Original article

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VASCULOGENIC POTENTIAL OF ADIPOSE-DERIVED MESENCHYMAL STEM CELLS *IN VITRO* INDUCED INTO OSTEOBLASTS APPLIED WITH PLATELET-RICH PLASMA IN AN ECTOPIC OSTEOGENIC MODEL

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Bone tissue deficiencies can be caused by fractures, bone loss or tumors. Insufficient vascularization is the main problem in successful bone tissue regeneration. In order to improve vascularization during bone tissue regeneration, a promising methods have been developed in the field of bone tissue engineering (BTE) by using adipose-derived stem cells (ADSCs). The aim of this study was to examine vasculogenic potential of ADSCs *in vitro* induced into osteoblasts (OBs) combined with platelet-rich plasma (PRP) and bone mineral matrix (BMM) in ectopic osteogenic implants, and compare it with implants consisting of uninduced ADSCs, PRP and BMM. ADSCs isolated from mice epididymal adipose tissue cultivated up to the third passage were divided into two groups: ADSCs *in vitro* induced into OBs and ADSCs expanded without osteoinduction. Based on biological triad principle, two types of implants were composed: implants containing BMM, PRP and ADSCs *in vitro* induced into OBs (BPO implants), and implants containing BMM, PRP and uninduced ADSCs (BPU implants). The BPO implants had higher expression of endothelial-related genes compared to the BPU implants. Additionally, VCAM-1 immunoexpression increases during *in vivo* experimental period in the BPO implants, while in the BPU implants VCAM-1 immunoexpression decreases during *in vivo* experimental period. Therefore, vasculogenic potential of ADSCs *in vitro* induced into OBs and combined with PRP and BMM in ectopic osteogenic implants is higher compared to the implants composed of uninduced ADSCs, PRP and BMM, which makes implants enriched with ADSCs induced into OBs good candidates for improving vascularization in bone tissue-engineered constructs.

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Introduction

Bone tissue deficiencies can be caused by fractures, bone loss or tumors (1). Insufficient vascularization is still the main problem in successful bone tissue regeneration. In order to improve vascularization during bone tissue regeneration, as an alternative to osteosynthetic stabilizing techniques and autologous bone transplantations (2), promising methods have been developed in the field of bone tissue engineering (BTE) (1). For the construction of successful bone grafts, it is of vital importance to improve angiogenesis in the early stage as well as in the long-term process of ossification (3). To accomplish this goal, different approaches have been applied (4-8).

A special place in BTE belongs to cell-based therapies that include the application of adipose-derived mesenchymal stem cells (ADSCs) (9-11). ADSCs secrete numerous growth factors (12) with a significant impact on tissue regeneration (13) so they can be applied *in vitro*-expanded, uninduced (14) for bone implant construction. On the other hand, ADSCs posses the ability for *in vitro* differentiation into various cell types including osteoblasts (OBs) (15, 16) which represents one more possibility for their application in BTE. ADSCs, whether induced into OBs or not, can be applied in BTE in combination with natural source of growth factors

and a biomaterial that represents bone mineral matrix (BMM) carrier for growth factors and cells. In this manner, the principle of biological triad is respected (16-20).

Data related to the impact of uninduced ADSCs as well as osteoinduced ADSCs on vascularization during bone tissue regeneration vary depending on the types of chosen experimental model, species of chosen experimental animals, types of biomaterial, source of growth factors, localization of fat taken for the isolation of ADSCs. Motif of this study was to see whether osteogenic induction of ADSCs is preferable over application of uninduced ADSCs in improving vascularization of ectopic osteogenic implants that were composed based on biological triad principle. Vascularization was observed via endothelial-related gene expression and immunoexpression of vascular cell adhesion protein-1 (VCAM-1) because endothelial cells and their progenitors are necessary for vascular network formation (21, 22), while VCAM-1 is a protein involved in molecular mechanism of blood vessel maturation (23).

Aim

The aim of this study was to examine vasculogenic potential of ADSCs *in vitro* induced into osteoblasts (OBs) combined with platelet-rich plasma (PRP) and BMM in ectopic osteogenic implants, and compare it with the implants consisting of cultivated, uninduced ADSCs, PRP and BMM.

Materials and methods

Experimental animals

The present research was done on syngeneic, male, BALB/c mice (Military Medical Academy, Belgrade, Serbia), each at the age of eight weeks and weighting between 22 g and 24 g. Local Ethical Committee approved all procedures that were done on the animals (approval number 01-2857-8). Whole research was conducted according to the Animal Welfare Act (Republic of Serbia). The mice were treated in accordance with the regulation of the "European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS no. 123 Appendix A)".

Isolation and expansion of ADSCs from stromal vascular fraction

ADSCs were obtained out of the adipose tissue that surrounds epididymis of BALB/c mice conforming to our earlier described protocols (16-19). In brief, after extraction from the animals, maceration and washing in the sterile conditions, adipose tissue was subjected to digestion in the water bath, at 37 °C, by using collagenase type I (Sigma-Aldrich, Hamburg, Germany) solution at the concentration of 2000 i.j. in low glucose Dulbecco's Modified Eagles Minimal Essential Medium (DMEM, PAA Laboratories, Pasching, Austria). When 45 minutes of digestion had passed, the process was stopped by using pre-warmed DMEM supplemented with 10% fetal bovine serum (FBS), 2 mmol/L of L-glutamine and 1% antibiotic-antimycotic solution (all from PAA Laboratories GmbH, Pasching, Austria)−complete DMEM (cDMEM). Isolated cells contained within stromal vascular fraction (SVF) were passed through 180 μm mesh, centrifuged (1500 rpm, 10 min, 4 °C) and after discarding white, "lipid" ring from the top of the tube, the cells were counted and seeded at the density of 10^6 per 25 cm^2 growth area of cell culture flask. Stromal vascular fraction cells (SVFs) were cultivated in cDMEM. In order to expand ADSCs from SVF and to eliminate other types of cells, nonadherent and terminally differentiated, SVFs were expanded up to the third passage (P03).

Osteogenic differentiation of ADSCs

At P03, the cells were counted and divided into two 24 well plates. Cell density per well was 1×10^4. In the first plate, ADSCs were subjected to osteogenic differentiation. Osteogenic media was prepared by adding the following supplements into cDMEM – 10^{-8} M dexamethasone, 50 μg/mL ascorbic acid and 10 mM β-glycerophosphate (24). The cells were *in vitro* induced into OBs for 15 days, since we have previously shown that osteoblast-related genes expression and osteocalcin immunoexpression are the highest at this time point during osteogenic differentiation (16). In the second plate, ADSCs were expanded in cDMEM for twelve days. These uninduced ADSCs were the control group. Both types of cell cultures were monitored on in--verted light microscope Axio Observer. Z1 that is equipped with AxioCam HR camera (Carl Zeiss, Oberkochen, Germany). After cultivation time ended, these two cell cultures were passaged, counted and used for the implant construction.

Preparation of implants and implantation procedure

Based on biological triad principle, implants were composed out of three components – biomaterial, source of growth factors and cells.

Deproteinized, sterilized bovine bone Bio-Oss®, size S (Geistlich-Pharma, Wolhusen, Switzerland) that represents a BMM was used as a carrier for growth factors and cells.

PRP was applied as a natural source of growth factors and prepared in two steps (25) in order to get 4-6 times higher platelets concentration in comparison with the one in physiological conditions. Platelets were counted manually in the Malassez counting chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Konigshofen, Germany) and established number of these blood components was $1.89 \pm 0.5 \times 10^6/\mu l$. We used 10% v/v of PRP in the liquid implant component since this concentration has shown to be the optimal for combining with ADSCs (14, 26, 27).

Two types of cell cultures were used in this experiment: uninduced ADSCs cultivated in cDMEM for 12 days after P03 and ADSCs *in vitro* induced into OBs for 15 days after P03. Regardless of the

used cell type, each implant contained 10 mg (~0.02 cm^3) of BMM and 20 µl of liquid component. Based on the used cell type, two types of implants were constructed:

1) BPU type of implants contained 10 mg of BMM, 2 µl of PRP (finally 10% v/v) and 1 × 10^4 of uninduced ADSCs in 18 µl of DMEM.

2) BPO type of implants contained 1 0mg of BMM, 2 µl of PRP (finally 10% v/v) and 1 × 10^4 of ADSCs *in vitro* induced into OBs.

The implants were prepared in the sterile, flat, glass plates. The cells were allowed to attach to BMM surface and fibrin fibers were allowed to form within implants for around 10-15 min before the implantation procedure (28). After that, each implant was shaped in a lump and implanted using sterile biopsy needle into the interscapular subcutaneous tissue of anaesthetized mice. In both groups, each mouse had four implants of the same type. Both experimental groups consisted of twenty animals (each). The extraction of implants was done one, two, four and eight weeks after implantations so that five animals per group were sacrificed per each single experimental period. The implants from each experi-mental period were placed in RNAlater® solution (RNA Stabilization Solution, Ambion, Life Technolo-gies, USA), at -80 °C, in order to preserve RNA until gene expression analysis. The implants extracted two and eight weeks after implantations were fixed by using 10% neutral buffered saline (NBF) for im-munohistochemical analysis.

Relative gene expression analysis

Concentration of total RNA isolated from the implants was determined by using RNeasy Mini Kit® (Qiagen, Hilden, Germany) and measured by using Qubit® RNA assay Kit and a Qubit® 2.0 fluorometer (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). The procedures were done in accordance with the manufacturers' recommendations. DNase I Rnase-free set (Qiagen, Hilden, Germany) was used for digestion of the residual DNA, after which isolated RNA was subjected to reverse transcription into cDNA by using High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) and in a thermal cycler SureCycler 8800 (Agilent Technologies, Santa Clara, CA, USA). Relative gene expression analysis was performed in a Stratagene MxPro-Mx3005P Real-Time thermal cycler (Agilent Technologies, Santa Clara, CA, USA). The reactions for endothelial-related genes (Table 1) expression were prepared by using Quanti-Tect primer assays (Qiagen, Hilden, Germany) and KapaSybr® Fast Universal 2 × qPCR Master Mix (Kapa Biosystems,Wilmington, MA, USA), in accordance with the manufacturers' instructions. The results were presented as relative to these genes' expression in PRP (calibrator sample). A housekeeping gene beta-actin was used as a gene normalizator (Table 1).

Table 1. List of primers used for qPCR

Genes	Gene symbol	QuantiTect Primer assay
beta-actin	*Actb*	Mm_Actb_2_SG, QT01136772
von Willebrand factor	*Vwf*	Mm_Vwf_1_SG, QT00116795
early growth response 1	*Egr1*	Mm_Egr1_1_SG, QT00265846
vascular endothelial growth factor receptor 1	*Flt1*	Mm_Flt1_1_SG, QT00096292
vascular cell adhesion molecule 1	*Vcam1*	Mm_Vcam1_1_SG, QT00128793

Immunohistochemistry

The samples of implants previously fixed with 10% NBF were decalcified in ethylenediaminetetraacetic acid solution (pH 7.4). Decalcified tissue was processed, embedded in paraffin and sliced at 4 µm on a Leica RM2235 microtome (Leica Microsystems, Solms, Germany). Prior to incubation with the primary antibody, heat-induced antigen-retrieval procedure was performed on sliced tissue sections by using 10 mmol/L of sodium citrate buffer (pH 6.0), in the microwave oven pre-warmed at 96 °C, for 30 min. Primary antibody used for the experiment was anti-VCAM-1 (1:1000, ab106777, Abcam, Cambridge, USA) and it was omitted in the negative controls. For visualization, rabbit-specific horseradish peroxidase/diaminobenzidine (HRP/DAB) detection Kit (ab64261, Abcam, Cambridge, USA) was used, in accordance with the manufacturer's instructions. Tissue sections were counterstained with Mayer's Haematoxylin (5 min, room temperature), mounted with VectaMount® (Vector Laboratories, Burlin-game, CA, USA) and analyzed on LEICA DMR light microscope (Leica Microsystems, Solms, Germany). Immunoreactivity in the sections was visualized as brown

colour and indicates immunopositivity for the applied antibody.

Statistical analysis

Statistical analysis was done in Microsoft Office Excel. The results are shown as mean value ± standard deviation. For the comparison of mean values, Student's T-test was applied.

The differences were considered significant for $p < 0.05$.

Results

Expression patterns and dynamics of endothelial-related genes *Vwf, Egr1, Flt1* and *Vcam1* in BPU and BPO implants extracted one, two, four and eight weeks after the implantations are presented in Figure 1.

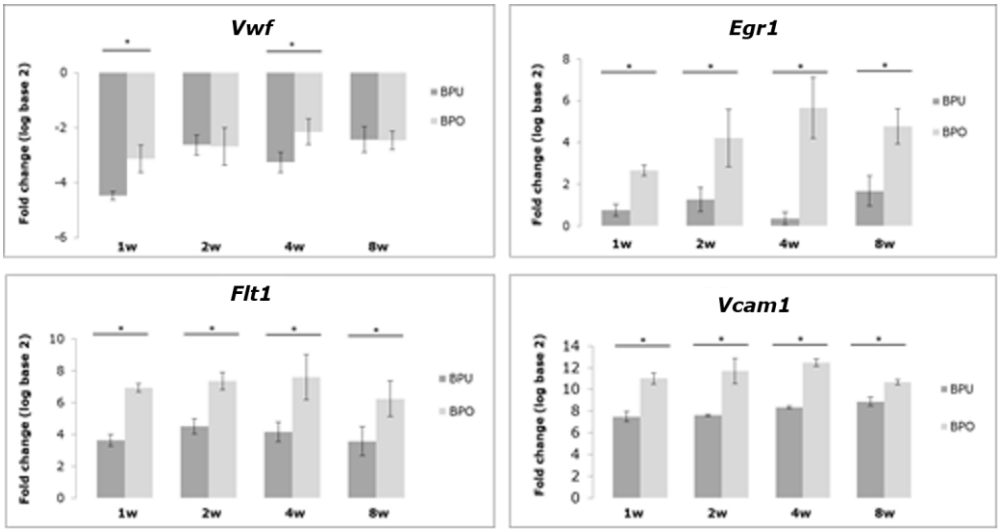

Figure 1. Patterns and dynamics of relative expression levels of endothelial-related genes in examined implants[1]

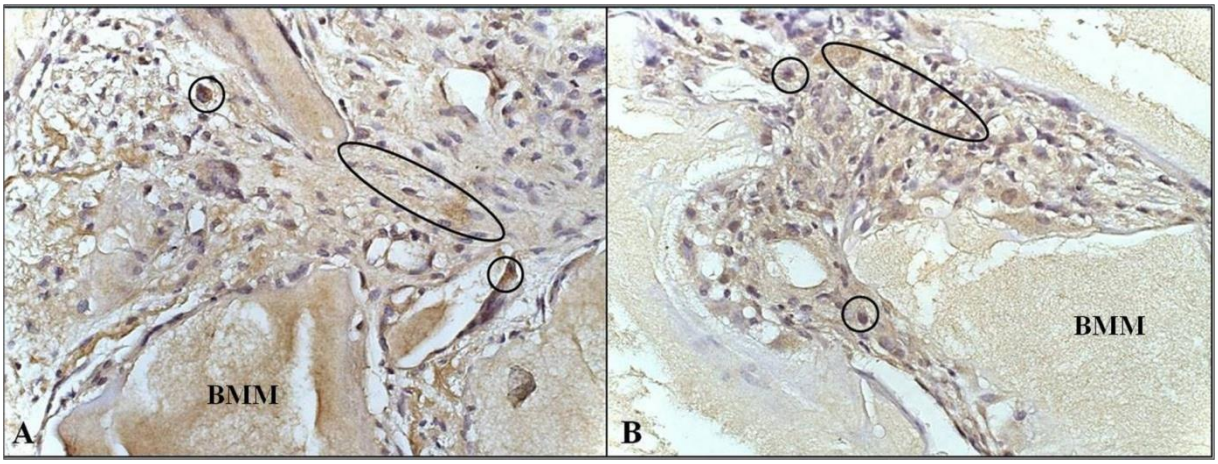

Figure 2. VCAM-1 immunoexpression at two weeks in BPU (A) and in BPO implants (B)[2]

[1] Patterns and dynamics of relative expression levels of endothelial-related genes: *Vwf, Egr1, Flt1* and *Vcam1* in BPU and BPO implants extracted at one, two, four and eight weeks of the *in vivo* experimental period. Significant differences between BPU and BPO groups of implants within the same experimental period: *$p < 0.05$.

[2] Ellipse presents VCAM-1 immunoexpression in the tissue between BMM granules. Circle presents VCAM-1 immunoexpression in the single cells. BMM - bone mineral matrix granules. Magnification: 400 ×. From: Jelena G. Najdanović. Uticaj mezenhimskih ćelija belog masnog tkiva miša, indukovanih *in vitro* ka endotelskim i osteogenim ćelijama, na vaskularizovanost ektopičnih osteogenih implanata, doktorska disertacija, Biološki fakultet, Univerzitet u Beogradu, 2016.

At one and four weeks, the expression of *Vwf* gene was significantly higher in BPO compared to the BPU implants ($p < 0.05$). At two and eight weeks, significant difference of *Vwf* gene expression between BPU and BPO groups was not detected and the level of *Vwf* expression was nearly the same in compared groups.

The *Egr1* gene expression was significantly higher ($p < 0.05$) in BPO compared to BPU implants at each single observation point.

At each observation point, the expression of *Flt1* was significantly higher ($p < 0.05$) in BPO than in the BPU group.

The expression of *Vcam1* gene was significantly elevated ($p < 0.05$) at each observation point in the BPO than in the BPU implants.

At two weeks, VCAM-1 immunoexpression in the BPU implants was intensive in the tissue between BMM granules (Figure 2A). At the same observation point, the tissue between BMM granules in the BPO type of implants had also strong VCAM-1 immunoexpression (Figure 2B).

In the BPU implants extracted eight weeks after implantations, VCAM-1 immunoexpression decreased (Figure 3A) compared to the earlier observation point (Figure 2A). However, VCAM-1 immunoexpression in the BPU implants was still present at eight weeks but mostly near BMM granules rather than between them (Figure 3A). Unlike in the BPU implants, VCAM-1 immunoexpression in the BPO type of implants increases at eight weeks (Figure 3B) in comparison with the earlier observation point within the same group (Figure 2B). VCAM-1 immunoexpression is noticeable in the tissue between BMM granules, in the single cells within the tissue as well as in the blood vessel wall cells (Figure 3B) and it is stronger compared to the BPU implants (Figure 3A) at the same observation point.

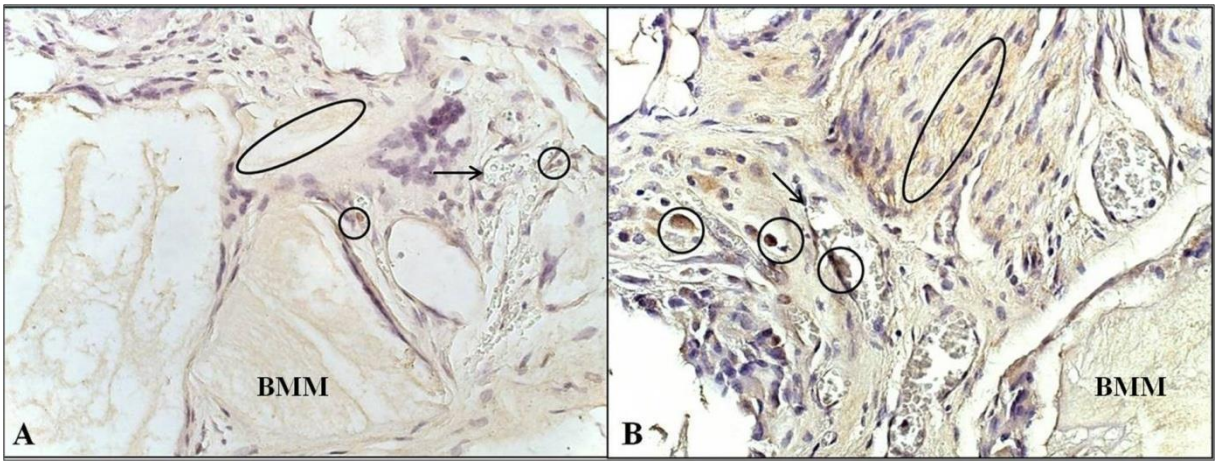

Figure 3. VCAM-1 immunoexpression at eight weeks in BPU (A) and in BPO implants (B)[3]

Discussion

The expression of *Vwf* was negatively regulated in both examined type of implants, at each observation point. *Vwf* is a gene that has a role in later stages of angiogenesis (29). This can be explained by the fact that, besides endothelial cells, *Vwf* gene is also expressed in platelets (30) and we evaluated gene expression in BPU and BPO implants relative to the gene expression in freshly isolated PRP. There was almost no difference in *Vwf* gene expression at two and eight weeks between BPO and BPU implants, but *Vwf* gene expression was significantly higher ($p < 0.05$) in BPO compared to the BPU implants at one and four weeks. This difference is a sign of better vasculogenic potential of ADSCs *in vitro* induced into OBs in comparison with ADSCs cultivated without osteoinductive factors.

The expression of *Egr1, Flt1* and *Vcam1* genes was positively regulated in both examined type of implants, at each single observation point. Positively regulated relative expression of endothelial related genes in BPU implants is probably influenced by biological factors released out of activated PRP. Biological factors released from activated platelets can accelerate *in vitro* and *in vivo* differentiation of ADSCs that were not previously cultivated in cell culture media inductive for differentiation towards certain cell line (14, 26, 31). However, the expression of all examined genes was lower in BPU in comparison with the BPO group.

[3] Ellipse presents VCAM-1 immunoexpression in the tissue between BMM granules. Circle presents VCAM-1 immunoexpression in the single cells. Blood vessels are presented with arrows. BMM - bone mineral matrix granules. Magnification: 400 ×. From: Jelena G. Najdanović. Uticaj mezenhimskih ćelija belog masnog tkiva miša, indukovanih in vitro ka endotelskim i osteogenim ćelijama, na vaskularizovanost ektopičnih osteogenih implanata, doktorska disertacija, Biološki fakultet, Univerzitet u Beogradu, 2016.

The *Egr1* gene can be positively regulated in response to hypoxia, cytokines and growth factors (32). It could be assumed that activation of *Egr1* gene in the examined implants was stimulated by growth factors released from cells that are their components.

The *Flt1* gene is specific receptor for vascular endothelial growth factor (VEGF), a "trigger" protein that controls differentiation of precursor cells towards endothelial and not hematopoietic cell line (33). Significantly higher ($p < 0.05$) expression of *Flt1* in BPO implants in comparison with BPU implants at each observation point can be related to more numerous endothelial cells and their progenitors (34) in BPO than in BPU implants.

The expression of *Vcam1*, a gene that encodes inducible endothelial cell adhesion molecule VCAM-1 which recruits leukocytes to the site of inflammation (35), was significantly elevated ($p < 0.05$) at each observation point in the BPO compared to the BPU implants. VCAM-1 is expressed on small and large blood vessels after stimulation of endothelial cells by cytokines (36) where it binds to its corresponding receptor on the leukocytes very late activation antigen-4 (VLA-4). However, it should be kept in mind that VCAM-1 and its receptor VLA-4 cannot be expressed on quiescent cells which indicates that, once the blood vessel formation is finished, these molecules are negatively regulated and are not necessary for the normal functioning of blood vessels in the quiescent phase (23).

VCAM-1 immunoexpression was strong at two weeks in BPU as well as in BPO implants which means that blood vessel formation had early onset in both examined types of implants. However, VCAM-1 immunoexpression decreases in the BPU implants during *in vivo* experimental period and, at eight weeks, it is weaker compared to the one observed in BPO implants. Numerous giant, multinucleated osteoclast-like cells were observed in both types of implants, at both observation points, but their presence was the most prominent in BPU implants at two weeks. Close to these cells, moderate VCAM-1 immunoexpression can be observed, which could be correlated not only with blood vessels formation but also with osteoclastogenesis process (37). The existence of osteoclast-like, giant multinucleated cells in BPU implants is probably the consequence of synergistic effect of PRP combined with uninduced ADSCs (9). On the model of bisphosphonate-related osteonecrosis of the rat jaw, combination of cultivated, uninduced ADSCs taken from the third passage and PRP led to higher osteoclasts number in the jaws of treated rats in comparison with the rats that haven't received ADSCs/PRP treatment for the prevention of bisphosphonate-related osteonecrosis (9).

Man and associates (14) used also an ectopic model and showed that blood vessel network is well developed after the implantation of uninduced ADSCs isolated from rabbit inguinal fat pads, PRP and alginate-based biomaterial. This effect could be attributed to the cytokines and chemokines secreted by ADSCs since these molecules are signals for the attraction of resident MSCs and precursor cells to the site of injury (1). The difference in the effect of this and our study is that ADSCs were obtained from adipose tissue of different localization−inguinal fat pads. Also, the implants were prepared in the different manner−the components of our implants were mixed just before the implantation procedure while the preparation of implants in the study of Man and associates included *in vitro* encapsulation of ADSCs and PRP with alginate biomaterial during three weeks.

Conejero and his team (38) compared the effects of uninduced ADSCs with those of osteoinduced ADSCs on the repair of rat palatal bone. They seeded uninduced ADSCs on poly-L-lactic acid scaffolds and, simultaneously, osteoinduced ADSCs on the same type of scaffolds. ADSCs induced into OBs triggered more significant bone tissue regeneration compared to the uninduced ADSCs. Higher bone mineral density, bone regeneration and vascular density was estimated in critical-size calvarial defects of male Lewis rats when the defects were filled with hydroxyapatite/poly(lactide-co-glycolide) [HA-PLG] seeded with ADSCs induced into OBs compared to the defects filled with uninduced ADSCs (39). Better vasculogenic potential of ADSCs induced into OBs than the one seen in uninduced ADSCs which we have shown in the present study, could explain more improved osteogenic process in two above mentioned and similar studies.

Conclusion

Vasculogenic potential of ADSCs *in vitro* induced into OBs and combined with PRP and BMM in ectopic osteogenic implants is higher compared to the implants composed of uninduced ADSCs, PRP and BMM, which makes implants enriched with ADSCs induced into OBs good candidates for improving vascularization in bone tissue-engineered constructs.

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VASKULOGENI POTENCIJAL MEZENHIMSKIH MATIČNIH ĆELIJA MASNOG TKIVA INDUKOVANIH *IN VITRO* U OSTEOBLASTE, PRIMENJENIH SA PLAZMOM OBOGAĆENOM TROMBOCITIMA U EKTOPIČNOM OSTEOGENOM MODELU

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Nedostaci koštanog tkiva mogu biti posledica povreda, gubitka kostiju ili tumora. Nedovoljna vaskularizacija glavni je problem za uspešnu regeneraciju koštanog tkiva. Kako bi vaskularizacija tokom regeneracije koštanog tkiva bila poboljšana, obećavajuće metode su razvijene u okviru tkivnog inženjerstva kosti (TIK), uz pomoć primene mezenhimskih matičnih ćelija (MMĆ). Cilj ovog istraživanja bio je da se ispita vaskulogeni potencijal MMĆ *in vitro* indukovanih u osteoblaste u kombinaciji sa plazmom obogaćenom trombocitima (PRP) i mineralnim matriksom kosti (MMK) u ektopičnom osteogenom modelu i uporedi sa implantima sastavljenim od neindukovanih MMĆ, PRP i MMK. MMĆ izolovane iz epididimalnog masnog tkiva miša kultivisane su do trećeg pasaža i potom podeljene u dve grupe: MMĆ *in vitro* indukovane u osteoblaste i ADSC ekspandirane bez osteogene indukcije. Na osnovu principa biološke trijade, sastavljena su dva tipa implanata: implanti koji su sadržali MMK, PRP i MMĆ *in vitro* indukovane u osteoblaste (BPO implanti) i implanti koji su sadržali MMK, PRP i neindukovane MMĆ (BPU implanti). BPO implanti imali su višu ekspresiju gena endotelnih ćelija u poređenju sa BPU implantima. Uz to, imunoekspresija VCAM 1 raste tokom *in vivo* eksperimentalnog perioda u BPO implantima, a opada u BPU implantima. Prema tome, vaskulogeni potencijal MMĆ indukovanih *in vitro* u osteoblaste i kombinovanih sa PRP i MMK u ektopičnom osteogenom modelu viši je u poređenju sa neindukovanim MMĆ, PRP i MMK na ovom modelu. To čini implante obogaćene MMĆ *in vitro* indukovanim u osteoblaste dobrim kandidatima za poboljšavanje vaskularizovanosti u tkivno inženjerisanim konstruktima kosti.

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Ključne reči: *ektopična osteogeneza, mezenhimske matične ćelije masnog tkiva, osteogena diferencijacija, ekspresija gena endotelnih ćelija, plazma obogaćena trombocitima*