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Monitoring angiogenesis in soft-tissue engineered constructs for calvarium bone regeneration: an in-vivo longitudinal DCE-MRI study

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**Running title:** DCE-MRI in tissue-engineered calvarium bone.
ABSTRACT

Tissue engineering is a promising technique for bone repair and can overcome the major drawbacks of conventional autogenous bone grafting. In this \textit{in-vivo} longitudinal study, we proposed a new tissue-engineering paradigm: inserting a biological soft-tissue construct within the bone defect to enhance angiogenesis for improved bone regeneration. The construct acts as a resorbable scaffold to support desired angiogenesis and cellular activity and as a vector of vascular endothelial growth factor, known to promote both vessel and bone growth. Dynamic contrast-enhanced magnetic resonance imaging was performed to investigate and characterize angiogenesis necessary for bone formation following the proposed paradigm of inserting a VEGF-impregnated tissue-engineered construct within the critical-sized calvarial defect in the membranous parietal bone of the rabbit. Results show that a model-free quantitative approach, the normalized initial area under the curve metric, provides sensitive and reproducible measures of vascularity that is consistent with known temporal evolution of angiogenesis during bone regeneration.

\textbf{Keywords:} Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI), tissue engineering, angiogenesis, bone regeneration.

\textbf{Abbreviations used:} DCE-MRI, dynamic contrast-enhanced magnetic resonance imaging, AIF, arterial input function, IAUC, initial area under the curve, VEGF, vascular endothelial growth factor, ACM, acellular matrix, HA, hyaluronic acid, IV, intravenous, FA, flip angle, BW, bandwidth, NEX, number of experiments, 3D, three dimensional, SPGR, spoiled gradient recalled echo, ROI, region of interest, micro-CT, micro-computed tomography.
GRAPHICAL ABSTRACT

Monitoring angiogenesis in soft-tissue engineered constructs for calvarium bone regeneration: an in-vivo longitudinal DCE-MRI study


DCE-MRI was performed to investigate and characterize angiogenesis necessary for bone formation following the proposed paradigm of inserting a VEGF-impregnated tissue-engineered construct within the critical-sized calvarial defect in the rabbit. Results show that a model-free quantitative approach, the normalized initial area under the curve metric, provides sensitive and reproducible measures of vascularity that is consistent with known temporal evolution of
INTRODUCTION

Autogenous bone grafting is considered the gold standard for repair and regeneration of extensive bony defects following trauma, cancer resection, or non-united fractures (1). However, grafting bone is non-ideal: a second surgical site, usually involving the iliac crest, tibia, calvarium, or scapula, is required; the bony skeleton does not offer a limitless supply of bone to harvest; the procedure is associated with increased operating room time usage and increased patient morbidity (2). Tissue engineering is one of the most promising techniques to overcome these major drawbacks of autogenous bone grafting (3). Successful tissue regeneration mainly depends on the establishment of a vascular bed, which connects the implanted construct to the host tissue and supplies necessary nutrients for bone regeneration (4). The different mechanisms underlying bone repair and regeneration have been extensively studied and identified (5,6), but very few studies have focused on monitoring the angiogenesis process during bone wound healing.

As a noninvasive imaging technique, MRI is well suited to in-vivo longitudinal evaluation of tissue repair following grafting procedures. In tissue-engineered constructs, MRI has been used to determine cellularization and maturation of the construct in vivo in cartilage (7,8), but bony constructs have been studied only in vitro (9,10). In either case, the vascular status and evolution of angiogenesis with tissue growth were not investigated. Angiogenesis in tissue-engineered constructs was first evaluated in bladder constructs in vivo (11-13) using dynamic contrast-enhanced MRI (DCE-MRI). The foray of DCE-MRI into tissue-engineering applications remains limited despite its well-established role in assessing the microvasculature in clinical and research studies (14). A few exceptions are found in in-vivo longitudinal studies conducted using DCE-MRI to evaluate the wound-healing process following subcutaneous
insertion of wound chambers (15) and massive bone allograft (16). In this latter study, revascularization was assessed at the osteotomy site but not at the graft site. To our knowledge, longitudinal in-vivo characterization of angiogenesis in bony constructs has never been reported.

In order to quantify angiogenesis using DCE-MRI derived data, one can use numerous analysis techniques. The quantitative approach using pharmacokinetic modeling of tracer concentration (17) provides parameters related to physiology, such as blood volume and vascular permeability. This approach depends on the knowledge of the tracer concentration evolution in the blood, or arterial input function (AIF). However, determination of the AIF may be compromised by tracer concentration-MR signal non-linearity, low temporal resolution, and partial volume effects, which are especially problematic in small animal brain imaging. Moreover, it has been shown that errors in AIF measurement can strongly influence pharmacokinetic parameter accuracy (18,19). To overcome this limitation, a model-free quantitative approach has been proposed (20), which uses the initial area under the tracer concentration-time curve (IAUC) as an empirical quantitative parameter. Change in the IAUC has been shown to correlate with vessel density (13) and tumor response to anti-vascular treatment (21,22). Also, this non-model-based analysis is very robust, and reproducibility is often comparable to or even better than that obtained with traditional model-based parameters (23-25).

In this in-vivo longitudinal study, we propose a new tissue-engineering paradigm: inserting a biological soft-tissue construct within the bone defect to enhance angiogenesis for improved bone regeneration. The construct acts as a resorbable scaffold to support desired angiogenesis and cellular activity and as a vector of vascular growth factor (VEGF), known to promote both vessel and bone growth (26). DCE-MRI was performed to investigate and characterize angiogenesis necessary for bone formation following the proposed paradigm of
inserting a VEGF-impregnated tissue-engineered construct within the critical-sized calvarial defect in the membranous parietal bone of the rabbit (27). Results show that an IAUC approach provides sensitive and reproducible measures of vascularity that is consistent with known temporal evolution of angiogenesis during bone regeneration.

**EXPERIMENTAL**

This study was approved by the institutional Animal Care Committee (protocol #7578).

*Construct Preparation and Implantation*

Tissue-engineered construct preparation began with the acellularization of urinary bladder harvested from 20-50 kg porcines. Bladders were washed with sterile phosphate buffer saline, longitudinally sectioned and stirred in hypotonic solution for 48 hours at 4°C to effectively break down cellular structures and inhibit proteases. Bladders were placed in hypertonic solution for 48 hours at 4°C to denature protein residues and degrade all DNA and RNA components. Bladders were washed with Hank’s Balanced Salt Solution (Invitrogen, #14175, USA) containing 2 U/mL Benzonase (Novagen, #: 70746, Germany) overnight at 37°C and transferred to 0.25% CHAPS-containing detergent based solution. Acellular matrices (ACMs) were repeatedly washed with sterile dH$_2$O and stored in 70% ethanol prior to use. H&E staining was used to confirm acellularity. ACMs were cut into 1.5 cm diameter discs, weighed, and immersed in gradually increasing concentrations of ethanol for full dehydration. ACMs were lyophilized (ViTis-temp, 120 millitorr and vacuum) for 24 hours, then rehydrated with increasing concentrations of HA (0.05, 0.1, 0.2 and 0.5 mg/100mL) (Sigma, product #H5388, USA). Alcian blue staining was used to confirm HA incorporation. In addition, HA-ACMs were dehydrated in alcohol, lyophilized, and rehydrated with VEGF$_{121}$ (Sigma, #V3388, USA) 10 ng/g of ACM.
Finally, a critical-sized (non-spontaneous healing) calvarial defect (15 mm in diameter) was created surgically in the parietal bones of the rabbits, the centre of the defect was 9.5 mm from the midline to allow 2 mm for the sagittal sinus. The tissue-engineered HA-VEGF containing construct was grafted to the calvarium.

**Experimental Protocol**

Surgery and MR imaging were performed on twelve New Zealand white rabbits (3.5-4 kg). Imaging sessions were held 1, 2, 3, 6 and 12 weeks after the surgery. All MR experiments were performed under anesthesia using two different protocols. Five rabbits were anesthetized with an intravenous (IV) injection of pentobarbital (25 mg/kg) through the ear vein. During the MRI (about 1 hour), anesthesia was maintained with an IV dose of 0.25 mg/kg/min; and the animals were kept under 100% oxygen with a face mask. For the other seven rabbits, anesthesia was performed with 5% of isoflurane for induction, and 2±0.2% for maintenance, in 100% oxygen using a face mask. All the animals were equipped with a catheter in the ear vein for the contrast agent injection (Gd-DTPA – Magnevist, Berlex Canada, Lachine, Canada) and continuous hydration (4 mL of saline/kg/h). Heart rate and pO₂ were monitored during the scan time. Five animals were euthanized after the fourth imaging session (6 weeks after surgery) while the remaining animals were euthanized after the last imaging session (12 weeks after surgery). In each animal, the calvarium was excised for subsequent histological procedures.

**MRI**

MRI was performed on a 1.5 T GE scanner (Signa EXCITE TwinSpeed; GE Healthcare, Milwaukee, WI, USA) using a three-inch diameter receive surface coil and a body transmit coil. Animals were placed in prone position and an axial orientation was chosen for all the slices. Construct localization was achieved using a gradient echo sequence: TR/TE = 6.5/2.3 ms,
FA = 30°, bandwidth (BW) = 23.4 kHz, 16 x 12 x 8 slices (thickness = 2 mm, space between slices = 1 mm), matrix = 192 x 192, FOV = 12 x 12 cm², number of experiments (NEX) = 2. A rapid 3D T₁-mapping method, based on variable flip angles and integrating B₁ correction (28), was employed prior to Gd-DTPA injection to acquire the baseline T₁ map: 3D fast spoiled gradient recalled echo (SPGR), TR/TE = 8.8/3.4 ms, FA = 2°, 10° and 20°, bandwidth (BW) = 31.2 kHz, matrix = 256 x 160 x 10, FOV = 10 x 10 x 3 cm³, number of experiments (NEX) = 4. Dynamic T₁-weighted images were acquired using a 3D fast SPGR sequence: TR/TE = 10.2/3.4 ms, FA = 60°, BW = 31.2 kHz, matrix = 256 x 128 x 10, FOV = 10 x 10 x 3 cm³, NEX = 0.75. After the acquisition of 5 baseline images, Gd-DTPA was administered as a rapid bolus (0.1 mmol of Gd/kg) and imaging continued for 6 min post-injection with a time resolution of 8.2 s (300 images in total).

**MRI data analysis**

All analyses were performed on a pixel-wise basis using in-house programs developed with Matlab (v.7.0, Mathworks Inc., Natick, MA, USA). Analysis of the 3D T₁ maps followed the method described previously (28). DCE-MRI data were converted into Gd concentration maps using the pre-injection T₁ map, the SPGR signal equation (29) and the Gd-DTPA relaxivity r₁ (4.1 s⁻¹.mM⁻¹, in plasma at 1.5 T and 37°C (30)). Finally, the initial area under the Gd concentration time curve for 60s after contrast agent injection (IAUC₆₀) was computed for each pixel. Only physiologically meaningful pixels were retained. Pixels were excluded if they met any of the following conditions: T₁ values outside of the range 0-4000 ms, Gd concentration mean value over time outside of the range 0-2 mM and for which the standard deviation of Gd concentration along the baseline was greater than 0.1 mM. Three regions of interest (ROI) were manually drawn. On the IAUC₆₀ map, two ROIs were defined on the construct: the periphery,
which corresponds to the early enhancing region, and the centre (Figure 1c); and one ROI surrounding the whole brain. For each ROI and for each MR acquisition time point, the mean IAUC$_{60}$ value was computed as well as the percentage of physiologically meaningful pixels in the ROI involved in this calculation. To minimize effects from inter-individual physiological variation and contrast agent dose variability, each IAUC$_{60}$ mean value was normalized to a reference, the IAUC$_{60}$ mean value measured in the brain.

Statistical analysis was based on the permutation test (also called randomization test, (31)), which, unlike the widely used $t$-test, is suited to data originating from small-sized groups and with unknown distributions. This test determines the significance of the difference of means $d$ between two populations $A$ and $B$ with $n_A$ and $n_B$ samples, respectively, as follows. A difference of means $d'$ is calculated between all possible groupings $A'$ and $B'$, where $A'$ and $B'$ represent a different partition of values from $A+B$ into two groups of size $n_A$ and $n_B$. The $P$-value $P$ is equal to the ratio of the number of events where $d' > d$ to the total number of events. Paired permutation tests were applied to compare IAUC$_{60}$ at different time points, and statistical significance was set at $P < 0.05$. Evolution of the IAUC$_{60}$ mean value with time was also determined for each individual animal and in each ROI by comparing the mean value of the estimate at week $(i)$ with the mean±SEM value at week $(i-1)$.

**Micro-Computed Tomography**

Calvarial specimens were scanned by an Explore Locus SP® micro-CT scanner (GE Medical Systems, London, Ontario, Canada) using the fast mode with 0.05 mm sections. Reconstruction of scanned images was done using Microview software (GE Medical Systems, London, Ontario, Canada) after calibrating using the bone water and air standard values. The reconstructed 3D image was then traced in 3 dimensions to the circumference of the original
defect margins. This allowed the creation of a 3D reconstruction of the defect, referred to as the ROI. A threshold level was selected manually based on 25% of the bone standard provided by the manufacturer.

RESULTS

MRI

Figure 1 illustrates that the soft-tissue construct was clearly identifiable on MR images even prior to contrast agent injection (Fig. 1a). The $T_1$-weighted MR signal increased in the whole construct after contrast agent injection with a more pronounced enhancement in the periphery than in the centre of the graft (Fig. 1b). Note that contrast uptake was observed from the first imaging time point (1 week after surgery) up to the last (12 weeks after surgery) in all rabbits. The IAUC$_{60}$ map provided the best support to draw the different ROIs with very clean contours of the construct (Fig. 1c).

Table 1 shows the evolution of the percentage of physiologically meaningful pixels in these ROIs that are retained for DCE-MRI analysis. The percentage of pixels retained is generally highest ($\geq 91\%$) in the construct periphery compared to the centre or normal brain tissue. An exception occurs at 12 weeks, where fewer useful pixels are detected in the entire construct. In the centre, the percentage of retained pixels increases with angiogenic growth. In the normal brain where contrast agent uptake is lowest, at least 75% of pixels were retained consistently throughout the 12 weeks, which supports the robustness of the proposed method for pixel selection. Table 2 reports the evolution of the size of the ROIs normalized to week 1. For both periphery and centre of the construct, the size of the ROI 6 weeks after the surgery is almost half of the initial size. The size of the ROI defined on the brain remains constant with time.
Figure 2 illustrates in one rabbit typical uptake curves in the periphery and the centre of the construct at four time points (1, 3, 6 and 12 weeks after surgery). The uptake curves in the periphery were characteristic of highly permeable vessels usually found in presence of active angiogenesis. At the early time point, the periphery enhancement curve (Fig.2, Week 1) exhibits the most rapid initial uptake followed by a distinct washout. Later time points may also show significant enhancement, but the initial uptake is more gradual and washout is not always evident (e.g. Fig.2, Week 6). In the centre of the construct, the uptake dynamics were distinct from those in the periphery, characterized by a much lower and gradual uptake. Both ROIs exhibited less Gd uptake at the last time point of 12 weeks after graft implantation.

Figure 3 shows the evolution of the mean IAUC$_{60}$ values normalized to the reference (IAUC$_{60}$ in the brain) in both the periphery and centre of the construct throughout the 12 weeks. The periphery exhibited the highest value at the early time point, followed by a plateau and a two-fold decrease at 12 weeks post-surgery. The centre of the construct exhibited different characteristics. Normalized IAUC$_{60}$ values were significantly lower at all time points, and a progressive increase was observed at early times up to 3 weeks post-surgery. The one common trait observed in both the centre and periphery was a decrease in uptake at 12 weeks. Values for all animals are shown in Table 3.

Figure 4 shows how angiogenesis evolves in individual animals at time points where significant changes were observed, namely, between weeks 1 and 3 and weeks 6 and 12. It also distinguishes groups administered different anesthetics (solid versus dashed lines). Several key results are noted in this figure. First, the proposed DCE-MRI approach yields reproducible trends in angiogenic development in most animals: a significant early decrease in the periphery versus an increase in the centre (in each case, the trend was observed on 8 animals out of 12), followed
by a decrease in the entire construct at 12 weeks. Second, this approach is insensitive to differential vascular response to anesthesia that is independent of angiogenesis. An exception to this is seen at 12 weeks (Table 3), where normalized IAUC_{60} values are significantly higher in the isoflurane group. Although the sample size was small (n=2), the possibility of anesthesia influence on vascular development at 12 weeks could not be disregarded, and these two animals were excluded from the twelve weeks mean values in Fig. 3 and Table 3. It is important to note that aside from the 12-week discrepancy, these two animals followed the general trend at earlier time points and were part of the 8 out of 12 animals showing the same behavior between week 1 and week 3. Further investigations are required to confirm this hypothesis regarding anesthesia-induced differences.

**Micro-Computed Tomography**

Figure 5 shows representative micro-CT images of excised calvarium. Micro-CT evaluation supported the MRI findings. There was new bone noted at 6 weeks, mostly at the periphery of the bony defects containing the HA-VEGF constructs, while at 12 weeks islands of newly formed bone had appeared in the center of the defects.

**DISCUSSION**

Tissue engineering is a promising technique for bone repair and can overcome the major drawbacks of conventional autogenous bone grafting. Instead of using a surgically harvested bone graft, a biological soft-tissue construct is placed within the bony defect. This construct acts as a resorbable scaffold to support two intimately linked processes during tissue regeneration: cellular colonization and angiogenesis. Monitoring angiogenesis longitudinally, for instance, using DCE-MRI, provides a means to assess bone development non-invasively. In this study, a
model-free analysis of DCE-MRI data was used to investigate and characterize angiogenesis in a VEGF-impregnated tissue-engineered construct within the rabbit calvarial defect. Vessel network initiation and establishment within the construct, leading to successful bone regeneration, were demonstrated \textit{in vivo} using normalized IAUC$_{60}$ measurements. The proposed approach provides reproducible and sensitive results and is robust to challenging experimental conditions.

Natural wound healing and bone regeneration after autogeneous bone grafting have been extensively described in the literature. Bone regeneration results from a combination of different processes, or phases, which occur usually in a dynamic equilibrium (6). Although our proposed paradigm uses a soft-tissue construct instead of a bone graft, the same phases are required to ensure successful bone regeneration. In fact, the collagen-based scaffold may be more easily resorbed than traditional bone grafts to allow new bone formation. Also, the soft-tissue construct provides the ideal three-dimensional framework to guide desired angiogenesis (4), which is a major determinant to bone formation. The different processes involved in bone formation are as follows. The early inflammatory phase is characterized by the different components of wound healing: inflammation, hemorrhage, hematoma, and blood clot formation. These combined phenomena stimulate macrophages to release growth factors (such as VEGF), which induces angiogenesis (32). Vascularization is the second stage in graft healing and is strongly associated with the previous phase. It starts shortly after graft implantation, and the mature vessel network is established within 2 to 3 weeks following surgery. The third phase significantly overlaps with the vascularization phase and may last up to 6 weeks. During this time span, the graft is colonized by cells, nutrients, and other growth factors necessary for new bone formation via the newly established vasculature. The final phase, the remodeling phase, is initiated between the
fourth and the sixth week post-surgery and continues for several months. It involves remodeling and mineralization of the immature randomly-oriented bone. The two latter phases are also accompanied by the resorption of the graft material (6).

In our study, periphery and centre regions of the soft-tissue construct exhibited different behaviors with regards to angiogenic activity, but in each case, the different phases observed were consistent with expected angiogenic progression, as described in the literature. In the periphery, the high IAUC value observed one week after the surgery is consistent with the first two phases of inflammation and angiogenesis, since both lead to high contrast agent uptake due to high vessel leakiness. Then, between the second and the sixth week after insertion, when both angiogenesis and the third phase are expected to occur, the IAUC is lower and remains relatively constant. The lower contrast agent uptake is consistent with the completion of the inflammatory phase. The plateau in IAUC needs to be interpreted carefully, since different uptake curve dynamics (at weeks 3 and 6) suggest distinct vascular behavior that possibly indicates a transition from phase 2 to 3. The IAUC contribution comes mainly from inflammatory/angiogenic activity initially and later from a mature vasculature that is functioning fully to supply the graft with elements to the new bony tissue. Finally, the drop at twelve weeks is expected since the remodeling phase no longer requires a large vascular supply, perhaps resulting in subsequent vessel pruning (33). In the centre, the inflammation phase is not distinguishable because of the distance from the actual surgery site. As for the three other phases, they are clearly separated: the slow IAUC increase reflects the growing vessels during angiogenesis, the plateau corresponds to the new bone formation phase, and the decrease is the same as in the periphery. Nevertheless, it should be pointed out that the uptake curve pattern observed in the centre may reflect primarily diffusion of the contrast agent from the construct
periphery to the core and is not a purely vascular-driven response. In fact, diffusion is a fundamental means of transport in tissue regeneration since some elements, like cells, are not vehicle from the host tissue onto the graft via the blood, but using diffusion along the scaffold. Finally, the size of ROIs defined on the construct decreased significantly from week 1, indicating resorption of the graft while new bony tissue is created as observed on the micro-CT results.

In this work, the IAUC approach was preferred to more common model-based approaches to analyze DCE-MRI data. Indeed, our experimental conditions (small animal brain imaging with clinical magnetic field, low temporal resolution) rendered the determination of the AIF very challenging and would have compromised a model-based analysis (19). Although empirical approaches do not have clear physiological associations, our results show that our normalized IAUC$_{60}$ metric is very sensitive to the different stages that occur during graft healing and bone regeneration, and can monitor the regeneration process and its success. Moreover, when quantitative model-based approaches are used, numerous factors (AIF errors, limited temporal resolution, choice of analysis model) may impair robustness and accuracy (18). When using an IAUC metric, several approaches have been taken. Walker-Samuel et al. (34) recommended the use of IAUC$_{90}$ for in-vivo studies. But they also mentioned that the integration period does not matter if the interval is long enough to ensure good signal-to-noise ratio. In another study, Morgan et al. (24) showed there were no major changes by using IAUC$_{60}$ or IAUC$_{180}$. Furthermore, they demonstrated that reproducibility was improved when IAUC was computed from the $T_1$ values rather than from the MR signal intensities. Finally, Evelhoch et al. (21) proposed normalizing the data with an AIF for longitudinal studies. Considering all these elements, we used normalized IAUC$_{60}$ values derived from the Gd concentration time curve to achieve accuracy. Although our normalization was performed using the whole brain IAUC$_{60}$
value rather than the AIF one, the automatic selection of enhancing pixels implemented provided results sensitive enough to minimize effects from inter-individual physiological variation and contrast agent dose variability. Muscle tissue was not chosen as a reference in this study because of low signal-to-noise ratio due to greater distance from the surface coil. The good sensitivity of the IAUC approach demonstrated in this study warrants further investigation to achieve improved distinction of uptake curve patterns.

The presented normalized IAUC\textsubscript{60} method also demonstrated robustness and insensitivity to the anesthetic procedure (one exception occurred at the last time point), whereas the non-normalized IAUC\textsubscript{60} values presented significant differences (results not shown). Indeed, the two anesthetics used in our protocol are well known to induce antagonistic physiological effects, mainly on the vasculature (35). Those effects have been investigated using MRI and they have been shown to influence the MR signal (36). As demonstrated by Hendrich et al. (37), using arterial spin labeling on rat, cerebral blood flow, as well as pO\textsubscript{2} and mean arterial blood pressure, measured on animals undergoing isoflurane anesthesia are higher than the ones of animals anesthetized with pentobarbital. If the proposed approach successfully homogenized the two groups without hiding the relevant changes in angiogenesis activity, a discrepancy appeared at 12 weeks after surgery. Several hypotheses may be raised to explain this result. First, one can suggest that the sample size of the isoflurane group at week 12 (n = 2), is not large enough to reflect an actual difference, and this result may be biased by inter-animal variability. Another hypothesis, as mentioned above, is long-term remodeling of the newly established vasculature, which normally leads to partial vessel pruning. The repeated isoflurane anesthesia could have an anti-pruning effect on the vasculature, resulting in a higher blood volume and, thus, a higher normalized IAUC\textsubscript{60} value. This explanation is likely, since isoflurane is known for having
preconditioning properties, as shown in stroke (38). Finally, as isoflurane and pentobarbital generate vasodilatation and vasoconstriction, respectively, the trend observed may be simply an emphasis of the natural trend, because vessels are more mature and more reactive to those chemical stimulations. A combination of these three hypotheses may also explain the reported difference. Understanding this phenomenon requires further investigation.

In conclusion, this study provides the first report of an in vivo longitudinal DCE-MRI study in tissue-engineered calvarium bone. The soft-tissue construct acts as a resorbable support for promoting angiogenesis and achieving complete bone regeneration. The normalized IAUC\textsubscript{60} approach used for the DCE-MRI analysis was capable of characterizing angiogenesis and discriminating between the different phases of bone regeneration, and was robust to challenging experimental conditions.

ACKNOWLEDGMENTS

We thank Marvin Estrada for animal care support and Tammy Rayner, Ruth Weiss, and Garry Detzler for technical assistance during MR scanning.
REFERENCES


**Table 1:** Evolution of the percentage of physiologically meaningful pixels (within the ranges of validity defined in *MRI data analysis*).

<table>
<thead>
<tr>
<th>ROI</th>
<th>n</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>6</th>
<th>12</th>
<th>n</th>
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</thead>
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<tr>
<td>Periphery</td>
<td>12</td>
<td>94 ± 2</td>
<td>94 ± 3</td>
<td>92 ± 1</td>
<td>91 ± 2</td>
<td>75 ± 6</td>
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<tr>
<td>Centre</td>
<td>12</td>
<td>77 ± 4</td>
<td>80 ± 5</td>
<td>93 ± 3</td>
<td>91 ± 4</td>
<td>69 ± 6</td>
<td>5</td>
</tr>
<tr>
<td>Brain</td>
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<td>75 ± 2</td>
<td>75 ± 2</td>
<td>71 ± 3</td>
<td>78 ± 2</td>
<td>77 ± 3</td>
<td>5</td>
</tr>
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Table 2: Evolution of the size of the ROI normalized to week 1.

<table>
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<th>3</th>
<th>6</th>
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</thead>
<tbody>
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<td>Periphery</td>
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<td>100</td>
<td>88 ± 6 *</td>
<td>88 ± 6</td>
<td>57 ± 6 *</td>
<td>70 ± 12</td>
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<tr>
<td>Centre</td>
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<td>100</td>
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<td>72 ± 10</td>
<td>56 ± 7 *</td>
<td>34 ± 6</td>
<td>5</td>
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<tr>
<td>Brain</td>
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<td>100</td>
<td>101 ± 1</td>
<td>103 ± 2</td>
<td>100 ± 2</td>
<td>102 ± 2</td>
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* P < 0.05 week (i) versus week (i-1)
Table 3: Evolution of normalized IAUC$_{60}$ values presented in all animals and in each anesthetic group.

<table>
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<tr>
<th>ROI</th>
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<th>2</th>
<th>3</th>
<th>6</th>
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<tbody>
<tr>
<td>All</td>
<td>12</td>
<td>8.42 ± 0.48</td>
<td>6.44 ± 0.47</td>
<td>6.56 ± 0.39</td>
<td>6.40 ± 0.57</td>
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<tr>
<td>Periphery Pentobarbital</td>
<td>5</td>
<td>7.93 ± 0.83</td>
<td>5.14 ± 0.41</td>
<td>* 6.73 ± 0.70</td>
<td>5.41 ± 0.44</td>
<td>3.44 ± 0.45 *</td>
<td>5</td>
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<tr>
<td>Isoflurane</td>
<td>7</td>
<td>8.78 ± 0.60</td>
<td>7.37 ± 0.53</td>
<td>* 6.44 ± 0.50</td>
<td>7.10 ± 0.86</td>
<td>7.46 ± 0.69 *</td>
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<tr>
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<td>1.49 ± 0.13</td>
<td>1.64 ± 0.13</td>
<td>2.13 ± 0.37</td>
<td>2.19 ± 0.73</td>
<td>1.35 ± 0.22 *</td>
<td>5</td>
</tr>
<tr>
<td>Isoflurane</td>
<td>7</td>
<td>2.23 ± 0.47</td>
<td>2.51 ± 0.39</td>
<td>2.65 ± 0.26</td>
<td>2.57 ± 0.32</td>
<td>2.95 ± 0.61 *</td>
<td>2</td>
</tr>
</tbody>
</table>

* P < 0.05 Pentobarbital versus Isoflurane
FIGURES LEGENDS

**Figure 1:** T1-weighted images (a) before, (b) 60s after Gd-DTPA injection and (c) the corresponding IAUC\(_{60}\) map. Imaging time is 3 weeks post-surgery. A zoomed-in version of the implanted VEGF-impregnated soft-tissue construct is shown in insets. Note the two distinct regions of the construct (periphery and centre) on images (b) and (c). The periphery ROI is delineated on the inset of (c).

**Figure 2:** Representative Gd-DTPA concentration-time curves in one animal between 1 and 12 weeks post-surgery. Mean values in the periphery and the centre of the implanted construct are shown.

**Figure 3:** Evolution of angiogenesis in the periphery and centre of the implanted construct in all animals (n=12, n=5 for 12 weeks) using the normalized IAUC\(_{60}\) metric. Results are presented as mean±SEM. \(P<0.05\): * different relative to week 1, # different relative to week 6.

**Figure 4:** Time points showing significant changes in angiogenic development in all animals. Evolution of the vascular response does not differ between the two anesthetic procedures (isoflurane (dashed lines) and pentobarbital (solid lines)). On each graph, the number of rabbits showing an increased (†), decreased (¶), or constant (=) change is indicated.

**Figure 5:** Evaluation of bone growth with micro-CT images. (a) At 6 weeks after the surgery, new bone is present mostly at the periphery of the bony defect containing the HA-VEGF construct. (b) At 12 weeks, there were islands of newly formed bone in the center of the defect. The dotted line indicates the midline and the circle is an estimate of the original defect.
Figure 1

Week 1

Week 3

Week 6

Week 12

Figure 2
Figure 3
Figure 4

(a) Periphery

(b) Centre

(c) Time after surgery (weeks)

(d) Time after surgery (weeks)

IAUC_{ROI} / IAUC_{brain} (a.u.)

Pentobarbital

Isoflurane

↑ 2 = 2 ↓ 8

↑ 8 = 1 ↓ 3

↑ 1 = 2 ↓ 4

↑ 0 = 1 ↓ 6
Figure 5