

Osteogenic potential of in vitro osteo-induced adipose-derived mesenchymal stem cells combined with platelet-rich plasma in an ectopic model

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Abstract

Purpose The osteogenic potential of in vitro osteo-induced adipose-derived mesenchymal stem cells (ADSCs) combined with platelet-rich plasma (PRP) and implanted on bone mineral matrix (BMM) carrier was examined in a subcutaneous model in Balb/c mice.

Methods In vitro osteogenic differentiation of ADSCs was monitored by relative bone-related gene expression and osteocalcin expression at the third, seventh and 15th day. Test implants consisting of in vitro osteo-induced ADSCs, PRP and BMM (OPC implants) and control implants consisting of PRP and BMM (PC implants) were examined. The relative expression of the bone-related genes encoding osterix, osteocalcin, collagen type I $\alpha 1$ and alkaline phosphatase was examined in implants extracted at one, two, four and eight weeks. Histochemical, immunohistochemical and histomorphometric analyses of implants extracted at two and eight weeks were performed.

Results The highest relative expression of bone-related genes and osteocalcin expression was found at the 15th day of

in vitro osteogenic induction of the ADSCs. Permanent and continuous increased expression of bone-related genes was noticed in OPC implants at eight weeks. Expression peaks of bone-related genes in PC implants were at two and four weeks, but they significantly decreased at eight weeks. The signs of resorption, formation of callus-like tissue positive for osteocalcin and increased presence of bone cells were found in OPC implants compared with PC implants. A higher percentage of infiltrated tissue and vascularisation was found in OPC than in PC implants.

Conclusions The combination of in vitro osteo-induced ADSCs and PRP on BMM carrier represents a promising approach for bone regeneration.

Keywords ADSCs · Bone-related gene expression · Ectopic osteogenesis · Osteogenic induction · PRP

Introduction

Adipose-derived mesenchymal stem cells (ADSCs) are multipotent cells [1, 2] that can successfully adhere to bone substitute materials, proliferate rapidly and have osteogenic potential [3, 4], which makes them good candidates for assisted bone tissue regeneration [2].

Bone substitute material and sources of growth and differentiation factors are of crucial importance for cell-based bone tissue reparation [2, 5]. Bone substitutes serve as cell carriers that imitate the natural bone environment, promote a rapid attachment, proliferation and differentiation of ADSCs [3, 4]. Platelet-rich plasma (PRP) is frequently used as a natural source of proliferation and growth factors [6]. Addition of the activated PRP stimulates in vivo differentiation and proliferation of implanted cells [7] and simulates natural conditions during bone injury.

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There is a need in clinical orthopaedic practice for an alternative to bone grafting, such as the combined use of stem cells, bone substitutes and growth and differentiation factors for bone regeneration [8]. So far, the specific roles of mesenchymal stem cells (MSCs) in bone fracture healing are not entirely clear [2]. In order to give closer insight into this concern, the osteogenic potential of in vitro osteo-induced ADSCs, combined with PRP and implanted on bone mineral matrix as a carrier, was examined in the subcutaneous mouse model. This model is a suitable and frequently used one [7] because it allows evaluation of in vivo osteogenic potential of ADSCs, thus avoiding the influence of the bone microenvironment [9].

Materials and methods

Isolation, expansion and osteogenic induction of ADSCs

Isolated epididymal adipose tissue from BALB/c mice was washed, macerated and digested using collagenase type I (Sigma-Aldrich, Hamburg, Germany) at a concentration of 2,000 I.U. per ml of Dulbecco's Modified Eagles Minimal Essential Medium (DMEM; PAA Laboratories, Pasching, Austria). Digestion lasted for 45 minutes at 37 °C and then the homogenate was filtered and centrifuged at 1,500 rpm, ten minutes. The pellet that contained the stromal vascular fraction (SVF) was re-suspended and cells were counted using the trypan blue dye exclusion method and seeded at a density 1×10^6 per flask (25 cm²). ADSCs derived from SVF were cultivated in DMEM, supplemented with 10 % fetal bovine serum, antibiotic-antimycotic solution and 2 mM L-glutamine (all from PAA Laboratories, Pasching, Austria), in a humidified atmosphere containing 5 % CO₂, at 37 °C. The media was replaced every two to three days until reaching confluency of 80 %.

At the third passage, ADSCs were subjected to osteogenic differentiation. The cells were seeded in 24-well plates (Greiner Bio-One, Frickenhausen, Germany), at density 1×10^4 per well, and cultivated in osteoinductive media (OM) up to 15 days prior to the preparation of implants for in vivo experiment. The OM contained 10⁻⁸ M dexamethasone, 50 µg/mL ascorbic acid and 10 mM β-glycerophosphate in DMEM supplemented with 10 % FBS, antibiotic-antimycotic solution and L-glutamine [10]. To monitor the change of bone-related gene expression during the osteogenic induction, cells were placed in RNAlater® (Ambion, Life Technologies, Paisley, UK) at the third, seventh and 15th day and stored at -80 °C until RNA purification step.

Immunocytochemistry

After the third passage, anti-integrin β1, CD29 (1:400, ab52971; Abcam, Cambridge, UK) antibody was used as a positive MSC marker. To assess the osteogenic phenotype at

the 15th day of in vitro osteogenic differentiation, antibody to osteocalcin (1:200, ab93876; Abcam) was used. Primary antibodies were applied overnight at 4 °C. An HRP/DAB detection kit (ab64261; Abcam) was used for visualisation, according to the manufacturer's instructions. Cells were counterstained with Mayer's haematoxylin (1 min, room temperature) and mounted.

Experimental animals

Syngeneic male Balb/c mice, 22–24 g in weight and 8 weeks old were used. During the experiment, animals were held in standard conditions and access to food and drink ad libitum. All procedures on animals were performed in accordance with the ethical standards of the Animal Welfare Act of the Republic of Serbia. Approval for the experiment was obtained by the Local Ethical Committee of the Faculty of Medicine, University of Niš, Serbia (approval 01-5518-7).

Preparation of PRP

The PRP was prepared by a two-step method [11]. Blood was collected from the orbital plexus of one group of mice into tubes with anticoagulant sodium citrate. Prepared PRP had two to six times higher concentration of platelets than normal physiological level. Platelets number was established by Malassez counting chamber (Paul Marienfeld, Lauda-Königshofen, Germany) and it was $1.48 \pm 0.3 \times 10^6 \mu\text{l}^{-1}$.

Preparation of implants and implantation procedure

Bone substitute material Bio-Oss®, size S (Geistlich-Pharma, Wolhusen, Switzerland), which represented the bone mineral matrix (BMM) was used as a cell carrier. Two types of implants were prepared: (1) OPC implants consisted of in vitro osteo-induced ADSCs, numbered $5 \times 10^5 \text{ ml}^{-1}$, mixed with activated PRP (activated with calcium chloride) and loaded onto BMM and (2) PC implants as a control group consisted of activated PRP mixed with DMEM and loaded onto BMM. Each implant consisted of 10 mg (0.02 cm³) of BMM. The PRP at concentration of 10 % was used per one implant as the most optimal concentration for combining with osteoprogenitor cells [5]. Before implantation the cells were allowed approximately 15 minutes to attach to BMM and fibrin fibres to be formed within the implants [12].

The animals were divided into two experimental groups, based on the type of implant, each consisted of 16 animals. We had four experimental periods (1, 2, 4 and 8 weeks) with four animals per each. Immediately after extraction, the implants were stored in the RNAlater® at -80 °C. For histochemical and immunohistochemical analysis we used implants extracted two and eight weeks after implantation.

Assessment of bone-related gene expression

The total RNA was isolated from the extracted implants and in vitro osteo-induced ADSCs by using RNeasy Mini Kit (Qiagen, Hilden, Germany) and Dnase I RNase-free set (Qiagen) for residual DNA digestion, according to the manufacturer's instructions. The RNA concentration was determined immediately after isolation by using Qubit[®] RNA assay kit and Qubit[®] 2.0 fluorometer (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), according to manufacturer's instructions.

Whole RNA was reversely transcribed into single-stranded cDNA by using a High-capacity cDNA Reverse Transcription Kit (Applied Biosystems[®], Thermo Fisher Scientific, Waltham, MA, USA). The reactions were performed and tracked in the PCR thermal cycler (SureCycler8800; Agilent Technologies, Santa Clara, CA, USA), according to the manufacturer's instructions.

Quantitative real-time PCR reactions were performed, monitored and analysed by the real-time thermal cycler, Stratagene MxPro-Mx3005P (Agilent Technologies, Santa Clara, CA, USA). The reactions were prepared by using Kapa Sybr[®] Fast Universal 2× qPCR Master Mix (Kapa Biosystems, Wilmington, MA, USA), according to manufacturer's instruction. A mix of reverse/forward primers for mice (Quantitect primer assays; Qiagen, Hilden, Germany) was used for bone-related genes transcription factor osterix (*SP7*), osteocalcin (*BGLAP*), collagen type I $\alpha 1$ chain (*COL1A1*) and tissue non-specific alkaline phosphatase (*ALPL*) (Table 1). The results were expressed relative to the bone-related gene expression levels in SVF (initial source of the cells) and normalised to the expression of housekeeping gene β -actin.

Histology

The extracted implants were fixed in 10 % neutral buffered formalin and decalcified in 10 % ethylenediaminetetra-acetic acid (EDTA) solution (pH 7.4). Tissue was processed and embedded in paraffin. Tissue blocks were cut at 4 μ m using a microtome Leica RM2235. Masson's trichrome and haematoxylin and eosin (H&E) staining were performed with tissue sections. The stained slides were analysed on the Leica System Microscope DM2500 equipped with Leica DFC490 Digital Camera (Leica Microsystems, Wetzlar, Germany).

Immunohistochemistry

After deparaffinisation, tissue sections were subjected to heat-induced antigen retrieval using sodium citrate. Primary antibody to osteocalcin (1:200, ab93876; Abcam, Cambridge, UK) was applied overnight at 4 °C. For visualisation, HRP/DAB detection kit (ab64261; Abcam) was used according to the manufacturer's instructions. Tissue sections were

counterstained with Mayer's haematoxylin (5 min, room temperature) and mounted.

Histomorphometry

Histomorphometrical measurements were performed in NIS-Elements software version 3.2 (Nikon, Chiyoda, Japan). The images were obtained by using the microscope Axio Observer.Z1 equipped with the camera AxioCam HR (Carl Zeiss, Oberkochen, Germany) at $\times 100$ magnification. Total area of explants, total vessel area and area of material granules on H&E stained tissue sections were measured using the "Annotations and Measurements" software tool. The percentage of vascularisation was calculated as follows: (total vessel area / total area of explants) $\times 100$, while the percentage of infiltrated tissue was calculated as follows: ((total area of explants – total area of material granules) / total area of explants) $\times 100$.

Statistical analysis

The mean values of relative gene expression, percentage of infiltrated tissue and vascularisation \pm SD, were used for analysis. Statistical analyses were conducted using the Kruskal-Wallis ANOVA and a post hoc Mann-Whitney tests in SPSS software version 15.0 (SPSS, Chicago, IL, USA). Statistically significant differences were considered for $p < 0.05$.

Results

Characterisation and evaluation of osteogenic differentiation of ADSCs

Isolated ADSCs expressed the CD29 marker (Fig. 1a, b). On the 15th day, ADSCs cultivated in OM exhibited pronouncedly the highest relative expression levels of bone-related genes (Fig. 1e–h) and were positive for osteocalcin (Fig. 1c, d).

qRT-PCR analysis of relative bone-related gene expression pattern in extracted implants

Dynamics and pattern of relative expression of bone-related genes in OPC and PC implants are shown and explained in Fig. 2.

Histochemical and immunohistochemical analysis

Histochemical and immunohistochemical analysis of the OPC and PC implants are shown and explained in Figs. 3 and 4.

Table 1 List of the primers used for quantitative real-time PCR

Gene name	QuantiTect Primer assay	Detected transcript ID and length	Entrez Gene ID
<i>SP7</i>	Mm_Sp7_1_SG, QT00293181	NM_130458 (2284 bp)	170574
<i>BGLAP</i>	Mm_Bglap1_va.1_SG, QT01057049	NM_001037939 (351 bp)	12096
<i>COL1A1</i>	Mm_Coll1a1_1_SG, QT00162204	NM_007742 (4709 bp)	12842
<i>ALPL</i>	Mm_Alpl_2_SG, QT01740221	NM_007431 (2521 bp)	11647
<i>ACTB</i>	Mm_Actb_2_SG, QT01136772	NM_007393 (1889 bp)	11461

Histomorphometrical analysis

The percentage of infiltrated tissue and vascularisation in OPC and PC implants are shown and explained in Fig. 5.

Discussion

At the third passage after isolation, the purified fraction of cells expressed CD29, a highly specific MSC marker [13] (Fig. 1a, b). Based on the literature data [1, 14], in vitro osteogenic differentiation of ADSCs was evaluated by the expression of osteocalcin and expression levels of *SP7*, *BGLAP*, *COL1A1* and *ALPL*. Positive immunocytochemical staining for osteocalcin (Fig. 1c,

d), which is a highly specific marker of bone cells [14], and pronouncedly upregulated expression of bone-related genes at the 15th day of in vitro osteogenic induction (Fig. 1e–h) indicated that ADSCs in our study were successfully induced towards osteoblasts. Our results are consistent with other studies which reported that differentiation occurred in two weeks [15].

Different dynamic and pattern of the bone-related gene expression was seen in OPC and PC implants (Fig. 2). The gene for osteoblast-specific transcription factor osterix [16] was expressed earlier in PC than in OPC implants with nearly steady expression pattern. On the other hand, a stable gradual increase in the expression level of *SP7* was exhibited in OPC implants from the first to the eighth week. Low expression levels of *BGLAP* in implants of both groups at one and two weeks are in accordance with the fact that osteocalcin is mostly expressed during the late stage of osteoprogenitor differentiation [14].

The expression pattern of *SP7* and *BGLAP* in OPC implants can be explained in two ways. First, after implantations, the implants were populated by resident osteoprogenitor cells under the influence of the implant components [17, 18]. Thus, the recruited cells modulated gene expression pattern in the OPC implants. Second, the ADSCs downregulated the expression levels of bone-related genes because their environment was changed by implant construction and in vivo application. This kind of response in osteoprogenitor cells was noticed earlier by Lian and Stein [19]. The in vitro osteo-induced ADSCs needed time to adapt, multiply and continue differentiation process after

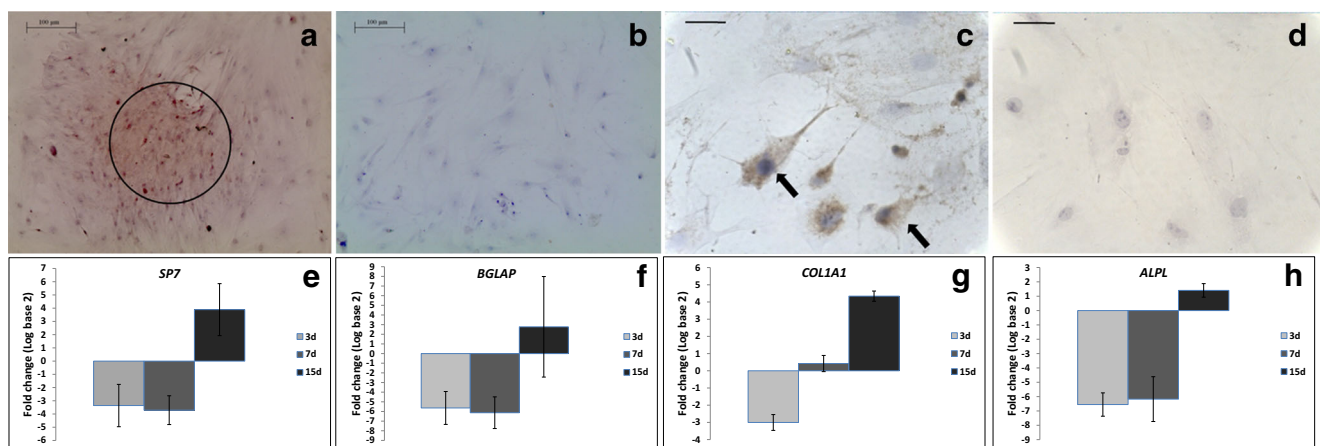
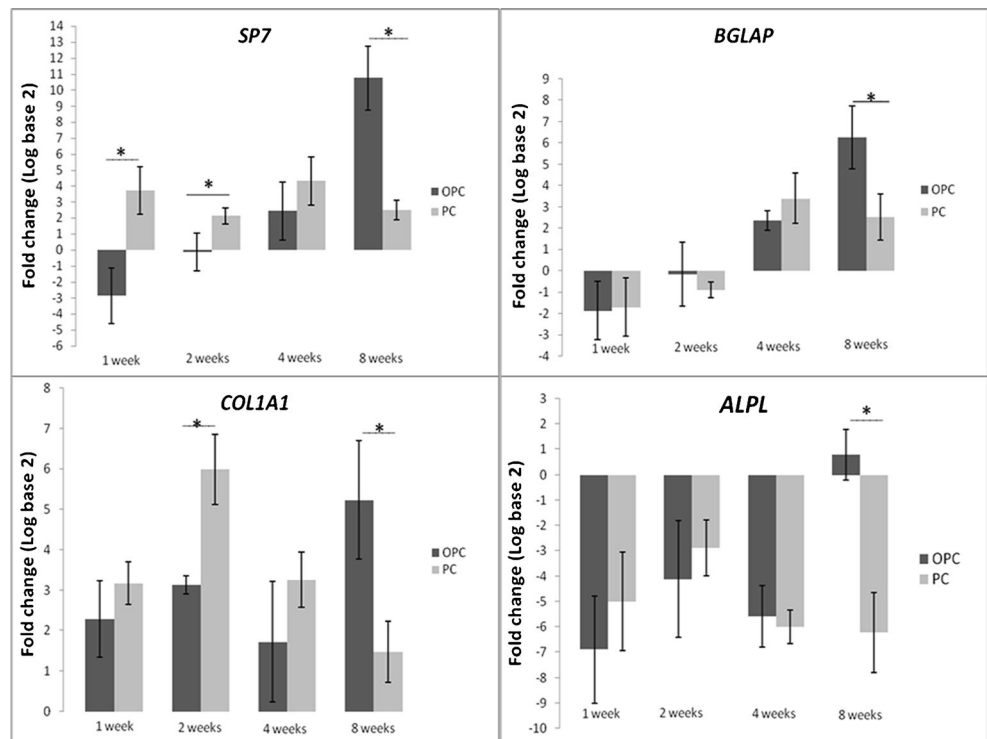


Fig. 1 Characterisation and evaluation of osteogenic differentiation of ADSCs. **a** ADSCs at the third passage were CD29 positive (circle); **b** negative control (without primary antibody). Bar 100 μ m; magnification 200 \times . **c** ADSCs cultivated in OM for 15 days were positive for osteocalcin; **(d)** negative control (without primary antibody). Bar

20 μ m; magnification 400 \times . **e-h** Prior to implantation, the dynamics of *SP7*, *BGLAP*, *COL1A1* and *ALPL* expression within in vitro osteo-induced ADSCs was examined at the 3rd (3d), 7th (7d) and 15th day (15d)

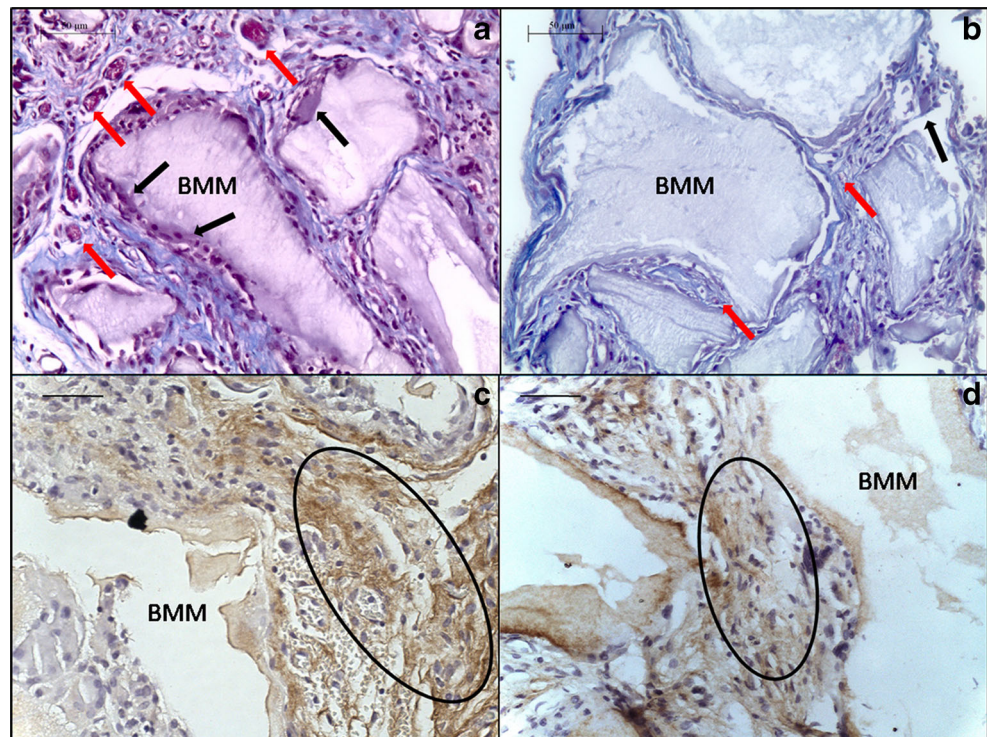
Fig. 2 Dynamics and pattern of relative expression levels of *SP7*, *BGLAP*, *COL1A1* and *ALPL* in OPC and PC implants extracted at one, two, four and eight weeks of the in vivo experimental period. Significant differences between groups within the same experimental period: $*p < 0.05$. At one and two weeks, significantly elevated expression level of *SP7* was exhibited in the PC implants compared with the OPC implants while at eight weeks it was vice versa. Significantly higher expression of *BGLAP* was noticed in the OPC than in the PC implants at eight weeks. At two weeks, the expression of *COL1A1* was significantly higher in the PC implants than in the OPC, while at eight weeks it was vice versa. Significantly elevated expression level of *ALPL* was observed in the OPC implants in comparison with PC at eight weeks



implantation. Nevertheless, once the expression of *SP7* and *BGLAP* was initiated in OPC implants, it progressed gradually and continuously to the eighth week. The dynamics and patterns of the *SP7* and

BGLAP expression which are seen in the PC implants are not odd. Some studies have shown that the combination of PRP only with a bone substitute material has osteoinductive properties [6, 20]. Thus, the expression

Fig. 3 Sections of the OPC and PC implants extracted at two weeks of in vivo experimental period. Masson's trichrome staining of the OPC (a) and the PC implants (b). *BMM* bovine bone mineral matrix granules. Greater presence of multinucleated osteoclast-like cells and blood vessels (arrows) was noticed in the OPC implants compared with PC implants. More collagen fibres between *BMM* granules were noticed in the PC than in OPC implants. Magnification 200×; bar 50 μm. Expression of osteocalcin in the OPC implants (c) and in the PC implants (d). Expression of osteocalcin was a bit more intensive in the OPC than in PC implants (showed with ellipses). Magnification 400×; bars 20 μm



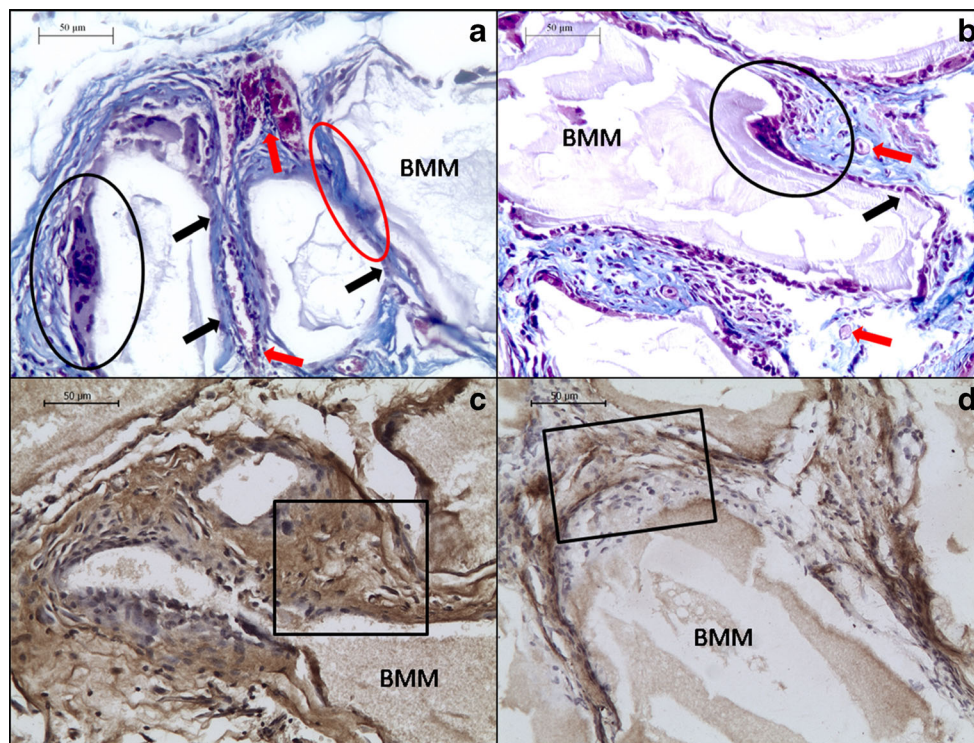


Fig. 4 Sections of the OPC and PC implants extracted at eight weeks of in vivo experimental period. Masson's trichrome staining of OPC (a) and PC implants (b). BMM bovine bone mineral matrix granules. Increased presence of the flattened bone lining osteoblast-like cells and larger blood vessels (arrows) were noticed in the OPC implants in comparison to the PC implants. The signs of resorption of BMM granules were more intensive (ellipses) in the OPC implants compared with the PC. In the OPC

implants, the matrix consisted of mature, dense collagen fibres which formed callus-like tissue were noticed (red ellipses), while in the PC implants collagen was mostly loose. Expression of osteocalcin in the OPC implants (c) and in the PC implants (d). Stronger expression of osteocalcin at the edge of BMM granules and in extracellular matrix was noticed in the OPC compared with PC implants (squares). Magnification 200×; bars 50 μm

levels of *SP7* and *BGLAP* was initiated earlier in the PC implants but expression decreased from the fourth to the eighth week.

COL1A1 expression is an important early marker of the osteogenic process [14]. An early expression of *COL1A1* was exhibited in both examined groups of implants. The expression peak occurred at two weeks in PC implants and

further decreased to the eighth week, while in OPC implants expression was steady at one, two and four weeks but further increased to the eighth week. This suggests different patterns of osteogenic process in the OPC and the PC implants.

An elevated expression of alkaline phosphatase is characteristic for pre-osteoblasts and differentiated osteoblasts [14]

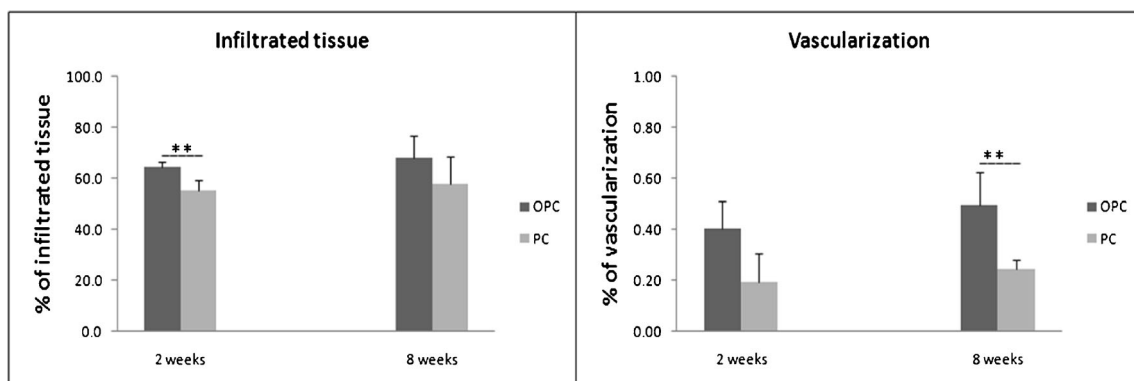


Fig. 5 The percentage of infiltrated tissue and vascularisation in the OPC and PC implants extracted at two and eight weeks of in vivo experimental period. Significant differences between groups: $**p < 0.01$. Percentage of infiltrated tissue was higher in the OPC implants compared with PC

implants but statistical significance was observed only at two weeks. Percentage of vascularisation was higher in the OPC than in the PC implants but statistical significance was observed only at eight weeks

are mandatory for mineralisation [21]. Significantly elevated *ALPL* expression in the OPC implants at eight weeks suggests that the osteogenic process was more advanced in the OPC than in the PC implants. Negative *ALPL* expression in both groups indicates that freshly isolated and untreated ADSCs (calibrator) have a high expression level of *ALPL*. This could be due to the fact that *ALPL* encodes tissue non-specific alkaline phosphatase which is also present in other tissues besides bone. Despite that, *ALPL* was frequently used for evaluation of osteogenic process [22, 23].

The presence of in vitro osteo-induced ADSCs in OPC implants induced significantly higher percentage of infiltrated tissue compared with PC implants (Fig. 5). The characteristics of infiltrated tissue in both groups of implants had been changed over time. More collagen fibres were noticed within PC implants than OPC at two weeks (Fig. 3a, b), which is consistent with *COL1A1* expression at this point. At eight weeks, collagen was present in both groups of implants, but the callus-like formation which is characteristic of early osteogenesis [24], was observed only in the OPC implants. This is supported with stronger expression of osteocalcin near BMM granules and in the matrix of OPC implants (Fig. 4). At this point, weak expression of osteocalcin in loose collagen matrix was seen in PC implants. In addition, the signs of intensive resorption and increased presence of osteoblast-like and multinucleated osteoclast-like cells were noticed in OPC compared with PC implants at each observation point (Figs. 3 and 4). The expression peaks of *SP7*, *BGLAP* and *ALPL* at eight weeks in OPC implants support the observation of higher activity and presence of osteoblast-like cells. The development of osteogenic process from the second to the eighth week in OPC implants was followed by a significant increase in the percentage of vascularisation (Fig. 5). All of these findings suggest a well-balanced osteogenesis in OPC implants. On the other hand, the absence of callus-like tissue, less presence of bone cells and significantly lower percentage of vascularisation indicate that osteogenic process was less expressed in PC than in OPC implants (Figs. 3, 4 and 5).

In summary, pronouncedly the highest relative expression of bone-related genes and osteocalcin expression were found at the 15th day of in vitro osteogenic induction of ADSCs. Therefore, ADSCs induced in that way were combined with PRP and BMM to evaluate their in vivo osteogenic potential in the ectopic model. Our results indicate that this combination triggered a more advanced and well-balanced osteogenic process than in the control group of implants consisting only of PRP and BMM. Thus, the combination of in vitro osteo-induced ADSCs for 15 days, PRP and BMM should be taken into consideration for the treatment of bone defects as an alternative to bone grafting. Only a few successful clinical studies, with implementation of ADSCs, were conducted [18]. Our study set the foundation for further investigation that is needed before the final translation of the presented approach to the clinical application.

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Conflict of interest The authors declare that they have no conflicts of interest.

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