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Bone Marrow Aspiration Concentrate and Platelet Rich Plasma for Osteochondral Repair in a Porcine Osteochondral Defect Model

Marcel Betsch[1], Johannes Schneppendahl[1]*, Simon Thuns[1], Monika Herten[2], Martin Sager[3], Pascal Jungbluth[1], Mohssen Hakimi[1], Michael Wild[4]

1 Department of Trauma and Hand Surgery, University Hospital Duesseldorf, Duesseldorf, Germany, **2** Clinic for Vascular and Endovascular Surgery, University Hospital Muenster, Muenster, Germany, **3** Central Animal Research Facility, University Hospital Duesseldorf, Duesseldorf, Germany, **4** Department of Trauma and Orthopaedic Surgery, Klinikum Darmstadt, Darmstadt, Germany

Abstract

Background: Bone marrow aspiration concentrate (BMAC) may possess a high potency for cartilage and osseous defect healing because it contains stem cells and multiple growth factors. Alternatively, platelet rich plasma (PRP), which contains a cocktail of multiple growth factors released from enriched activated thrombocytes may potentially stimulate the mesenchymal stem cells (MSCs) in bone marrow to proliferate and differentiate.

Methods: A critical size osteochondral defect (10×6 mm) in both medial femoral condyles was created in 14 Goettinger mini-pigs. All animals were randomized into the following four groups: biphasic scaffold alone (TRUFIT BGS, Smith & Nephew, USA), scaffold with PRP, scaffold with BMAC and scaffold in combination with BMAC and PRP. After 26 weeks all animals were euthanized and histological slides were cut, stained and evaluated using a histological score and immunohistochemistry.

Results: The thrombocyte number was significantly increased ($p = 0.049$) in PRP compared to whole blood. In addition the concentration of the measured growth factors in PRP such as BMP-2, BMP-7, VEGF, TGF-β1 and PDGF were significantly increased when compared to whole blood ($p < 0.05$). In the defects of the therapy groups areas of chondrogenic tissue were present, which stained blue with toluidine blue and positively for collagen type II. Adding BMAC or PRP in a biphasic scaffold led to a significant improvement of the histological score compared to the control group, but the combination of BMAC and PRP did not further enhance the histological score.

Conclusions: The clinical application of BMAC or PRP in osteochondral defect healing is attractive because of their autologous origin and cost-effectiveness. Adding either PRP or BMAC to a biphasic scaffold led to a significantly better healing of osteochondral defects compared with the control group. However, the combination of both therapies did not further enhance healing.

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* E-mail: johannes.schneppendahl@med.uni-duesseldorf.de

Introduction

Although autologous chondrocyte transplantation is a clinically successful strategy to treat chondral defects, the restoration of functional articular cartilage and subchondral bone is questionable [1]. Autologous osteochondral grafting is currently one of the few surgical options to address subchondral bone and articular cartilage at once [2]. However, autologous osteochondral grafts have limitations in size, a limited number of available implants and donor site morbidity, which may cause a future deterioration at the healthy donor site [3]. Additionally, the clinical outcomes of both techniques in prospective randomized trials are inconsistent [4,5].

Using a resorbable biphasic scaffold for osteochondral repair would allow a single-step treatment of subchondral bone and articular cartilage. Furthermore, it would reduce donor-site morbidity, and permit long-term storage and on-demand use [6]. A combination of bone-marrow stimulation and resorbable scaffolds, which provide initial mechanical stability and allow a homogenous three-dimensional cell distribution, could be a promising treatment option [6] as indicated in multiple studies using a large variety of different scaffolds for osteochondral repair

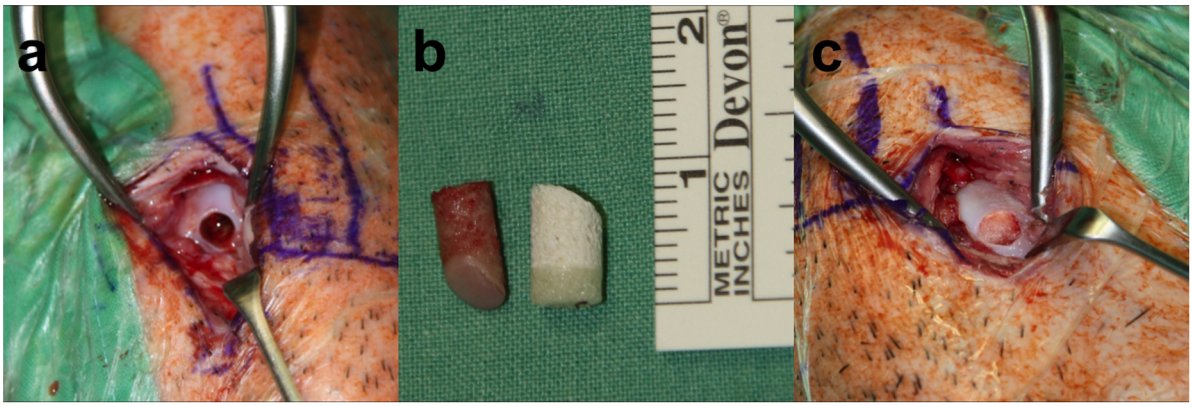

Figure 1. Osteochondral defect. In all animals, a 6×10 mm cylindrical osteochondral defect in the medial femoral condyles of both knee joints was surgically created with a cylindrical chisel (a). The osteochondral graft (left) from the defect was measured and then the biphasic scaffold (right) was cut to the respective length of the graft prior to implantation (b). The cut scaffold was then supplemented with the respective supplement and implanted into the condyle (c).
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[7–9]. Mesenchymal stem cells (MSCs) are a promising cell type for osteochondral repair, because once obtained, e.g. from the iliac crest, they can differentiate into chondrocytes and osteoblasts [10]. However, in vitro expansion of MSCs holds various problems, e.g. sterility of the cell culture, high costs, use of fetal bovine serum and the length of the cultivation, which does require a time-delayed second operation [11]. A possible alternative could be the use of a perioperative stem cell concentrate, as a single-step procedure by means of density gradient centrifugation of autologous bone marrow [12,13]. Cell-based therapy options using intraoperative, one-step procedures with progenitor cells from bone marrow have shown promising results in musculoskeletal tissues [13,14]. Platelet rich plasma (PRP), which is defined as a fraction of autologous blood having a platelet concentration above baseline [15], contains a high concentration of growth factors with a positive effect on tissue healing and regeneration [16,17]. Due of its autologous origin and low cost it has significant advantages over other therapies with recombinant growth factors. PRP extracted from autologous blood is less immunogenic and more biocompatible than recombinant growth factors, which often are descended from other species [18]. Furthermore, there is no risk of transmissible diseases and it can easily be obtained on the day of surgery from whole blood.

Since recent studies indicated that growth factors derived from platelets can stimulate the chondrogenic differentiation of bone marrow stem cells (BMSCs), enhance chondrocyte proliferation and extracellular matrix biosynthesis [19–22] combining both bone marrow aspiration concentrate (BMAC) and PRP to stimulate bone and chondrogenic healing in osteochondral defects may be effective. Therefore, the purpose of this present study was to evaluate the potential of PRP in combination with bone marrow aspiration concentrate BMAC in a scaffold for the treatment of an osteochondral defect in a large animal model.

Materials and Methods

Animals

Fourteen adult female Goettinger mini-pigs (aged 18–30 months, weight 25–35 kg) were used in this study. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The local Animal Care and Use Committee of the Heinrich Heine University and local government of Duesseldorf (permit number: 87–51.04.2010.A140) approved the animal selection, management and the surgery protocol. All surgery was performed under sodium pentobarbital anaesthesia, and all efforts were made to minimize suffering.

Bone Marrow Harvest and Concentration

Bone marrow (BM) aspirate was harvested from both iliac crests of 7 mini-pigs by Jamchidi vacuum aspiration. After aspiration the mononucleated cells were concentrated using a point-of-care device (MarrowStim® mini concentration system, Biomet Biologics, Inc., USA). One 30 ml syringe was filled with 6 ml of citrate anticoagulant (ACD-A, Anticoagulant Citrate Dextrose Solution, Biomet Biologics) and 2×12 ml of bone marrow. According to the manufacturers instructions this solution was centrifuged at 600×g for 15 min., then 3–4 ml of the nucleated cell concentrate (NCC) was separated from the cell free plasma/ACD-A mixture and from red blood cells. The concentration of the mononucleated cells in the original BM aspirate and in the separated NCC of the BMAC group and in the BMAC of the BMAC+PRP group prior to mixture was analysed with an automatic counter (ADVIAs 120®, Bayer Diagnostics GmbH, Germany). The concentration factor of the NCC was calculated from the quotient of mononuclear cells in BM/NCC of the BMAC and BMAC+PRP group.

Platelet Rich Plasma (PRP) Preparation

PRP was prepared from 60 ml autologous whole blood, which was retrieved from the auricular vein of the mini-pigs directly prior to the surgery. The thrombocyte concentrate was produced using the "GPS II Platelet Separation System®" (Biomet Biologics, USA). Following the manufacturer's protocol, to 6 ml of citrate anticoagulant 54 ml of autologous whole blood were added and then centrifuged at 3200 RPM for 15 minutes resulting in three different layers. The platelet-poor plasma (PPP) was then separated resulting in 10 ml of PRP. PRP was activated with autologous thrombin, which was produced from 7 ml of whole blood by centrifugation, and 2 ml of 10% calcium chloride. The thrombocyte count in whole blood and in the resulting PRP was analysed in an automatic cell counter. In addition, the concentration factor of PRP was calculated from the quotient of the thrombocyte count in whole blood and in PRP. Additionally, BMP-2, BMP-7, VEGF, TGF-β and PDGF were quantified in

Table 1. Modified O'Driscoll score.

	Score
Nature of the predominant tissue	
Cellular morphology	
Hyaline articular cartilage	4
Incompletely differentiated mesenchyme	2
Fibrous tissue	0
Safranin-O staining of the matrix	
Normal or nearly normal	3
Moderate	2
Slight	1
None	0
Structural characteristics	
Surface regularity	
Smooth and intact	3
Superficial horizontal lamination	2
Fissures: 25–100% of the thickness	1
Severe disruption, including fibrillation	0
Structural integrity	
Normal	2
Slight disruption, including cysts	1
Severe disintegration	0
Thickness	
100% of normal adjacent cartilage	2
50–100% of normal cartilage	1
<50% of normal cartilage	0
Bonding to the adjacent cartilage	
Bonded at both ends of graft	2
Bonded at one end, or partially at both ends	1
Not bonded	0
Absence of cellular changes resulting from degeneration	
Hypocellularity	
Normal cellularity	3
Slight hypocellularity	2
Moderate hypocellularity	1
Severe hypocellularity	0
Chondrocyte clustering	
No clusters	2
<25% of the cells	1
5–100% of the cells	0
Absence of degenerative changes in adjacent cartilage	
Normal cellularity, no clusters, normal staining	3
Normal cellularity, mild clusters, moderate staining	2
Mild or moderate hypocellularity, slight staining	1
Severe hypocellularity, poor or no staining	0
Total	24

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serum or plasma and in PRP using commercially available ELISA kits (Quantikine®, ELISA KITS, R&D Systems, USA).

Animal Model and Surgery

All mini-pigs were acclimated for one week before the surgery and were randomized, using a sealed envelope system, into the following four treatment groups: biphasic scaffold alone (control group), scaffold with PRP, scaffold with BMAC and scaffold with PRP and BMAC. Both knee joints of the 14 animals were used for the experiments resulting in a total number of 28 defects. In all animals, a 6×10 mm cylindrical osteochondral defect in the medial femoral condyle was surgically created with a cylindrical chisel. The osteochondral defect was filled in all groups with a biphasic scaffold (TRUFIT BGS©, Smith & Nephew, USA), which contains 50% non-crystalline amorphous poly-D,L-lactide-co-glycolide (PLGA), 10% poly(glycolic acid) fibres and 40% calcium sulfate (Figure 1). We chose this scaffold because previous studies have demonstrated promising results using this scaffold for osteochondral repair and because of its hydrophilic nature that facilitates absorption of PRP and BMAC [23,24]. PRP, BMAC or the combination of PRP and BMAC were added to the scaffold prior to implantation. After activation of the PRP with autologous thrombin the scaffold was immersed in 2 ml of PRP for 5 minutes to ensure that PRP had been completely soaked up by the scaffold before polymerizing it to a platelet-rich gel. In the BMAC group, 2 ml of BMAC was added to the scaffold for 5 minutes, and in the PRP with BMAC group 1 ml BMAC and 1 ml PRP were mixed first and then after activation of the PRP, the scaffold was immersed for 5 minutes in this combined solution. In the control group the defects were filled with the biphasic scaffold alone.

One experienced surgeon performed all surgical procedures in aseptic conditions with the mini-pigs under general anaesthesia. The skin of both hind knee joints was scrubbed and disinfected. A midline skin incision was made and the medial femoral condyle was exposed through a medial parapatellar approach. A cylindrical osteochondral defect (diameter 6 mm, depth 10.0 mm) was made in the medial femoral condyle using a 6 mm mosaicplasty digging tool to ensure that the size of all defects was comparable. The biphasic osteochondral implant was press-fit into the defect until the scaffold was at the same level with the surface of the surrounding articular cartilage. Wound closure was accomplished with bioresorbable sutures, and after the procedure the animals were returned to the enclosure and monitored until fully recovered from the anaesthesia.

Postoperative Management and Euthanasia

All mini-pigs were allowed to fully weight bear and to move freely without any constraints postoperatively. After 26 weeks the animals were euthanized with an overdose of 3% sodium pentobarbital (Eutha 77®, Essex Pharma GmbH, Germany). The knee joints were opened and macroscopically evaluated for signs of inflammation, such as tissue reddening, hypertrophy of the villous part of the synovial membrane, tissue adhesions and clarity and colour of the synovial fluid. The macroscopic appearance and quality of healing were assessed blindly using a scoring system. Thereafter, the medial femoral condyles of both knees were excised and fixed in 10% neutral buffered formalin solution for 14 days.

Staining and Evaluating Specimens

The specimens were dehydrated using ascending grades of alcohol and xylene, then infiltrated, and embedded in methylmethacrylate for non-decalcified sectioning. Any negative influence of heat was avoided by performing controlled polymerization in a cold atmosphere (-4°C). Each specimen was cut in the sagittal direction using a diamond wire saw (Exakt®, Apparatebau, Norderstedt, Germany). Serial sections were prepared from the

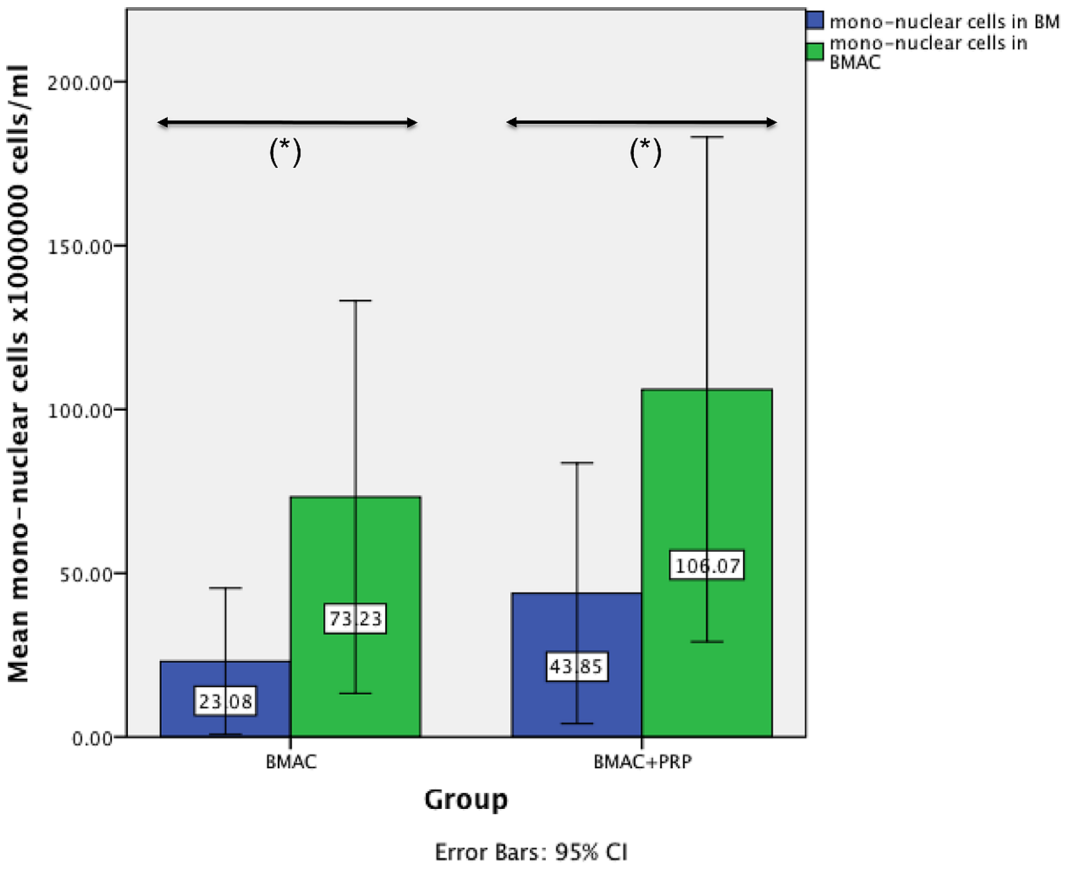

Figure 2. Mean mononuclear cell count. The mean mononuclear cell count of both treatment groups (BMAC and BMAC+PRP) in bone marrow (BM) and bone marrow aspiration concentrate (BMAC). In the BMAC group there was a 3.17 ($p = 0.021$) fold increase of mononuclear cells in BMAC compared to BM, and in the BMAC+PRP a 2.42 ($p = 0.006$) fold increase of the mononuclear cells.
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central parts of the defect areas, resulting in three sections each of approximately 320 µm thickness. All specimens were glued with acrylic cement (Technovit 7210 VLC®, Heraeus Kulzer) to oversize plastic slides (size: 50×100×2 mm, Dia-Plus®, Walter Messner GmbH, Germany), and ground to a final thickness of approximately 60 µm. The sections were stained with toluidine blue and safranin-o to evaluate the glycosaminoglycan (GAG) content and for histological assessment. Furthermore, a mouse monoclonal antibody reactive with porcine collagen II (1:400, Acris Antibodies GmbH, Germany) and the corresponding nonspecific antibody (mouse IgG1) as a negative control were used to stain for collagen II in the sections. The macroscopic appearance of the defects was evaluated using a macroscopic scoring system introduced by Rudert et al [25]. For histological examination a modified O'Driscoll score (Table 1) was used [26]. Two independent experts double-blinded, who were familiar with the histology of cartilage repair, evaluated the defects. All scores were the means of the two independent evaluations.

To evaluate the quality of the BMAC and PRP, the following in vitro tests were performed.

MSC Culture

The mononucleated cells from BM and from NCC were cultured in standard cell culture medium DMEM, with 1 g/l glucose, 20% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine (PAA Laboratories GmbH, Austria). Culture conditions were 5% CO_2 and 37°C.

CFU Assay

The proliferation potency of the mononucleated cells was measured in colony-forming units (CFU). Equal concentrations of mononucleated cells from BM and from NCC were seeded in 24 - well plates at cell densities of 1×10^5 cells/cm^2. After 7 and 14 days of culture the cells were incubated for 60 min at room temperature with hematoxylin to determine the number of CFU-fibroblast-like cells (CFU-F).

FACS Analysis

Due to the limited availability of commercial antibodies cross-reacting with the species pig, only the MSC markers CD14, CD44, CD90 and the hematopoietic markers CD45 and CD34 were measured by flow cytometry (Cytomics FC 500 FACS; Beckman Coulter, Germany) for phenotypic characterization of the cultivated cells.

Lineage Differentiation and Corresponding Histological Staining

Adherent cells of the first passage were further cultivated for osteogenic, chondrogenic and adipogenic differentiation as described previously [27].

Statistical Analysis

Unifactorial ANOVA was calculated to check for differences in the histological scores between the four groups. A t-Test was used

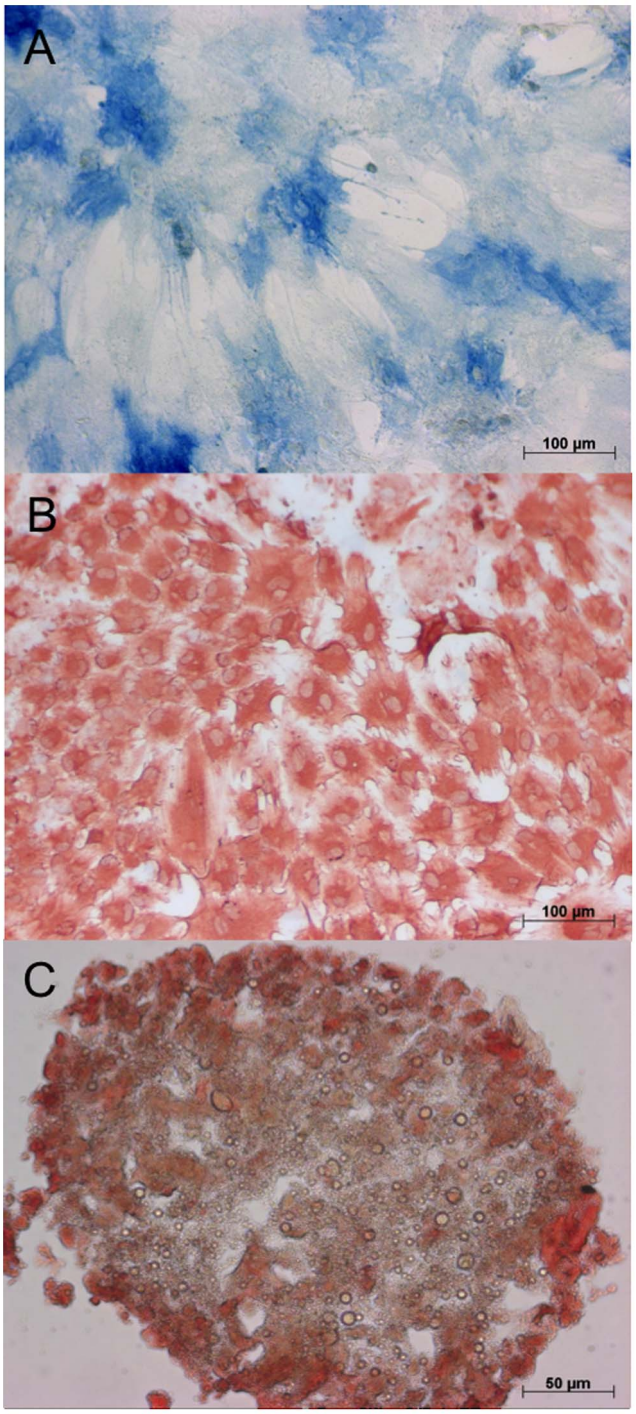

Figure 3. Differentiation assays on day 21. The differentiation of the mesenchymal stem cells from the bone marrow aspiration concentrate into osteoblasts became apparent by an intense blue staining for alkaline phosphatase activity (A). The adipocytes became apparent by the accumulation of lipid-rich vacuoles stained with PPAR (B) Cells cultured in chondrogenic medium showed a positive stain for chondrocyte markers over an increasing proportion of the pellet from 10 to 21 days (C).
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to evaluate for differences in the growth factors. The level of significance was set at $p<0.05$. Statistical analysis and graphic presentation were prepared using software SPSS 20.0® (SPSS Inc., Chicago, USA).

Results

In vitro Analysis

The quality of the used BMAC was evaluated by a calculation of the concentration factor of the mononuclear cells in both BMAC groups compared to the mononuclear cells in bone marrow, the formation of colony forming units, a cell characterization using FACS analysis and the trilineage differentiation of the cells. The mean value of the mononuclear cells in the BM of the BMAC group was 23.08×10^6 cells/ml ±24.12 and 43.85×10^6 cells/ml ±43.00 in the PRP with BMAC group (Figure 2). In the BMAC group the mean concentration of mononuclear cells was 73.23×10^6 cells/ml ±64.75 and 106.07×10^6 cells/ml ±83.27 in the PRP with BMAC group. The resulting concentration factors of the mononuclear cells in BMAC was 3.17 ($p=0.021$) in the BMAC only group and 2.42 ($p=0.006$) in the PRP with BMAC group, a statistically significant higher level of mononuclear cells within the BMAC fraction compared with the BM in both groups. For all animals of the BMAC group, adherent cells reached confluence after 16–21 days. The number of CFU-F was counted in colonies consisting of more than 40 cells with a defined center. After 7 days the mean number of CFU-F in BM was 0.3 ± 0.69 in 1×10^5 cells/cm^2, while in BMAC the mean CFU-F was 1.9 ± 2.5 in 1×10^5 cells/cm^2 ($p=0.144$). On day 14 the mean number of CFU-F in BM was 3.21 ± 3.55 in 1×10^5 cells/cm^2, while in BMAC the mean CFU-F was 5.09 ± 5.21 in 1×10^5 cells/cm^2 ($p=0.456$). In BM there was no significant difference ($p=0.078$) found between the mean CFU-F on day 7 and day 14. However, we found a significant difference of the CFU-F in BMAC between day 7 and 14 ($p=0.035$). FACS analysis revealed positive staining for CD44 in more than 90% ±8.3 of all viable single cells. For CD14 91.2% ±7.6 of all viable single cells were stained positively, which is comparable to CD90 (89% ±11.3). The hematopoietic markers CD45 and CD34 could be detected on only 5.9% ±1.70 and 2.5% ±0.9 cells, respectively. The data demonstrate a predominant expression of cell surface molecules associated with MSC and a minimal expression of hematopoietic cell surface molecules. Cells could be successfully differentiated into osteogenic, chondrogenic and adipogenic cells in all animals. The differentiation of the cells into osteoblasts became apparent by an intense blue staining for alkaline phosphatase activity. Positive staining for the osteocalcin (red) was detected in more than 90% of all adherent cells as shown by counterstaining with hemalaun (Figure 3). The adipocytes became apparent by the accumulation of lipid-rich vacuoles. Cells cultured in chondrogenic medium showed a positive stain for chondrocyte markers over an increasing proportion of the pellet from 10 to 21 days. In the PRP group the platelet number was significantly increased ($p=0.049$; $2.2\pm0.57\times10^6$ cells) compared with the platelet number in whole blood ($0.426\pm0.06\times10^6$ cells), which was on average a 5.24-fold increase (Figure 4). In the PRP with BMAC group the platelet number was also significantly increased ($p<0.001$) in PRP ($1.6\pm0.42\times10^6$ cells) compared with the platelet number in whole blood ($0.38\pm0.09\times10^6$ cells), a 4.29-fold increase (Figure 4). The mean concentration of BMP-2 was 12.87 ± 2.06 pg/ml in plasma and 167.7 ± 83.42 pg/ml in PRP ($p=0.001$), the mean concentration of BMP-7 was 95.85 ± 37.28 pg/ml in plasma and 178.86 ± 128.98 pg/ml in PRP ($p=0.003$), the mean concentration of VEGF was 11.97 ± 3.6 pg/ml in plasma and 77.35 ± 3.6 pg/ml in PRP ($p=0.001$), the mean concentration of TGF-β1 was 2899.71 ± 855.50 pg/ml in plasma and

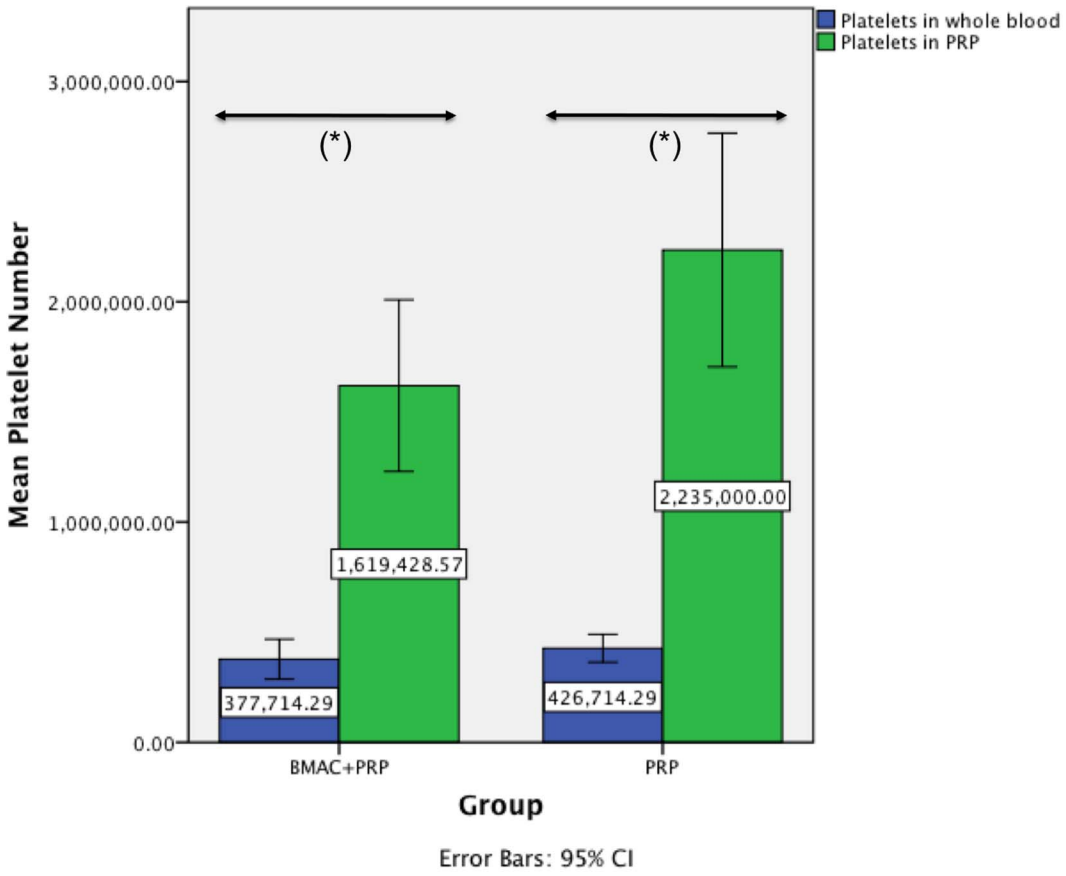

Figure 4. Mean number of platelets. The mean number of platelets in whole blood and in PRP in both treatment groups. In both groups we measured a significant increase ($p<0.001$ *and* $p=0.049$) of the platelet number in PRP compared to whole blood.
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40904.84±5468.71 pg/ml in PRP ($p<0.001$) and the mean concentration of PDGF was 107.69±194.45 pg/ml in plasma and 13962.29±5127.81 pg/ml in PRP ($p<0.001$).

All animals were ambulatory after the surgery, indicating the feasibility of using a bilateral defect model. Gross examination of the knee joints revealed that there were no abrasions on the opposing articulating surfaces, and no inflammation of the synovial membrane or other joint tissues were noted in any defects. None of the implanted scaffolds appeared dislodged. In no defect was the biphasic scaffold sitting above the native cartilage (Figure 5). Macroscopic examination of the explanted knee joints showed no lesions on the corresponding articular surfaces, and no inflammation of the synovial membrane was recognized. None of the 28 implanted scaffolds was dislodged. We found a mean macroscopic score for the control group of 5.57±1.62, of 5.86±1.77 for the PRP group, 6.57±1.13 for the BMAC group and 5.86±1.35 for the BMAC+PRP group. No significant differences ($p>0.05$) were found between the groups for the macroscopic score.

Histology

The newly formed tissue in the cartilage area of the defects in the therapy groups stained blue with toluidine blue and contained collagen II on the basis of positive immunostaining (Figure 6 and Figure 7). In contrast, mostly fibrous tissue was found in defects of the control group, which did not stain for collagen type II or sulphated glycosaminoglycans (sGAG). Remnants of the implanted scaffolds were consistently present in the osseous phase of the defects, while the cartilage phase was completely replaced after 26 weeks. The repair tissue in the bony phase was fibrous with vascularisation and giant cells, indicating an on-going degradation process of the scaffold at 26 weeks. In ten of the twenty-eight defects a subchondral cyst was noted in the subchondral layer. Furthermore, the normal architecture of the cancellous bone has not been restored after 26 weeks. The lowest score in the modified O'Driscoll score (12.86±3.24) was found for the biphasic scaffold alone (control group) (Figure 8). Adding bone marrow aspiration concentrate (16.86±2.67) or platelet rich plasma (17.43±3.05) to the scaffold led to a significant increase in the score ($p<0.05$) compared with the control group. The combination of BMAC and PRP with the biphasic scaffold (17.43±2.44) also led to a significant increase of the score ($p<0.05$) as compared to the control group, but it did not further enhance the histological score when compared to the BMAC and PRP group (Figure 8).

Discussion

Articular cartilage has a limited capacity for self-repair because of the low mitotic activity of chondrocytes and the avascularity of hyaline cartilage. In weight bearing joints large articular cartilage defects fail to heal spontaneously and may often result in progressive deterioration and osteoarthritis. The goal of current treatments is still only to prevent or delay the development of osteoarthritis [28]. The results of this study show that the combination of BMAC or PRP in a biphasic scaffold can significantly improve osteochondral healing in mini-pigs. Howev-

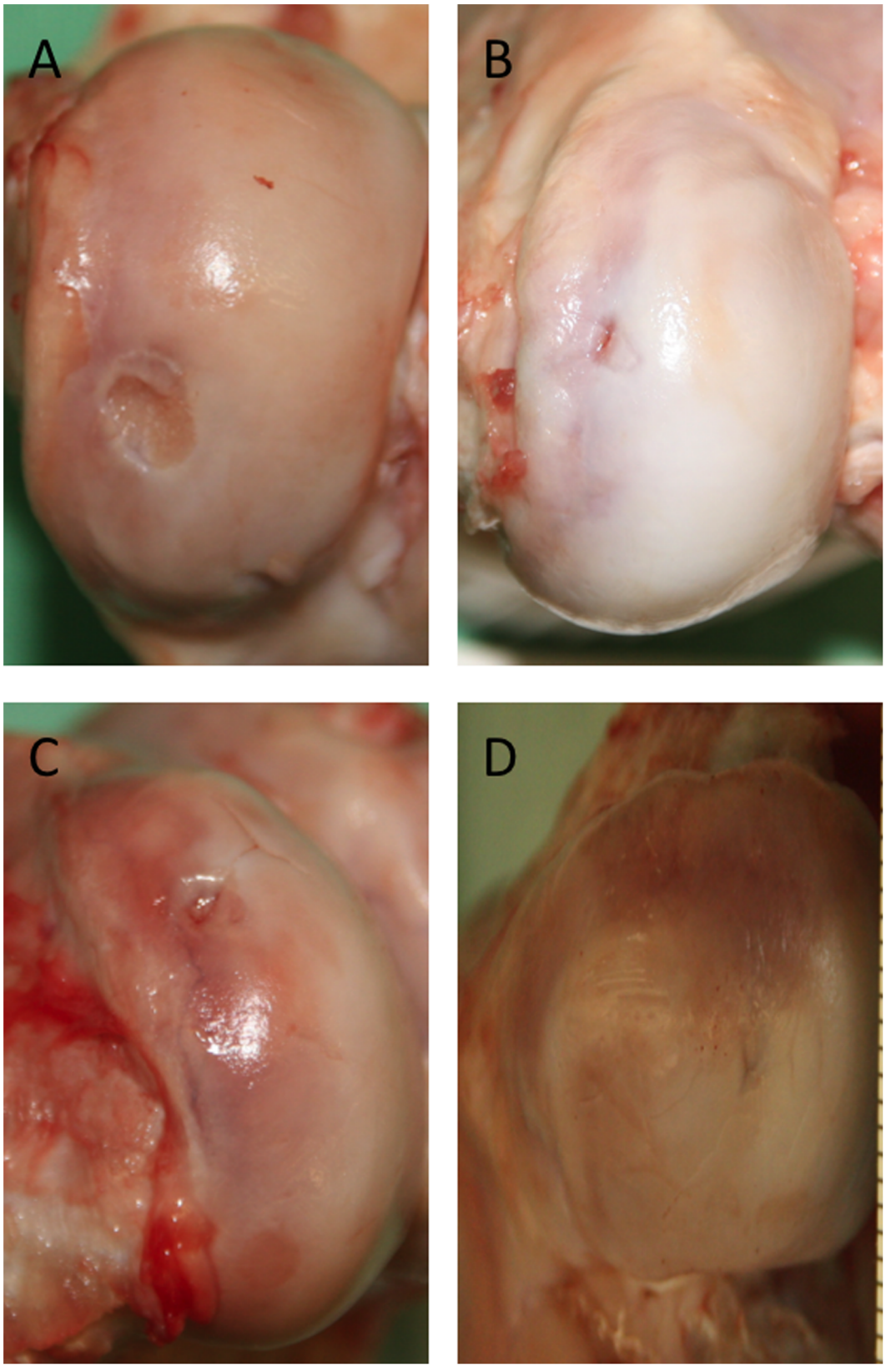

Figure 5. Representative macroscopic pictures of all four groups. In several cases a central depression was noted in the group, where the TRUFIT plug was implanted without any further supplement (A). In some cases, smaller residues of the defect were still visible in the PRP (B), BMAC (C) or BMAC+PRP (D) treatment groups.
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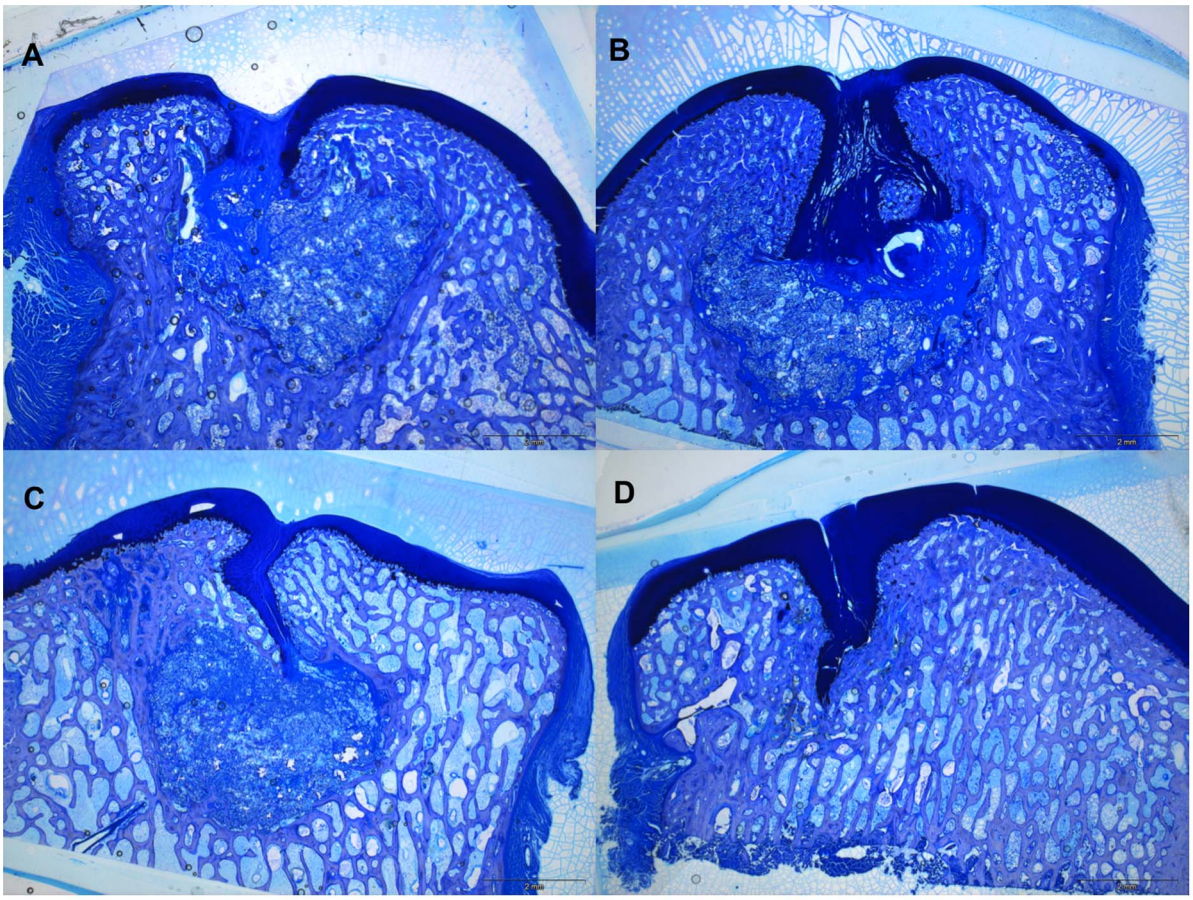

Figure 6. Representative histological slides. Representative histological slides (stained with toluidine-blue) of each of the investigated groups, a) scaffold only b) PRP c) BMAC d) BMAC with PRP. In the treatment groups the regenerated tissue stained positive for sGAGs.
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er, the combination of both BMAC and PRP did not further enhance the histological score. We used a bilateral defect model in the medial femoral condyles of mini-pigs with a diameter of 6 mm and a depth of 10 mm to evaluate the influence of BMAC and PRP on the osteochondral defect healing. Following Jung et al. [29] defects of 10 mm depth and 5.4 mm diameter are considered a critical osteochondral defect in this species. We chose the mini-pig as an animal model because its knee joint resembles the human knee joint dimensionally, and it also has a 1.5–2.0 mm thick cartilage surface, which makes it useful for the evaluation of cartilage repair [30,31]. The poly lactic-glycolic acid (PLGA) biphasic scaffold used in our study is commercially available in Europe and approved for use in osteochondral defects. This scaffold is a multiphasic implant, composed of polyglycolic acid and poly-D,L-lactide–co-glycolide fibers preferentially aligned to provide a structural scaffold with pores, which provide space to allow cellular ingrowth. Calcium sulfate forms 10% of the plug, and there are also trace amounts of surfactant within the scaffold. This bilayer design provides cartilage and bone phases yielding comparable mechanical properties to the host tissue. In the cartilage phase, the material is softer and malleable enough to be physically contoured to the joint surface. The results of this study show that the newly formed tissue integrated well with the native cartilage. The implant is designed to be completely resorbed over time, allowing for a complete filling of the defect with repair tissue [32]. However, in our study and in a study by Joshi et al. [33] remnants of the scaffold in the bony phase were still present, possibly indicating its failure to restore the subchondral bone. These findings are in accordance with the work of Jiang et al. [34] who observed a substantial amount of a DL-poly-lactide-co-glycolide and b-tricalcium phosphate biphasic scaffold remaining in the osseous phase after 26 weeks, while the chondral phase of the implant was completely replaced by regenerative tissue. The newly formed tissue in the bony phase had an immature and disorganized structure with an infiltration of mononuclear and plasmatic cells. In ten of the twenty-eight defects, a cyst was found in the subchondral bone at the site of the scaffold. This could indicate that even after 26 weeks the cancellous bone adjacent to the implanted material is still undergoing a remodeling process and that the remnants of the scaffold itself could have impaired the histological results. Getgood et al. in their osteochondral defect study noted subchondral bone cysts in groups treated with a PLGA scaffold and suggested this as part of the healing pattern in ostechondral defects in the early phase of regeneration at 26 weeks [35]. The degradation of PLGA is caused by hydrolytic cleavage of ester bonds, which releases lactic and glycolic acid into the microenvironment within the material [36]. Getgood et al. [35] suggested that the decrease in pH may have a detrimental effect on cells and acid-sensitive calcium-phosphate precipitates in the osseous compartment, which could cause subchondral bone cysts during the course of scaffold resorption.

Platelet rich plasma (PRP) is defined as a fraction of autologous blood having a platelet concentration above baseline [15]. It is a rich source of autologous growth factors, such as PDGF, TGF- β,

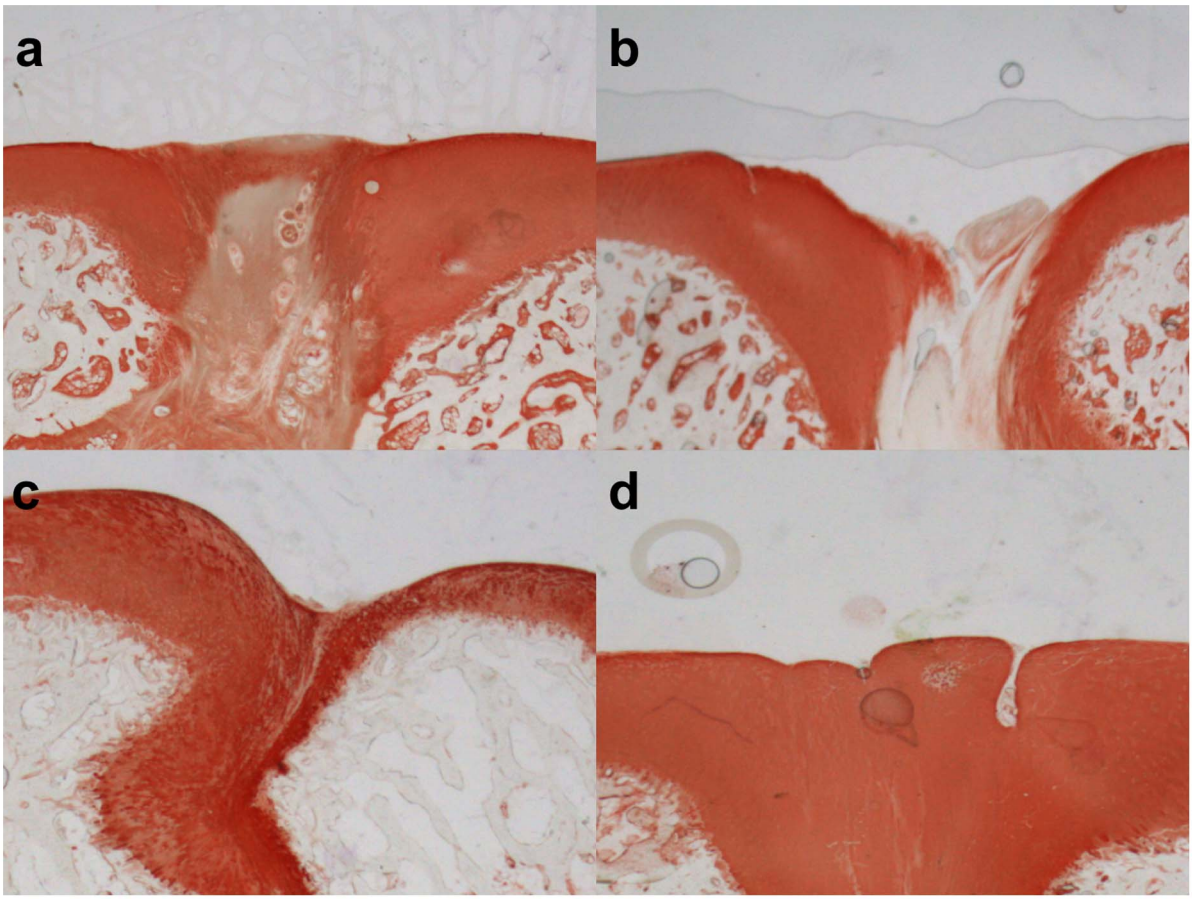

Figure 7. Immunohistochemical analysis for collagen II. Immunohistochemical analysis for collagen II of the regenerate cartilage in representative slides of all four groups. a) scaffold only b) PRP c) BMAC d) BMAC with PRP. No signs of degenerative changes in the adjacent cartilage were found. In the defects of the therapy groups, areas of chondrogenic tissue, which contained collagen II on the basis of positive immunostaining, were present. However, in the control group the regenerative tissue was generally fibrous. This tissue was deficient in collagen type II as shown by specific staining.
doi:10.1371/journal.pone.0071602.g007

bFGF, IGF, VEGF, epithelial cell growth factor (ECGF) [2], as well as other factors [37] and can therefore be used as an alternative option to recombinant growth factors. PRP can be easily obtained on the day of surgery from autologous whole blood without a risk of disease transmission or immunogenic reaction. Multiple in vitro studies have shown a positive effect of PRP on the maintenance of the chondrocyte phenotype during expansion [38] as well as on chondrocyte proliferation and matrix production [19,39]. Positive effects of PRP on cartilage restoration and glycosaminoglycan content were also shown in a rabbit chondral defect study using PRP in addition to a bilayer scaffold [40]. In a study by Milano et al. [41] the combination of microfracture with PRP showed a significant improvement in cartilage repair. Furthermore, Sun et al. [42] demonstrated in their osteochondral defect rabbit study that defects treated with a combination of PRP in a PLGA scaffold healed significantly better than the scaffold alone. PRP preparation is not standardized due to the use of multiple centrifugation protocols, different quantities of blood harvested and different quantities of PRP obtained, which all can result in a variability of PRP performance and growth factor concentration [43,44]. Kon et al. e.g. found negative effects of growth factors from platelet rich plasma, when used in combination with a hydroxyapatite-collagen scaffold in sheep [45]. However, they did not report the number of platelets in the PRP that was used in their study, which is important since platelet concentrations in PRP between three to five times higher than in whole blood seem to be advantageous in tissue healing and higher or lower concentrations seem to have adverse effects [46,47]. The point-of-care device that was used in our study led to a platelet concentration between 4.3 to 5.2 fold in PRP compared to whole blood indicating a beneficial concentration of the platelets in PRP. In addition the concentration of the measured growth factors in PRP such as BMP-2, BMP-7, VEGF, TGF-β1 and PDGF were significantly increased when compared with whole blood. The analysis of the PRP used in this study indicates that it was sufficiently replete with concentrated growth factors to allow the investigation of its effects on osteochondral healing. In future studies we also recommend that the number of leukocytes in PRP should be determined since studies have shown a potential influence of elevated numbers of leukocytes in PRP on the clinical outcome [48,49].

Recent studies demonstrated that growth factors derived by platelets could stimulate chondrogenic differentiation of bone marrow derived mesenchymal stem cells, enhance chondrocyte proliferation and extracellular matrix biosynthesis [19–22,50–52]. BMAC has been shown to be a good source for MSCs and growth factors in the regeneration of cartilage and hard tissues [53,54]. CFU-F counts were used as a quality marker for MSCs since they

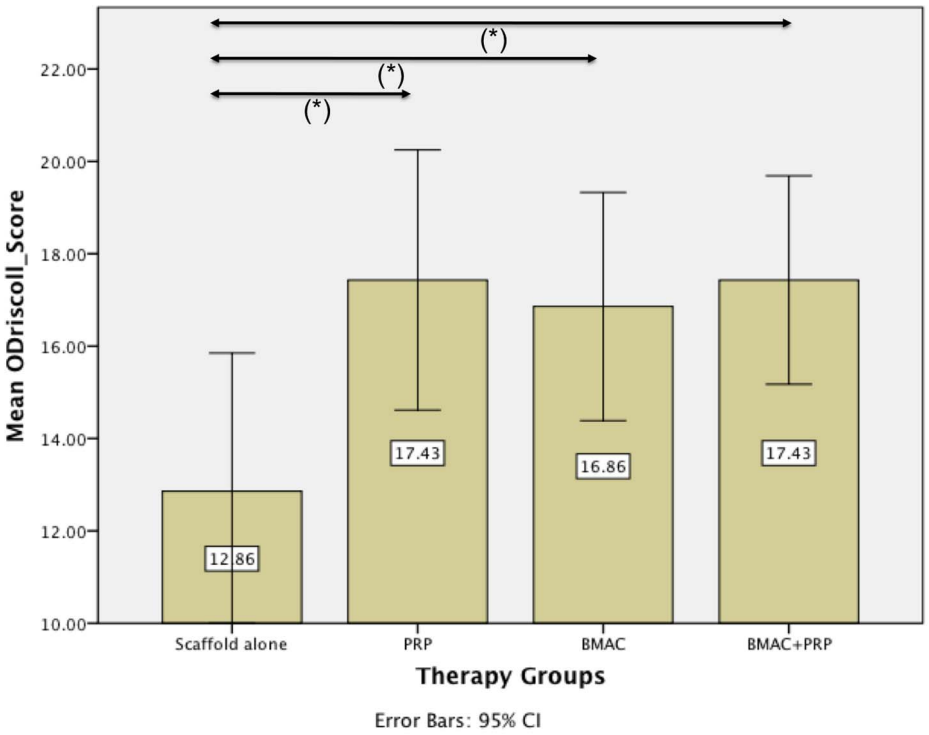

Figure 8. Results modified O'Driscoll histological scoring. The lowest score in the histological score (12.86±3.24) was found for the biphasic scaffold alone (control group). Adding bone marrow aspiration concentrate (16.86±2.67) or platelet rich plasma (17.43±3.05) to the scaffold led to a significant increase in the histological score ($p<0.05$). However, the combination of BMAC and PRP with the biphasic scaffold (17.43±2.44) did not further enhance the histological score.
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indicate the number of transplanted progenitor cells [55]. In our study the number of CFU-F in BMAC (5.09±5.21 in 1×105 cells/cm^2) was significantly higher than BM. These results are in accordance with the findings of other studies where mean values between 4.1 and 5.5 CFU-F/cm^2 were measured in BMAC [13,56]. The higher number of CFU-F from BMAC compared with BM is presumably due to the higher amount of mesenchymal progenitor cells and the reduction in the number of other cell types in the BMAC, created by the gradient centrifugation. The concentration factor of mononuclear cells in both BMAC groups was between 2.42 and 3.17, which was a significant increase compared to BM for both groups. Jäger et al. and Hermann et al. had a 5.2 and 4.4 fold increase in mononuclear cells in human BMAC compared to BM using the same concentration device, respectively [13,57]. The lower number of mononuclear cells in our study may be due to species differences in cell size and density between humans and pigs. We were able to demonstrate the quality of the MSCs in BMAC by chondro-, osteo- and adipogenesis of the MSCs and positive staining for mesenchymal stem cell surface markers using FACS analysis.

The kinetics of cartilage and bone regrowth exceeds a 6-month evaluation period. Therefore, it must be considered that the osteochondral repair is probably still in evolution at the time of the evaluation in our study. However, we believe that a 6-month follow-up is sufficient to provide an indication of the ongoing regenerative process. A limitation of our study is that we evaluated the osteochondral repair in an acute, but not chronic mini-pig model. This has to be kept in mind when interpreting the results of our study, especially since in humans most of the defects that need treatment are of chronic nature. The addition of BMAC or PRP and the combination of BMAC with PRP in a biphasic scaffold led to a significant improvement of the osteochondral defect healing compared with the biphasic scaffold alone. Despite this significant improvement in the histological score, the main repair tissue in the cartilage layer was mostly fibrocartilage. Interestingly, the combination of BMAC with PRP did not further enhance the histological score when compared to the BMAC or PRP group. An explanation for this could be that both BMAC and PRP contain high levels of growth factors and in PRP it was demonstrated that too high levels of growth factors could have adverse effects on tissue healing [58]. A further explanation could be that we used a combination of 1 ml of BMAC and 1 ml of PRP in the BMAC+PRP group, instead of 2 ml in the respective BMAC and PRP alone groups, which would result in a lower total cellularity in the BMAC+PRP group.

Conclusions

The biphasic scaffold used in this study led to mostly fibrous tissue in the defects of the control group. However, adding either PRP or BMAC resulted in a significantly better healing of the osteochondral defect compared with the control group. The clinical application of BMAC or PRP is attractive because of their autologous origin and cost-effectiveness, but the combination of both therapies did not further enhance healing in our study. In future studies different scaffolds should be evaluated as alternatives to the TRUFIT implant since the scaffold-only group had not produced a substantial repair.

Author Contributions

Conceived and designed the experiments: MB M. Hakimi MW. Performed the experiments: MB JS ST M. Herten MS. Analyzed the data: MB ST

MW PJ. Contributed reagents/materials/analysis tools: PJ M. Herten JS M. Hakimi. Wrote the paper: MB MW.

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