Biomaterials 31 (2010) 3572–3579

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Comparison of mesenchymal stem cells from bone marrow and adipose tissue for bone regeneration in a critical size defect of the sheep tibia and the influence of platelet-rich plasma

Philipp Niemeyer [a], Katharina Fechner [b], Stefan Milz [c], Wiltrud Richter [b], Norbert P. Suedkamp [a], Alexander T. Mehlhorn [a], Simon Pearce [c], Philip Kasten [b,d,*]

[a] Department of Orthopedic Surgery and Traumatology, Freiburg University Hospital, Freiburg, Germany
[b] Department of Orthopedic Surgery, Heidelberg University Hospital, Heidelberg, Germany
[c] AO Research Institute, AO Foundation, Davos, Switzerland
[d] Department of Orthopedic Surgery, University of Dresden, Dresden, Germany

ARTICLE INFO

Article history:
Received 17 December 2009
Accepted 13 January 2010
Available online 11 February 2010

Keywords:
Bone, tissue engineering
Bone regeneration
Platelet
Growth factors
Stem cell
Adipose tissue

ABSTRACT

Aim of the present study was to compare the osteogenic potential of bone marrow derived mesenchymal stem cells (BMSC) and adipose-tissue derived stem cells (ASC) and to evaluate the influence of platelet-rich plasma (PRP) on the osteogenic capacity of ASC in a large animal model.

Ovine BMSC (BMSC-group) and ASC (ASC-group) were seeded on mineralized collagen sponges and implanted into a critical size defect of the sheep tibia ($n = 5$ each). In an additional group, platelet-rich plasma (PRP) was used in combination with ASC (PRP-group). Unloaded mineralized collagen (EMPTY-group) served as control ($n = 5$ each). Radiographic evaluation was performed every 2 weeks, after 26 weeks histological analysis was performed.

Radiographic evaluation revealed a significantly higher amount of newly formed bone in the BMSC-group compared to the ASC-group at week 10 and compared to EMPTY-group from week 12 (all $p < 0.05$). A superiority on radiographic level concerning bone formation of the PRP-group versus the empty control group was found ($p < 0.05$), but not for the ASC-group. Histological analysis confirmed radiographic evaluation finding analogous significances.

In conclusion, ASC seem to be inferior to BMSC in terms of their osteogenic potential but that can partially be compensated by the addition of PRP.

1. Introduction

Mesenchymal stem cells (MSC) are an attractive cell population for regeneration of mesenchymal tissue such as bone. Various studies have been published demonstrating the bone-building capacity of mesenchymal stem cells and even their usefulness in treating critical size bone defects. Most of these studies were conducted with MSC derived from bone marrow (BMSC). However, other sources have recently been described to contain corresponding cell populations. MSC with characteristics similar to bone marrow derived cells can be obtained from human adipose tissue [8], ligaments [9], and lung [10]. MSC derived from adipose tissue (ASC), in particular, are considered to be an attractive alternative to MSC from bone marrow, because of their abundant availability and excellent expansion and proliferation capacities. Although ASC have now been sufficiently characterized, and their differentiation potential has been described extensively, there is still a lack of *in vivo* studies comparing the regenerative potential of ASC with those of BMSC in an orthotopic large animal model. The hypothesis was that ASC have an equivalent osteogenic potential as BMSC.

In addition, the influence of platelet-rich plasma (PRP) on bone healing using adipose-tissue derived mesenchymal stem cells (ASC) was subject of the present study. PRP has been of increasing interest in recent years and the data available in literature are controversial. While some studies report a significant improvement of bone healing in presence of PRP [1–6], other studies were not able to detect any positive influence [7–10]. Nevertheless, the influence of PRP on mesenchymal stem cells has only been investigated on cells derived from bone marrow (BMSC). Since adipose-derived stem

* Corresponding author at: Division of Shoulder and Elbow Surgery and Sports Medicine, Department of Orthopaedic Surgery, Dresden University Hospital Carl Gustav Carus, University of Dresden, Fetscherstrasse 74, 01307 Dresden, Germany. Tel.: +49 351 458 5006; fax: +49 351 458 4376.
E-mail address: philip.kasten@uniklinikum-dresden.de (P. Kasten).

0142-9612/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2010.01.085

cells obviously behave differently in a biological environment, the influence of PRP on ASC has to be evaluated separately. Accordingly, the second hypothesis was that the addition of PRP to ASC is beneficial for bone healing.

2. Materials & methods

2.1. Isolation and expansion of ovine MSC from bone marrow aspirates

Ovine MSC were obtained from the iliac crest under local anesthesia and procedural sedation for a later autogenous transplantation. The anterior superior iliac spine was aspirated yielding between 15 and 40 ml bone marrow. BMSC were isolated as published elsewhere [11] with minor variations *at a density of* $1 \times 10^5/cm^2$. Briefly, bone marrow mononuclear cells were obtained by Biocoll density gradient centrifugation ($d = 1.077$ g/cm^3; Biochrom) and plated in fibronectin-coated tissue culture flasks (Nunc). The expansion medium used was 58% low-glucose DMEM (Cambrex), 40% MCDB201 (Sigma), 2% FCS (Stemcell Technologies, Inc.), supplemented with 2 mM L-glutamine, 100 U/ml Pen/Strep, ITS, linoleic acid, 10 nM dexamethasone, 0.1 mM L-ascorbic-acid-2-phosphate (all from Sigma), PDGF-bb and EGF (10 ng/ml each, R&D Systems).

2.2. Isolation of ovine MSC from adipose tissue

Adipose tissue (starting quantity 5–7 g) for the autogenous isolation of ovine MSC was obtained from experimental animals under local anesthesia (2–5 ml lidocaine s.c) and analgosedation (2 mg/kg ketamine, 0.05 mg/kg, detomidine i.m., circa 5–10 μg fentanyl/kg i.v.). A lumbar paravertebral excision site was chosen. After a 3–5 cm skin incision, the subcutaneous fat tissue was dissected and removed using scalpel and scissor, and placed directly into a correspondingly labeled falcon flask containing phosphate buffered saline (PBS) with 20 mg/ml bovine serum albumin (BSA) and 1% penicillin/streptomycin. Ovine MSC were obtained by enzymatic digestion with collagenase according to standing protocols [12,13]. The adipose tissue was then washed twice with PBS containing 20 mg/ml BSA. The samples were then placed in petri dishes to reduce the adipose tissue to portions approximately 0.5 cm in size and to dissect and remove fibrous material and blood vessels. A 0.6 mg/ml collagenase solution (collagenase type B (Roche diagnostics, derived from Clostridium hydrolyticum) with a specific activity of >0.15 U/mg) was obtained by dilution with Krebs–Ringer buffer containing HEPES and the adipose tissue was incubated with an equivalent volume of collagenase solution and agitated lightly at 37 °C for 90 min. Undigested tissue and endothelial cell aggregates were largely removed by subsequent filtration through a polypropylene membrane with a pore size of 150 μm. After centrifugation of the cells at 600×g for 10 min at room temperature, the supernatant together with the lipid layer were discarded and the cell pellet was washed twice with PBS. The cells were resuspended in expansion medium and seeded into 25 cm^2 cell culture bottles (Nunc, Denmark). Cells were expanded at 37 °C and 6% CO_2 with medium according to Verfaille as published previously [11,14]. One or two days after seeding the adherent cells were thoroughly rinsed with PBS to remove non-adherent cells from the culture.

2.3. Verification of the MSC character of BMSC and ASC

To verify their MSC character, BMSC as well as ASC were successfully differentiated into bone, cartilage and fat according to standard protocols [11,15]. For this purpose, after differentiation into different lineages, cells were stained for collagen type II (after chondrogenic differentiation; antibody: ICN, Aurora, Ohio, USA) as well as with van Kossa (after osteogenic differentiation; Sigma, Taufkirchen, Germany) and with oil red staining (after adipogenic differentiation; Sigma, Taufkirchen, Germany). All stains were performed according to the manufacturer's protocols. For both cell types, cells from passages 2–4 (corresponds to 16–20 doublings) were used for experiments.

2.4. Seeding and 3D cultivation on mineralized collagen

Two rectangular pieces of mineralized collagen (total volume 5 cm^3 corresponding to the bone defect size) per animal were placed into individual wells of a Petri dish, with care being taken to see that they were not in direct contact with the surrounding well border. The mineralized collagen sponge (Healos[a], DePuy, USA) used in this study is a soft lyophilized, 3-dimensional matrix constructed of cross-linked collagen-1 fibers coated with non-crystalline hydroxyapatite. The principal components of the matrix are type I bovine collagen and non-ceramic hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$]. Pore sizes vary from 4 μm to 200 μm. The overall porosity is >95%. The Ca/PO_4 ratio is 1.67. The mineralized collagen sponge contains approximately 30% mineral by weight. All matrices were loaded with 2×10^7 cells as published earlier [16]. In brief, trypsinized MSC were resuspended and 2×10^7 cells per animal were applied to each of the matrices (viability >95% as determined by trypan blue staining). After one hour of sedimentation, the dishes were filled up with expansion medium [11] and incubated at 37 °C until transplantation. In all cases the transplantation was performed within 24 h after seeding.

2.5. Preparation and characterization of platelet-rich plasma

Allogeneic human leukocyte-depleted PRP was obtained from a local blood bank. The PRP was prepared at room temperature by centrifugation (20 min, 150 × g) of a pool of 3 buffy coats (mean age 29, standard deviation (SD) 7) derived from whole blood donations that were 1:10 anticoagulated with acid-citrate-phosphate-dextrose (Compoflex, Fresenius HemoCare, Bad Homburg, Germany). After a second centrifugation at 2200 × g for 15 min at room temperature, a mean platelet count in the leukocyte-depleted PRP to 1.0×10^9/ml corresponding to a 4–5 fold increase of the platelet count in whole blood was adjusted. The platelet-rich supernatant was leukocyte-depleted via filtration (Fresenius Bio P Plus, Fresenius HemoCare). The residual leukocytes were less than 1×10^6 per unit of PRP. PRP from a total of 3 donors was mixed to achieve representative and standardized values of the growth factors. The PRP was frozen 2–3 h after donation in 50 ml falcons at −20 °C and stored until needed. To characterize the content of the growth factors in the PRP, the PDGF-AB and TGF-β1 contents of ten donors were quantified by a commercial enzyme-linked immunoassay (ELISA) (Quantikine Human R&D Systems, Minneapolis, USA). The platelets were degranulated to release the growth factors by freezing and thawing [6]. The content of PDGF-AB was 185.67 ng/ml and of TGF-β1 97.11 ng/ml.

The construct of the PRP-group received 40 ml of PRP just before implantation into the defect and was activated with 5 ml 10% CaCl solution with 20 IE thrombin (Tissucol Duo (Baxter)/ml CaCl).

2.6. Animal model and group size

Five animals were assigned to each group and euthanized 6 months post-operatively. There were four different groups in this experiment (see Table 1). Whereas unloaded mineralized collagen served as control in the EMPTY-group, in the BMSC-group 2×10^7 autogenous ovine BMSC were transplanted on mineralized collagen, and in the ASC-group collagen scaffolds were seeded with the same amount of autogenous ovine ASC. In the PRP-group, ASC were applied in the same fashion as in the ASC-group with the exception that additionally 40 ml activated PRP was applied into the defect after positioning of the cell-scaffold construct. Three year old, approximately 65 kg heavy female non-pregnant Swiss alpine sheep were used for this study. Prior to the beginning of the study, all animals were routinely dewormed with 0.2 mg/kg p.o. moxidectin (Cydectin®, Wyeth Pharmaceuticals, Münster, Germany) and 0.2 mg/kg s.c. doramectin (Dectomax®, Pfizer, Karlsruhe, Germany). The animals were fasted 24–36 h preoperatively with ad libitum access to drinking water. Premedication consisted of 0.3 mg/kg i.v. diazepam (Valium®, Roche) and 0.08 mg/kg i.v. butorphanol (Morphasol®, Gräub, Bern, Switzerland).

2.7. Animal surgery

For the operation the animals were put under general anesthesia and prepared for surgery as published previously [17]. In the mid diaphysis (7 cm measured from the medial malleolus of the tibia) the bone was marked over a length of three cm. After that, a CF-PEEK plate (38% carbon fibre reinforced polyether-ether-ketone plate, according to its characteristic shape later called 'Snakeplate', custom made by icotec AG, Altstätten, Switzerland) was placed and the holes 1, 2, 6 and 7 were drilled. Holes 3, 4 and 5 were left empty as they corresponded to the defect zone. The plate was removed and a 2.7 mm custom-made spacer was placed and fixated with a monocortical screw in the middle of the pre-determined defect zone. A full diameter section of the bone was cut out using an oscillating saw. Care was taken that all of the periosteum was removed. In addition, the periosteum was removed at both sides of the osteotomy for additional 3 mm. After placing the Snakeplate again the threads were cut, measured, and screws, made of the same material, but also including tantalum fibers, which makes them visible on radiographs, were introduced. The screws were tightened with a force of 1.5 N m. Following that, a 7 hole LCP plate (Synthes GmbH, Oberdorf, Switzerland) was fixed to the cranial aspect of the tibia (Synthes GmbH, Oberdorf, Switzerland). Care was taken that the plates

Table 1
Different experimental groups used in the current study, survival time for all animals was 6 months after surgery.

Study group	Implanted material	Number of animals treated
EMPTY-group	unloaded mineralized collagen	5
BMSC-group	2×10^7 autogenous MSC from bone marrow (BMSC) seeded on mineralized collagen	5
ASC-group	2×10^7 autogenous MSC from adipose tissue (ASC) seeded on mineralized collagen	5
PRP-group	2×10^7 autogenous ASC seeded on mineralized collagen and addition of 40 ml PRP	5

were in a 90° angle to each other, that all screws were placed bicortically, and that the screws did not interfere with each other. The defect was a full-size 3 cm mid diaphyseal defect with the periosteum completely removed.

2.8. Postoperative management and monitoring

Postoperative analgesia was provided by intramuscular injection of carprofen (Rimadyl® 4 mg/kg) and subcutaneous injection of buprenorphine (Temgesic® 0.01 mg/kg) at the end of surgery. The sheep also received buprenorphine three times daily for 3 days and carprofen once a day for five days. No antibiotics were administered. During the duration of the study, the sheep were checked every 24 h by an experienced animal caretaker. For eight postoperative weeks, the sheep were kept in suspension slings which allowed them to stand and to bear full weight on all legs, but protects them from high loading forces, especially during lying down. After that, the sheep were kept in single boxes. After six months the sheep were euthanized by means of an intravenous overdose of barbiturate (pentobarbital, Vetanarcol®). Afterwards, both hind legs were exarticulated at the knee and the tibia was dissected, the implants were removed, and the samples were processed and analyzed.

2.9. Radiographic analysis

Correct positioning of the osteosynthesis was confirmed by radiography immediately postoperatively, then every two weeks, and also post mortem. A digital radiographic suite was used (Philips, Trauma Diagnost). The animals were sedated using 0.03 mg/kg detomidine i.m. (Domosedan®, Pfizer, Karlsruhe) and fixated by an assistant. Biplanar radiographs were performed: anterior/posterior (48 kV/15 mA s) and laterolateral (53 kV/32 mA s), for which the sheep were positioned in a lateral recumbent position. Quantification of osteoneogenesis was performed using a method developed by our group which was compared to alternative methods with regard to reliability, variability and objectivity in a previous study [18]. The laterolateral radiographs were analyzed with public domain computer software (GIMP, GNU General Public License). Using this software, it is possible to quantify the osteoneogenesis based on histogram analysis. Quantification of newly formed bone is performed as follows: A rectangular area of pre-defined size (300 × 300 pixels) is selected to define the "region of interest" (ROI). Then, the pixel zone representing ossification is defined: the lower end of the range is created using 3 individual measurements of the connective tissue using the color selector pipette tool. This creates the lower limit of the interval defined as the ossification zone. Correspondingly, pixel values of cortical bone are used to set the upper limit. The entire ossification zone was divided into three equally sized regions defined as beginning, good, and very good ossification, thus providing improved and simplified assessment. The histogram function was then used to assess the pixel values of the three regions within the bone defect. The regions of "good" and "very good" ossification were considered newly formed bone for the present study.

2.10. Histological evaluation

The explanted tibia samples were split longitudinally, and one half was embedded in methylmethacrylate (MMA, Fluka, Switzerland) for histological evaluation as published previously [17]. In brief, the samples were fixed in 70% methanol, dehydrated using a series of increasing concentrations of alcohol (70%, 96% und 100% ethanol), and embedded in methylmethacrylate monomer (MMA). After polymerization the samples were cut longitudinally using a sawing microtome (Leica SP 1600). Two to three centimeters of the original bone tissue proximal and distal to the defect were included in the sample. *Giemsa–Eosin staining* was performed to enable the morphological analysis of the non-decalcified polished MMA sections with Giemsa solution (15%) and eosin counterstaining as published previously [17].

2.11. Fluorescence marking

Fluorescent *in vivo* marking was performed during the postoperative phase of the study at selected time points to allow analysis of new bone formation. Animals were marked with 60 mL of calcein green injected subcutaneously in the eighth week and with 60 mL of xylenol orange in the tenth week. These dyes are safe for the animals and are incorporated into the bone formed at the time of bioavailability. This enables time-locking when the bone samples are assessed histologically using a fluorescent light source (Leica M165 FC, 450 nm).

2.12. Histological evaluation

Special attention was paid to heterotopic ossifications and signs of inflammation or potential tissue rejection reaction during the descriptive evaluation. The localization of newly formed bone was also noted. Quantitative evaluation was performed in accordance to the Mosheiff Score [19] and the Werntz Score [20], both of them established for assessing experimental bone defects. Furthermore, the area of newly formed bone was analyzed with public domain image processing software GIMP (GNU General Public License) (in equivalent fashion as the radiologic evaluation mentioned above) and put into relation to the total area of the bone defect. To calculate the Mosheiff Score [19] ranges of interest (ROI) were generated that could be superimposed on the images and which demarcated nine small fields of predefined size (25 mm^2, three proximal, three medial, and three distal). These ROIs was then superimposed on the scanned digital histological images and each of the nine small areas was independently given a score ranging from zero (no ossification) to two (good ossification). These values were assessed individually for each slice and then added to give a representative measure of total ossification. In the Werntz score [20], different qualities of the bone regions are taken into consideration and points are given for each quality. Zero to five points can be given for the category "bone formation" and zero to four points each for the categories "union" and "remodelling." The individual categories are analyzed independently and also after addition of the points.

2.13. Statistical evaluation

After data collection, separate data existed from radiographic follow up and histological assessments. Radiographic data from 14 time points existed at evaluation. There was data from histological evaluation corresponding to the three different methods of quantification. All data and measurements from the three test groups passed tests for normal distribution, thereby fulfilling prerequisites for subsequent statistical analyses. The results were examined by a multifactorial variance analysis (ANOVA). Afterwards, differences between the independent variables were checked in Turkey post-hoc tests. Alpha error was consequently adjusted, p-values <0.05 were taken to be significant. All tests were two tailed. In addition, a paired t-test was also performed to examine radiological differences for each group over the course of the experiment. Statistical analysis was performed using the SPSS statistics package version 15.0.

3. Results

3.1. Radiographic quantification of newly formed bone

A significant superiority of the BMSC-group versus the ASC-group could be detected from week 10 until the end of the study period (week 26) (Figs. 1 and 2). In addition, significantly more bone formation could be detected in the BMSC-group compared to

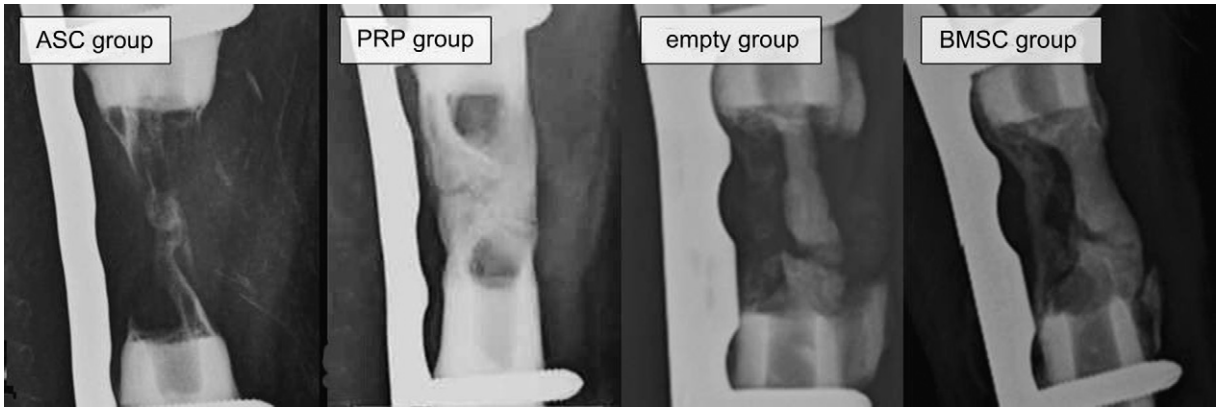

Fig. 1. Representative radiographs 26 weeks after surgery (lateral view) of the best animal of each group (from left to right): ASC-group, EMPTY-group, PRP-group; BMSC-group.

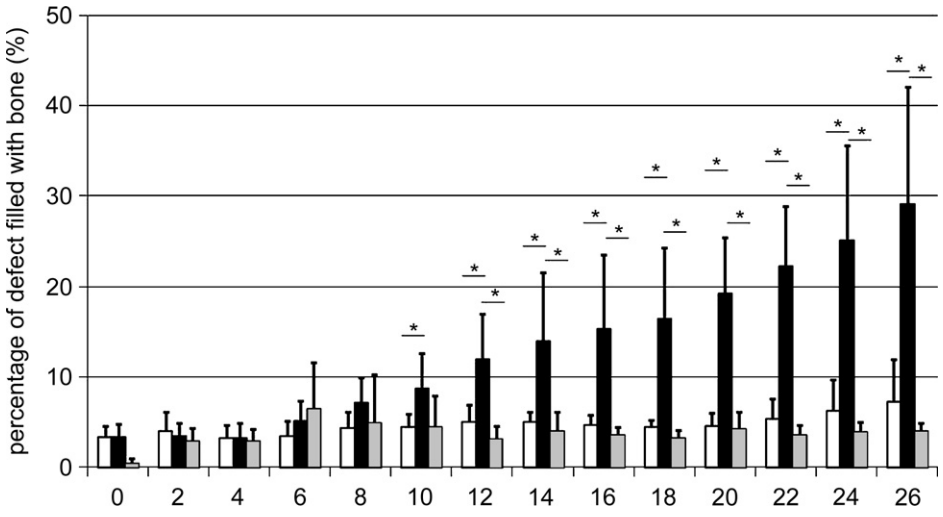

Fig. 2. Quantitative radiographic assessment of bone formation in the defect zone in the clinical course following surgery comparing BMSC, ASC and the empty-group: The BMSC-group (black columns) shows significant more bone formation from week 10 to the end of the study period compared to the ASC-group (white columns) and from week 12 to week 26 compared to the EMPTY-group: (grey columns); significant differences ($p < 0.05$ are indicated by "*").

the EMPTY-group between weeks 12 and 26. Concerning the influence of PRP, a trend to more bone formation in the PRP-group compared to the EMPTY-group was detected between week 20 and week 26 (p-values between 0.1 and 0.05), but this observation lacked statistical significance (Fig. 3).

Compared to postoperative percentage of bone in the defect, in the BMSC-group, significantly more bone compared to immediately postoperative observations (day 1) was detected from week 8 until the end of the study period (all p-values < 0.05, Fig. 4). While in the ASC-group, no significant bone formation compared to day 1 could be detected at any of the time points investigated, in the PRP-group, significantly larger amounts of bone were found in weeks 22 and 26 (p-values < 0.05).

3.2. Histological evaluation

Analysis of all histological samples of the BMSC-group and of 2 out of 5 samples of the PRP-group exhibited bridging of the defect. In none of the animals that were assigned either to the EMPTY-group or to the ASC-group a bridging of the defect was achieved. Representative histological samples of different treatment groups are given in Fig. 5. Detailed descriptive evaluation of the histological samples revealed the presence of osteoid and "bone lining cells" as histological characteristics of mainly direct ossification (Fig. 6). Partially, also histological signs of indirect ossification (enchondral ossification) could be detected. In the majority of the animals, bone formation started at the proximal and distal osteotomy. Bone in the defect seemed to be less mature and this was mainly observed in the animals in which bridging occurred late within the study period. In addition, fluorescence marking revealing an earlier fusion in the PRP-group compared to the BMSC-group. This was clearly visible in those animals in which osseus bridging occurred (Fig. 7). In all animals treated with cell-loaded implants (all groups except for the EMPTY-group) areas of newly formed bone without contact to the edges of the osteotomy zone were found. In the ASC-group areas of cells with large vacuoles similar to a adipogen phenotype were observed (Fig. 8).

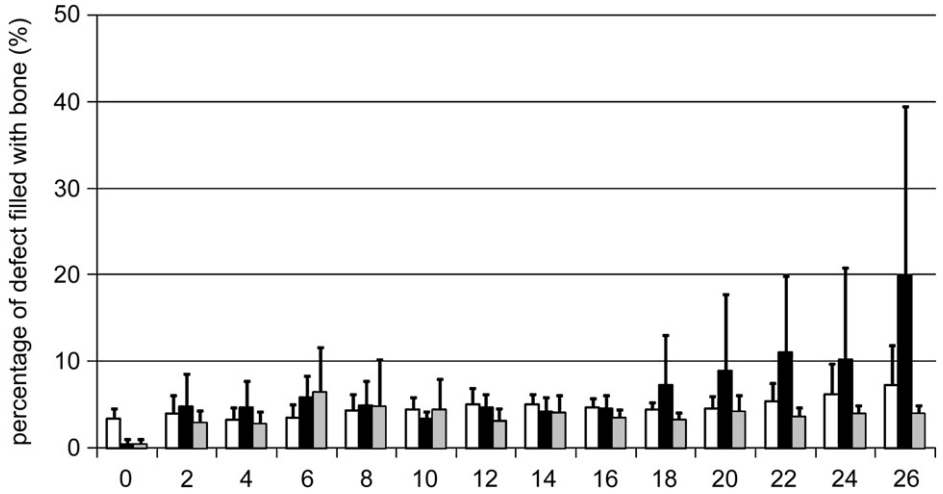

Fig. 3. Quantitative radiographic assessment of bone formation in the defect zone in the clinical course following surgery comparing PRP, ASC and the empty-group: In the mean, the PRP group (black columns) shows more bone formation compared to the ASC-group (white columns), and compared to empty scaffolds (EMPTY-group: grey columns); nevertheless, probably due to a heterogeneity between different individuals from the PRP-group (represented by a high standard deviation), this observation lacked of statistical significance.

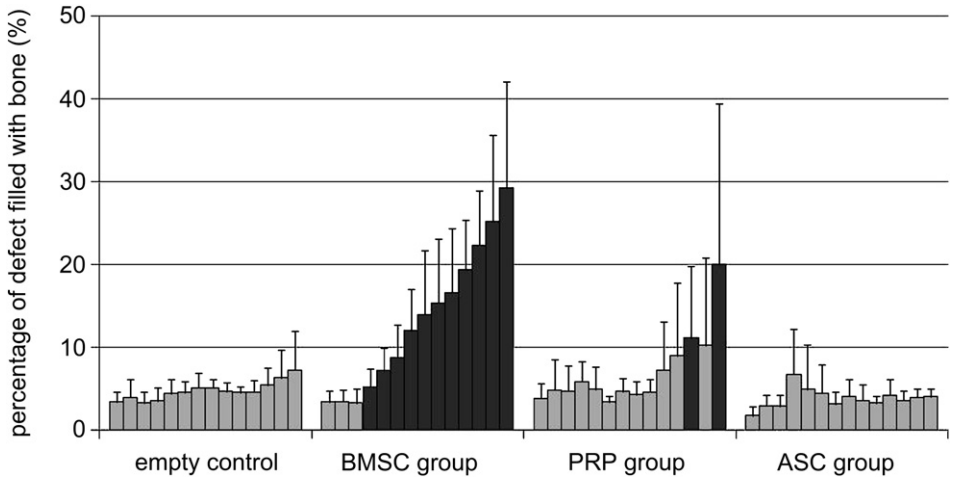

Fig. 4. Quantitative evaluation of newly formed bone in the defect (in %), with regard to differences compared to "day 0" in dependence of group assignment. Percentage of defect filled with bone in % is visualized from day 0 (left column) to week 26 (right column) of every group. Significances ($p < 0.05$) compared to day 0 are visualized by black columns.

3.3. *Quantitative analysis of histology*

For quantitative histological assessment of newly formed bone, imaging software describing the percentage of newly formed bone in the defect was used as well as two established semi-quantitative scoring systems. All three methods demonstrated analogous results. Concerning the quantitative determination of newly formed bone, at the end of the study a period with significantly higher amount of bone was detected in the BMSC-group compared to the ASC-group ($p = 0.010$). Concerning the influence of PRP, a larger amount of bone in the PRP-group compared to the ASC-group was detected as a trend but failed to reach statistical significance ($p = 0.084$). In addition, animals treated with autogenous BMSC showed higher amounts of bone compared to unloaded controls ($p = 0.023$). Results are given in Fig. 9 part A. Using the Werntz score, superiority of BMSC compared to ASC was found as a trend ($p = 0.080$). The sub-score "union" revealed a superiority of BMSC versus ASC ($p = 0.049$, Fig. 9 part B). According to the semi-quantitative scoring system used by Sarkar, significantly more bone formation was detected in the BMSC-group versus the EMPTY-group ($p = 0.032$) and the ASC-group ($p = 0.042$, Fig. 9 part C).

4. Discussion

In vivo data regarding the osteogenic potential of adipose mesenchymal stem cells (ASC) to heal bone defects remain scarce [21–24]. These cells are enormously attractive for clinical use because of their ready availability and easy accessibility. Prior to clinical use, however, it must be proven that their osteogenic potential is not inferior to that of bone marrow cells. At the moment, there are few experimental studies using bone defect models addressing this issue [22,25,26]. These studies suggest that undifferentiated ASC lead to functionally inferior regenerated tissues compared to MSC derived from bone marrow or periost. With the exception of the Peterson study, which examined the efficacy of BMP-2 transfected ASC, the results of these studies are of only limited relevance for various reasons. One problem was that in the study of Hui et al. the cells were applied within a fibrin glue suspension [25]. From our perspective, this approach is limited concerning its lack of biomechanical stability which is needed in a weight-bearing bone defect. Another problem is that a rabbit model which has a high rate of spontaneous healing was employed, and furthermore the defects were no critical size bone defects, but rather partial defects only [25]. In the study by Hayashi et al. a small animal model (rat defect model) was used, which only allows a very limited extrapolation of results to the human patients situation and therefore must be regarded inferior to a defect model in a large animal [26]. Nonetheless, these studies alert the reader to possible problems which could arise with the use of adipose stem cells and thereby justify the separate and comparative analysis of the regeneration potential of these cells with autogenous BMSC. The *in vivo* bone defect model studies also showed that the initially comparative studies, which demonstrated essentially identical properties of adipose and bone marrow stem cells at the *in vitro* level [27,28] and after heterotopic *in vivo* transplantation [29], cannot uncritically be transferred to the regeneration capacity of orthotopic bone.

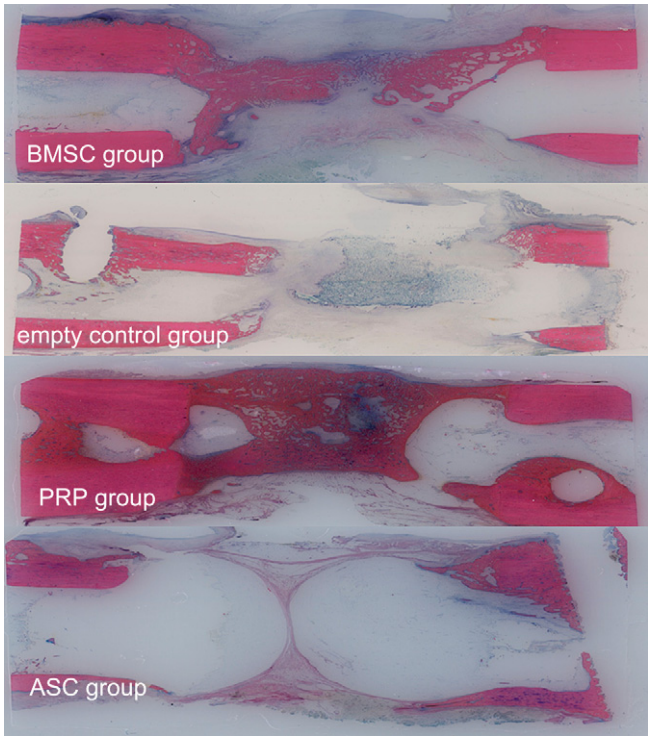

Fig. 5. Representative histological slides of animals assigned to different treatment groups (overview).

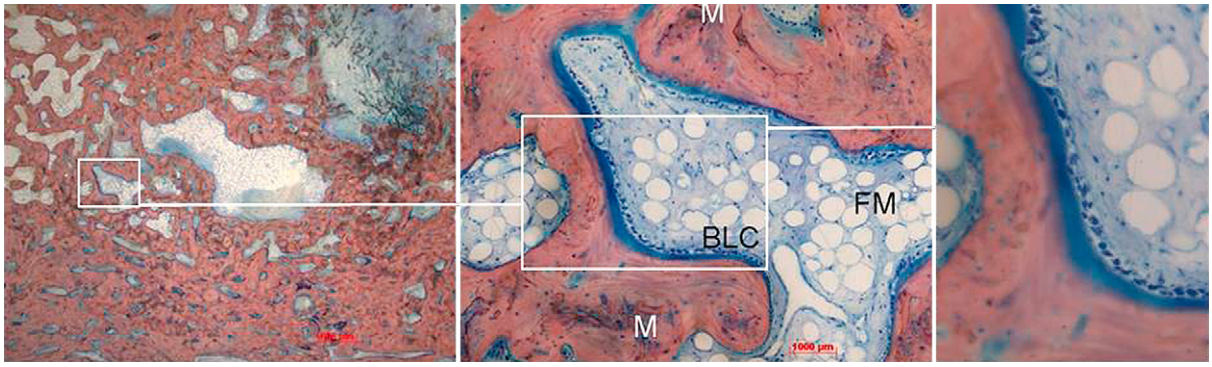

Fig. 6. Representative histological slide (Giemsa–Eosine staining in different magnifications) of an animal treated with ASC in combination with PRP (PRP-group). The presence of bone lining cells (BLC) and osteoide demonstrate a direct ossification, while parts of the mineralized collagen are well incorporated and still visible after 6 months (M). FM indicate fat cells as a sign of mature bone formation.

For the present study a critical size defect of the sheep tibia was chosen, since there are a lot of studies proving this animal model to be a valid and reliable model for evaluation of bone regeneration [30]. In addition, the large amount of previously performed studies using this model provides the opportunity to better compare results of the present study to other experiments. In our eyes, the most important issue when choosing an animal model for translational research is the transferability of the conclusions to the human patients situation. According to the work of Metak et al., this is optimal in bone defects in sheep [31], because anatomy and biomechanical properties of the sheep tibia are comparable to the human counterpart. In addition walking and maximal weight bearing of the sheep tibia is also comparable to the human situation as demonstrated by Lanyon and coworkers as early as 1975 [32].

Concerning the results of the present study, osteoneogenesis as assessed by radiography was insufficient in the group treated with mineralized collagen colonized with adipose stem cells (ASC-group). In contrast to the groups treated with autogenous BMSC, ASC therapy provided significantly inferior results after week 12 ($p < 0.001$). Regarding osteoneogenesis no advantage in the ASC-group as compared to the empty control group could be detected by radiographic assessment at any time point. Furthermore, histology following euthanasia of the animals also revealed no benefit of ASC administration compared to non-colonized control groups in terms of quantitative assessment of the newly formed bone and in terms of semi-quantitative scores. Analogously to radiographic evidence, there was also histological evidence both in terms of the area filled with bone ($p < 0.01$) as well as assessment of osteoneogenesis as a sub-score of the semi-quantitative score developed by Werntz [20], as well as the score developed by Mosheiff [19] which all showed the clear inferiority of ASC compared to BMSC ($p_{Werntz\ BF} = 0.046$; $p_{Mosheiff} = 0.042$).

Furthermore, in examining the histological samples, it became evident that more adipose-like cells with large intracellular vacuoles were detected in the ASC-Group, and that the tissue filling the defect in this group resembled adipose tissue. It remains possible that undifferentiated stem cells from fat differentiate spontaneously toward adipocytes without additional exogenous osteoinductive factors, while MSC isolated from bone marrow are determined to generate bone and do not appear to require additional factors to influence them. Studies by other groups appear to refute our data on bone marrow stem cells [33,34] and report a benefit from prior *in vitro* differentiation when using adipose stem cells [21]. The results presented here, as well as the impression of spontaneous adipogenic differentiation seen in the descriptive analysis of histological samples, support this theory. Our results demonstrate — using an animal model which is both biomechanically sound and transferable to humans — that undifferentiated adipose stem cells are inferior to bone marrow stem cells with regard to their osteogenic potential.

The use of high-dose thrombocyte concentrates as osteoinductive components for bone regeneration is a recent focus of research in the field of tissue engineering. "Platelet-rich plasma" (PRP) was first used in the 1990s as a pool of autogenous growth factors for bone regeneration [35]. There are now numerous studies showing the effect of PRP on bone regeneration. Most of these, however, focus on BMSC or bone marrow in general. Many studies showing the effect of PRP on bone regeneration were carried out in the fields of maxillofacial surgery and implantology. The state of the data regarding the supportive efficacy of PRP can be considered

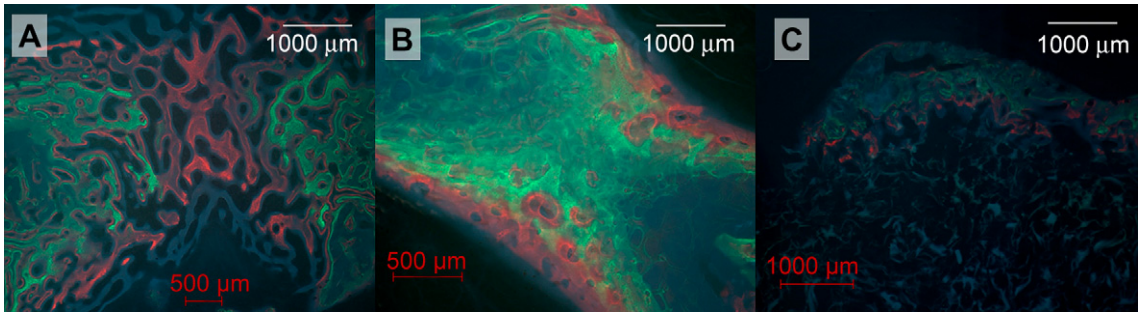

Fig. 7. Fluorescence labelling revealed an earlier bridging of the osseous defect in the PRP-group (image B) during the calcein green labelling in week 8 compared to the bone marrow group (BMSC-group) in which fusion occurred during the xylene orange labelling after week 10. No significant bone formation could be observed in the EMPTY-group; (image C).

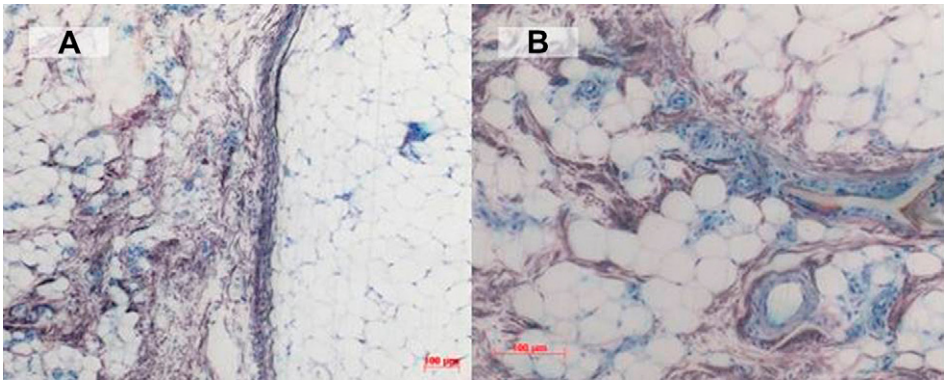

Fig. 8. In those animals treated with ASC, a spontaneous differentiation toward the adipogen lineage was observed as demonstrated by multiple cells with characteristic intracellular vacuoles.

controversial. While many studies were able to demonstrate a positive effect [1–6], others could not [7–10]. The latter observations may possibly be the result of insufficient thrombocyte concentrations (the thrombocyte concentrations correlate with the osteogenic inductive capacity of PRP [36]) and are sometimes not even mentioned in the original studies, as well as suboptimal production and activation protocols, suboptimal observation timeframes or the presence of potent osteoinductive factors which overshadow the PRP effect [9,37]. Even if these studies from the field of maxillofacial surgery present important fundamentals for the use of PRP for bone regeneration, studies using load-bearing long bone models are a more germane basis for the present study. Dallari compared BMSC, PRP, and freeze dried bone allografts (FDBA) individually or in all possible combinations in a critical-size defect model in rabbit femur [38]. He used bilateral cylindrical burr holes into the distal femoral condyle as a cancellous bone defect. The best healing rate — calculated using the area of the remaining defect — was obtained by using all three components together, which led to significantly better results at two weeks than BMSC or PRP alone. Compared to femoral condyles of healthy bone, the groups treated with FDBA, BMSC, or PRP alone showed significant differences in bone volume, trabecular thickness and separation, whereas the group treated with the combination of all three closely approximated healthy bone. Wiltfang et al. used a critical-size defect applied to the forehead of miniature pigs. He applied various xenogenic carrier materials and autogenous bone, as well as additional treatment with PRP produced by two different methods and assessed the defects at two, four, and 12 weeks using radiology and immunohistochemistry. The Wiltfang group found positive effects of PRP only early on in the healing phases (at two weeks) and only in combination with autogenous bone [39]. Only Sarkar and Rai used a truly load-bearing critical-size defect model [10,40]. Whereas Sarkar found no differences at 12 weeks between treatment with a combination of a collagen carrier with PRP and control group (collagen carrier alone) in a sheep tibia critical-size defect model [10], Rai et al. found a beneficial effect of PRP in his most recent study using an 8 mm defect in the femur in a rat model [40]. Rai noted that the addition of PRP to the bone replacement material polylactone-TCP led to improved vascularization at three weeks and a significantly greater rate of completely healed femoral bones at 12 weeks, as well as increased stiffness upon torsion. In a recent study, PRP yielded better bone formation than an empty calcium-deficient hydroxyapatite scaffold as determined by both histology and micro-computer tomography ($p < 0.05$) in a critical-size diaphyseal radius defect in a rabbit model [6]. In contrast to other studies which postulated an increase in bone mass caused by PRP in the early weeks [40], in our study we only observed a significant difference between the groups with and without PRP treatment starting in weeks 18 and 20. This phenomenon might be explained by our method of assessing bone density in which, unlike with semi-quantitative scores, bone is only recognized as such at high Hounsfield values, and therefore more precise and more detailed compared to scoring systems in which only presence or absence of more mature bone is distinguished. This could explain why radiographically observed effects are seen later.

The PRP which caused the observed effects in the present study derived from pooled xenogenic PRP. Because PRP does not exhibit any significant immunogenicity, this method was also chosen to assure that the PRP we used was uniform and homogenous and that variations caused by inhomogeneous PRP could be avoided. Furthermore, producing PRP from sheep is difficult. Nonetheless, numerous authors prefer autogenous PRP and described it to be

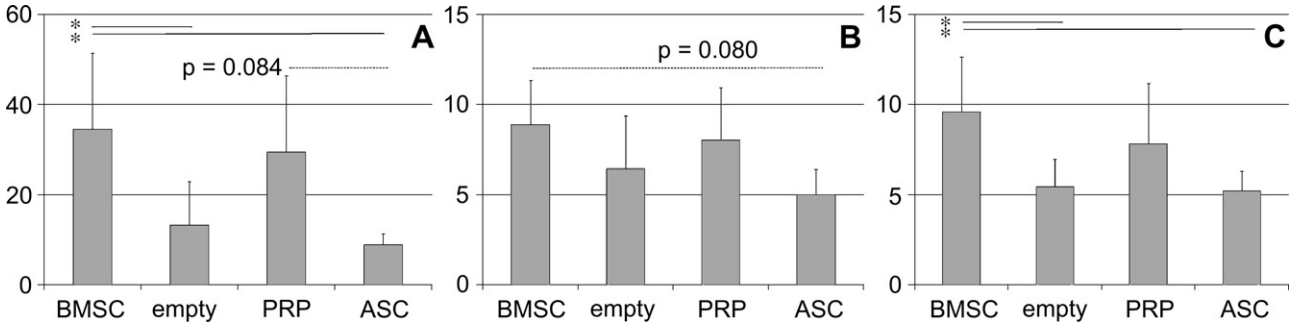

Fig. 9. Quantitative evaluation of newly formed bone on histological level (A: quantitative determination of defect filled with bone in %; B: semi-quantitative evaluation according to Werntz; C: semi-quantitative evaluation according to Sarkar); Significances are indicated by "*" ($p < 0.05$).

more potent [37]. Our results in the present study should be considered against this background. Furthermore, the efficacy of PRP could be further increased by using autogenous PRP. Nevertheless, the present study appears to provide fundamental proof of the efficacy of PRP on adipose stem cells used for osteogenesis.

5. Conclusions

ASC yielded inferior bone formation on a hydroxyapatite coated collagen scaffold than BMSC after implantation in the weight bearing sheep tibia. Most likely undifferentiated ASC are predetermined to differentiate toward adipose tissue in the orthotopic site as compared to BMSC that bridged the critical size defect in each case. The quantification of newly formed bone in the defect area over time using conventional radiography showed substantial benefits of the influence of PRP as compared to animals treated with adipose stem cells without adjuvant osteoinductive factors. Despite showing only a trend toward significance, from our perspective the data nevertheless can be interpreted that addition of PRP can partially compensate for the clearly inferior osteogenic potential of ASC, and also that the combination of PRP and adipose stem cells represents a promising therapeutic approach.

Appendix

Figures with essential colour discrimination. Many of the figures in this article have parts that are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.biomaterials.2010.01.085.

References

[1] Kassolis JD, Reynolds MA. Evaluation of the adjunctive benefits of platelet-rich plasma in subantral sinus augmentation. J Craniofac Surg 2005;16(2):280–7.
[2] Kassolis JD, Rosen PS, Reynolds MA. Alveolar ridge and sinus augmentation utilizing platelet-rich plasma in combination with freeze-dried bone allograft: case series. J Periodontol 2000;71(10):1654–61.
[3] Kim SG, Kim WK, Park JC, Kim HJ. A comparative study of osseointegration of Avana implants in a demineralized freeze-dried bone alone or with platelet-rich plasma. J Oral Maxillofac Surg 2002;60(9):1018–25.
[4] Kitoh H, Kitakoji T, Tsuchiya H, Katoh M, Ishiguro N. Transplantation of culture expanded bone marrow cells and platelet rich plasma in distraction osteogenesis of the long bones. Bone 2007;40(2):522–8.
[5] Kasten P, Vogel J, Beyen I, Weiss S, Niemeyer P, Leo A, et al. Effect of platelet-rich plasma on the in vitro proliferation and osteogenic differentiation of human mesenchymal stem cells on distinct calcium phosphate scaffolds: the specific surface area makes a difference. J Biomater Appl 2008;23(2):169–88.
[6] Kasten P, Vogel J, Geiger F, Niemeyer P, Luginbuhl R, Szalay K. The effect of platelet-rich plasma on healing in critical-size long-bone defects. Biomaterials 2008;29(29):3983–92.
[7] Froum SJ, Wallace SS, Tarnow DP, Cho SC. Effect of platelet-rich plasma on bone growth and osseointegration in human maxillary sinus grafts: three bilateral case reports. Int J Periodontics Restorative Dent 2002;22(1):45–53.
[8] Ranly DM, Lohmann CH, Andreacchio D, Boyan BD, Schwartz Z. Platelet-rich plasma inhibits demineralized bone matrix-induced bone formation in nude mice. J Bone Joint Surg Am 2007;89(1):139–47.
[9] Jensen TB, Rahbek O, Overgaard S, Soballe K. No effect of platelet-rich plasma with frozen or processed bone allograft around noncemented implants. Int Orthop 2005;29(2):67–72.
[10] Sarkar MR, Augat P, Shefelbine SJ, Schorlemmer S, Huber-Lang M, Claes L, et al. Bone formation in a long bone defect model using a platelet-rich plasma-loaded collagen scaffold. Biomaterials 2006;27(9):1817–23.
[11] Reyes M, Lund T, Lenvik T, Aguiar D, Koodie L, Verfaillie CM. Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. 2001;98(9):2615–25.
[12] Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, et al. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 2002;13(12):4279–95.
[13] Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, et al. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 2001;7(2):211–28.
[14] Winter A, Breit S, Parsch D, Benz K, Steck E, Hauner H, et al. Cartilage-like gene expression in differentiated human stem cell spheroids: a comparison of bone marrow-derived and adipose tissue-derived stromal cells. Arthritis Rheum 2003;48(2):418–29.
[15] Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells 1999;284(5411):143–7.
[16] Niemeyer P, Krause U, Fellenberg J, Kasten P, Seckinger A, Ho AD, et al. Evaluation of mineralized collagen and alpha-tricalcium phosphate as scaffolds for tissue engineering of bone using human mesenchymal stem cells. Cells Tissues Organs 2004;177(2):68–78.
[17] Niemeyer P, Schonberger TS, Hahn J, Kasten P, Fellenberg J, Suedkamp N, et al. Xenogenic transplantation of human mesenchymal stem cells in a critical size defect of the sheep tibia for bone regeneration. Tissue Eng Part A; 2009 [Epub ahead of print].
[18] Schönberger T, Hahn J, Kasten P, Südkamp NP, Fechner K, Pearce S, et al. A novel software-based evaluation method for objective quantification of bone regeneration in experimental bone defects. European Cells and Materials Journal 2007;14(S1).
[19] Mosheiff R, Friedman A, Friedman M, Liebergall M. Quantification of guided regeneration of weight-bearing bones. Orthopedics 2003;26(8):789–94.
[20] Werntz JR, Lane JM, Burstein AH, Justin R, Klein R, Tomin E. Qualitative and quantitative analysis of orthotopic bone regeneration by marrow. J Orthop Res 1996;14(1):85–93.
[21] Yoon E, Dhar S, Chun DE, Gharibjanian NA, Evans GR. In vivo osteogenic potential of human adipose-derived stem cells/poly lactide-co-glycolic acid constructs for bone regeneration in a rat critical-sized calvarial defect model. Tissue Eng 2007;13(3):619–27.
[22] Peterson B, Zhang J, Iglesias R, Kabo M, Hedrick M, Benhaim P, et al. Healing of critically sized femoral defects, using genetically modified mesenchymal stem cells from human adipose tissue. Tissue Eng 2005;11(1–2):120–9.
[23] Follmar KE, Prichard HL, DeCroos FC, Wang HT, Levin LS, Klitzman B, et al. Combined bone allograft and adipose-derived stem cell autograft in a rabbit model. Ann Plast Surg 2007;58(5):561–5.
[24] Cui L, Liu B, Liu G, Zhang W, Cen L, Sun J, et al. Repair of cranial bone defects with adipose derived stem cells and coral scaffold in a canine model. Biomaterials 2007;28(36):5477–86.
[25] Hui JH, Li L, Teo YH, Ouyang HW, Lee EH. Comparative study of the ability of mesenchymal stem cells derived from bone marrow, periosteum, and adipose tissue in treatment of partial growth arrest in rabbit. Tissue Eng 2005;11(5–6):904–12.
[26] Hayashi O, Katsube Y, Hirose M, Ohgushi H, Ito H. Comparison of osteogenic ability of rat mesenchymal stem cells from bone marrow, periosteum, and adipose tissue. Calcif Tissue Int 2008;82(3):238–47.
[27] Xu Y, Malladi P, Wagner DR, Longaker MT. Adipose-derived mesenchymal cells as a potential cell source for skeletal regeneration. Curr Opin Mol Ther 2005;7(4):300–5.
[28] Wagner W, Wein F, Seckinger A, Frankhauser M, Wirkner U, Krause U, et al. Comparative characteristics of mesenchymal stem cells from human bone marrow, adipose tissue, and umbilical cord blood. Exp Hematol 2005;33(11):1402–16.
[29] Hattori H, Sato M, Masuoka K, Ishihara M, Kikuchi T, Matsui T, et al. Osteogenic potential of human adipose tissue-derived stromal cells as an alternative stem cell source. Cells Tissues Organs 2004;178(1):2–12.
[30] Marcacci M, Kon E, Zaffagnini S, Giardino R, Rocca M, Corsi A, et al. Reconstruction of extensive long-bone defects in sheep using porous hydroxyapatite sponges. Calcif Tissue Int 1999;64(1):83–90.
[31] Metak G, Gomoll A, Wolter W, Barth G, Ascherl R Interspecies comparison of healing standardized bone defects with and without autogenous bone transplantation. Langenbecks Arch Chir Suppl Kongressbd 1998;115(Suppl. I):25–30.
[32] Lanyon LE, Hampson WG, Goodship AE, Shah JS. Bone deformation recorded in vivo from strain gauges attached to the human tibial shaft. Acta Orthop Scand 1975;46(2):256–68.
[33] Niemeyer P, Kasten P, Simank HG, Fellenberg J, Seckinger A, Kreuz PC, et al. Transplantation of mesenchymal stromal cells on mineralized collagen leads to ectopic matrix synthesis in vivo independently from prior in vitro differentiation. Cytotherapy 2006;8(4):354–66.
[34] Kasten P, Vogel J, Luginbuhl R, Niemeyer P, Tonak M, Lorenz H, et al. Ectopic bone formation associated with mesenchymal stem cells in a resorbable calcium deficient hydroxyapatite carrier. Biomaterials 2005;26(29):5879–89.
[35] Marx RE, Carlson ER, Eichstaedt RM, Schimmele SR, Strauss JE, Georgeff KR. Platelet-rich plasma: growth factor enhancement for bone grafts. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1998;85(6):638–46.
[36] Arpornmaeklong P, Kochel M, Depprich R, Kubler NR, Wurzler KK. Influence of platelet-rich plasma (PRP) on osteogenic differentiation of rat bone marrow stromal cells. An in vitro study. Int J Oral Maxillofac Surg 2004;33(1):60–70.
[37] Marx RE. Platelet-rich plasma: evidence to support its use. J Oral Maxillofac Surg 2004;62(4):489–96.
[38] Dallari D, Fini M, Stagni C, Torricelli P, Nicoli Aldini N, Giavaresi G, et al. In vivo study on the healing of bone defects treated with bone marrow stromal cells, platelet-rich plasma, and freeze-dried bone allografts, alone and in combination. J Orthop Res 2006;24(5):877–88.
[39] Wiltfang J, Schlegel KA, Schultze-Mosgau S, Nkenke E, Zimmermann R, Kessler P. Sinus floor augmentation with beta-tricalciumphosphate (beta-TCP): does platelet-rich plasma promote its osseous integration and degradation? Clin Oral Implants Res 2003;14(2):213–8.
[40] Rai B, Ho KH, Lei Y, Si-Hoe KM, Jeremy Teo CM, Yacob KB, et al. Polycaprolactone–20% tricalcium phosphate scaffolds in combination with platelet-rich plasma for the treatment of critical-sized defects of the mandible: a pilot study. J Oral Maxillofac Surg 2007;65(11):2195–205.