

OSTEOGENIC EFFECT OF INFLAMMATORY MACROPHAGES LOADED ONTO MINERAL BONE SUBSTITUTE IN SUBCUTANEOUS IMPLANTS

Jelena M. Živković^{1,*}, Stevo J. Najman¹, Marija Đ. Vukelić¹, Sanja Stojanović¹, Milena V. Aleksić²,
Milica N. Stanisavljević² and Jelena G. Najdanović¹

¹ Institute of Biology and Human Genetics, Faculty of Medicine, University of Niš, Boulevard Dr Zoran Đinđić 81,
18000 Niš, Serbia

² Department of Biology and Ecology, Faculty of Science and Mathematics, University of Niš, Višegradska 33,
18000 Niš, Serbia

*Corresponding author: jelena.zivkovicmf@yahoo.com

Abstract - We analyzed the influence of inflammatory macrophages on the osteogenic process in subcutaneous implants composed of mineral bone substitute. Thioglycollate-elicited peritoneal macrophages (TEPMs) were characterized as inflammatory. This was confirmed microscopically by the nitroblue tetrazolium (NBT) test and the production of tumor necrosis factor α (TNF- α). The implants (M-type) were made of mineral bone substitute (Bio-Oss[®]) mixed with TEPMs and blood clot. Implants without macrophages served as the control (C-type). Subcutaneous implantation in the interscapular area was performed on BALB/c mice. Implants were extracted after 2 and 8 weeks. In M-type implants, phagocytosis and angiogenesis were more pronounced, and osteoblast-like cells aligned onto granules of implanted material and osteoid structures can be seen. The observed higher osteocalcin and lower osteopontin immunoreexpression in M-type implants when compared to the control after 8 weeks suggest a more advanced osteogenic process. Our results indicate that the presence of inflammatory macrophages in the composition of an implant may have a beneficial effect on the osteogenic process.

Key words: macrophages; osteogenesis; subcutaneous implantation; mineral bone substitute; blood clot

Received September 15, 2014; **Accepted** October 6, 2014

INTRODUCTION

In clinical practice, there are often situations where major injuries, diseases or congenital malformations cause large bone defects (Khan et al., 2005). The repair of large bone defects has always been a challenge for orthopedics and surgery in general. Fracture healing involves the coordination of migration, proliferation, differentiation and activation of many cell types (Colnot et al., 2005).

The bone fracture healing process has three stages: inflammation, renewal and remodeling (Einhorn, 1998; Dimitriou et al., 2005). Therefore, bone fracture reparation is preceded by initial inflammatory response whose main participants are macrophages (Andrew et al., 1994). They are known to release a variety of factors that affect bone, such as proinflammatory cytokines and reactive oxygen species (Christou et al., 1987; Forman and Torres, 2002; Kanczler et al., 2003; Schett, 2011). Opinions differ as to how

macrophages and their products affect bone and osteogenesis (Grundnes and Reikeraas, 2000; Champagne, 2002; Mountziaris and Mikos, 2008; Lacey et al., 2009; Soltan et al., 2012; Bhat et al., 2013).

Biomaterials that are commonly used as “helpers” in regeneration and reparation processes are natural and synthetic hydroxyapatite-based mineral bone substitutes (Schwartz et al., 2000). We hypothesized that the inclusion of inflammatory macrophages in the composition of the implant based on mineral bone substitute could have an effect on osteogenic process, especially in initial phase. Since bone tissue is well vascularized, bone fractures often lead to bleeding. Therefore, it is of great importance to use blood as one of the components of experimental implant composition. In this way, the blood clot and macrophages affect the initiation of the osteogenic process, which could be a simulation of what happens in the healing of bone fractures. Nowadays there it believed that the molecules of the inflammatory phase and the formation of blood clots are essential for initiating the reparative process (Shapiro et al., 2008).

In this paper, we evaluated the role of inflammatory macrophages in the initiation of osteogenic processes on subcutaneous implantation model. To the best of our knowledge, there are no data describing the effects of inflammatory peritoneal macrophages obtained from elicited mouse peritoneum on ectopic osteogenic processes.

MATERIALS AND METHODS

Mineral bone substitute

The mineral bone substitute that has been used in this experiment is Bio-Oss[®]. Bio-Oss[®] (Geistlich-Pharma, Wolhusen, Switzerland) is a biocompatible biomaterial that is structurally most similar to hydroxyapatite in bone.

Experimental animals

BALB/c mice were obtained from the Military Med-

ical Academy (Belgrade, Serbia). The study was performed according to the Animal Welfare Act (Republic of Serbia) and European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS no. 123), with the approval of the Institutional Ethics Committee (Medical Faculty, University of Niš). In our experiment, males weighing between 22 and 24 g, at 10 to 12 weeks of age were used. Animals were kept in standard conditions and had access to food and water *ad libitum*.

Peritoneal macrophage collection

Thioglycollate-elicited peritoneal macrophages (TEPMs) were obtained from 10- to 12-week-old male BALB/c mice, as described previously (Stewart et al., 1975), with some modifications. Briefly, five mice were injected intraperitoneally with 0.5 ml of 3% thioglycollate (Fluka, Munich, Germany) to induce aseptic peritonitis. After 4 days, the animals were anesthetized with 10% ketamidol (Richter Pharma, Wels, Austria) and peritoneal lavage was performed with cold, sterile RPMI 1640 medium (PAA Laboratories, Pasching, Austria) supplemented with 25 U/ml heparin (Galenika, Belgrade, Serbia). The peritoneal cavity was washed twice with 5 ml heparinized RPMI medium. Fluid recovery was approximately 3 ml per mouse. Cells were centrifuged twice (1 200 rpm, 4°C, 10 min) and diluted in RPMI without heparin. Resident peritoneal macrophages (RPMs) were obtained in the same manner without thioglycollate elicitation of macrophages.

Preparation of macrophage conditioned medium (CM)

Macrophage-conditioned medium (CM) was prepared according to previously published protocols, with some modifications (Sassa et al., 1983; McKenna et al., 1988). RPMs or TEPMs were centrifuged and density was adjusted to 2×10^6 cells/ml in RPMI supplemented with 2% fetal bovine serum (FBS; PAA Laboratories) and an antibiotic-antimycotic solution (PAA Laboratories). One milliliter of cell suspension per well was seeded into 12-well plates (Greiner Bio-

One, Frickenhausen, Germany) and incubated for 3 h to let the cells adhere to the wells in a humidified atmosphere containing 5% CO₂ at 37°C. The non-adherent cells were removed by washing with phosphate buffered saline (PBS). Two ml of fresh medium was added to each well and cells were incubated for additional 24 h. CM media were collected from wells and centrifuged (3000 rpm, room temperature, 10 min) to remove particulate material. The CM were filtered through a 0.2- μ m-pore sized filter (Minisart[®] Sartorius, Wien, Austria), aliquoted and stored on -20°C until further testing.

Microscopic NBT test

After the CM was removed, cells were washed with fresh medium. The NBT (nitroblue tetrazolium) test is conventionally used to determine the intracellular production of reactive oxygen species in phagocytic cells through their ability to reduce the NBT substance (Choi et al., 2006). We performed a microscopic NBT test according to the principles of the Gifford and Malawista (1972) method, with some modifications. The NBT solution was prepared by dissolving the NBT powder (nitrotetrazolium blue chloride; Sigma-Aldrich, Munich, Germany) in PBS (pH 7.4), filtered through a 0.2- μ m-pore sized filter and added to macrophages at concentration 0.1% (w/v). To stimulate the reduction of NBT we added phorbol myristate acetate (PMA; Sigma-Aldrich) at the final concentration of 10⁻⁶ M. Cells were incubated with the NBT-PMA solution for 30 min at 37°C. Macrophages reduced the yellow dye NBT to blue formazan crystals. After the incubation period, the cells were washed in PBS and fixed in methanol (Merck, Darmstadt, Germany) for 5 min at room temperature. Fixed cells were then washed in distilled water and air-dried. We observed the cells with an inverted light microscope (Observer Z1, Carl Zeiss, Jena, Germany), previously covered by a small volume of PBS, to see the granules clearly without counterstaining. Cell imaging was performed with the appropriate software (AxioVision, Carl Zeiss). For the semiquantitative analysis of the NBT test, we used an arbitrary scale, 0 through 3. With "0" are marked cells without formazan deposits, "1" are cells

with a small amount of powdered formazan, "2" are cells with several dense formazan clusters and "3" are cell filled cells with formazan crystals. The percentage of each class of cells was calculated after counting at least 200 cells in *quadruplicate*. Class "0" presented NBT-negative cells, and other classes in the sum presented NBT-positive cells.

L929 bioassay for TNF- α determination

The presence of TNF- α in the CM of TEPMs and RPMs was determined by a cytotoxicity evaluation on the L929 cell line (murine fibroblasts), the most common cell line used for the evaluation of TNF- α presence due to its high sensitivity to TNF- α (Shiau et al., 2001) and the ability of TNF- α to cause cell death in this cell line. L929 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; PAA Laboratories) containing 10% FBS, 2 mM L-glutamine (PAA Laboratories) and the antibiotic-antimycotic solution at 37°C in a humidified atmosphere containing 5% CO₂. Replacement of medium was carried out every 2-3 days. After reaching the appropriate confluence, cells were detached with Trypsin-EDTA solution (PAA Laboratories) and centrifuged at 4°C for 10 min at 1000 rpm. Cells were then washed and the appropriate density was adjusted. In this assay, 2.5 x 10⁴ cells per well were seeded in 96-well plates in DMEM containing supplements as above. After 24 h of cultivation, the medium was removed and the CM, containing FBS at final concentration 10% and 2 mM L-glutamine, was added to the cells. Fresh RPMI medium containing supplements as above (control medium) was added to the cells that were used as control. Cells were incubated in CM or control medium for the next 24 h. Cells were microscopically analyzed at the end of incubation period and then the MTT cytotoxicity test was performed.

MTT test

The MTT test is based on the reduction of yellow MTT (tetrazolium salt 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; Carl Roth, Karlsruhe, Germany) by mitochondrial dehydrogenases of viable cells to purple formazan, which is insoluble in

water. This test was performed according to the basic principles of Mosmann's method with modifications (Mosmann, 1983). After completion of the incubation period, cells were washed with PBS and then 100 μ l of MTT solution at a concentration of 1 mg/ml was added to each well. Cells were incubated in MTT solution for the next 3 h at 37°C. Formazan crystals were dissolved with 100 μ l of 2-propanol (Fisher Chemical, Pittsburgh, PA, USA) and the absorbance values were measured on ELISA plate reader (Thermo Labsystems, Milford, Massachusetts) at 540 nm. Results are presented as a percentage of cell viability calculated by the following formula: % of cell viability = absorbance value of cells in CM * 100 / absorbance value of cells in control medium. Statistical analysis was performed using absorbance values and included standard deviation, coefficient of variation and Student's t-test.

Animal experimental design

Blood collection

Blood was used to form a clot to serve as natural "fibrin glue" which enables implant components to become homogeneous. In addition, a blood clot simulates natural injury. Blood was collected from a retro-orbital plexuses of three anesthetized BALB/c mice and pooled. For implant preparation, blood was diluted with prepared macrophage cell suspension or saline in 1:4 ratio, which we have previously found to have the ability to form a clot.

Implants preparation

Every single implant contained 10 mg of Bio-Oss[®] and 30 μ l of diluted blood and was shaped in lumps after clotting. We made 2 types of implants: 1) M-type composed of Bio-Oss[®] mixed with blood diluted with TEPMs suspension, so the total number of macrophages was 2.5×10^5 per implant, and 2) C-type composed of Bio-Oss[®] mixed with blood diluted with saline which served as a control.

Implantation

For implantation, 24 BALB/c mice were divided

randomly into two experimental groups with 12 animals per group. Before the experiment, the animals were anesthetized with 10% ketamidol (0.1ml/10g b.w.). A surgical field was shaved and cleaned with foam and povidone-iodine solution. Implants were inserted in a subcutaneous pocket through a large, sterile biopsy needle, interscapularly. Each animal had four implants inserted of one type separated in four different places in the form of X in relation to the entry point. Two and 8 weeks after implantation the implants were removed (from 6 animals per experimental group and per implantation time), providing 24 samples per implant type and implantation time.

Histology

The removed implants were fixed in 10% neutral buffered formalin and decalcinated in 10% EDTA solution (pH 7.4), dehydrated in ascending concentrations of ethanol and embedded in paraffin. H&E (Hematoxylin and eosin) and MT (Masson trichrome) staining were performed on 2.5- μ m thick sample slices. Immunohistochemical staining was performed on 4- μ m thick sample slices. Sections were then analyzed and photographed under a light microscope (Leica DMLS).

Immunohistochemistry

We examined osteocalcin (OC) and osteopontin (OP) as osteogenic markers using the antibodies anti-osteocalcin (ab93876, Abcam, Burlingame, USA) and anti-osteopontin (ab8448, Abcam). For immunohistochemical staining, an HRP/DAB detection IHC kit (ab64261 Abcam) was used according to the manufacturer's protocol. Paraffin was removed with xylene and the sections were rehydrated in descending concentrations of ethanol. Sections were incubated with Hydrogen Peroxide Block for 10 min at room temperature to inactivate endogenous peroxidase, and Protein Block for 5 min at room temperature to prevent nonspecific background staining. Tissue sections were then incubated with the primary antibodies anti-osteocalcin (1:200) and anti-osteopontin (1:100) for 1 h at 37°C, washed with PBS and incu-

bated with biotinylated goat anti-rabbit IgG for 10 min at room temperature. After this, streptavidin peroxidase was added for 10 min at room temperature followed by incubation with DAB chromogen for 10 min at room temperature. Sections were counterstained with *Mayer's* hematoxylin, dehydrated and mounted with permount. As negative control, sections were treated in the same way with the exception of the primary antibodies.

RESULTS

Functional characterization of macrophages NBT reduction ability

Reactive oxygen species production in the PMA-stimulated TEPMs was higher compared to RPMs. We used the microscopic NBT test for characterization of macrophages after 24 h incubation in cell culture medium. Among the TEPMs was a larger number of NBT-positive TEPMs, as well as a larger amount of formazan deposits per cell (Fig. 1A). Among the RPM, 5.34% NBT-negative cells were observed as compared to only 0.96% of TEPMs. In addition, there were more RPMs than TEPMs in class "1", and in class "3", the opposite relation between these two types of macrophages (Fig. 1B).

TNF- α activity

TNF- α activity was measured by L929 bioassay. We measured cell viability using the MTT test and we presented the results as a percentage of cell viability in both conditioned media, TEPMs and RPMs, respectively (Fig. 2A).

The CM of TEPMs reduced the viability of L929 cells by 33.6% and was slightly cytotoxic, while the CM of RPMs was not cytotoxic on L929 cells and cell viability was reduced by only 3.3%, which means that the CM obtained from RPMs did not contain TNF- α in detectable levels according to this assay (Fig. 2A). Cell viability in the CM obtained from TEPMs was statistically significantly reduced ($p < 0.01$) compared to the CM obtained from RPMs and the control medium.

In Fig. 2B, the reduced number of L929 cells in the CM of TEPMs can be seen, while cell density in the CM of RPMs is similar to the control culture. Cells that were incubated in the CM of TEPMs had needle-shaped extensions that presented a sign of the cytotoxic effect.

Histological analysis of implants

Two weeks after implantation, a connective capsule was observed over the M-type implants. Bio-Oss[®] particles are big, but the process of degradation could be noticed. In some places, large, very active multinuclear phagocytes were observed (Figs. 3A and B). Between the Bio-Oss[®] particles immature connective tissue could be seen, which contained whole cells, was and rich in collagen; the observed spindle-shaped cells were arranged in layers on the particles' surfaces. Osteoblast-like cells were sporadically observed (Fig. 3). The implant was permeated mainly by smaller blood vessels, located within immature connective tissue (Fig. 3A).

Two weeks after implantation, a connective capsule was observed over the C-type implants. The structure of Bio-Oss[®] particles was more compact than in the M-type implants. Multinucleated phagocytes were present on the implant particles, resorbing them (Fig. 4A). Immature connective tissue between the particles of Bio-Oss[®] was characterized by high cell density. Collagen deposits were observed between particles. Some cells entered into the biomaterial (Fig. 4B). The implant was permeated mostly by small blood vessels whose number was smaller than in the M-type implants (Fig. 4A).

Eight weeks after implantation, biomaterial particles had not yet been fully resorbed in the M-type implants, but they were much reduced in size. Phagocytosis was very prominent, observed as increased numbers of giant multinuclear cells. The connective tissue between particles was present, but with reduced cellularity. Major blood vessels could be seen (Fig. 5, A) as well as osteoblast-like cells arranged as a palisade on the particle surface (Fig. 5, B). In addition, the forming osteoid tissue was seen

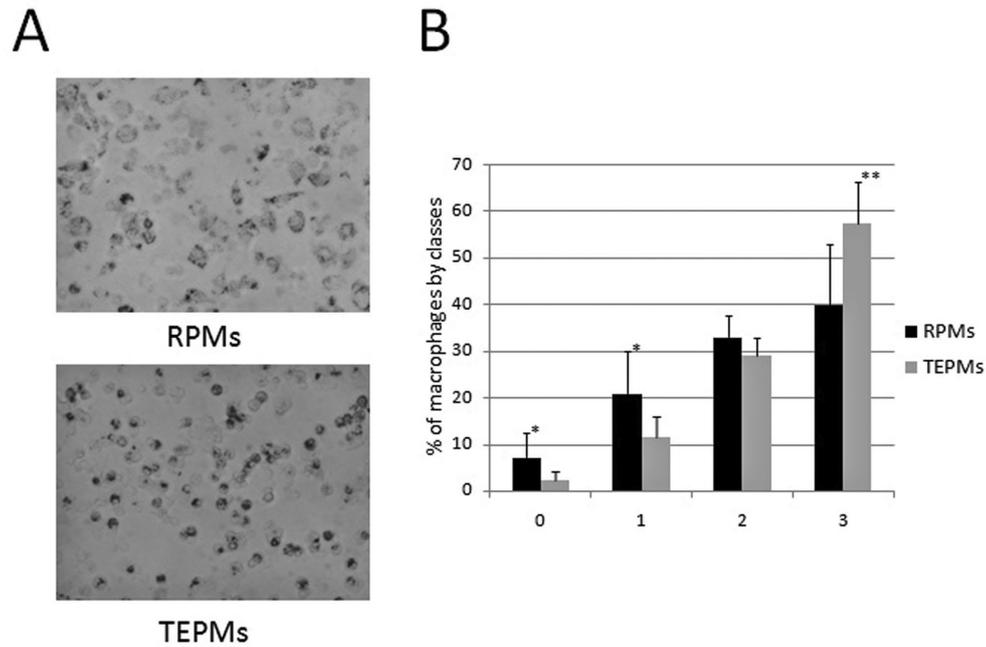


Fig. 1. Microscopic NBT test for characterization of peritoneal macrophages after 24 h incubation in cell culture medium; brightfield, 400x (A). Percentage of different classes of macrophages in relation to the presence of formazan after 24 h incubation in cell culture medium.

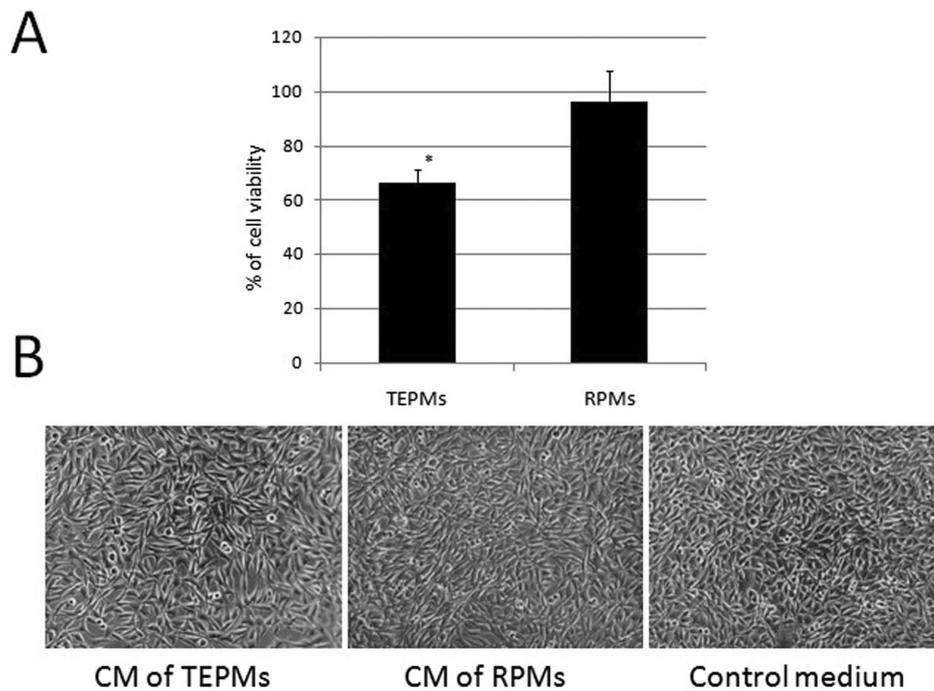


Fig. 2. Percentage of viability of L929 cells in CM obtained from TEPMs and RPMs, * $p < 0.01$ (A). Morphology of L929 cells after 24 h incubation in different media; phase contrast, 200x (B).

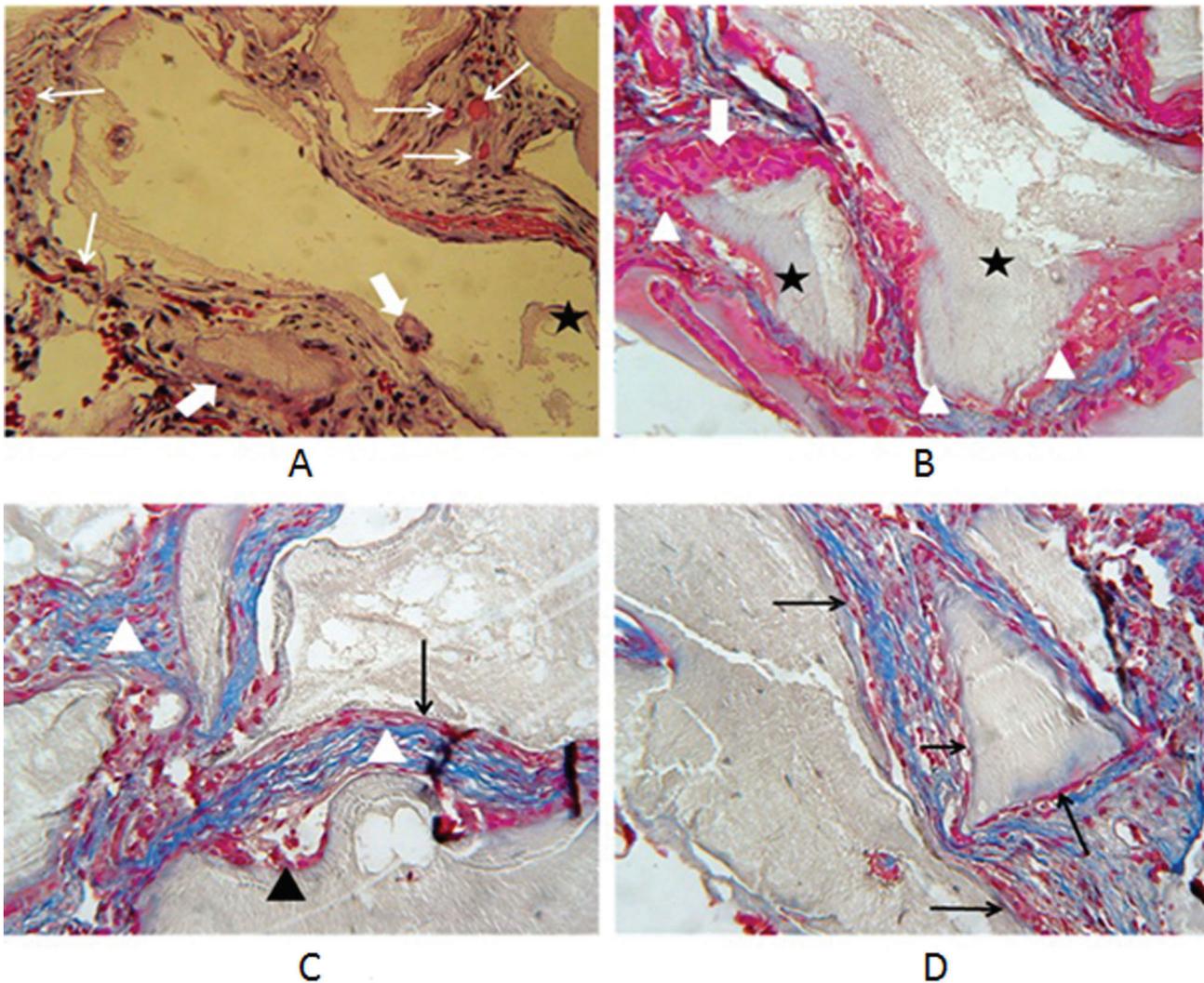


Fig. 3. M-type implants after 2 weeks of implantation. H&E, 400x (A); MT, 400x (B), (C), (D), respectively. Multinuclear phagocytes - thick white arrow; blood vessels - thin white arrow; collagen - white triangle; cell lining and attaching to Bio-Oss[®] particle - thin black arrows; osteoid formation - black triangle; Bio-Oss[®] particle - black star.

in the implant (Fig. 5, C). There was less collagen in the M-type implants (Fig. 5, D) than in the C-type implants (Fig. 6, A).

Eight weeks after implantation in C-type implants Bio-Oss[®] particles were still large and poorly resorbed. There were still some areas where the process of phagocytosis was active, but it seemed less pronounced than in the M-type implant. Mostly smaller osteoclast-like cells could be seen. The connective tissue between particles was more abundant

compared with the M-type implants. The implants were permeated with a well-developed collagen matrix (Fig. 6, A). Mononuclear osteoblast-like cells were sporadically distributed on biomaterial surfaces as well (Fig. 6, B).

Immunohistochemical analysis of implants

After both periods of observation, OP immunoreactivity seemed to be more expressed in the control implants. At eight weeks, small areas within the bone

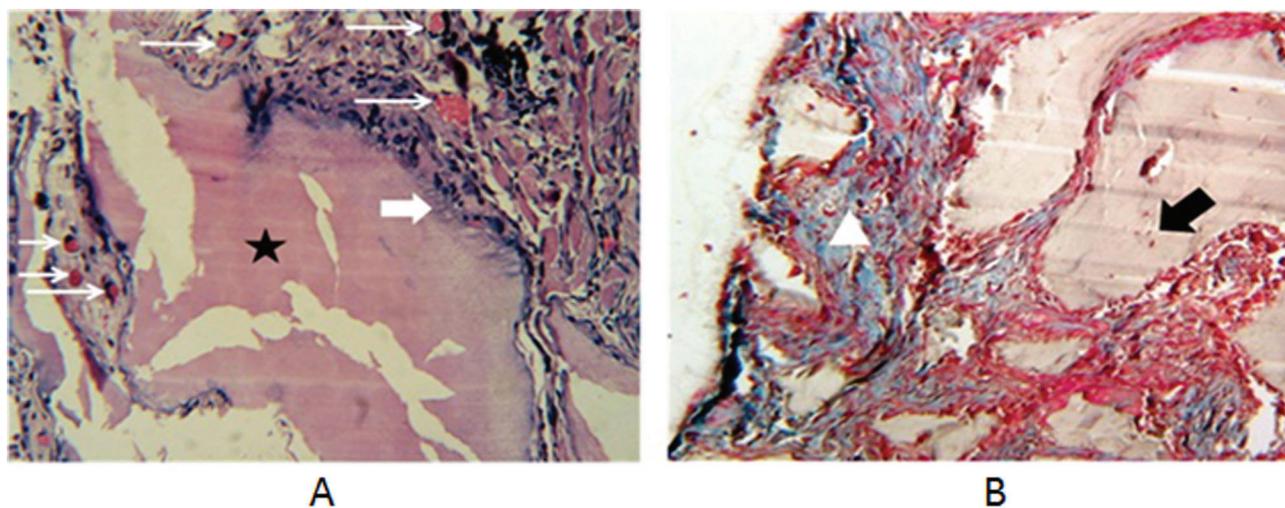


Fig. 4. C-type implants after 2 weeks of implantation. H&E, 400x (A); MT, 200x (B). Blood vessels - thin white arrow; surface resorbing of Bio-Oss[®] particle - thick white arrow; collagen - white triangle; cells in Bio-Oss[®] particle - thick black arrows.

matrix were immunolabeled for OP in the control implants, while in the M-type implants very light colored or no-colored fields could be found (Fig. 7). OC immunoreactivity could be seen in both types of implants in both analyzed periods, but it was more intense in the M-type implants (Fig. 7, F) compared to a control after eight weeks of implantation (Fig. 7, H).

DISCUSSION

Of all three stages of bone reparation, the inflammatory one is probably the most critical for fracture healing, similar to that of wound healing in soft tissues (Mizuno et al., 1990; Grundnes and Reikeras, 1993). During this phase, the secretion of proinflammatory cytokines and growth factors occurs, and the levels of several inflammatory mediators (such as TNF- α) are markedly increased. Most of them are synthesized and secreted by macrophages (Gerstenfeld et al., 2003; Rundle et al., 2006). Thus, osteogenesis commonly occurs in the inflammatory environment, no matter how the bone was previously injured, and macrophages are a crucial component of this environment. The peritoneal cavity is the most appropriate source for obtaining large amounts of macrophages and is often used in the examination of the inflammatory reaction that involves macrophage

cell lineage. In experiments on animals, thioglycollate is routinely used for macrophages elicitation (Spitalny, 1981; Chan et al., 1998).

Functional characterization of macrophage

The functional characterization of macrophages includes an analysis of oxygen reactive species production and cytokine secretion. The NBT test is a conventional method used in assays of phagocytic cell activity and oxygen reactive species production. Reactive products of NADPH/oxidase reduce NBT (yellow-colored) to formazan crystals (blue-colored) (Choi et al., 2006). The total activity of this enzyme increases proportionally with an increased level of cellular metabolism that leads to an increased conversion of tetrazolium salt to formazan, staining cells more intensely. One of the characteristics of inflammatory macrophages is the increased production of proinflammatory cytokines, including TNF- α . The L929 cell line is the most common cell line used for the evaluation of TNF- α cytotoxicity due to its high sensitivity to TNF- α (Shiau et al., 2001). Therefore, we decided to use this assay to evaluate the effect of TNF- α in macrophage CM. A relative number of NBT-positive cells and a larger amount of formazan deposits per cell in TEPMs, as well as a stronger cytotoxic effect of the CM of TEPMs compared to that

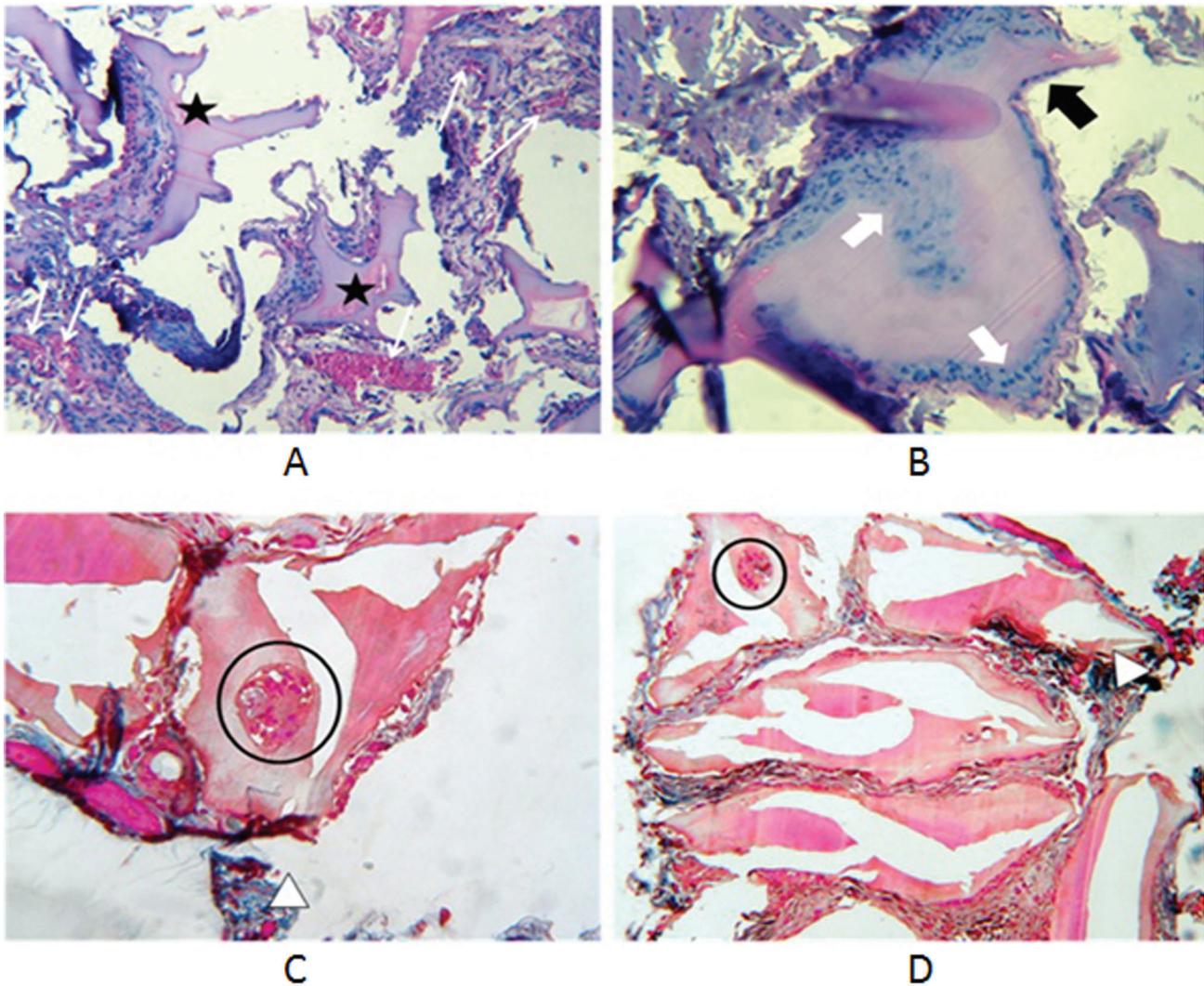


Fig. 5. M-type implants after 8 weeks of implantation. H&E, 200x (A); H&E, 400x (B); MT, 400x (C); MT, 200x (D), respectively. Bio-Oss[®] particle - black star; blood vessels - thin white arrow; multinuclear phagocytes - thick white arrow; osteoblast-like arrangement of cells - thick black arrows; osteoid formation - framed; collagen - white triangle.

of RPMs, indicate that TEPMs in our study had the characteristics of inflammatory cells.

Histological analysis of implants

The subcutaneous implantation model was chosen in our study because it allows the analysis of basic interactions of implant components, such as macrophages, blood clots and bone mineral substitute, without the impact of surrounding bone tissue. Induced ec-

topic osteogenesis can simply serve for examination of particular factors involved in the osteogenic process. The period of two weeks was chosen because the inflammatory phase ends within it, and the period of eight weeks because within it a new bone tissue is expected to be formed (Shapiro, 2008).

In both types of implants, after both analyzed periods, there was a connective capsule around the implants. The formation of a fibrous capsule is usu-

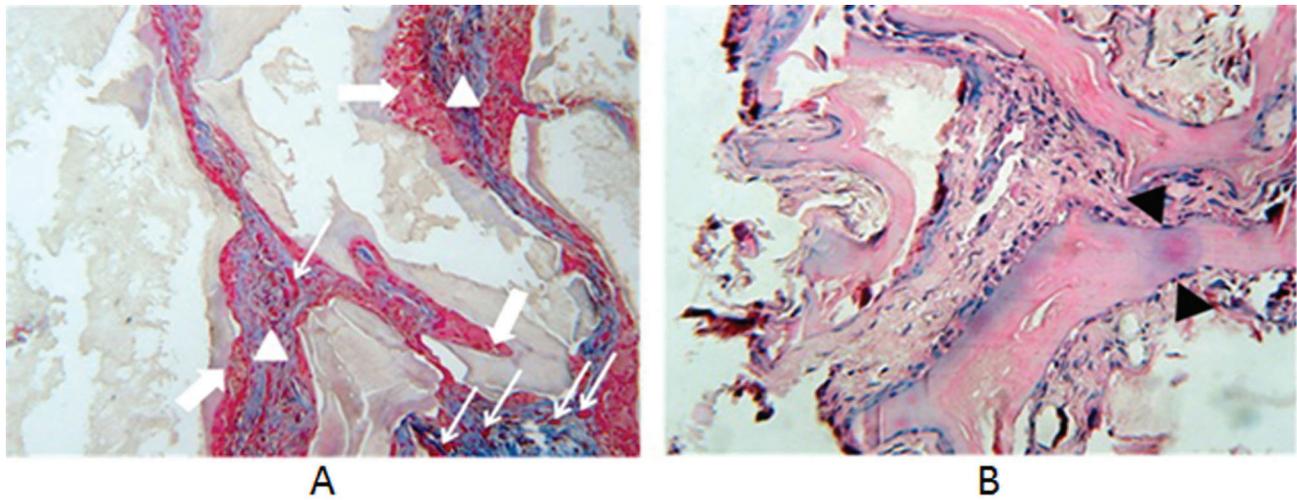


Fig. 6. C-type implants after 8 weeks of implantation. MT, 200x (A); H&E, 400x (B). Blood vessels - thin white arrow; multinuclear phagocytes - thick white arrow; collagen - white triangle; mononuclear cells on the material surface - black triangle.

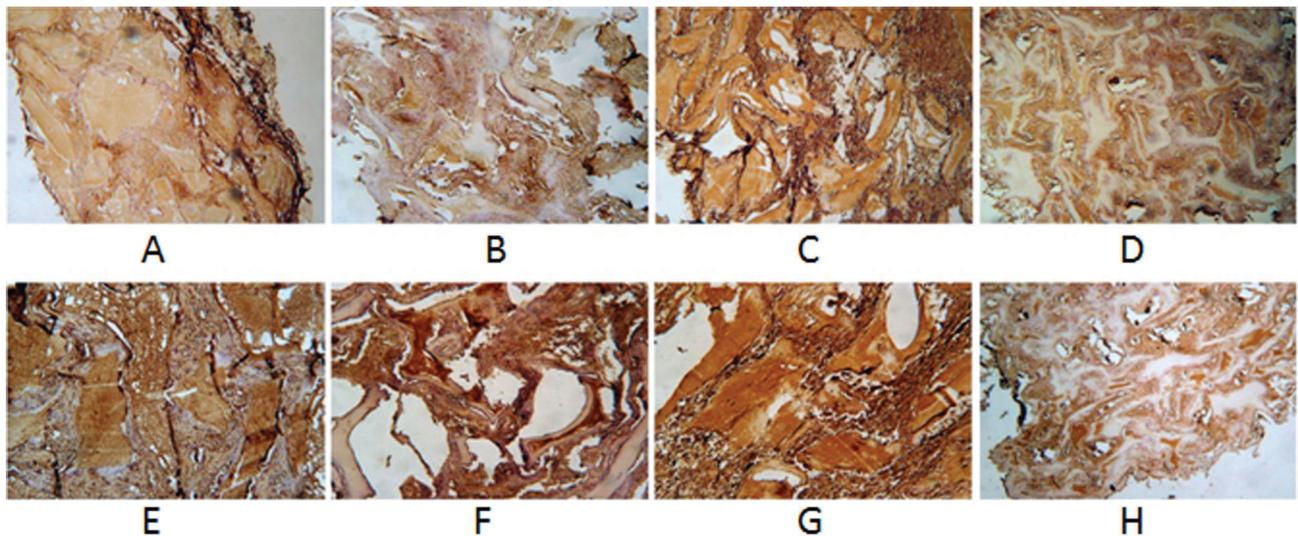


Fig. 7. OP and OC immunoreactivity in implants. OP immunoreactivity in: M-type implants after 2 weeks of implantation, 100x (A); M-type implants after 8 weeks of implantation, 100x (B); C-type implants after 2 weeks of implantation, 100x (C); C-type implants after 8 weeks of implantation, 100x (D). OC immunoreactivity in: M-type implants after 2 weeks of implantation, 200x (E); M-type implants after 8 weeks of implantation, 200x (F); C-type implants after 2 weeks of implantation, 200x (G); C-type implants after 8 weeks of implantation, 100x (H).

ally the final step in the reaction to implanted biomaterials (Anderson and McNally, 2011). The reason for this is that macrophages and other phagocytes cannot completely degrade the implanted material, and the organism acts in order to isolate the foreign body. It is best achieved by forming a thin capsule that prevents further interaction of the implant and the host (Kontinen et al., 2005).

In the M-type implants, as well as in the control implants, even after eight weeks of implantation, Bio-Oss® particles had not been fully resorbed, but there were visible signs of degradation. The presence of long-term phagocytosis, even after two months of implantation, may be caused by the hardness of the biomaterial (Najman et al., 2004). Resorption of M-type implants seems to be constant and even eight weeks

after implantation numerous, large osteoclast-like cells were detected, which means that the degrading process was still ongoing. The process of phagocytosis was “smoldering” all the time, in contrast to the control implants where phagocytosis seemed to be extinguished. This may be due to the macrophages’ ability to synthesize and release a number of cytokines that attract other new phagocytic cells. One such example is TNF- α (Heidenreich et al., 1988).

Vascularization and angiogenesis are essential for the maintenance and regeneration of bone tissue, because without them it quickly degenerates (Schmid et al., 1997). Angiogenesis is an important component of the inflammatory response and reparative processes (Kajdaniuk et al., 2011). Inadequate blood supply of a fracture site may lead to pseudoarthrosis, a condition which is defined as the nonunion of a fracture when the bone repair process has stopped, resulting in fibrous tissue formation at the fracture site (Helfet et al., 2003). The process of angiogenesis was more pronounced in the M-type implants, especially after eight weeks compared to control implants. The presence of numerous macrophage infiltrations could be connected to a stronger angiogenic process as previously reported (Najman et al., 2004). In an organism, an inflammatory response with macrophage influx is required stimulus for the wound healing (Leibovich and Ross, 1975). The angiogenic activity of macrophages is associated with their secretory activity. Stimulated macrophages release not only a series of angiogenic cytokines and growth factors (Sunderkotter et al., 1991), but a number of proteolytic enzymes as well (Shapiro et al., 1991) that “dig” channels that are to be populated with endothelial cells that form capillaries. In addition, it should be kept in mind that the implants in our study were formed by creating a blood clot, which is known to represent a rich source of angiogenic factors and other growth factors. Blood vessels that were seen in the C-type implants, especially after 2 weeks, could be attributed to the effect of blood clot. The abundance of blood vessels in the M-type implants certainly has to be attributed to the effect of macrophages whose activity can be further enhanced by components of blood clot (Davies, 2003).

The collagen matrix in both types of implants and after both examined periods was observed. However, in the later period of implantation, deposits of collagen were much more intense in the C-type implants compared to those in the M-type implants. It has been shown that macrophages decompose collagen with collagenases (Werb and Cordon, 1975). On the other hand, the strong collagen deposits in the control implants somehow conserved the Bio-Oss® granules, thus preventing the cells to reach the implanted biomaterial and establish closer contact with them. In this way, present macrophages may play an important role in regulating the amount of collagen and prevent the development of fibrous tissue.

The cellularity of both types of implants was reduced after eight weeks, which is probably due to their connective capsule isolation. However, there was obviously greater cellularity in the M-type implants. The immature connective tissue between the biomaterial particles was better aligned with the granules within the M-type implants. Osteoblast-like cells on biomaterial granule surface as well as osteoid structures could also be seen. The bone mineral matrix itself serves as a chemoattractant for cell recruitment, which further helps proinflammatory signals. Cells that will initiate the osteogenic process need to come from the environment (Nair et al., 2011). Rifas and his co-workers showed that the peritoneal macrophages of rats *in vitro* synthesize a mitogenic factor or factors influencing the osteoblast-like cell proliferation (Rifas et al., 1984; Rifas et al., 1989).

Immunohistochemical analysis of implants

OC and OP are non-collagenous bone matrix proteins. It has been shown that during the development of the skeletal system increased OP expression and lack of OC expression is characteristic of immature osteoblasts (Nakase et al., 1994; Stein et al., 1996). From all of the above, the lower OP and higher OC immunoreactivity in the M-type implants compared to the controls may indicate a greater maturity degree of the bone matrix in the M-type implants, and therefore point to the fact that the process of osteogenesis is more advanced. The osteogenic organic ex-

tracellular matrix is a prerequisite for the later stages of osteogenesis, such as mineralization and ultimately the formation of mature bone.

CONCLUSIONS

Our results indicate that presence of inflammatory macrophages in the composition of an implant may have a beneficial effect on the process of angiogenesis and formation of an organic bone matrix, which is a crucial prerequisite for successful osteogenesis. We can conclude that managing the macrophage response in an implant could be a tool for directing the process of bone regeneration. This may be of great importance for application in bone tissue engineering and clinical practice.

Acknowledgments - This paper is supported by Project Grant III41017 financed by the Ministry of Education, Science and Technological Development of the Republic of Serbia.

Authors' contributions

Jelena M. Živković, *in vitro* and *in vivo* experiments, IHC, analysis, writing; Stevo J. Najman, hypothesis, experiments supervising, analysis, writing; Marija Đ. Vukelić, *in vivo* experiments; Sanja T. Stojanović, *in vitro* experiments, statistics, analysis, writing; Milena V. Aleksić, processing of samples, histochemical staining; Milica N. Stanisavljević, *in vivo* experiments; Jelena G. Najdanović, *in vitro* experiments

Conflict of interest disclosure

The authors state that there is no conflict of interest.

REFERENCES

- Anderson, J. M. and A. K. McNally (2011). Biocompatibility of implants: lymphocyte/macrophage interactions. *Semin. Immunopathol.* **33**, 221-233.
- Andrew, J. G., Andrew, S. M., Freemont, A. J. and D. R. Marsh (1994). Inflammatory cells in normal human fracture healing. *Acta Orthop. Scand.* **65**, 462-466.
- Bhat, A., Wooten, R. M. and A. C. Jayasuriya (2013). Secretion of growth factors from macrophages when cultured with microparticles. *J. Biomed. Mater. Res., Part A.* **101**, 3170-3180.
- Carnes, D. L., Goldstein, M., Dean, D. D. and B. D. Boyan (2000). Ability of deproteinized cancellous bovine bone to induce new bone formation. *J. Periodontol.* **71**, 1258-1269.
- Champagne, C. M., Takebe, J., Offenbacher, S. and L. F. Cooper (2002). Macrophage cell lines produce osteoinductive signals that include bone morphogenetic protein-2. *Bone.* **30**, 26-31.
- Chan, J., Leenen, P. J., Bertinello, I., Nishikawa, S. I. and J. A. Hamilton (1998). Macrophage Lineage Cells in Inflammation: Characterization by Colony-Stimulating Factor-1 (CSF-1) Receptor (c-Fms), ER-MP58, and ER-MP20 (Ly-6C) Expression. *Blood.* **92**, 1423-1431.
- Choi, H. S., Kim, J. W., Cha, Y. N. and C. Kim (2006). A quantitative nitroblue tetrazolium assay for determining intracellular superoxide anion production in phagocytic cells. *J. Immunoass. Immunochem.* **27**, 31-44.
- Christou, N. V., Mannick, J. A., West, M. A. and D. L. Kasper (1987). Lymphocyte-macrophage interactions in the response to surgical infections. *Arch. Surg.* **122**, 239-251.
- Colnot, C., Romero, D. M., Huang, S. and J. A. Helms (2005). Mechanisms of action of demineralized bone matrix in the repair of cortical bone defects. *Clin. Orthop. Relat. Res.* **435**, 69-78.
- Davies, J. E. (2003). Understanding peri-implant endosseous healing. *J. Dent. Educ.* **67**, 932-949.
- Dimitriou, R., Tsiridi, E. and P. V. Giannoudis (2005). Current concepts of molecular aspects of bone healing. *Injury.* **36**, 1392-1404.
- Einhorn, T. A. (1998). The cell and molecular biology of fracture healing. *Clin. Orthop. Relat. Res.* **355** (Suppl), S7-21.
- Forman, H. J. and M. Torres (2002). Reactive oxygen species and cell signaling: respiratory burst in macrophage signaling. *Am. J. Respir. Crit. Care Med.* **166**, S4-S8.
- Gerstenfeld, L. C., Cullinane, D. M., Barnes, G. L., Graves, D. T. and T. A. Einhorn (2003). Fracture healing as a post-natal developmental process: molecular, spatial, and temporal aspects of its regulation. *J. Cell. Biochem.* **88**, 873-884.
- Gifford, R. H. and S. E. Malawista (1972). The Nitroblue Tetrazolium Reaction in Human Granulocytes Adherent to a Surface. *Yale J. Biol. Med.* **45**, 119-132.
- Grundnes, O. and O. Reikeras (1993). The importance of the hematoma for fracture healing in rats. *Acta Orthop. Scand.* **64**, 340-342.
- Grundnes, O. and O. Reikeraas (2000). Effects of macrophage activation on bone healing. *J. Orthop. Sci.* **5**, 243-247.
- Heidenreich, S., Weyers, M., Gong, J. H., Sprenger, H., Nain, M. and D. Gemsa (1988). Potentiation of lymphokine-in-

- duced macrophage activation by tumor necrosis factor- α . *J. Immunol.* **140**, 1511-1518.
- Helfet, D. L., Kloen, P., Anand, N. and H. S. Rosen (2003). Open reduction and internal fixation of delayed unions and nonunions of fractures of the distal part of the humerus. *J. Bone Jt. Surg., Am. Vol.* **85-A**, 33-40.
- Kajdaniuk, D., Marek, B., Foltyn, W. and B. Kos-Kudła (2011). Vascular endothelial growth factor (VEGF) - part 2: in endocrinology and oncology. *Endokrynol. Pol.* **62**, 456-464.
- Kanczler, J. M., Millar, T. M., Bodamyali, T., Blake, D. R. and C. R. Stevens (2003). Xanthine oxidase mediates cytokine-induced, but not hormone-induced bone resorption. *Free Rad. Res.* **37**, 179-187.
- Khan, S. N., Cammisa Jr, F. P., Sandhu, H. S., Diwan, A. D., Girardi, F. P. and J. M. Lane (2005). The biology of bone grafting. *J. Am. Acad. Orthop. Surg.* **13**, 77-86.
- Konttinen, Y. T., Zhao, D., Beklen, A., Ma, G., Takaqi, M., Kivelä-Rajamäki, M., Ashammakhi, N. and S. Santavirta (2005). The microenvironment around total hip replacement prostheses. *Clin. Orthop. Relat. Res.* **430**, 28-38.
- Lacey, D. C., Simmons, P. J., Graves, S. E. and J. A. Hamilton (2009). Proinflammatory cytokines inhibit osteogenic differentiation from stem cells: implications for bone repair during inflammation. *Osteoarthr. Cart.* **17**, 735-742.
- Leibovich, S. J. and R. Ross (1975). The role of the macrophage in wound repair. A study with hydrocortisone and antimacrophage serum. *Am. J. Pathol.* **78**, 71-100.
- McKenna, T. M., Reusch, D. W. and C. O. Simpkins (1988). Macrophage-conditioned medium and interleukin 1 suppress vascular contractility. *Circ. Shock.* **25**, 187-196.
- Mizuno, K., Mineo, K., Tachibana, T., Sumi, M., Matsubara, T. and K. Hirohata (1990). The osteogenic potential of fracture hematoma. Subperiosteal and intramuscular transplantation of the haematoma. *J. Bone Jt. Surg., Br. Vol.* **72**, 822-829.
- Mosmann, T. (1983). Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. *J. Immunol. Methods.* **65**, 55-63.
- Mountziaris, P. M. and A. G. Mikos (2008). Modulation of the Inflammatory Response for Enhanced Bone Tissue Regeneration. *Tissue Eng., Part B.* **14**, 179-186.
- Nair A., Shen J., Lotfi P., Ko C. Y., Zhang C. C. and L. Tang (2011). Biomaterial Implants Mediate Autologous Stem Cell Recruitment In Mice. *Acta Biomater.* **7**, 3887-3895.
- Najman, S., Savić, V., Đorđević, Lj., Ignjatović, N. and D. Uskoković (2004). Biological evaluation of hydroxyapatite/poly-L-lactide (HAp/PLLA) composite biomaterials with poly-L-lactide of different molecular weights intraperitoneally implanted into mice. *Bio-Med. Mater. Eng.* **14**, 61-70.
- Nakase, T., Takaoka, K., Hirakawa, K., Hirota, S., Takemura, T., Onoue, H., Takebayashi, K., Kitamura, Y. and S. Nomura (1994). Alterations in the expression of osteonectin, osteopontin and osteocalcin mRNAs during the development of skeletal tissues in vivo. *Bone Miner.* **26**, 109-122.
- Rifas, L., Shen, V., Mitchell, K. and W. A. Peck (1984). Macrophage-derived growth factor for osteoblast-like cells and chondrocytes (bone cells/coupling factor/bone formation). *Proc. Natl. Acad. Sci. U S A.* **81**, 4558-4562.
- Rifas, L., Cheng, S. L., Shen, V. and W. A. Peck (1989). Monokines produced by macrophages stimulate the growth of osteoblasts. *Connect. Tissue Res.* **23**, 163-178.
- Rundle, C. H., Wang, H., Yu, H., Chadwick, R. B., Davis, E. I., Wegedal, J. E., Lau, K. H., Mohan, S., Ryaby, J. T. and D. J. Baylink (2006). Microarray analysis of gene expression during the inflammation and endochondral bone formation stages of rat femur fracture repair. *Bone.* **38**, 521-529.
- Sassa, S., Kawakami, M. and A. Cerami (1983). Inhibition of the growth and differentiation of erythroid precursor cells by an endotoxin-induced mediator from peritoneal macrophages. *Proc. Natl. Acad. Sci. U S A.* **80**, 1717-1720.
- Schett, G. (2011). Effects of inflammatory and anti-inflammatory cytokines on the bone. *Eur. J. Clin. Invest.* **41**, 1361-1366.
- Schmid, J., Wallkamm, B., Hammerle, C. H., Gogolewski, S. and N. P. Lang (1997). The significance of angiogenesis in guided bone regeneration. A case report of a rabbit experiment. *Clin. Orthop. Relat. Res.* **8**, 244-248.
- Schwartz, Z., Weesner, T., van Dijk, S., Cochran, D. L., Melloning, J. T., Lohmann, C. H., Carnes, D. L., Goldstein, M., Dean, D. D. and B. D. Boyan (2000). Ability of deproteinized cancellous bovine bone to induce new bone formation. *J. Periodontol.* **71**, 1258-1269.
- Shapiro, S. D., Campbell, E. J., Senior, R. M. and H. G. Welgus (1991). Proteinases secreted by human mononuclear phagocytes. *J. Rheumatol., Suppl.* **27**, 95-98.
- Shapiro, F. (2008). Bone development and its relation to fracture repair. The role of mesenchymal osteoblasts and surface osteoblasts. *Eur. Cells Mater.* **15**, 53-76.
- Shiau, M. Y., Chiou, H. L., Lee, Y. L., Kuo and T. M. Y. H. Chang (2001). Establishment of a consistent L929 bioassay system for TNF- α quantitation to evaluate the effect of lipopolysaccharide, phytomitogens and cytodifferentiation agents on cytotoxicity of TNF- α secreted by adherent human mononuclear cells. *Mediators Inflammation.* **10**, 199-208.
- Soltan, M., Rohrer, M. D. and H. S. Prasad (2012). Monocytes: super cells for bone regeneration. *Implant Dent.* **21**, 13-20.

- Spitalny, G. L.* (1981). Dissociation of bactericidal activity from other functions of activated macrophages in exudates induced by thioglycollate medium. *Infect. Immun.* **34**, 274-284.
- Stein, G. S., Lian, J. B., Stein, J. L., van Wijnen, A. J., Frenkel, B.* and *M. Montecino* (1996). Mechanisms regulating osteoblast proliferation and differentiation, In: *Principles of bone biology*, (Eds. J. P. Bilezikian, L. G. Raisz, and G. A. Rodan), 69-86. Academic Press, San Diego.
- Stewart, C. C., Lin, H.* and *C. Adles* (1975). Proliferation and colony-forming ability of peritoneal exudate cells in liquid culture. *J. Exp. Med.* **141**, 1114-1132.
- Sunderkotter, C., Goebeler, M., Schulze-Osthoff, K., Bhardwaj, R.* and *C. Sorg* (1991). Macrophage-derived angiogenesis factors. *Pharmacol. Ther.* **51**, 195-216.
- Werb, Z.* and *S. Cordon* (1975). Secretion of a specific collagenase by stimulated macrophages. *J. Exp. Med.* **142**, 346-360.