Bone formation in peri-implant defects grafted with microparticles: a pilot animal experimental study


Abstract

Aim: This study aimed to evaluate the healing of peri-implant defects grafted with microparticles (MPs).

Material and Methods: Six domestic pigs received nine standardized defects at the calvaria, and an implant was inserted in the middle of each defect. The space between the implant and lateral bone portion was filled with MP pellets (n = 18) or MP supernatant (n = 18) or left unfilled (n = 18). After 14 and 28 days, three animals were sacrificed and specimens removed for further processing. Samples were microradiographically and histologically analysed. In addition, we immunohistochemically stained for anti-vWF as a marker of angiogenesis.

Results: In the case of bone regeneration and vessel formation, the null hypothesis can be partially rejected. After 14 and 28 days, no significant difference was observed within groups regarding de novo bone formation, bone density and osseointegration. However, superior vessel formation was found at both time points.

Conclusion: Microparticles represent a promising treatment option to accelerate peri-implant vessel formation. Further studies are needed to investigate the regenerative properties of MPs more precisely.

Conflict of interest and source of funding statement

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Dental implants are routinely used for the reconstruction of edentulous or partially edentulous jaws with predictable success and long-term stability. Nevertheless, local bone deficiencies due to dentoalveolar atrophy, tumour or trauma can compromise the clinical use and success of dental implants (Esposito et al. 2006). For osseous reconstruction purposes, bony defects can be filled with bone substitute materials (BSM) (Calori et al. 2011, Hernandez-Alfaro et al. 2012, Slotte et al. 2013, Yip et al. 2014). In general, BSM are mainly osteoconductive and mostly lacking osteoinductive properties.

The use of autologous bone as an osteoinductive graft material is associated with surgery-related risks and donor site morbidities and is limited in size (Dimitriou et al. 2011). Therefore, current research focuses on new applications and techniques to support local tissue repair, especially de novo bone formation in implant-related treatments.

De novo bone formation is a complex mechanism that occurs in simultaneous processes. Platelets rep-
resent a physiological barrier, limiting blood loss and accelerating the generation of thrombin to intensify the coagulation process. Due to the release of local mediators, platelets interact with the progress of inflammation, wound healing and tissue repair (Barnes et al. 1999, Devescovi et al. 2008). The activation of platelets by physiological agonists, such as thrombin, apoptosis and mechanical stress, also results in the release of small (1 mM) membrane vesicles from the platelet surface by exocytosis, so-called microparticles (MPs). Endothelial cells, leucocytes, megakaryocytes and erythrocytes are also able to shed MPs from their plasma membranes into the circulation (Horstman & Ahn 1999). However, platelet MPs represent the most abundant MPs in the bloodstream, constituting approximately 70–90% of circulating MPs (Horstman & Ahn 1999, Berckmans et al. 2001, Joop et al. 2001). Studies show that MPs interact in various ways with their environment. Experimental data suggest that platelets play an integral role in modulating innate and adaptive immunity (Semple & Freedman 2010), haemostasis, thrombosis (Castaman et al. 1996, 1997, Barry et al. 1998) and angiogenesis (Kim et al. 2004, Brill et al. 2005). Furthermore, MPs have been shown to stimulate mitogenic activity and the proliferation of bone-forming cells (Gruber et al. 2002). Angiogenesis and the activation of bone-forming cells are mandatory processes in hard tissue regeneration. Thus, we hypothesized that MP application represents a promising method for enhancing the regeneration of peri-implant defects.

The aim of the present preclinical animal study was to evaluate the impact of MPs on bone formation, bone density, implant osseointegration and vessel formation in standardized circumferential peri-implant defects after 14 and 28 days.

Material and Methods

Animals

Six female pigs were included in this animal study. The research project was approved by a state Animal Research Committee in Bavaria (approval no. 54-2532.1-45/12, Ans-

Preparation of microparticles

The MPs were prepared by the Bio-Products & Bio-Engineering AG (Vienna, Austria). Human platelets were disrupted using an ultrasonic method, spontaneously forming MPs. After separation by centrifugation at 2000 g, the pellet with corpuscular proportions and the remaining supernatant with MPs could be distinguished. A fibrin matrix (0.4%) was used as a carrier substance, which clotted due to thrombin addition during defect application procedures.

Surgical procedures

All surgical procedures were performed using intravenous anaesthesia with Midazolam and ketamine HCl (Ketavets, Ratiopharm, Ulm, Germany). After local anaesthesia, a sagittal incision was made at the forehead and the soft tissue and peri-osteum mobilized. Using a trephine drill (1 × 1 cm, Roland Schmid, Fürth, Germany), nine bony defects (10 mm diameter, 8 mm depth) were created in the calvariae (os frontale). To guarantee standardized defect preparation, defect depth was controlled with a ruler. A chisel was used for additional defect preparation. The defect sizes represent critical size defects (Schlegel et al. 2006a, Werner et al. 2012, 2013). The defects were positioned at least 10 mm apart to avoid biological interactions. Implant beds were prepared at the caudal aspect in the middle of each defect (3.4 mm diameter, 7 mm depth). Implants (NobelReplace Select Straight TiUnite® NP 3.5 × 15 mm, Nobel Biocare Services AG, Zurich, Switzerland) were inserted with primary stability (7 mm depth) at the caudal defect portion, and the cranial part of the implant was free in the middle of the created defects. In total, 54 calvarian defects (six animals × 9 defects) were created and allocated to three groups: two experimental groups and one control group (N = 18 each group). Experimental group B contained supernatant MPs and group C contained the corpuscular platelet proportion (i.e. pellet) with MPs. The defects of the experimental groups were completely filled with the prepared solutions. The defects of the control group (group A) were left unfilled (Fig. 1). All defects were randomly assigned based on a computer generated list (Rand-List®, DatInf GmbH, Tübingen, Germany). After treating the peri-

Fig. 1. Stylized presentation of defect location (grey: empty control defect, red: MP-Pellet, yellow: MP supernatant) and circumferential peri-implant defects configuration.
implant bony defects, the periosteum and skin over the defects were sutured (Vicryl 3.0®, Vicryl® 1.0; Ethicon Co., Norderstedt, Germany) in two layers. During the first three postoperative days, animals received Streptomycin® (0.5 g/day i.v.; Gruhenthal GmbH, Stolberg, Germany).

Animal euthanasia
To investigate the influence of MPs on early bone formation, three randomly selected animals were sacrificed on day 14 and the remaining animals on day 28. The animals were initially sedated with a mixture of Azaperone® and Midazolam® (1 mg/kg, i.m.). Subsequently, 20% Pentobarbital® solution (Dermocal AG, Buenos Aires, Argentina) was delivered into the ear vein until cardiac arrest occurred.

Specimen preparation
The ossa frontalia was harvested and immediately stored at −80°C. Afterwards, the specimens were dissected from the ossa frontalia and subsequently fixed by immersion in 1.4% paraformaldehyde (4°C). Specimens were dehydrated in increasing concentrations of alcohol at room temperature in a dehydration unit (Shandon Citadel 1000, Shandon GmbH, Frankfurt, Germany). Xylol was used as an intermediary fixation. Specimens were embedded in Technovit 9100 (Heraeus Kulzer, Kulzer Division, Werheim, Germany), which is suitable for the cutting and grinding technique described by Donath and Breuner (Donath & Breuner 1982). After fixation, the specimens were cut in the middle of the implant through the longitudinal axis using a precision saw (Exakt Gerätebau, Norderstedt, Germany). One half of the specimen was processed for microradiographic/light-microscopic evaluation and the other half for immunohistochemistry.

Microradiography
Using a grinding machine (Exakt Gerätebau, Norderstedt, Germany), one half of each embedded specimen was reduced to 150–180 μm for microradiographic examination. Specimens were X-rayed in a Faxitron cabinet (Faxitron, Fa; Wheeling Illinois, IL, USA) for 6 min. at 13 kV and 2.5 mA. The X-rays (Kodak, Stuttgart, Germany) were digitized with an Epson scanner (Epson Perfection 4900 Photo, Seiko Epson Corporation, Nagano, Japan) at 2400 dpi and 8-bit grey-scale and stored in TIFF format (Fig. 2).

Microradiographic analysis
Peri-implant bone formation and bone density rates were evaluated using microradiographs (Schlegel et al. 2004, 2006b, Thorwarth et al. 2007, von Wilmowsky et al. 2010, Ramazanoglu et al. 2013). We used
Bioquant Osteo software 2013 v13.2.6 for microradiographic analyses. Microradiographs showed distinct defect preparation margins, whereby the proportion of newly formed bone within the defect was precisely distinguished and evaluated. The proportion of newly formed bone was stated as a per cent of total defect volume. Bone density was determined based on the newly formed bone mass and the mineralized bone fraction within the newly formed bone examined and stated as a per cent.

Light microscopy

Light microscopy allows qualitative analysis of the osseous integration of implants in local and newly formed bone (Fenner et al. 2009, Lutz et al. 2010, Ramazanoglu et al. 2011, von Wilmowsky et al. 2011, Schlegel et al. 2013). The slices were reduced to 30-μm thickness, high gloss polished, and transferred to 10% H₂O₂ solution for 5 min. To obtain uniform specimen sizes for standardized evaluation, thickness was controlled (Mitutoyo, Neuss, Germany) to ± 3 μm. After rinsing under cold running water, the specimens were stained with toluidin blue O for 10 min. (Sigma-Aldrich Chemie GmbH, Munich, Germany). Excess stain was removed by rinsing the specimens under running water. The stained specimens were examined under a light microscope (Axio Imager.A1; Zeiss, Jena, Germany) by digitizing the specimens with an attached video camera (QICAM FAST 1394, Qimaging, Burnaby, Canada). The pictures were stored in TIFF format (Fig. 2).

Toluidin blue O analysis

To determine the percentage of direct contact between mineralized bone and the implant surface [bone-to-implant contact (BIC)], four regions of interest (ROIs) were evaluated for each implant using Bioquant Osteo software 2013 v13.2.6 (Fenner et al. 2009, Lutz et al. 2010, Ramazanoglu et al. 2011, von Wilmowsky et al. 2011, Schlegel et al. 2013). Two ROIs were located in the crestal and two in the apical implant region (Fig. 3).

Immunohistochemistry

In half of each specimen, the implants were removed using tapered flat pliers after crestal peri-implant preparation. During all procedures, care was taken that the peri-implant bone was not damaged. Specimens were re-embedded and finally cut into 3-μm sections using a microtome (Leica microsystems, Heidelberg, Germany). Specimen preparation included rehydration with ethanol, de-acrylation in 2-methoxyethylacetate, decalcification in ethylenediaminetetraacetate (EDTA) and unmasking of the antigens in citrate at 99°C. To determine vessel formation, tissue sections were immunostained with primary antibodies directed against vWF (DAKO, Hamburg, Germany; concentration 1:3000). The staining procedures were performed using the labelled streptavidin-biotin method and an Autostainer (Cytomation Autostainers plus, Dako TM, Glostrup, Denmark) (Schlegel et al. 2006c, 2013, Park et al. 2007). A secondary antibody (DAKO Diagnostics GmbH, Germany) was added to complex the primary antibody. Finally, the addition of StreptAB/HRP (DAKO Diagnostics GmbH, Germany) enabled the binding of the actual dye, AEC+ (DAKO Diagnostics GmbH, Germany). The procedure was completed using nuclear haematoxylin counterstaining. Each specimen was accompanied by a negative control.

Immunohistochemical analysis

As ROIs, standardized image sections were selected within the newly formed bone in the bony defects. Stained vessels were counted in the ROIs and presented as the number of vessels per square millimetre.

Statistical analysis

Mean values and standard deviations were calculated for each variable and group using the animal as statistical unit (N = 3). Due to the limited number of animals, only descriptive
statistical analyses of the evaluated parameter were applied.

Results

Microradiographic findings

Fourteen days after surgery, 34.08 ± 5.66% of the defect was filled with de novo bone in the control group (group A), 29.91 ± 9.75% in group B and 28.88 ± 13.68% in group C. (Fig. 4, Table 1). Twenty-eight days after surgery, 61.53 ± 14.1% of the critical size defects of group A had regenerated with newly formed bone. The bone regeneration in groups B was 65.27 ± 12.6% and in group C 64.51 ± 15.11% (Fig. 4, Table 1).

Fourteen days after surgery, 52.17 ± 10.89% of the newly formed bone was mineralized in group A. This proportion of mineralized tissue was higher than in group B (52.77 ± 7.12%) and lower than in group C (42.84 ± 18.64%) (Fig. 4, Table 1). Twenty-eight days after surgery, 59.48 ± 11.16% bone density was detected in group A, 50.93 ± 13.69% in group B and 50.83 ± 7.41% in group C (Fig. 4, Table 1).

Light microscopy findings

Fourteen days after surgery, group A showed 8.62 ± 7.24% BIC in the defect area compared to 6.34 ± 4.92% and 7.15 ± 6.37% in groups B and C respectively (Fig. 5, Table 1). In the local bone portion, a BIC rate of 65.27 ± 26.04% was detected in group A, 65.47 ± 19.99% in group B and 58.50 ± 28.28% in group C (Fig. 5, Table 1).

After 28 days, the BIC in group A was 9.36 ± 11.09% in the defect area. The BIC in group B was 9.78 ± 10.67% and in group C 4.21 ± 5.28% (Fig. 6, Table 1). In the local bone portion, the BIC rate was 60.17 ± 34.77% in group A, 73.23 ± 28.64% in group B and 65.05 ± 27.37% in group C (Fig. 5, Table 1).

General observations of defect healing

The microradiographic and histological evaluation showed bone regeneration in defects with and without MP modification. As expected, wound regeneration began from the margins of the defect. However, the use of MPs did not seem to have any osseoinductive influence in the region of the implants. An invasion of inflammatory cells (i.e. neutrophil granulocytes, lymphocytes or macrophages) was not observed.

Immunohistochemical findings

Fourteen days after the surgical procedure, 22 ± 4 vessels/mm² were counted in the newly formed bone in empty control defects, 20 ± 7 vessels/mm² in the defects in group B, and 59 ± 43 vessels/mm² in the defects in group C (Fig. 6, Table 1).

Twenty-eight days after surgery, 18 ± 3 vessels/mm² were counted in the newly formed bone in the control group. In groups B and C, 16 ± 4 vessels/mm² and 26 ± 8 vessels/mm², respectively, were counted in the area of newly formed bone (Fig. 6, Table 1).

Discussion

The stimulation of prerequisite factors for de novo bone formation represents a promising treatment option to induce osseointegration and peri-implant bone formation. Mandatory factors in bone regeneration, which
are affected by MPs, are the ability to stimulate vessel formation, mitogenic activity and the proliferation of bone-forming cells (Gruber et al. 2002, Martinez & Andriantsitohaina 2011). Therefore, MPs can potentially contribute to the regeneration of mineralized tissue.

The applied animal model is well investigated and cited to investigate the regeneration of critical size bone defects and the regeneration of peri-implant defects (Schlegel et al. 2004, 2006a, Thorwarth et al. 2006, 2007, Park et al. 2007, Lutz et al. 2008, Tudor et al. 2008, Stockmann et al. 2012, Wehrhan et al. 2012). One advantage of the chosen animal model is that the forehead region of the pig provides desmal bone, which has a regeneration rate of 1.2–1.5 μm/day, which is comparable to that of humans (1–1.5 μm/day) (Eitel et al. 1981, Schlegel et al. 2006a). Because intra-oral factors, such as mastication, plaque accumulation and disproportionate implant loading, can negatively influence peri-implant bone formation, an extra-oral model was used. Therefore, the chosen animal model provides ideal conditions for investigating bone regeneration and implant osseointegration (Schlegel et al. 2006a) and is sufficient for investigating our hypotheses and obtain results comparable to those of other investigators (Rupprecht et al. 2003, Thorwarth et al. 2005, Tudor et al. 2008). However, the different type of bone compared to the intra-oral context makes additional studies in intra-oral animal models necessary prior to a clinical study.

Based on the current literature, we hypothesized that the application of MPs initiates increased BIC of the exposed implant surfaces. However, after 14 and 28 days no significant osseointegration of the implants into the defects was measured based on BIC. No superior BIC were found in or between any of the groups in this study. The literature shows that the osseointegration of exposed implant surfaces achieved by grafting materials, such as xenografts (Machado et al. 1999, 2000, Nociti et al. 2000, Schou et al. 2003a), resorbable HA (Hurzeler et al. 1997) and autogenous bone (Schou et al. 2003b,c) is significantly higher than the BIC we observed for defects grafted with MPs. However, the comparability is limited due to the selected time points: 14 and 28 days. The difference between the standardized grafting materials reported in the literature and the application of MPs for defect modification in this study is a gain of graft quality and, therefore, the ability to enhance bone regeneration. Xenografts or alloplastic BSM have osteoconductive properties, whereas autologous bone has osteoinductive and osteogenic properties. The applied MPs have no osteoconductive properties, but they do have an osteoinductive effect (Gruber et al. 2002) as long as bone-forming or precursor cells are in direct contact with the MP solution. In our study, MPs were applied in conjunction with a fibrin matrix, which is neither osteoconductive nor osteoinductive. The fact that osseointegration largely depends on the presence of osteoconductive BSM has been described many times in the literature (Hurzeler et al. 1997, Hall et al.)

Fig. 5. Mean BIC in the defect and local bone for the empty control, microparticle (MP) supernatant and MP pellet group (N = 18 defects per group) 14 days and 28 days after surgery. Error bars indicate standard deviation.

Fig. 6. Mean number of vessels/mm² in the newly formed bone for the empty control, microparticle (MP) supernatant, MP pellet group and the local bone (N = 18 defects per group) 14 days and 28 days after surgery. Error bars indicate standard deviation.
Due to the capability of MPs, enhanced bone formation was hypostatized (Gruber et al. 2002, Kim et al. 2004) for defects filled with MPs. Fourteen days after surgery, the mean bone formation values for the peri-implant defects filled with the MP pellet or MP supernatant were inferior to the bone formation in the unfilled control defects. These outcomes were unexpected due to the angiogenic (Hayon et al. 2012a,b, Ohtsuka et al. 2013) and osteoinductive (Gruber et al. 2002) potential of MPs reported in the literature. One possible reason is the early phase of investigation. Fourteen days is probably too early to observe the induced bone regeneration effect of MPs. Furthermore, the fibrin carrier represents a potential barrier that restricts MP effects at an early stage of wound healing. In the empty control defects, mesenchymal pluripotent stem cells or bone-forming cells could have more easily migrated from bony walls into the defect area. The described cell migration is mandatory for bone regeneration and could have been restricted by the fibrin carrier (Kallas 2001, Dimitriou et al. 2005). Therefore, the regenerative potential of a blood-filled circumferential peri-implant defect is higher than the regeneration potential of a MP-fibrin solution-filled defect after 14 days of regeneration. Two weeks after defect preparation, 34.08 ± 5.66% of the empty control defects were filled with newly formed bone. This value correlates with those from the current literature whereby the model can be accepted as valid (Schlegel et al. 2006a). Twenty-eight days after defect preparation, the unfilled control defects were inferior to the MP test groups. Higher rates of newly formed bone were detected in the MP supernatant group (group B) compared to the pellet group (group C). Thus, after 28 days the angiogenic and osteoinductive potential of MPs lead to increased bone formation rates compared to the other groups. The potential for vessel formation by MPs was detected by immunohistochemical staining for vWF expression (Hofmann et al. 2008, Bir et al. 2009, Wang et al. 2010). At both evaluation time points, higher vWF expression and higher vessel formation rates were shown in the MP pellet group (group C). We exclude inflammatory induced vessel formation due to xenogen MP application because inflammatory cell invasion was not observed 14 and 28 days after defect preparation. The vessel forming effect of MPs can be explained by their influence on the number of haematopoietic stem/progenitor cells (Janowska-Wieczorek et al. 2001) and their capability to maximize the adhesion of circulating angiogenic cells (CAC) to endothelial cells and the migration of CACs to stromal cell-derived factor-1α (SDF-1α), thereby accelerating re-endothelialization of the denuded endothelial cells (Mause et al. 2010). Furthermore, MPs contain a variety of growth and trophic factors, which are essential to angiogenesis and tissue regeneration (Hayon et al. 2012b). Therefore, MP application seems to represent a new strategy for increasing the effects of therapeutic vessel formation for limb ischaemia in atherosclerotic patients (Ohtsuka et al. 2013) or to support the rehabilitation of ischaemic brain tissues (Hayon et al. 2012a,b). In this study, the influence of MPs on peri-implant hard tissue regeneration was investigated in a randomized, controlled in vivo study for the first time. Although the investigation period of 28 days was too short to describe the regeneration potential of MPs in total, superior vessel formation was obvious in the MP groups compared to the control group. Increased vessel formation is associated with higher metabolic activity, and accelerated soft and hard tissue regeneration can be assumed. Because the functional properties cannot be described immunohistochemically, and due to the limited specimen number (54 defects), the evidence given by the results is limited. To describe the full potential of MPs in bone regeneration, further studies with longer periods of investigation are necessary.

In addition to bone quantity, the density of newly formed bone was also evaluated. After 14 days, the evaluation showed almost identical results for the unfilled control and MP groups. Twenty-eight days after surgical intervention, greater bone density was present in the control defects than in the experimental groups. Therefore, the assumption that MP application is associated with increased bone density after 14 and 28 days of healing must be rejected.

Conclusion

The application of MPs leads to superior vessel formation, which is mandatory for soft and hard tissue regeneration. Due to the short study period, the entire influence of MPs on bone regeneration cannot be scrutinized. This pilot study indicates that MPs may represent a promising treatment option.

References


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Clinical Relevance

**Scientific rationale for the study:** The clinical application of microparticles has already shown promising results for the vascularization of soft tissues. However, the impact of microparticles on peri-implant hard tissue regeneration remains unknown.

**Principal findings:** At the early stage of wound healing, microparticles did not have any impact on osseointegration, de novo bone formation, or bone density in this animal defect model. However, vessel formation was superior.

**Practical implications:** The positive effects of microparticles on vessel formation were already apparent at an early stage of wound healing. However, the long-term effect on de novo bone formation should be investigated.

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