Onset and Maintenance of Angiogenesis in Biomaterials: 
*In Vivo* Assessment by Dynamic Contrast-Enhanced MRI

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**Objective:** To describe dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) as a practical tool for longitudinal assessment of angiogenesis in biomaterials.

**Background:** There is a lack of suitable methods for *in vivo* evaluation of the integration of biomaterials in a clinical setting. In oncology, DCE-MRI is used for the longitudinal monitoring of altered tumor angiogenesis during therapy. Thus, we investigated whether DCE-MRI enables to assess the integration of biomaterials over time.

**Methods:** The tested material was bovine bone matrix applied in a bilateral sinus lift procedure in combination with concentrated mononuclear cells, including mesenchymal stem cells and autologous thrombin. To assess the development of new blood vessels inside the biomaterial, DCE-MRI was carried out before and 11, 25, 53, and 104 days after surgery. Perfusion parameters were calculated according to the model of Tofts.

**Results:** Analysis of the data revealed increasing parameters for perfusion and blood supply within the transplant over time. It was possible to determine the values for each transplantation site and each point of time separately.

**Conclusion:** DCE-MRI is appropriate to repetitively survey angiogenesis and integration of biomaterials in patients. It seems appropriate as a valuable indicator of treatment response or failure, with consecutive adaption of the therapy regime.

**Introduction**

**Sinus floor elevation** and insertion of dental implants are standard procedures to rehabilitate the atrophic maxilla.1,2 Density and quality of the bone correlate significantly with the success of dental implant treatment.3,4 In the ongoing search for the appropriate augmentation material, one approach uses the osteoconductive properties of bone substitutes. They act as space fillers and provide a guiding structure for osteoprogenitor cells and blood vessels originating from the local bone. The integration of biomaterials can be evaluated histologically in animal models after sacrifice. Thus, there is no appropriate tool for repetitive *in vivo* evaluation in clinical trials with patients.

The conventional imaging method for examination of bone or teeth is based on X-rays, because the tissue is dense and injuries and disease often change the density. Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) has been used successfully to assess parameters of tissue perfusion particularly in oncology.5 Its advantage is based on the high sensitivity of contrast agents and on the absence of ionizing radiation (in contrast to X-rays), which facilitates longitudinal monitoring of angiogenesis in patients. DCE-MRI may help to assess the early formation and preservation of new blood vessels in patients with transplanted biomaterials.

The aim of this study was to evaluate the feasibility to monitor the transformation of the transplanted biomaterial especially with focus on the formation of the vascular system using DCE-MRI.

**Materials and Methods**

Approval by the local ethics committee was obtained.

**Patient**

Both maxillary sinus were augmented in a 68-year-old, female, edentulous patient with a bilateral maxillary
Evaluated application of biomaterial

Harvesting of mononuclear cells. The pelvic bone was punctured at the superior posterior iliac spine (Bone Marrow Aspiration Pack; Harvest Technologies Corporation, Plymouth, MA). About 120 mL of bone marrow was collected, pooled, and anticoagulated with 5 mL of heparin solution (Heparin-Natrium, 10,000 U/mL, diluted with NaCl to 1000 U/mL; both from B. Braun, Melsungen, Germany). Bone marrow cells were isolated using the bone marrow aspirate concentrate (BMAC) system (Bone Marrow Procedure Pack; Harvest Technologies Corporation) according to manufacturer’s instructions.

Hemocytometry and flow cytometrical (FACS) analysis. The bone marrow aspirate (BMA) and the BMAC were analyzed hemocytometrically (Sysmex Europe GMBH, Norderstedt, Germany). Flow cytometry was carried out with the same samples. Cells were stained simultaneously with PerCP-conjugated monoclonal antibody to CD 34 or CD 34 and CD 45, APC-conjugated monoclonal antibody to CD 45, FITC-conjugated monoclonal antibody to CD 44, and PE-conjugated monoclonal antibody to CD 73 (Becton Dickenson Biosciences, San Jose, CA) and then analyzed (FACScalibur; BD Biosciences, San Jose, CA).

Proof of pluripotency. The cells from the bone marrow concentrate were amplified and differentiated into chondrogenic, adipogenic, and osteogenic cell lineages according to the methods of Pittenger et al.6 Adipocytes were stained with oil red O. Chondrogenic potential was confirmed by immunostaining with mouse anti-human aggregcan antibodies. Osteogenic cells were characterized histologically by their expression of alkaline phosphatase, collagen type I, and calcification marked with the van Kossa staining.

Surgical procedure. Under general anesthesia the osteotomy of the lateral sinus wall was outlined with an irrigated round burr, and the Schneiderian membrane was lifted.1 The site was augmented with bovine bone mineral (BBM; BioOss®, Geistlich Biomaterials, Wolhusen, Switzerland) and enriched with mononuclear cells in thrombin. The lateral sinus wall defect was covered with a collagen membrane (Bio-Gide®, Geistlich Pharma AG).

After a 3.8-month healing time six implants (Straumann Standard Plus, Basel, Switzerland), between 4.1 and 4.8 mm in diameter and 14 mm in length, were inserted under local anesthesia. Before implant insertion cylindrical biopsies from the augmented region were taken with a trephine burr (Gebr. Brasseler GmbH & Co. KG, Lemgo, Germany), fixed in formalin, and prepared according to Schenk’s method.7 Three months later the implants were exposed under local anesthesia, and healing screws were inserted. Prosthetic impression was taken 3 weeks after implant uncovering (Impregum, Espe, Seefeld, Germany). Prosthetic loading followed 4 weeks after implant impression.

Histology. The histomorphometric examination was done with a light microscope (Axiovert 135; Zeiss, Koehren, Germany). The BBM particles were marked, and the new formed bone around the particles was measured with the computer software AnalySIS® Soft Imaging system (Olympus Europa GmbH, Hamburg, Germany).

In vivo evaluation of new angiogenesis

MRI measurements were performed 2h before the intervention to obtain baseline values (1.5 T, standard head coil, Sonata; Siemens Medical Solutions, Erlangen, Germany). The MR-protocol included morphologic images and DCE examinations. To monitor the transformation of the transplanted material and the development of angiogenesis, examinations were repeated on days 11, 25, 53, and 104 (d11–d104) after the intervention.

MR examinations. The protocol included a morphologic T1-weighted (T1w-tse) sequence (TE = 12 ms; TR = 465 ms) in transverse, coronal, and sagittal orientation. Transversal images, including fat-saturation, were also acquired after the application of contrast media (CM).

DCE measurements were performed in one transversal slice of 10 mm thickness through both sinuses. A time series of inversion recovery balanced SSFP (TrueFISP) images were acquired (t = 40 ms; TE = TR/2 = 1.24 ms)8,9 to obtain the dynamic change of the T1 relaxation rate for a period of 5:30 min with temporal resolution of 3 s. The measurement started 36 s before the administration of CM to gain baseline values. Consecutively, Gd-DTPA (Magnevist; Bayer-Schering Pharma, Berlin, Germany) at a dosage of 0.1 mmol/kg body weight was injected into an antecubital vein at a flow rate of 3 mL/s (power injector; Medrad, Inc., Warrendale, PA).

DCE-MRI analysis. A custom-built software package, developed under Matlab (http://www.mathworks.com), was used for data analysis. CM concentration–time courses were computed of T1 relaxation rates10,11 for a region of interest (ROI). The initial area under concentration curve for the first 60 s after the bolus reached the tissue (IAUC60) is a model-independent parameter. It measures the uptake of CM in the ROI and depends on blood supply, permeability, and surface area of the vessel wall and leakage space. Applying the two-compartment model of Tofts and Kermode,12 we evaluated the volume transfer constant (ktrans)12 and the volume of extravascular extracellular space (ve) as quantitative pharmacokinetic parameters. The physiological interpretation of ktrans depends on the behavior of the tissue microvessels, especially on the scale of blood flow compared to capillary permeability.13

ROIs were chosen in the left/right sinus and for comparison in muscle. For the ROI analysis the mean MR signal over the ROI is the input for the analysis process.

To obtain more spatial information about the heterogeneous development of the transplanted material, an analysis for each volumetric pixel of the image slice was also performed if signal-to-noise ratio was sufficient.

Results

The patient recovered well from the surgical procedure. The augmented regions showed a vertical bone height greater than 13 mm in the augmented maxillary sinus region. The volume of the transplants and of the sinus can be viewed...
in the upper section of Table 1. Implants showed primary stability.

**Hemocytometry and flow cytometrical (FACS) analysis**

The BMA contained $9.7 \times 10^3$/µL white blood cells, $3.05 \times 10^6$/µL red blood cells, and $120 \times 10^3$/µL platelets. The BMAC was enriched with $48.1 \times 10^3$/µL white blood cells, $1.0 \times 10^6$/µL red blood cells, and $687 \times 10^3$/µL platelets. The FACS analysis showed a distinct population of CD 34 and CD 45 negative cells that were positive for CD 44 and CD 73 in the BMAC as in the cultured cells.

**Proof of pluripotency**

The cultured MSCs could be differentiated successfully into adipocytes as shown by oil red O staining, chondrocytes as shown by aggrecan immuno staining, and osteoblasts as shown by calcification, alkaline phosphatase, and collagen type I activity.

**Histological analysis**

There were no signs of inflammation. New formed lamellae appeared as vital bone tissue containing osteocytes inside the bone lacunae. The biomaterial could be easily identified by its size, shape, and color. The new formed bone lamellae connected the biomaterial particles, stabilizing the grafted complex, and integrated well in the surrounding host bone. Blood vessels could be detected in the biopsy. There was more new bone formation and less fibrous tissue in the right sinus than in the left one (Table 1).

| Table 1. Distribution of the Volumetric and Histomorphometric Parameters |
|---------------------------------------------------------------|---|---|
| **Volume** | **Left** | **Right** |
| Sinus volume (mL) | 13.7 | 11.4 |
| Graft volume (mL) | 5.5 | 2.8 |

<table>
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<tr>
<th><strong>Histology</strong></th>
<th><strong>Left</strong></th>
<th><strong>Right</strong></th>
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<tbody>
<tr>
<td>New bone (%)</td>
<td>11.3</td>
<td>13</td>
</tr>
<tr>
<td>Biomaterial (%)</td>
<td>25.5</td>
<td>32.9</td>
</tr>
<tr>
<td>Marrow space (%)</td>
<td>63.2</td>
<td>54.1</td>
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DCE-MRI

The transplanted material has a hyperintense (bright) signal intensity in the T1w-tse images, whereas bone provides less signal (hypointens). With proceeding time after the surgery (d25–d104) the hypointense region grows to the center and the hyperintense part reduces accordingly, representing bone tissue forming from the border to the center of the transplant (Figs. 1 and 2).

Figure 2 shows images of one representative transversal slice, for each day native (n) and past (p) CM administration. For d11–d104, only a detail including the sinus and the nose is displayed. Right side and left side of the sinus turn out to vary in size and progress. The nose is highly supplied with blood; therefore, it shows a strong signal enhancement caused by the CM. The border of the sinus is covered with mucosa, which also shows slightly enhanced signal past CM (d0). After the surgery, the mucosa is swollen and takes up more CM in this acute state. The signal enhancement in a broad band at the border of the sinus at d11 highlights the swollen mucosa. At d25 it is very difficult to distinguish between the freshly build bone at the rim of the transplant and the swollen mucosa, but it is quite obvious that the mucosa returns to the initial volume with increasing time after the surgery (d25–d104).

No CM reaches the central hyperintense region of the transplant, showing no signal enhancement past CM. At days 53 (d53) and 104 (d104) the new evolved bone tissue takes up CM, which means a vascular system had been built in that region.

In contrast to DCE-MRI, the comparison of native and past CM MR images is a qualitative method. Figure 3 shows the curves of the mean CM concentration in an ROI containing both sides of the sinus. Because it is likely that the ROIs include some mucosa at the edges, little CM uptake could be measured even before the surgery, when the sinus was still empty. It is quite obvious that the CM uptake increases drastically after the surgery. Simultaneously, the shape of the concentration curve changes.

Figure 4 shows the results of the quantitative analysis of the concentration curves. For comparison, also an ROI in muscle tissue that was not effected by the surgery was evaluated. Compared to muscle tissue iAUC60, ktrans and ve of the sinus ROI start with a lower value at d0, but cross the muscle level later on reaching a considerable higher value 104 days after the transplantation. The parameters of the left and the right sinus show in principle a similar behavior, but in detail, differences. This suits to the observation made at the T1w-tse images (Fig. 2).

Figure 5 shows iAUC60-parametric maps (in color) of the ROIs in the sinus before and after the intervention. Native TrueFISP images were used as background in gray scale to demonstrate the location of the chosen ROIs. The maps show clearly the formation of the vascular system from the rim to the center of the transplant. The results of the ROI analysis (Figs. 3 and 4) were derived with the same ROIs used for the pixel-wise analysis (Fig. 5).

Discussion

Histomorphometrical analysis and radiologic imaging are standard methods for the in vivo evaluation of biomaterial integration over time. Animal research requires sacrifice to obtain histological specimens at a set timeframe preventing intra-individual comparison. Human biopsies can only be harvested in limited indications, that is, point of implantation. The standard imaging method in oral preprosthetic surgery is volume tomography. The privilege of MRI is the variety of possible contrasts and the potential to demonstrate dynamic function without using ionizing radiation. Therefore, it is qualified for repeating follow-ups to monitor the progress of building new bone and its vascularization.

There are a few publications about using conventional morphologic MRI associated with sinus lift augmentation. Three case reports15,16 with cancellous bone graft, one with a biomaterial (Surgicel®) as graft material17 and a small study of six patients, in which allogeneic cancellous bone chips were
are described. Both groups used preoperative images, if acquired, for planning of the operation. Postoperative images were used to measure the increased height of the maxilla.

Badhe et al. used MRI to evaluate the long-term outcome of porcine dermal collagen grafts in the treatment of rotator cuff tears. Ramponi et al. used contrast-enhanced MRI to show that fibroblast growth factor improves angiogenesis in collagen sponges, placed subcutaneously in mice. They compared relative values of enhanced uptake of two different contrast agents.

DCE-MRI is utilized in oncologic studies to evaluate the success of anti-angiogenic therapy over time. The method provides quantitative evaluation that can be compared over time or subject. Ehrhart et al. employed a semiquantitative version of DCE-MRI to observe increasing vascularization after femoral osteotomy in a canine femur model. Bone marrow surrounding the osteotomy site showed an increased CM uptake 3 weeks postoperative, in contrast to nearby muscle.

The aim of this present study was to investigate the feasibility to monitor the transformation of the transplanted.
material to normal bone tissue especially with focus on the formation of the vascular system.

The morphologic T1w-tse images and the DCE-MRI demonstrate the formation of the bone tissue and the vascular system from the rim of the transplant to the center. The high signal of the material enclosed by this rim in the T1w-tse images (Figs. 1 and 2) can be evidence for a relative high concentration of cells or molecules (e.g., proteins), causing a short T1 relaxation time.

Because of the surgery, the mucosa was swollen and had a high uptake of CM in this acute state. The transplant was surrounded by the mucosa except for the interface to the residual maxillary bone. It is very important to keep this in mind—especially when reading the images 11 and 25 days after the surgery. A quite possible interpretation of the DCE-MRI data, including the native and post-CM T1w-tse images, would be that at day 11 the transplant has not yet build a perfused vascular system and only the swollen mucosa has taken up CM. At day 25 the swell of the mucosa is reduced and in the rim of the transplant a perfused vascular system has developed. Because the CM uptake in the ROI is in that case partly of the mucosa and partly of the new build and perfused bone tissue, the concentration curve (Fig. 3) has not increased between day 11 and day 25 but started to change its form. After day 25 the mucosa has gone down reaching normal state at least at day 104. At the same period new bone tissue is formed toward the center of the transplant, provided with a new build vascular system. Therefore, the influence of the mucosa to the concentration curve has been drastically reduced, but the mean value itself has highly increased. The kind of plateau between day 11 and day 25 prior further increase of iAUC60, ktrans, and ve support this interpretation (Fig. 4).

Leakage space (ve) and IAUC60 (uptake of CM) rise (Fig. 4) with increasing vascularization in the ROI. No final plateau has been reached yet at day 104. Because the metallic implants that are placed in the center of the interesting region would cause artifacts in the images, no later MR examinations were performed. To interpret the volume transfer constant ktrans in the case under consideration is more complicated. The parameter ktrans depends on the blood flow, the permeability, and the surface of the capillary walls. New vessels not yet reached maturity are leakier than in their final mature state. The surface increases with growing vascularization, but whether the blood flow changes is not known.

In the present study the evaluation of the conventional volume tomography revealed a larger left sinus that was...
treated with a larger transplant that extended further into the posterior maxilla than on the right side. The center of the left sinus showed less radiopacity, which indicates less density of this region. Histomorphometrical evaluation showed that the biomaterial was less densely packed and that there was slightly less new bone formation in the left sinus. The new bone formation rate in both sinuses is according to numbers given in literature. The connection between the biomaterial particles and the newly formed bone matrix gives stability within the augmentation, which is important for successful integration. Yildirim et al. report the successful augmentation of the maxillary sinus in 11 patients by using BBM and venous blood. In histomorphometric analysis they found a new bone formation rate of 14.7% after an average time of 6–8 months postaugmentation. In the present case it was 11.3% and 13% after only 3.5 months when using concentrated mononuclear cells from BMA instead of venous blood.

The topographic aspect of contrast agent uptake indicates that vessel ingrowth into the biomaterial originates from the periphery—no matter if there was contact to residual bone caudally or to the Schneiderian membrane cranially. These two structures ensured that the graft was surrounded by vital tissue. Because DCE-MRI could measure both sides separately, it was possible to detect that the parameters for angiogenesis increased slower for the ROI on the left side. These results combined with the histological findings indicate that a larger augmented volume needs more time to integrate than a smaller one. It is known that new bone formation follows angiogenesis. The new bone is built around the vessels concentrically. This leads to the formation of the Haversian system.

The augmentation method itself is currently under evaluation. Jaquière et al. found evidence for the osteoinductive property of BioOss® and MSCs when implanting constructs subcutaneously in nude mice.

For further studies the MR protocol should be extended by sequences with additional contrasts to reach a better distinction between regions of swollen mucosa and new build bone tissue at the border of the transplant.

Conclusion

It is possible to observe the transformation of the transplanted material with DCE-MRI. The findings correlate to histological parameters. DCE-MRI offers quantitative parameters to monitor the increasing vascularity of the transplant. DCE-MRI is a suitable, noninvasive, clinical tool for the repetitive evaluation of angiogenesis in biomaterials. It could be used for research purposes or to improve the predictability of therapies in regenerative medicine.

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Disclosure Statement

No competing financial interests exist.

References


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