blood. Blood in implants was allowed to clot before implantation. The number of BMCs was adjusted at 2.5×10^5 cells per implant. MB-type implants served as controls.

Each of two animal experimental groups consisted of 18 animals. Mice were anesthetized with 10% ketamidor (0.1ml/10g b.w.) and 4 implants of one type were inserted into the interscapular subcutaneous pocket. Implants were removed after 2, 8 and 12 weeks of implantation and fixed in 10% neutral buffered formalin. Decalcification was carried out in 10% formic acid. After standard tissue processing, H&E (Haematoxylin and eosin) staining was performed.

Results

Histological analysis two weeks after implantation

At 2-week observation point (Figure 1), MMB-type implants are characterised by high cell density of connective tissue between MMC particles. Among numerous cell types, inflammatory ones stand out in the form of multinuclear cells, polymorphonuclear cells and other leukocytes. The tissue between the particles shows an exceptionally good vascular support. Multinuclear cells are at the surface of particles, but are also penetrating into particles accompanied by a heterogeneous cell population resembling the hematopoietic process. In some spots, osteoblast-like
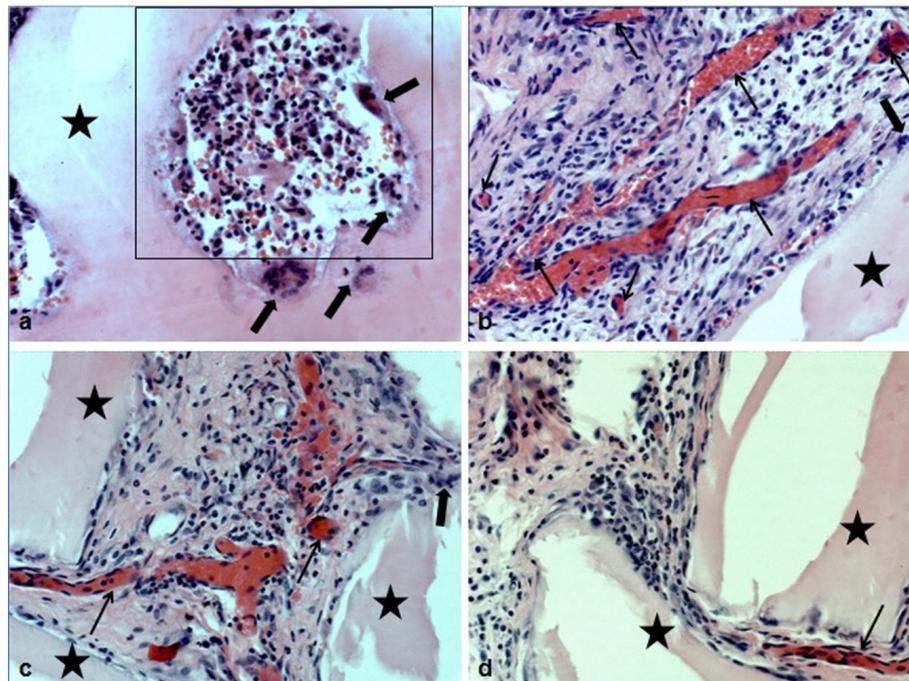

Figure 1. Microscopic images of implants two weeks after implantation.

(a, b) MMB-type implants, H&E, magnification 400x,
(c, d) MB-type implants, H&E, magnification 400x.
Legend: Mineral matrix carrier (star), phagocytes (thick arrow), blood vessel (thin arrow), cell infiltration (square).
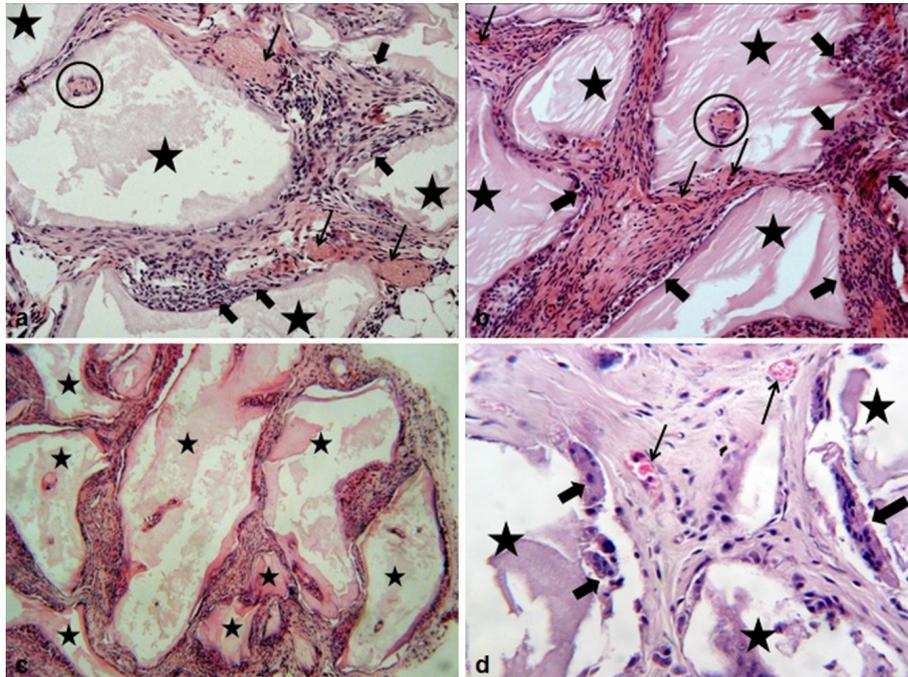
Figure 2. Microscopic images of implants eight weeks after implantation

(a, b) MMB-type implants, H&E, magnification 200x,
(c) MB-type implants, H&E, magnification 100x,
(d) MB-type implants, H&E, magnification 400x.
Legend: Mineral matrix carrier (star), phagocytes (thick arrow), blood vessel (thin arrow), osteon-like structure (circle)
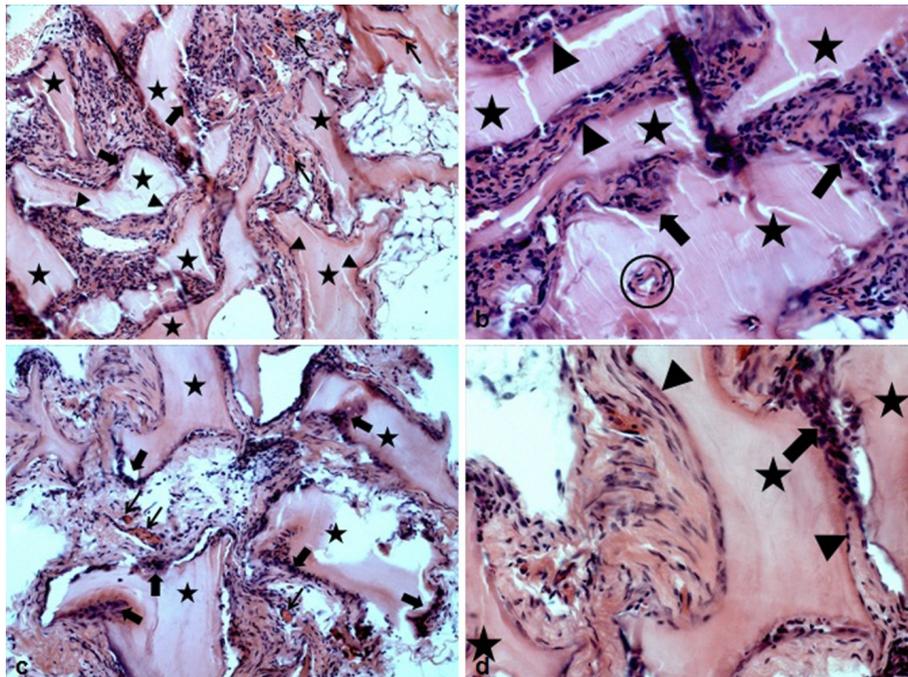

Figure 3. Microscopic images of implants twelve weeks after implantation

(a) MMB-type implants, H&E, magnification 200x,
(b) MMB-type implants, H&E, magnification 400x,
(c) MB-type implants, H&E, magnification 200x, (d) MB-type implants, H&E, magnification 400x.
Legend: Mineral matrix carrier (star), phagocytes (thick arrow), blood vessel (thin arrow), osteoblast-like cells (arrowheads), osteon-like structure (circle)

cells form cell monolayer on the particle surface. In the same period of observation, phagocytes can be found predominantly on the surface of particles in MB-type implants. Well developed connective tissue and a blood network are also seen.

Histological analysis eight weeks after implantation

Strong resorption of MMC particles persists in MMB-type implants during this period, evidenced by the presence of giant multinuclear cells. The tissue between the particles, rich in cells, is permeated with numerous blood vessels (some of them very large). Highly organised collagen fibers surround the MMC particles. Osteon-like structures can be seen in some particles. In MB-type implants, good tissue cellularity is preserved. Thanks to numerous, giant, multinucleated cells, the resorption process is in full swing. The connective tissue between the particles is permeated with blood vessels which are obviously smaller in diameter compared to those observed in MMB-type implants (Figure 2).

Histological analysis twelve weeks after implantation

The particles of MMC in MMB-type implants are reduced in size compared to the previous period of observation. The connective tissue contains blood vessels, numerous cells and fibers. Osteon-like structures, as well as osteoblast-like cells, are present. The particles in MB-type implants are thinned and in some places a strong resorption process takes place. Blood vessels permeate the tissue between MMC particles. Connective tissue cell density is reduced, compared with MMB-type implants and MB-type implants from the previous observation points. At some spots osteoblast-like cells are found (Figure 3).

Discussion

The basic principle of tissue engineering involves the use of three components in order to achieve good tissue regeneration: cells, scaffolds and regulatory signals (19). Bone fractures do not involve only the injury to the bone tissue, but other structures such as bone marrow and blood vessels as well. As mentioned above, bone marrow and blood are the source of different types of cells (stem cells among others), as well as regulatory signals (cytokines and growth factors). At the fracture site, due to blood vessel injury, the formation of blood clots (hematoma) occurs, which together with bone marrow, periosteum and surrounding tissues participate in proper bone tissue healing (20).

An implantation of biomaterial is naturally followed by the resorption, which is a critical step that allows the creation of space to be filled with bone and vascular tissues (21). Both types of implants are characterized by the presence of

phagocytes during the 12-week period of observation. However, in MMB-type implants, a stronger resorption process is noticeable 2 and 8 weeks after implantation, decreasing thereafter, compared to MB-type implants in which resorption increases in 8th week and persists in 12th week after implantation. It is well documented that bone marrow accomodates bone marrow-derived macrophages (22), while blood contains monocyte- macrophage precursors (23). The differences in the resorption rate between these two examined types of implants could therefore be explained with the initial presence of both cell types in MMB-type implants, compared to MB-type implants (which initially contain blood monocytes only). Further, bone marrow and blood are the reservoir of different cytokines e.g. tumor necrosis factor-alpha (TNF-alpha) and Interleukin-6 (IL-6) (24, 25), responsible for phagocyte support and attraction. Even 12 weeks after implantation MMC is not fully resorbed in any of the implant types, which is in correlation with fact that resorption of inorganic biomaterials can last for many years (26).

Both types of implants are characterized by the presence of good vascular support 2 weeks after implantation. In this regard, greater differences are noticed, especially at 8-weeks observation point. Again, these differences could be attributed to the initial content of these two types of implants we studied. Bone marrow is a rich source of proangiogenic factors, as it is shown that conditioned BMCs supernatants increase proliferation, migration and tube formation of human coronary artery endothelial cells (27). Likewise, endothelial progenitor cells exist in bone marrow (28), while platelet secretory factors have a chemoattractant activity on endothelial cells and promote growth of new blood vessels (29). Platelets promote mobilization of BMCs and their recruitment into the growing vasculature, according to the results obtained from hypoxia-induced angiogenesis models (30). Angiogenesis and vasculogenesis are the basis of proper osteogenesis and bone fracture healing, because bloodstream is the means of transportation for different cells and regulatory signals which are the key for successful osteogenic and reparation processes (8, 31). Good vascular support in MMB-type implants through 12-weeks observation period and initial presence of not only BMCs and blood cells, but also cytokines with chemoattractant influence, may have a role in preserved tissue cellularity between the particles in these implants. Unlike MMB-type, in MB-type implants tissue cellularity decreased at 12-weeks observation point. Osteoblast-like cells, osteon-like structures, as well as hematopoietic fields have been observed in MMB-type implants, which can be (although not solely) attributed to the initial presence of MSCs and HSCs.

Conclusion

The inclusion of bone marrow and blood clot in ectopic implant composition enhances angioge-

nesis and neovascularization and enables long-term tissue cellularization, all of which could have a beneficial effect on the ectopic osteogenic process. Our results indicate that bone marrow in blood clot loaded on a mineral matrix carrier can be promising for bone tissue engineering.

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Originalni rad

TKIVNO INŽENJERSTVO KOSTI BAZIRANO NA KOSTNOJ SRŽI U KRVNOM UGRUŠKU NA MINERALNOM MATRIKSU KAO NOSAČU: EKSPERIMENTALNA STUDIJA NA SUBKUTANOM MODELU MIŠA

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Reparacija koštanih defekata može biti potpomognuta odgovarajućim bio-materijalima, ćelijama i regulatornim signalima, što jeste osnovni princip tkivnog inženjerstva kosti. Komponente kostne srži i krvnog ugruška dolaze u bliski kontakt sa koštanim defektima nakon povrede. Imajući u vidu da ove strukture predstavljaju izvore različitih ćelija i regulatornih signala, želeli smo da istražimo njihov efekat na osteoreparatorni proces u ektopičnim implantima. Subkutana implantacija je izvedena na miševima Balb/c soja. Biomaterijal Bio-Oss® u funkciji mineralnog matriksa kombinovan je sa krvlju razblaženom suspenzijom ćelija kostne srži (MMB implanti) ili samo sa krvlju razblaženom fiziološkim rastvorom (MB implanti, kontrola). Implanti su ekstrahovani dve, osam i 12 nedelja nakon implantacije. MMB implante karakteriše izraženiji početak resorpcije u odnosu na kontrolu. U MMB implantima je takođe prisutna velika gustina vezivnog tkiva tokom 12-nedeljnog perioda posmatranja sve vreme, kao i dobra vaskularizovanost, koja je najizraženija 8 nedelja nakon implantacije. Uočavaju se i ćelije nalik osteoblastima i strukture nalik osteonu. Navedeni rezultati ukazuju na to da komponente kostne srži, zajedno sa krvnim ugruškom, mogu imati podsticajan efekat na osteoreparatorni proces. *Acta Medica Medianae 2017;56(3):5-11.*

Ključne reči: *kostna srž, krvni ugrušak, mineralni matriks, implant, osteoreparatorni proces*