

Journal of Visualized Experiments

Calvarial model of bone augmentation in the rabbit for the assessment of bone growth and neovascularization in bone substitution materials --Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59976R1
Full Title:	Calvarial model of bone augmentation in the rabbit for the assessment of bone growth and neovascularization in bone substitution materials
Keywords:	Animal experiment, surgical model, bone augmentation, rabbit calvarium, bone growth, neovascularization.
Corresponding Author:	Stephane Durual SWITZERLAND
Corresponding Author's Institution:	
Corresponding Author E-Mail:	Stephane.Durual@unige.ch
Order of Authors:	Laurine Marger Antonio Barone Carla P Martinelli-Kläy Leandra Schaub Malin Strasding Mustapha Mekki Irena Sailer Susanne S Scherrer Stephane Durual
Additional Information:	
Question	Response
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US\$2,400)
Please indicate the city, state/province, and country where this article will be filmed . Please do not use abbreviations.	Geneva, Switzerland

TITLE:

Calvarial Model of Bone Augmentation in Rabbit for Assessment of Bone Growth and Neovascularization in Bone Substitution Materials

AUTHORS & AFFILIATIONS:

Laurine Marger¹, Antonio Barone², Carla P Martinelli-Kläy³, Leandra Schaub¹, Malin Strasding⁴, Mustapha Mekki¹, Irena Sailer⁴, Susanne S Scherrer¹ and Stéphane Durual¹

¹Division of Fixed Prosthodontics and Biomaterials, Biomaterials Laboratory, University of Geneva, University Clinics of Dental Medicine, 1, Rue Michel Servet, 1204 Geneva, Switzerland

²Department of Surgery, Division of Oral and Maxillofacial Surgery (HUG), Unity of Oral Surgery and Implantology, University of Geneva, University Clinics of Dental Medicine, 1, Rue Michel Servet, 1204 Geneva, Switzerland

³Department of Surgery, Division of Oral and Maxillofacial surgery (HUG), Laboratory of Oral & Maxillofacial Pathology, University of Geneva, University Clinics of Dental Medicine, 1, Rue Michel Servet, 1204 Geneva, Switzerland

⁴Division of Fixed Prosthodontics and Biomaterials, University of Geneva, University Clinics of Dental Medicine, 1, Rue Michel Servet, 1204 Geneva, Switzerland

Corresponding author:

Stéphane Durual (stephane.durual@unige.ch)

Email addresses of co-authors:

L. Marger	(laurine.marger@unige.ch)
A. Barone	(antonio.barone@unige.ch)
C. P. Martinelli-Kläy	(CarlaPatricia.Martinelli-Klay@hcuge.ch)
L. Schaub	(leandra.schaub@etu.unige.ch)
M. Strasding	(malin.strasding@unige.ch)
M. Mekki	(mustapha.mekki@unige.ch)
I. Sailer	(irena.sailer@unige.ch)
S. S. Scherrer	(susanne.scherrer@unige.ch)

KEYWORDS

Animal experiment, surgical model, bone augmentation, rabbit calvarium, bone growth, neovascularization

SUMMARY:

Here we present a surgical protocol in rabbits with the aim to assess bone substitution materials in terms of bone regeneration capacities. By using PEEK cylinders fixed onto rabbit skulls, osteoconduction, osteoinduction, osteogenesis and vasculogenesis induced by the materials may be evaluated either on live or euthanized animals.

ABSTRACT:

The basic principle of the rabbit calvarial model is to grow new bone tissue vertically on top of the cortical part of the skull. This model allows assessment of bone substitution materials for oral and craniofacial bone regeneration in terms of bone growth and neovascularization support. Once animals are anesthetized and ventilated (endotracheal intubation), four cylinders made of polyether ether ketone (PEEK) are screwed onto the skull, on both sides of the median and coronal sutures. Five intramedullary holes are drilled within the bone area delimited by each cylinder, allowing influx of bone marrow cells. The material samples are placed into the cylinders which are then closed. Finally, the surgical site is sutured, and animals are awakened. Bone growth may be assessed on live animals by using microtomography. Once animals are euthanized, bone growth and neovascularization may be evaluated by using microtomography, immune-histology and immunofluorescence. As the evaluation of a material requires maximum standardization and calibration, the calvarial model appears ideal. Access is very easy, calibration and standardization are facilitated by the use of defined cylinders and four samples may be assessed simultaneously. Furthermore, live tomography may be used and ultimately a large decrease in animals to be euthanized may be anticipated.

INTRODUCTION:

The calvarial model of bone augmentation was developed in the 90's with the aim to optimize the concept of guided bone regeneration (GBR) in the oral and craniofacial surgical domain. The basic principle of this model is to grow new bone tissue vertically on top of the cortical part of the skull. To do so, a reactor (e.g., titanium -dome, -cylinder or -cage) is fixed onto the skull to protect the bone regeneration conducted by a graft (e.g., hydrogel, bone substitute, etc.). With the aid of this model, titanium or ceramic cages¹⁻⁶, GBR membranes⁷⁻¹⁰, osteogenic factors¹¹⁻¹⁷, new bone substitutes^{12,16-29} or the mechanism of neovascularization during the bone regeneration process³⁰ were assessed.

From a translational point of view, the calvarial model represents a one-wall defect that can be compared to a class IV defect in the jaw³¹. The aim is to grow new bone above a cortical area, without any lateral support from endogenous bone walls. The model is thus extremely stringent and assesses the real potential of vertical osteoconduction over the cortical part of the bone. If the model described herein is primarily dedicated to the assessment of osteoconduction in bone substitutes, osteogenesis and/or osteoinduction may be also assessed, as well as vasculogenesis¹⁻³⁰.

Essentially for ethical, practical and economic reasons, the calvarial model was developed in the rabbit in which the bone metabolism and structure are quite relevant when compared to human³². Of the 30 references cited above, 80% used the rabbit calvarial model^{1-15,17,22,23,26-30,33}, thus demonstrating the relevance of this animal model. In 2008, the Busenlechner group transferred the calvarial model to the pig, to allow the comparison of eight bone substitutes simultaneously²⁰ (as compared to two bone substitutes with the rabbit). On the other hand, our group transferred the rabbit calvarial model to sheep. In brief, titanium domes were placed on

sheep skulls to characterize the osteoconduction of a new 3D-printed bone substitute. These studies allowed us to develop and master the calvarial model and its analysis^{16,21}.

The last three studies cited^{16,20,21}, together with several other investigations^{12,17-19,22-24,26-29}, confirmed the great potential of the calvarial model as a screening and characterization model. However, even though the results obtained were quite satisfactory, they also pointed out some limitations: (1) The use of titanium domes, which prevented radiotracer diffusion and in turn live micro-CT use. These could not be removed before histological processing, forcing the researchers to embed the samples in poly(methyl methacrylate) resin (PMMA). The resulting analyses were therefore largely limited to topography. (2) High financial costs especially because of the cost of the animals, and costs related to the logistics, maintenance and the surgery of the animals. (3) Difficulties to obtain ethical approvals for large animals.

A recent study by Polo, et al.²⁶ largely improved the model on the rabbit. Titanium domes were replaced by closable cylinders that could be filled with a constant volume of material. Four of these cylinders were placed on rabbit skulls. At completion, the cylinders could be removed so that biopsies were metal-free, introducing much more flexibility concerning sample processing. The rabbit calvarial model became attractive for simultaneous testing with lower costs, easy animal handling and facilitation of sample processing. Taking advantage of these recent developments, we have further improved the model by replacing titanium with PEEK to produce cylinders, thereby allowing radiotracer diffusion and the use of microtomography on live animals.

In this article, we will describe the anesthesia and surgery processes and show examples of outputs that may be obtained using this protocol, i.e., (immuno-) histology, histomorphometry, live and ex vivo microtomography to evaluate the mechanisms of bone regeneration and quantify the new bone synthesis supported by bone substitute materials.

PROTOCOL:

In line with Swiss legal requirements, the protocol was approved by an academic committee and supervised by the cantonal and federal veterinary agencies (authorizations n° GE/165/16 and GE/100/18).

1. Specific devices and animals

1.1. Cylinders

1.1.1. Machine cylinders with lateral stabilizing tabs out of PEEK to have inner diameter of 5 mm, outer diameter of 8 mm and a height of 5 mm (**Figure 1**).

1.1.2. Machine PEEK caps were design them to clip precisely onto the top of the cylinder (thickness 1 mm).

1.1.3. Sterilize PEEK cylinders and caps by autoclaving before surgery.

1.2. Screws

1.2.1. Use self-drilling micro screws (made of commercial pure titanium (grade 5)) to fix the cylinders (1.2 mm in diameter, 4 mm in length). Sterilize by autoclaving before surgery.

1.3 Animals

1.3.1. Purchase three-month-old New Zealand white rabbits (male or female), weighing ~2.5 kg each.

NOTE: We obtained rabbits by breeding at the University of Geneva.

2. Surgery

2.1. Surgical tray

2.1.1. Keep scalpels, scissors, two forceps, periosteal elevator, syringes (1, 2, 5, 50 mL), surgical motor, round surgical burs (0.8 mm diameter), needles, sterile saline, four cylinders, eight screws, and screwdriver ready.

2.2. Preclinical treatment

2.2.1. Acclimate the animals one week prior to surgery.

2.2.2. Provide a prophylactic antibiotic daily (5–10 mg/kg by mouth (PO)) starting 2 h before surgery up to 3 days after surgery.

2.3. Anesthesia and intubation

2.3.1. Sedate the animals by intramuscular (IM) injection of ketamine (25 mg/kg, 50 mg/mL, 0.5 mL/kg) + xylazine (3 mg/kg, 20 mg/mL, 0.15 mL/kg). Wait ~20 min for the animals to sleep deeply (complete muscular atony).

NOTE: This premedication will allow a simple, fast and painless intubation process. Deep analgesia and anesthesia is induced as described in step 2.3.8.

2.3.2. Place an intravenous (IV) cannula into the marginal vein from the ear and keep it closed until intubation is completed.

NOTE: This IV line will serve to perfuse fentanyl and propofol for deep analgesia and anesthesia, respectively (see step 2.3.8).

2.3.3. Maintain anesthesia by supplying 5% sevoflurane in pure oxygen until intubation is performed.

NOTE: This step is necessary only if the animal shows signs of awakening (eye movements, muscular contractions).

2.3.4. Anesthetize the trachea locally by spraying 10% lidocaine. Place the rabbit in prone position and maintain its head in vertical extension.

2.3.5. Slide the first endotracheal tube of small diameter (2.5 mm) into the rabbit trachea until airflow can be heard in the tube. This will open the larynx and facilitate the insertion of the definitive tube.

2.3.6. Insert a guide (intubation catheter) in the tube to fix the position of the tube into the trachea. Remove the small diameter tube and slide the definitive endotracheal tube (4.9 mm) on the guide.

2.3.7. Remove the guide and inflate the balloon at the end of the endotracheal tube to seal and block the device into the trachea. Immediately ventilate (7 mL/kg, frequency of 40/min) the animal with 3% sevoflurane in pure oxygen.

2.3.8. Continuously perfuse (ear vein) fentanyl (0.01 mg/mL, 2–4 mL/h) to induce analgesia, 2–4 mg/kg of (2%) propofol (20 mg/mL, 4–8 mL/h) to induce anesthesia, and 4 mL/kg/h of Ringer's acetate to maintain iso-volumetric conditions.

2.3.9. Place a rectal temperature probe. Also monitor heart function, temperature and oxygen saturation during the entire process.

2.3.10. Control the depth of anesthesia by monitoring autonomous breathing; if the animal shows signs of autonomous breathing, dispense a small bolus of propofol and fentanyl.

2.4. Site preparation

2.4.1. Place the rabbit on a heated pad (39 °C) on the surgery table. Shave the scalp.

2.4.2. Disinfect the site by scrubbing the skin with povidone iodine (10%). Then drape the rabbit with a sterile surgical drape and cut out an access area for the skull.

2.4.3. Disinfect the surgical site with povidone iodine (10%) for a second time. Apply a lubricating gel on the eyes to avoid irritation and dryness.

2.4.4. Prepare a draped table (sterile drape) on which to place the complete surgical tray.

2.5. Surgical site opening

2.5.1. Anesthetize locally with a subcutaneous (SC) injection of lidocaine on the skull.

2.5.2 Incise through the skin (with a scalpel) along the calvarial sagittal line, from the orbits to the external occipital protuberance (~4 cm in length). Ensure that the periosteum is incised.

2.5.3 Gently elevate the periosteum (with a periosteal elevator) on both side of the incision. Rinse the site with sterile saline.

2.6. Cylinder placement

2.6.1. Locate the median and coronal sutures on the skull (**Figure 2A,B**). Note that these anatomical lines form a cross. The cylinders will be placed in each of the quadrants defined by the cross, ensuring that the edge of the cylinder is not over the suture (**Figure 2C**).

2.6.2. Place the first cylinder on the left upper quadrant (left frontal bone), and try to lay the device flat. Fix in the position with strong hand pressure and screw a micro-screw, until resistance is felt. Ensure that the screw head is flush with the surface of the cylinder tab.

2.6.3. Repeat the same procedure on the other tab to fix the cylinder tightly onto the skull. Ensure that the cylinder is hermetically fixed to the bone.

2.6.4. Repeat the procedure on the right upper quarter (right frontal bone), left lower quarter (left parietal bone) and right lower quarter (right parietal bone).

2.7. Bone drilling of 5 intramedullary holes within the area circumscribed by the cylinders (Figure 1)

2.7.1. Drill an intramedullary hole under saline irrigation (0.8 mm in diameter, ~1 mm in depth) with a round bur on the bone, in the center of the area circumscribed by the cylinder. Ensure that bleeding appears.

2.7.2. Drill two more intramedullary holes along the axis passing through the two tab screws, at the inner edges of the cylinder. Along the perpendicular axe, drill two more intramedullary holes at the inner edges of the cylinder. Ensure that bleeding appears.

2.7.3. Repeat the operation within the three other cylinders.

2.8. Filling cylinders with material samples and capping (Figure 3)

2.8.1. Prepare the desired bone substitute material according to manufacturer instructions or material specifications.

2.8.2. Fill the first cylinder to the brim with the material sample and close the cylinder by fitting the cap. Repeat the process in the 3 other cylinders.

2.9. Surgical site closure

2.9.1. Close the skin above the cylinders with an intermittent non-resorbable suture.

2.9.2. Apply a sprayable dressing onto the wound.

3. Post-surgical treatment

3.1. Stop analgesia and anesthesia (propofol and fentanyl perfusion arrest) supply and check the recovery of autonomous breathing.

3.2. Stop the ventilation once the animal has recovered autonomous breathing. Maintain the animal under pure oxygen before complete awakening.

3.3. Inject buprenorphine hydrochloride SC (0.02 mg/kg, 0.03 mg/mL, 0.67 mL/kg) and repeat the injection every 6 h for 3 days as post-surgical analgesia.

3.4. Transfer the animal into its usual housing with water and complete feeding.

3.5. Remove the sutures after about 10 days of wound healing.

REPRESENTATIVE RESULTS:

The model described herein is dedicated to the assessment of osteoconduction in bone substitutes. Osteogenesis and/or osteoinduction of bone substitutes either (pre-)cellularized or loaded with bioactive molecules may be also assessed, as well as vasculogenesis¹⁻³⁰. A kinetic study may be used, from 3 days up to 3 months after surgery depending on the mechanisms and outputs to be analyzed. A classical timeline allowing descriptions at early and mid-time is: 2, 4, 6, 8 and 12 weeks. Note that a minimum of 6 samples per time point is mandatory to obtain significant results. Each sample to be tested needs to be placed at least one time in each position onto the skull per time point (random allocation). Finally, sham samples (e.g., cylinders filled with coagulated blood) have to be included in the protocol³⁴.

Once the surgery is completed, bone growth may be monitored at different time points by using bone tomography on live animals. An example is shown in **Figure 4A,B**. Additional analysis requires animals to be sacrificed (lethal intravenous injection of 150 mg/kg pentobarbital (100 mg/mL). After euthanasia, samples are sectioned and cylinders are carefully removed (**Figure 5**). Biopsies are fixed with a solution of phosphate-buffered saline and 4% formaldehyde. Bone growth may then be assessed by using microtomography (**Figure 4 C,D**). Samples may also be processed for (immune-) histological staining. Histomorphometric analysis and specific stainings are then possible to complete the analysis more specifically (**Figure 6**).

FIGURE LEGENDS:

Figure 1: Specifications of PEEK cylinders. Two holes (0.8 mm in diameter) were drilled on the lateral stabilizing tabs for screwing. The positions of the 5 intramedullary holes (0.8 mm in diameter) to be drilled on the skull within the area delimited by the cylinder are marked using red circles.

Figure 2: Representative image of the rabbit skull and placement of the cylinders. Pictures showing the median and coronal sutures on the rabbit skull delineating the left-right parietal and frontal bones (A,B). Placement of the cylinders on both side of the sutures (C). Scale bars = 5 mm.

Figure 3: Representative image of cylinders fixed, filled and capped. Picture showing four cylinders fixed onto the skull of a rabbit with titanium screws. Within the area delimited by each cylinder, 5 intramedullary holes (0.8 mm in diameter, ~1 mm in depth) were drilled under irrigation with a round bur to allow bone cell migration. Cylinders were filled with different bone substitute samples (calibrated volumes) before capping (one closed cylinder is shown only). Scale bar = 5 mm.

Figure 4: Representative images from microtomographic (micro-CT) analysis. With the final goal to assess bone growth conducted by bone substitutes, 4 cylinders were fixed onto a rabbit skull with titanium screws and filled with bone substitute materials. (A) Live imaging: two-dimensional transverse scan (14 min, 99 kV/88 μ A with a resolution of 20 μ m) of a cylinder at 12 weeks. (B) three-dimensional (3D) reconstruction from live micro-CT analysis at 4 weeks (red circles: bone substitutes in cylinders; red arrow: control in which the cylinder is filled with coagulated blood). (C,D) After euthanasia (12 weeks), cylinders were removed before fixation and micro-CT analysis. (C) 2D transverse scan (57 min, 99 kV/88 μ A with a resolution of 10 μ m) of a cylinder and 3D-reconstruction of the total new bone in the cylinder (D). Bone substitute particles (red), new bone (green) and bone bed (yellow) are shown. Scale bars = 2 mm

Figure 5: Representative images of a biopsy at 4 weeks. After euthanasia (4 weeks), samples were block-sectioned and cylinders were removed before fixation in 4% formalin, micro-CT analysis and histological processing. Scale bar = 5mm.

Figure 6: Representative pictures of (immune-)histological sections. With the final goal to assess bone growth and neovascularization conducted by bone substitutes, 4 cylinders were fixed onto a rabbit skull with titanium screws and filled with bone substitutes. After euthanasia (12 weeks), cylinders were removed before fixation and histological processing. (A) Masson-Goldner staining (50x): bone substitute appears as mauve particles surrounded by new bone in green. (B) The slices were scanned and processed for digital extraction of bone substitute material so that the new bone (red) could be quantified easily. (C) Immunostaining of CD31 (arrows), a typical marker of endothelial cells and the neovascularization process. (D) Immunofluorescent staining (green) of a highly neovascularized zone in which some new capillaries highly express CD31 (arrow).

DISCUSSION:

The model described herein is simple and should be developed quite easily as long as all the steps are followed and the equipment is suitable. As the protocol described is a surgical method, all

the steps appear critical and must be followed properly. It is critical to be trained for animal experiments, especially in rabbit handling and anesthesia. Do not hesitate to ask for professional anesthetist and veterinary help. It is critical to insist on the daily visual monitoring of animals before and after suture removal. Even if the skin from the skull is thick, abundant and loose, the cylinders' fixation induces large tensions. If the sutures are removed too early, the wound might reopen and will require one more week of suturing and a new antibiotic treatment. In case of dehiscence out of the wound line, the surgeon will have to consider if suturing is possible or not. If not, sample rejection will have to be considered.

In addition to the critical steps described in the protocol section, the details below may be help in the proper implementation of this protocol. From a technical point of view, if the rabbits used are young and small, it is important to use a two-step intubation, as described in the protocol. The second (and final) tube is too large to be used in first intubation, and there is a real risk of "wrong way" that may be deleterious or even fatal. Depending on the materials tested, it may be interesting to take some fresh blood sample from the ear IV line that is already placed. This could provide a good method to pre-infuse the bone substitute material with natural cells and growth factors. Freshly coagulated blood may also provide an ideal sham sample.

The way the intramedullary holes are drilled should be very helpful for future histological processing and histomorphometrical analysis. In effect, as long as the holes are (i) in the same location, (ii) the biopsies are similarly oriented and (iii) the cutting process is standardized (i.e., same thickness, same cut level), the fields that are evaluated are equivalent and their comparison is highly pertinent. As a matter of standardization, it may be of real interest to calibrate the volume-quantity of material to fill the cylinder, and to prepare it in advance, in a sterile way.

From a scientific point of view, depending on the products or hypothesis tested, it may be important to avoid placing the cylinders onto bone sutures. Some specific stem cells are present in these structures³⁵, different from the mesenchymal bone stem cells that are involved in the mechanisms of intramembranous ossification, i.e., the bone regeneration process encountered in the orofacial area. Therefore, a real bias may occur in case of misplacement.

One of the major advantages of the calvarial model is the use of live microtomography by which the bone growth may be followed on a single animal as a longitudinal study. This strategy may largely reduce the number of euthanized animals and therefore respect the "3R rule"³⁶. Depending on the microtomograph device used (resolution, space available for the animal), variations will occur in terms of strategy of anesthesia (IV, gas, etc.), as well as in the image resolution and the relevance of the resulting analysis.

We routinely use a microtomograph dedicated to animal experiments (e.g., Quantum GX) for routine checks. A typical experiment starts with a sedation, as described in step 2.3.1. Anesthesia is then maintained with 2% isoflurane in pure oxygen. This allows the animal to breathe calmly during a scanning time of about 14 min (99 kV/88 μ A with a resolution of 20 μ m); this is sufficient for checking basic parameters (engraftment, cylinder fixation, semiquantitative analysis of the

bone growth, etc.). When looking for a precise qualitative and quantitative analysis, a very fine tuning of the scanning time, resolution, anesthesia and animal positioning will be needed.

Existing models developed for the assesement of osteoconduction, osteogenesis, osteoinduction as well as vasculogenesis, are numerous, especially in the orofacial field. Due to ethical and economic considerations, our purpose will be limited to rabbit or smaller animals. Apart from the skull, the locations in which the material may be tested are the mandibulla³⁷⁻⁴⁷, the diastema⁴⁸ or the incisive sockets (after teeth extraction)⁴⁹⁻⁵¹. Rats, mice, guinea pigs and rabbits may be used for each of the models described below. Briefly, a critical defect is drilled (or a socket is created by incisive extraction), filled with the material sample and then covered by a membrane. Apart from the obvious fact that only two samples can be tested in each of these locations (one sample per mandible side or per incisive socket), the surgical procedures are also largely more difficult and invasive. Accesses to the site are limited and if one is using rat or mice, the difficulty is further increased by the size of the animals. Finally, the critical size of the defects (i.e., dimensions that do not allow a spontaneous bone regeneration) is not well-defined and varies from one animal to another.

Besides these generalities, specific constraints may arise depending on the anatomical location subjected to the defect and to the subsequent analysis. As an example, in the mandibular model, teeth roots are present within the defect. This may interfere with the material and modify the bone regeneration process. The defect could be placed more distally on the ramus, but in that case, the bone would be very thin (two corticals affixed with an extremely thin medullary space) and the resulting defect volume may be too small⁴⁵⁻⁴⁷. Given these examples of limitations and depending on the model, large deviations may be anticipated in terms of material volume to be tested, quality and quantity of data collected.

As the evaluation of a material requires a maximum of standardization and calibration, the calvarial model appears ideal. Access is very easy, calibration and standardization are facilitated by the use of defined cylinders and 4 samples may be assessed simultaneously. Furthermore, live tomography may be used and finally a large decrease in animals to be euthanized may be anticipated.

ACKNOWLEDGEMENTS

The authors are indebted to Geistlich AG (Wolhusen, CH) and the Osteology foundation (Lucerne, CH) (grant n°18-049) for their support, as well as Global D (Brignais, FR) for providing the screws. A particular thanks goes to Dr B. Schaefer from Geistlich. We are also grateful to Eliane Dubois and Claire Herrmann for their excellent histological processing and their precious advices. Finally, we warmly acknowledge Xavier Belin, Sylvie Roulet and the entire team of Pr Walid Habre, “experimental surgery Dpt” ,for their remarkable technical assistance.

DISCLOSURES

The authors have nothing to disclose.

REFERENCES

- 1 Anderud, J., et al. Guided bone augmentation using a ceramic space-maintaining device. *Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology*. **118** (5), 532-538, doi:S2212-4403(14)00637-3, 10.1016/j.oooo.2014.06.011, (2014).
- 2 Lundgren, A. K., Lundgren, D., Hammerle, C. H., Nyman, S., Sennerby, L. Influence of decortication of the donor bone on guided bone augmentation. An experimental study in the rabbit skull bone. *Clinical Oral Implants Research*. **11** (2), 99-106, doi:clr110202, , (2000).
- 3 Lundgren, D., Lundgren, A. K., Sennerby, L., Nyman, S. Augmentation of intramembraneous bone beyond the skeletal envelope using an occlusive titanium barrier. An experimental study in the rabbit. *Clinical Oral Implants Research*. **6** (2), 67-72 (1995).
- 4 Slotte, C., Lundgren, D. Impact of cortical perforations of contiguous donor bone in a guided bone augmentation procedure: an experimental study in the rabbit skull. *Clinical Implant Dentistry and Relat Research*. **4** (1), 1-10 (2002).
- 5 Tamura, T., et al. Three-dimensional evaluation for augmented bone using guided bone regeneration. *Journal of Periodontal Research*. **40** (3), 269-276, doi:JRE802, 10.1111/j.1600-0765.2005.00802.x, (2005).
- 6 Yamada, Y., et al. Correlation in the densities of augmented and existing bone in guided bone augmentation. *Clinical Oral Implants Research*. **23** (7), 837-845, doi:10.1111/j.1600-0501.2011.02204.x, (2012).
- 7 Chierico, A., et al. Electrically charged GTAM membranes stimulate osteogenesis in rabbit calvarial defects. *Clinical Oral Implants Research*. **10** (5), 415-424 (1999).
- 8 Ikeno, M., Hibi, H., Kinoshita, K., Hattori, H., Ueda, M. Effects of the permeability of shields with autologous bone grafts on bone augmentation. *International Journal of Oral and Maxillofacial Implants*. **28** (6), e386-392 (2013).
- 9 Ito, K., Nanba, K., Murai, S. Effects of bioabsorbable and non-resorbable barrier membranes on bone augmentation in rabbit calvaria. *Journal of Periodontology*. **69** (11), 1229-1237, doi:10.1902/jop.1998.69.11.1229, (1998).
- 10 Lee, Y. M., et al. Enhanced bone augmentation by controlled release of recombinant human bone morphogenetic protein-2 from bioabsorbable membranes. *Journal of Periodontology*. **74** (6), 865-872, doi:10.1902/jop.2003.74.6.865, (2003).
- 11 Fugl, A., et al. S-nitroso albumin enhances bone formation in a rabbit calvaria model. *International Journal of Oral and Maxillofacial Surgery*. **43** (3), 381-386, doi:S0901-5027(13)01066-7, 10.1016/j.ijom.2013.06.018, (2014).
- 12 Ikeno, M., Hibi, H., Kinoshita, K., Hattori, H., Ueda, M. Effects of self-assembling peptide hydrogel scaffold on bone regeneration with recombinant human bone morphogenetic protein-2. *International Journal of Oral and Maxillofacial Implants*. **28** (5), e283-289 (2013).
- 13 Ito, K., et al. Effects of ipriflavone on augmented bone using a guided bone regeneration procedure. *Clinical Oral Implants Research*. **18** (1), 60-68, doi:CLR1284, 10.1111/j.1600-0501.2006.01284.x, (2007).
- 14 Jung, R. E., Hammerle, C. H., Kokovic, V., Weber, F. E. Bone regeneration using a synthetic matrix containing a parathyroid hormone peptide combined with a grafting material. *International Journal of Oral and Maxillofacial Implants*. **22** (2), 258-266 (2007).

482 15 Minegishi, T., et al. Effects of ipriflavone on bone augmentation within a titanium cap in
483 rabbit calvaria. *Journal of Oral Science*. **44** (1), 7-11 (2002).

484 16 Moussa, M., et al. Medium-Term Function of a 3D Printed TCP/HA Structure as a New
485 Osteoconductive Scaffold for Vertical Bone Augmentation: A Simulation by BMP-2 Activation.
486 *Materials*. **8** (5), 2174-2190, doi:10.3390/ma8052174, (2015).

487 17 Thoma, D. S., Kruse, A., Ghayor, C., Jung, R. E., Weber, F. E. Bone augmentation using a
488 synthetic hydroxyapatite/silica oxide-based and a xenogenic hydroxyapatite-based bone
489 substitute materials with and without recombinant human bone morphogenetic protein-2.
490 *Clinical Oral Implants Research*. **26** (5), 592-598, doi:10.1111/clr.12469, (2014).

491 18 Busenlechner, D., et al. Resorption of deproteinized bovine bone mineral in a porcine
492 calvaria augmentation model. *Clinical Oral Implants Research*. **23** (1), 95-99, doi:10.1111/j.1600-
493 0501.2011.02198.x, (2012).

494 19 Busenlechner, D., et al. Paste-like inorganic bone matrix: preclinical testing of a prototype
495 preparation in the porcine calvaria. *Clinical Oral Implants Research*. **20** (10), 1099-1104,
496 doi:CLR1743, 10.1111/j.1600-0501.2009.01743.x, (2009).

497 20 Busenlechner, D., et al. Simultaneous in vivo comparison of bone substitutes in a guided
498 bone regeneration model. *Biomaterials*. **29** (22), 3195-3200, doi:S0142-9612(08)00264-0,
499 10.1016/j.biomaterials.2008.04.021, (2008).

500 21 Carrel, J. P., et al. A 3D printed TCP/HA structure as a new osteoconductive scaffold for
501 vertical bone augmentation. *Clinical Oral Implants Research*. **27** (1), 55-62,
502 doi:10.1111/clr.12503, (2016).

503 22 Murai, M., et al. Effects of different sizes of beta-tricalcium phosphate particles on bone
504 augmentation within a titanium cap in rabbit calvarium. *Dental Materials Journal*. **25** (1), 87-96
505 (2006).

506 23 Nishida, T., et al. Effects of bioactive glass on bone augmentation within a titanium cap in
507 rabbit parietal bone. *Journal of Periodontology*. **77** (6), 983-989, doi:10.1902/jop.2006.050170,
508 (2006).

509 24 Nyan, M., et al. Feasibility of alpha tricalcium phosphate for vertical bone augmentation.
510 *Journal of Investigating and Clinical Dentistry*. **5** (2), 109-116, doi:10.1111/jicd.12022, (2012).

511 25 Polimeni, G., et al. Histopathological observations of a polylactic acid-based device
512 intended for guided bone/tissue regeneration. *Clinical Implant Dentistry and Related Research*.
513 **10** (2), 99-105, doi:CID067, 10.1111/j.1708-8208.2007.00067.x, (2008).

514 26 Polo, C. I., et al. Effect of recombinant human bone morphogenetic protein 2 associated
515 with a variety of bone substitutes on vertical guided bone regeneration in rabbit calvarium.
516 *Journal of Periodontology*. **84** (3), 360-370, doi:10.1902/jop.2012.110674, (2013).

517 27 Slotte, C., Lundgren, D., Burgos, P. M. Placement of autogeneic bone chips or bovine bone
518 mineral in guided bone augmentation: a rabbit skull study. *International Journal of Oral and*
519 *Maxillofacial Implants*. **18** (6), 795-806 (2003).

520 28 Tamimi, F. M., et al. Bone augmentation in rabbit calvariae: comparative study between
521 Bio-Oss and a novel beta-TCP/DCPD granulate. *Journal of Clinical Periodontology*. **33** (12), 922-
522 928, doi:CPE1004, 10.1111/j.1600-051X.2006.01004.x, (2006).

523 29 Torres, J., et al. Effect of solely applied platelet-rich plasma on osseous regeneration
524 compared to Bio-Oss: a morphometric and densitometric study on rabbit calvaria. *Clinical*

525 *Implant Dentistry and Related Research*. **10** (2), 106-112, doi:CID068, 10.1111/j.1708-
526 8208.2007.00068.x, (2008).

527 30 Yamada, Y., et al. Angiogenesis in newly augmented bone observed in rabbit calvarium
528 using a titanium cap. *Clinical Oral Implants Research*. **19** (10), 1003-1009, doi:CLR1554,
529 10.1111/j.1600-0501.2008.01554.x, (2008).

530 31 Cordaro, L., Terheyden, H. in *ITI Treatment Guide Vol. 7* eds D. Wismeijer, S. Chen, & D.
531 Buser) (Quintessence, 2014).

532 32 Pearce, A. I., Richards, R. G., Milz, S., Schneider, E., Pearce, S. G. Animal models for implant
533 biomaterial research in bone: A review. *European Cells & Materials*. **13** 1-10 (2007).

534 33 Min, S., et al. Effects of marrow penetration on bone augmentation within a titanium cap
535 in rabbit calvarium. *Journal of Periodontology*. **78** (10), 1978-1984, doi:10.1902/jop.2007.070086,
536 (2007).

537 34 Braun, T. M. in *Osteology guidelines for oral and maxillofacial regeneration Preclinical*
538 *models for translational research* eds WV Giannobile & M Nevins) Ch. 4, 31-43 (Quintessence
539 publishing, 2011).

540 35 Doro, D. H., Grigoriadis, A. E., Liu, K. J. Calvarial Suture-Derived Stem Cells and Their
541 Contribution to Cranial Bone Repair. *Frontiers in Physiology*. **8** 956,
542 doi:10.3389/fphys.2017.00956, (2017).

543 36 Russel, W., Burch, R. *The principles of humane experimental technique*. . (Universities
544 Federation for Animal Welfare, 1959).

545 37 Asvanund, P., Chunhabundit, P. Alveolar bone regeneration by implantation of nacre and
546 B-tricalcium phosphate in guinea pig. *Implant Dentistry*. **21** (3), 248-253,
547 doi:10.1097/ID.0b013e3182563ae000008505-201206000-00017, , (2012).

548 38 Gielkens, P. F., et al. Vivosorb, Bio-Gide, and Gore-Tex as barrier membranes in rat
549 mandibular defects: an evaluation by microradiography and micro-CT. *Clinical Oral Implants*
550 *Research*. **19** (5), 516-521, doi:CLR1511, 10.1111/j.1600-0501.2007.01511.x, (2008).

551 39 Lioubavina, N., Kostopoulos, L., Wenzel, A., Karring, T. Long-term stability of jaw bone
552 tuberosities formed by "guided tissue regeneration". *Clinical Oral Implants Research*. **10** (6), 477-
553 486 (1999).

554 40 Mardas, N., Kostopoulos, L., Stavropoulos, A., Karring, T. Osteogenesis by guided tissue
555 regeneration and demineralized bone matrix. *Journal of Clinical Periodontology*. **30** (3), 176-183,
556 doi:20031, , (2003).

557 41 Stavropoulos, A., Kostopoulos, L., Mardas, N., Nyengaard, J. R., Karring, T. Deproteinized
558 bovine bone used as an adjunct to guided bone augmentation: an experimental study in the rat.
559 *Clinical Implant Dentistry and Related Research*. **3** (3), 156-165 (2001).

560 42 Stavropoulos, A., Kostopoulos, L., Nyengaard, J. R., Karring, T. Deproteinized bovine bone
561 (Bio-Oss) and bioactive glass (Biogran) arrest bone formation when used as an adjunct to guided
562 tissue regeneration (GTR): an experimental study in the rat. *Journal of Clinical Periodontology*. **30**
563 (7), 636-643, doi:093, , (2003).

564 43 Stavropoulos, A., Kostopoulos, L., Nyengaard, J. R., Karring, T. Fate of bone formed by
565 guided tissue regeneration with or without grafting of Bio-Oss or Biogran. An experimental study
566 in the rat. *Journal of Clinical Periodontology*. **31** (1), 30-39 (2004).

567 44 Stavropoulos, A., Nyengaard, J. R., Kostopoulos, L., Karring, T. Implant placement in bone
568 formed beyond the skeletal envelope by means of guided tissue regeneration: an experimental

study in the rat. *Journal of Clinical Periodontology*. **32** (10), 1108-1115, doi:CPE756, 10.1111/j.1600-051X.2005.00756.x, (2005).

45 Thomaidis, V., et al. Comparative study of 5 different membranes for guided bone regeneration of rabbit mandibular defects beyond critical size. *Medical Science Monitor*. **14** (4), BR67-73, doi:850281, (2008).

46 Zhang, J. C., et al. The repair of critical-size defects with porous hydroxyapatite/polyamide nanocomposite: an experimental study in rabbit mandibles. *International Journal of Oral and Maxillofacial Surgery*. **39** (5), 469-477, doi:S0901-5027(10)00016-0, 10.1016/j.ijom.2010.01.013, (2010).

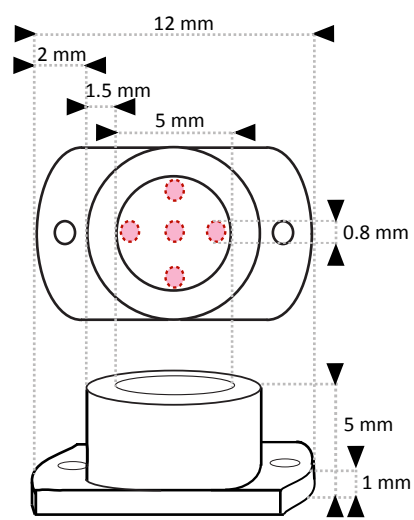
47 Zhang, X., et al. Osteoconductive effectiveness of bone graft derived from antler cancellous bone: an experimental study in the rabbit mandible defect model. *International Journal of Oral and Maxillofacial Surgery*. **41** (11), 1330-1337, doi:S0901-5027(12)00216-0, 10.1016/j.ijom.2012.05.014, (2012).

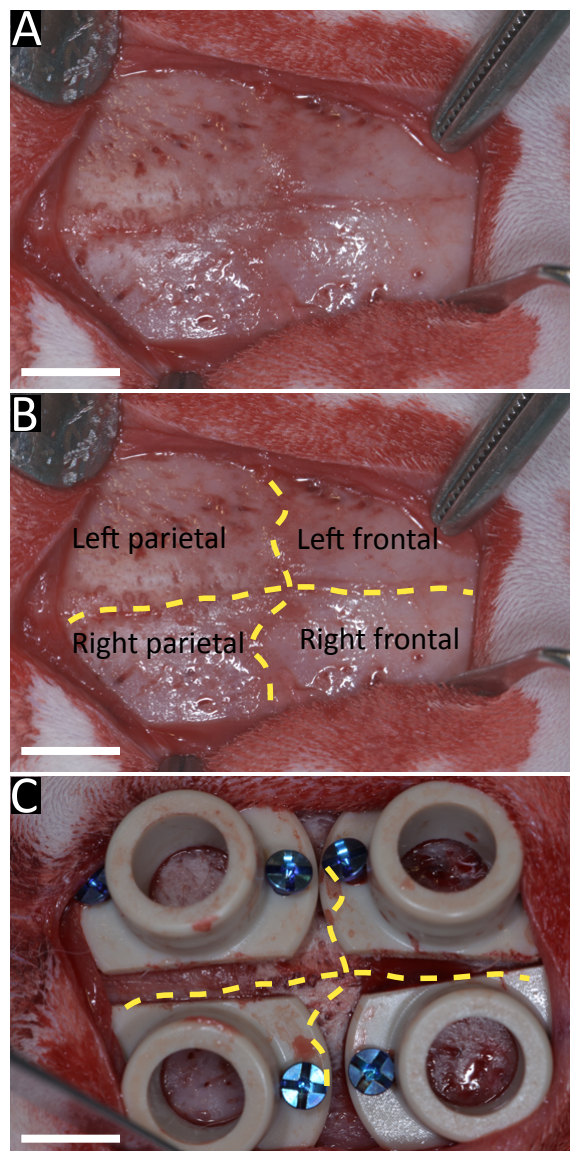
48 Bronoosh, P., et al. Effects of low-intensity pulsed ultrasound on healing of mandibular bone defects: an experimental study in rabbits. *International Journal of Oral and Maxillofacial Surgery*. **44** (2), 277-284, doi:S0901-5027(14)00354-3, 10.1016/j.ijom.2014.09.020, (2015).

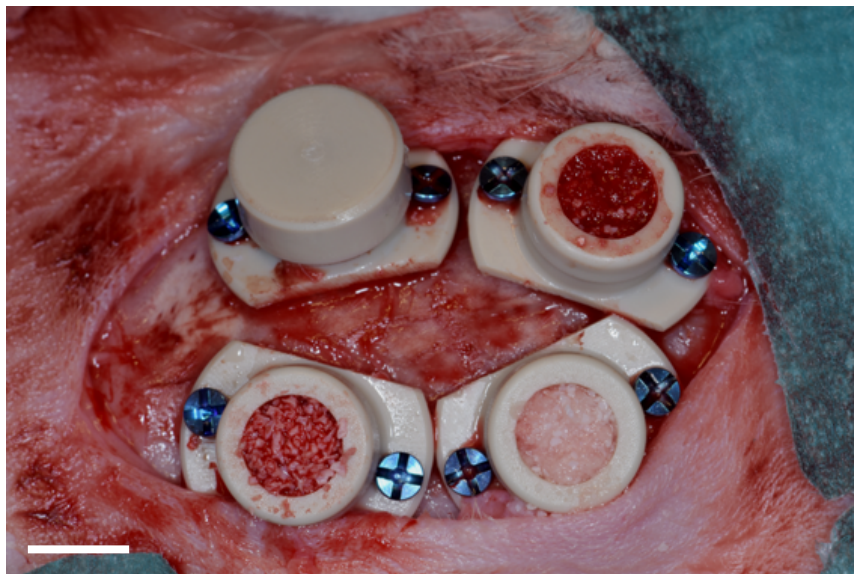
49 Gomes, F. V., et al. Low-level laser therapy improves peri-implant bone formation: resonance frequency, electron microscopy, and stereology findings in a rabbit model. *International Journal of Oral and Maxillofacial Surgery*. **44** (2), 245-251, doi:S0901-5027(14)00343-9, 10.1016/j.ijom.2014.09.010, (2014).

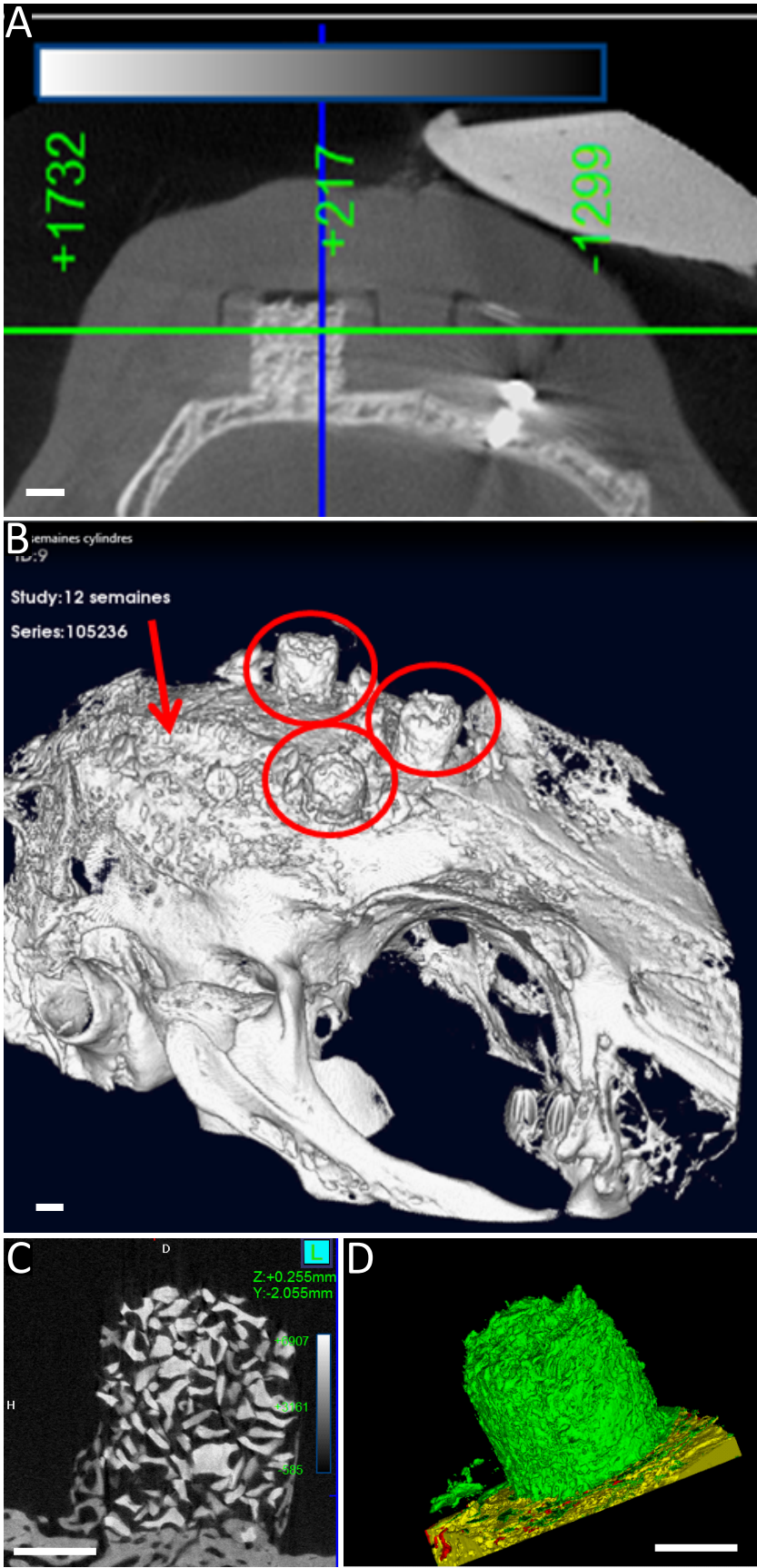
50 Lalani, Z., et al. Spatial and temporal localization of secretory IgA in healing tooth extraction sockets in a rabbit model. *Journal of Oral and Maxillofacial Surgery*. **62** (4), 466-472, doi:S0278239103012692, , (2004).

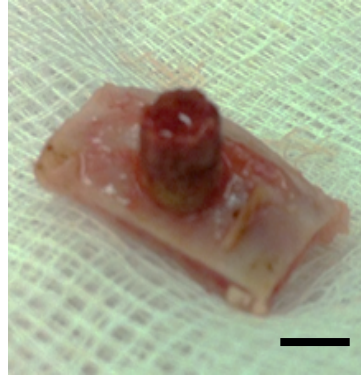
51 Osorio, L. B., et al. Post-extraction evaluation of sockets with one plate loss--a microtomographic and histological study. *Clinical Oral Implants Research*. **27** (1), 31-38, doi:10.1111/clr.12510, (2014).

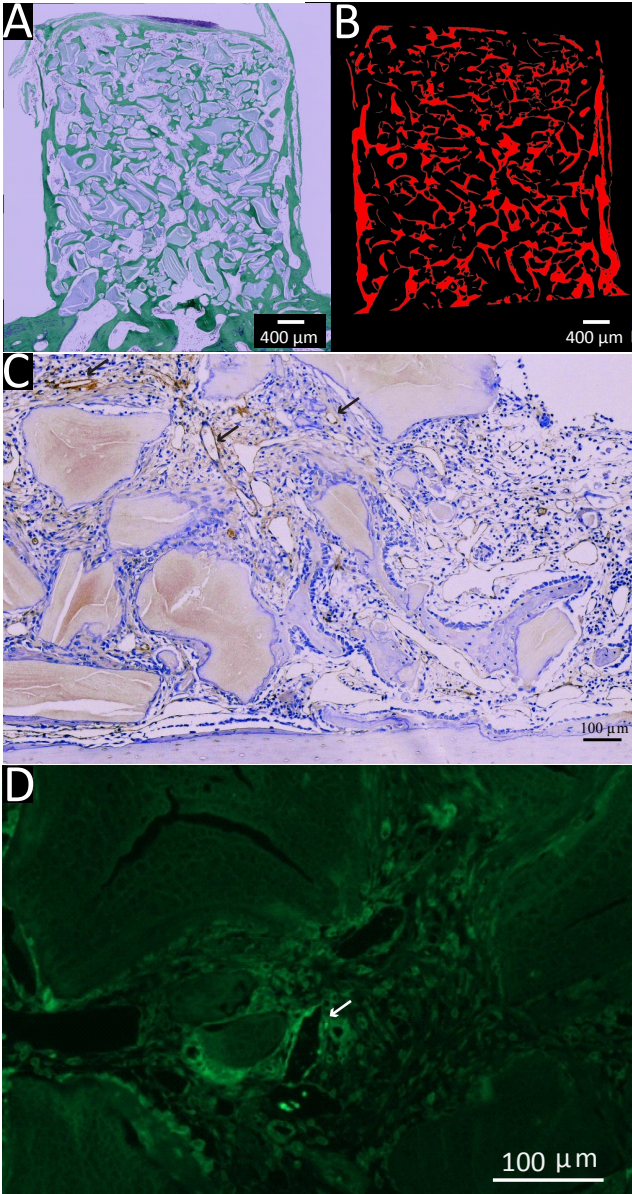












Name of Reagent/ Equipment	Company	Catalog Number
Drugs		
Enrofloxacin Baytril 10%	Bayer	
Fentanyl	Bischel	
Ketalar 50mg/ml	Pfizer	
Lidohex	Bichsel	
Opsite	Smith and Nephew	66004978
Povidone iodine 10%, Betadine	Mundipharma	
Propofol 2%	Braun	3538710
Rapidocain 2%	sintetica	
Ringer-acetate	Fresenius Kabi	
Rompun 2%	Bayer	
Sevoflurane 5%	Abbvie	
Sterile saline	Sintetica	
Temgesic	Reckitt Benckiser	
Thiopental Inresa	Ospediala	
Xylocaine 10% spray	Astra Zeneca	
Equipment		
Fresenius Vial pilot C	Imexmed	
Heated pad	Harvard Apparatus	
Suction dominant 50	Medela	
Suction tubing Optimus	Promedical	80342.2
Surgical motor	Schick dental	Qube
Ventilation	Maquet Servo1	
Material		
Cylinders and caps	Boutyplast	Customized
Manual self-retaining shaft	GlobalD	ACT1K
Mobile handle for self-retaining shaft	GlobalD	MTM
Self- drilling screws	GlobalD	VA1.2KL4

Surgical tray

Endotracheal tube Shiley diameter 2,5mm	Covidien	86233
Endotracheal tube Shiley diameter 4,9mm	Covidien	107-35G
Ethicon prolene 4-0	Ehticon	8581H
Forceps	Marcel Blanc	BD027R
Intubation catheter	Cook medical	
Needlle holder	Marcel Blanc	BM008R
Needles BD Microlance3	Becton Dickinson	300300/304622
Periosteal	HU-Friedy	P9X
Round surgical burs	Patterson	78000
Scalpel	Swann-Morton	
Scissors	Marcel Blanc	00657
Syringes Omnifix	Braun	4616057V
Venflon G22	Braun	42690985-01

Comments/Description

Antibiotic

For analgesia

Ketamine for anesthesia

Lubricating gel for the eyes

Sprayable dressing

anti-infective agent

For anesthesia

Local anesthesia

Volume compensation

Xylazin for anesthesia

For anesthesia

Buprenorphine hydrochloride, analgesia

For anesthesia

For intubation

Infusion pump

Drilling of intramedullary holes

composition: PEEK (poly ether ether ketone)

cross-drive screws composed by Titanium grade5, ISO 5832-3

For intubation

For intubation

Non-resorbable suture

145 mm

Guide for intubation

26G; 18G

0.8 mm in diameter, Drilling of intramedullary holes

n°10 and n°15

180 mm

5ml, 10ml and 50ml

Vasofix safety for the ear iv line



1 Alexia Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:

Calvarial model of bone augmentation in the rabbit for the assessment of bone growth and neovascularization in bone substitution materials.

Author(s):

L. Ray, A. Berone, C. Martinek, L. Schaub, T. Strandberg, R. Netti, I. Sailer, S. Scherer, S. Durrach

Item 1: The Author elects to have the Materials be made available (as described at <http://www.jove.com/publish>) via:

☒ Standard Access

☐ Open Access

Item 2: Please select one of the following items:

☒ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: **"Agreement"** means this Article and Video License Agreement; **"Article"** means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; **"Author"** means the author who is a signatory to this Agreement; **"Collective Work"** means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; **"CRC License"** means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: <http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode>; **"Derivative Work"** means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; **"Institution"** means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; **"JoVE"** means MyJoVE Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; **"Materials"** means the Article and / or the Video; **"Parties"** means the Author and JoVE; **"Video"** means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion

of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4 and 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum

rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to

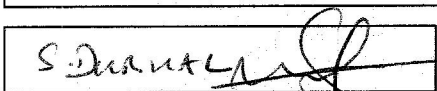
the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US\$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to be one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	DURUAL Stephane	
Department:	Laboratory of Biomaterials	
Institution:	University clinics of dental medicine, University of Geneva, CH	
Title:	Senior lecturer	
Signature:		Date: 03-07-2019

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140



UNIVERSITÉ
DE GENÈVE

FACULTÉ DE MÉDECINE

Clinique universitaire de
médecine dentaire

DIVISION de PROTHESE FIXE et
BIOMATERIAUX

S. Durual, PhD-PD
Senior lecturer

Tél. +41 22 379 40 93 – Fax +41 22 379 40 52
stephane.durual@unige.ch

Dr Vineeta Bajaj
JoVE, review editor

Geneva, April 15th 2019.

Object Revision, JoVE59976 Calvarial model of bone augmentation in the rabbit for the assessment of bone growth and neovascularization in bone substitution materials

Dear Dr Bajaj

Thank you for your thorough review of our submission and your quite positive assessment of our work.

The comments/criticisms/requests for amendments were addressed as follows:

For a sake of clarity, as numerous changes were demanded, we did not highlight the modifications within the manuscript, especially with respect to the filmed protocol already highlighted in yellow. Introduction and discussion were also largely edited (even if the content did not change substantially), the reason why we preferred not to highlight the whole section. The changes are listed below.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

✓ **Done**

2. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to ..."

✓ **Done: "Here we present a surgical protocol in the rabbit with the aim to assess bone substitution materials in terms of bone regeneration capacities. By using PEEK cylinders fixed on rabbit skulls, osteoconduction, osteoinduction, osteogenesis and vasculogenesis induced by the materials may be evaluated either on live or sacrificed animals."**



3. Please define all abbreviations during the first-time use.

✓ **Done**

- **PEEK, long abstract p2**
- **PMMA, introduction p3**
- **PEEK, introduction p3**
- **Im, 2.3.1 p4**
- **Iv, 2.3.2 p4**

4. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: Boutyplast, Leyment, FR; Global D, Brignais, FR; UNIGE breeding, Arare, 126 CH;

✓ **Done, all the commercial mentions were transferred in the MM table.**

6. Please revise the Introduction to include all of the following:

- a) A clear statement of the overall goal of this method
- b) The rationale behind the development and/or use of this technique
- c) The advantages over alternative techniques with applicable references to previous studies
- d) A description of the context of the technique in the wider body of literature
- e) Information to help readers to determine whether the method is appropriate for their application

✓ **Done, Introduction was rephrased and re-paragraphed. Goals and opened possibilities for interested people appear clearly.**

7. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.

✓ **Done, this was already included in section 1.3.**

8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly.

✓ **Done**

- **2.3.1 p4 "Note that this premedication will allow a simple, fast and painless intubation process...."**
- **2.6.3 p5 "Ensure that the screw head is adjusted with the surface of the cylinder tab."**
- **2.6.4 p5 "Ensure that the cylinder is hermetically fixed to the bone."**

9. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step.

✓ **Done**

- **Except for sections 2.3.1, 2.3.3, 2.3.6 (p4) that require some explications and clarifications.**

10. All steps should be numbered. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.

✓ **Done**

11. The Protocol should contain only action items that direct the reader to do something and should be in complete sentence describing all specific details related to the action.

✓ **Done,**

- **Except for sections 2.3.1, 2.3.3, 2.3.6 (p4) that require some explications and clarifications.**

12. Please ensure that is a single line space between each step, substep, notes and caution of the protocol section.

✓ **Done**

13. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?

- ✓ **Done, please note that anesthesia and surgery will be filmed. Some details will appear much clearer as soon as the video support will take place.**

14. 2.3.1 How do you ensure the depth of anesthesia?

- ✓ **Done:** “2.3.1 Sedate the animals by intramuscular (im) injection of ketamine (25 mg/kg, 50mg/ml, 0.5ml/kg) + xylazin (3mg/kg, 20mg/ml, 0.15ml/kg). Wait ca. 20 min for the animals to sleep deeply (**complete muscular atony**). Note that this premedication will allow a simple, fast and painless intubation process. (**Deep analgesia and anesthesia is induced as described in 2.3.11.**)”

15. 2.3.2.: Do you first perform sedation and then anesthesia? What is the significance of doing both?

- ✓ **Done,** “2.3.1 Sedate the animals by intramuscular (im) injection of ketamine (25 mg/kg, 50mg/ml, 0.5ml/kg) + xylazin (3mg/kg, 20mg/ml, 0.15ml/kg). Wait ca. 20 min for the animals to sleep deeply (complete muscular atony). **Note that this premedication will allow a simple, fast and painless intubation process. (Deep analgesia and anesthesia is induced as described in 2.3.11.)**”

16. 2.4.2: Do you perform the shaving on the surgical site? How do you maintain the sterility of the area?

- ✓ **Done, some more precisions were added from 2.4.2 to 2.4.5 (p5):**

“2.4.2 Shave the skull

2.4.3 Disinfect the site by scrubbing the skin with povidone iodine (10%).

2.4.4 Drap the rabbit with a sterile surgical drape and cut out an access area for the skull.

2.4.5 Disinfect another time the surgical site with povidone iodine (10%).”

17. 2.5.2: How big is the incision? How do you perform the incision?

- ✓ **P5, Done:** “2.5.2 Incise through the skin (with a scalpel) along the calvarial sagittal line, from the orbits to the external occipital protuberance (ca. 4 cm in length). Ensure that the periosteum is incised.”

18. 2.6.1: when was this suture performed?

- ✓ **Done, p5, 2.6.: “Locate the median and coronal sutures on the skull (Fig. 2 A-B). Note that these anatomical lines draw a cross. The cylinders will be placed in each of the quarters defined by the cross, avoiding that the light of the cylinder is above the suture (Fig. 2C).”**

19. 3: Stop how?

- ✓ **Done p6: “3.1 Stop analgesia and anesthesia (propofol and fentanyl perfusion arrest) and check the recovery of autonomous breathing.”**

20. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

- ✓ **Done, from sections 2.3 to 3.5. Some modifications were achieved in this section and appear highlighted in green. Please consider all the section from 2.3-3.5 to be filmed.**

21. Will the animal be available on the day of filming?

- ✓ **Yes. We will need to plan ca. 2-3 months in advance to order the animals, rent the surgical room and plan the animal housing.**

22. Please consider moving all the materials to the materials section.

- ✓ **Done: Sections 1.1, 1.2 and 1.3 are essential for the protocol and part of the development we added to the model. Therefore, it's necessary that these sections are maintained into the text. A transfer into the MM table would “dilute” the importance of these 3 points.**

23. Please ensure that the figures are described in order. Figure 1 should be described before figure 2.

- ✓ **Done**

24. Please discuss all figures in the Representative Results. However, for figures showing the experimental set-up, please reference them in the Protocol.

- ✓ **Done**

25. Please include a title and a description of each figure and/or table. All figures and/or tables showing data must include measurement definitions, scale bars, and error bars (if applicable). Please include all the Figure Legends together at the end of the Representative Results in the manuscript text.

- ✓ **Done**

26. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”



- ✓ **The figure 1 from the initial version was removed, as proposed by reviewer 2. No need for copyright permission anymore.**

27. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

- a) Critical steps within the protocol
- b) Any modifications and troubleshooting of the technique
- c) Any limitations of the technique
- d) The significance with respect to existing methods
- e) Any future applications of the technique

- ✓ **Done: the discussion was revised and clarified. The protocol is discussed from a technical and a scientific point of view and compared to other existing models. A mention was added for the postoperative care and the accompaniment by professional anesthetists and veterinaries.**

28. Please expand the journal titles in the reference section.

- ✓ **Done**

29. Please alphabetically sort the materials table.

- ✓ **Done. The sections were preserved and ordered alphabetically. Within each section, the products-materials were ordered alphabetically.**



Reviewers' comments:

Reviewer #1:

Minor Concerns:

The indication of the scale is not very clear on some figures.

✓ **Done, the scale bars were updated, especially on figure 6**

Reviewer #2:

Minor Concerns:

1. There are two "2.3.4" in the text.

✓ Modified

2. Figure 1 has less relevant to the paper.

✓ **The figure 1 was suppressed from the revised version**

3. In figure 2, it is suggested to add more information. For example, the height of the cylinder, the size of the screws, and the size of the five small holes.

✓ **Done**

4. In 2.1, periosteal is listed, but it seems not being used in the whole process. Please have a check.

The periosteal is needed to elevate the periosteum, as mentioned in section 2.5.3 :

"2.5.3 Gently elevate the periosteum (with a periosteal) on both side of the incision."

Dr. S. Durual