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Abstract:

Introduction: Many studies have aimed to investigate the regeneration potential of bone substitutes through animal models at different defect sites, where the bone healing mechanism varies due to developmental, structural and functional differences. This study aims to develop a rabbit model with two functionally different (non-load-bearing calvarias and load-bearing mandibular) critical-sized defects (CSD) in one rabbit.

Material & Method: The comparison of the "gold standard" autograft to a sham (no graft) control was undertaken in order to validate this model; at the same time, a 3D-printed biphasic calcium phosphate scaffold was implanted to test its utility in the evaluation of new bone substitute materials. Twenty rabbits were selected with both a 10 mm calvaria defect and a 11 mm bicortical semi-cylindrical mandibular defect. The animals were euthanized at 4 and 12 weeks once surgery, microcomputed tomography and histological analysis had been performed.

Results: In the case of the calvaria, the results for the non-healing sham group compared with the healing of those that had undergone the autograft validated the CSD model. But the mandibular defect was not validated, due to the particularity of mandible high mechanical stress and infectious risk.

Discussion: This study showed for the first time that rabbits have a high tolerance for the bilateral double-site CSD model under consideration; and further studies are essential to modify and improve the design of mandibular CSD.

Keywords: Bone regeneration; Rabbit model; Calvaria defect; Mandible defect; Micro-computed tomography

Introduction

The reconstruction of large bone defects poses many challenges in implant dentistry and cranio-maxillofacial surgery. There are several critical-sized defect (CSD) models used for assessing the bone regeneration in different anatomical regions such as the cranium [1–3] and the alveolar bone (maxilla and mandible) [4,5] that have distinct bone healing processes due to structural, functional and developmental differences between the two [6]. Moreover, mandibular reconstruction is influenced by a combination of high mechanical stress and infectious risk due to the proximity of the septic oral cavity and the presence of the teeth. Overall, the calvarial defect model and mandibular alveolar bone defect model are well described for non-load-bearing and load-bearing patterns, respectively.

Rabbits rank first among all animal models used in evaluating specific tissue responses, tissue regeneration and the drug delivery of the biomaterial in the cranio-maxillofacial region, due to their short cycle of breeding and skeletal maturity, ease of handling and low housing cost [7]. There are various studies in the literature employing either the calvarial or mandible defect to evaluate the safety and efficacy of bone substitutes. However, considering the differences structurally and functionally between a defect of the non-load-bearing calvaria and load-bearing mandibular defects [6], it is necessary to evaluate the developed bone substitute biomaterials in both types of defect, to confirm the bone repair efficacy and applicability of tested material in cranio-maxillofacial region. However, the study of two defect sites requires the doubling of animals considering a single site defect model (not co-existing in one rabbit), and comparing the same material in different bone defect sites could be influenced by inter-individual variability or evaluation bias. Moreover, the existing models also vary by breed/age of animal or shape, size and

location of the defect. The validation and standardization of specific defect designs are therefore necessary.

This study is the first to develop a rabbit model with two functionally different (non-load-bearing calvaria and load-bearing mandibular) CSD in one rabbit. The validation of this double-site CSD model for bone regeneration research has been conducted by comparing the “gold standard” autograft with a sham, in which no graft has taken place. At the same time, a 3D-printed biphasic calcium phosphate (3D-BCP) scaffold has been implanted to test the utility of this model in the evaluation of bone substitute materials and tissue engineering constructs. A comprehensive understanding of the benefits and eventual limitations of this new model will aid in rigorously evaluating a novel bone implant material and device.

Materials and Methods

Biomaterial graft

A custom-designed 3D-BCP scaffold was used to test the effectiveness of the developed animal model. This was generously provided by CryoCeram. In brief, the implantable samples were fabricated from synthetized BCP powder (40 wt% hydroxyapatite - 60 wt% β-tricalcium phosphate) using a 3D printer. The CryoCeram 3D printer shapes ceramic through a photo-polymerization process. Two types of 3D-BCP samples in the form of a macroporous mesh were produced to fit the dimension of the calvarial and mandibular defects, respectively: disks of 9.5 mm diameter for a thickness of 1.5 mm, and half-disks of 10 mm diameter, height of 4.8 mm and a maximal thickness of 2.8 mm.

Animals and surgical procedures

All experimental animal procedures were carried out in the Animal Facility of the University of Lille "Plateforme Ressources Expérimentales - D.H.U.R.E", according to the current European regulations regarding the protection of animals used for scientific purposes (Directive 2010/63/EU). Protocols and surgical procedures have been evaluated and approved by the French Minister of Research and the local Ethical Committee for Animal Experimentation as animal use project No. 11664.

Twenty adult male white New Zealand rabbits, weighing 3.5–4 kg were used, and they were divided into two main groups according to the type of graft material: autograft and 3D-BCP. Each group was then separated into two groups based on when they were euthanized after the operation (4 weeks or 12 weeks): each with n=5. For both calvarial and mandibular defects, the graft (*autograft* or *3D-BCP*) was implanted on the left side, and the right side was left *empty* (n=20).

Immediately prior to surgery, each animal received an intramuscular injection of buprenorphine hydrochloride (0.1 mg/kg body weight) for postoperative analgesia and a subcutaneous injection of 0.2 mL 2.5% enrofloxacin solution for perioperative antibiotic coverage. Intramuscular injections of ketamine hydrochloride at 35 mg/kg body weight and xylazine hydrochloride at 5 mg/kg body weight were administered as a pre-anesthetic induction. General inhalation anesthesia was induced by inserting a V-Gel supraglottic airway device and delivering a 2.5–3% isoflurane/O_2 gas mixture and the anesthetic state was maintained with 1.5–2% Isoflurane/O_2 gas mixture. The calvarial and perimandibular areas were shaved and scrubbed with Chlorhexidine solution.

Mandible defect (rabbit in supine position): A shallow midline incision along the inferior surface of the mandible from the chin to a line perpendicular to the gonial angles of the mandible was made, and the superficial cervical fascia was then incised to expose the masticatory muscles. Both sides were then sequentially operated. A second incision through the periosteum along the inferior border of the hemimandible was performed so that a subperiosteal flap could be developed to help elevate and retract the soft tissues. To avoid periosteum osteogenesis interference, the buccal periosteum at the surgical site was totally removed during surgery. A 11mm trephine burr was used to create a bicortical semi-cylindrical osteotomy at the mandibular basal edge (Figure 1) using a surgical handpiece operating at 15,000 rpm along with copious saline irrigation.

The important landmarks for locating the defect were as follows: distal border of the defect at 5 mm from the preangular notch; and the superior border of the defect at 6 mm from the basal edge (Figure 1). On the left side, the defect was filled with a 3D-BCP scaffold or autograft (bone fragments harvested during the homolateral defect creation), while the other side was left empty.

The muscle and subcutaneous layers were closed with continuously resorbable 3-0 Monocryl Plus sutures and the skin closed with interrupted 3-0 Vicryl sutures.

Calvaria defect (rabbit in prone position): A midline cranial incision was performed up to the periosteum. The overlying periosteum was then excised to expose both parietal bones. A bicortical circular osteotomy was performed on each side of the midline using a 10 mm diameter trephine burr on a surgical handpiece operating at 15,000 rpm along with copious saline irrigation. The landmarks for locating the defect were as follows: 2 mm externally to the midline and 2 mm distally to the frontoparietal suture (Figure 2). During the osteotomy, care was taken not to injure the dura mater under the bone. Then, using a thin osteotome, the circular bicortical bone segment was mobilized and luxated. On one side the defect was left empty, while on the other side, the defect was filled with 3D-BCP disks or with bone autograft (bone fragments harvested during the homolateral defect creation). Continuously resorbable 3-0 Monocryl Plus sutures and interrupted 3-0 Vicryl sutures were used to close the overlying soft tissues and skin in layers.

Four injections of buprenorphine (0.02 mg/kg) and enrofloxacine (5 mg/kg) were given 8–12 h apart after surgery for continued postoperative analgesia and antibiotic prophylaxis. Food and water were distributed *ad libitum*. All animals were closely monitored and weighed regularly for 10 days following surgery to evaluate any acute weight loss.

Tissue harvesting

At 4 and 12 weeks following surgery, rabbits were euthanized by intravenous administration of 1 mL of T61 solution. The mandibular and calvarial bone specimens were harvested with the aid of a dental diamond disc and placed in 10% neutral buffered formalin for 48 to 72 hours.

Micro-computed tomography (micro-CT) imaging

The harvested mandibular and calvarial bone specimens were imaged using a Skyscan 1172 X-ray microtomography. Datasets were reconstructed and analyzed using NRecon and CTAn software. From axial cross-sections, the region of interest was defined as a circle of 10 mm diameter centered on the defect center; a reference cross-section was determined as a median cross section from a volume that includes all bone within the region of interest. A total of 100 cross sections around the reference cross section were used as our volume of interest. The reoriented datasets were filtered with Gaussian filter for 3D-BCP and Anisotropic Diffusion filter for autograft and empty samples. Filtered datasets were binarized with Otsu algorithm [8]. 3D models were rendered from binarized datasets with the Marching Cubes 33 algorithm [9]. Morphometric measurements were calculated from 3D models for bone volume (BV), tissue volume (TV) and BCP scaffold volume.

DataViewer and CTVox software was used for 2D and 3D illustration.

Histological evaluation

Following the micro-CT analysis, the samples of 4 and 12 weeks were decalcified with ethylene-diamine-tetra-acetic acid (EDTA) for 3 weeks for histological evaluation. Each sample was rinsed thoroughly with phosphate-buffered saline (PBS), and subsequently dehydrated with a graded series of ethanol and embedded in paraffin blocks. The longitudinal sections of 4 μm thickness were prepared with a microtome. Sections were stained with hematoxylin-eosin-saffron and then examined qualitatively with optical microscopy.

Statistical methods

Statistical analysis was performed with Prism v5.03.0001 regarding the micro-CT data. Results were expressed as mean and standard deviation for each experimental group. Non-parametric

analysis using Kruskal-Wallis ANOVA was used to assess the difference between the three groups. The α risk was set at 5%.

Results

Animal health and macroscopic observations.

All animals recovered after surgery. As the bicortical semi-cylindrical defect at the mandible damaged the tooth roots bilaterally, their quality of life was affected. Food intake and weight gain in each animal gradually improved within 10 days, thus implying that the rabbits had a good tolerance for the studied bilateral double-site CSD model.

At predetermined time points of 4 and 12 weeks, the calvaria and mandible were harvested and studied macroscopically. Partial healing of the calvaria at 4 weeks and almost complete healing at 12 weeks in the autograft group, while there was only a fibrous cap at 4 weeks and 12 weeks (associated with scattered ossified areas) in the empty group (Figure 2). In the 3D-BCP group, there was a good integration of the mesh disk at 4 weeks and a partial covering by osseous tissue at 12 weeks. No signs of inflammation and necrosis were observed in any group of fresh samples. Both autograft and the 3D-BCP were integrated well and were stable at the defect site.

Concerning the mandibular site (Figure 2), the autograft fragments were not stable at the defect site and some small fragments of the autografts were displaced from the defect, while 3D-BCP were stable at the defect site without any displacement. The bone healed from the defect edges at 4 and 12 weeks both in the autograft and empty groups. In the 3D-BCP group, the half-disks were well integrated at 4 weeks and partially covered by osseous tissue at 12 weeks.

Micro-CT imaging

Micro-CT was used to demonstrate the qualitative evaluation of healing in calvarial and mandibular defects. (Figure 3). The quantitative data for the bone regeneration rate was calculated and represented as a BV/TV ratio (Figure 4).

Calvaria: BV/TV ratio in the autograft group (4 weeks: 36.8%, SD 1.7%; 12 weeks: 42.1%, SD 3.1%,) was statistically significantly higher ($p<0.05$) than the empty group (4 weeks: 15.1%, SD 1.17%; 12 weeks : 23.08%, SD 1.8%) and the 3D-BCP (4 weeks: 17.08%, SD 0.08%; 12 weeks: 22.1%, SD 1.2%) group. The 3D-BCP group showed a slightly higher BV/TV ratio without a statistical difference from the empty group. More bone tissue was therefore found in the autograft group. The results followed the same evolution in each group between 4 weeks and 12 weeks. Thus, the efficacy of the autograft is confirmed. The model of CSD in the calvaria is therefore validated. Naked 3D-BCP reconstruction remains inferior to autograft regarding bone-repair efficacy.

Mandible: The BV/TV ratio at 4 weeks in the autograft (28.4%, SD 8.57%) and empty groups (24.01%, SD 8.35%) were almost the same ($p>0.05$). Furthermore, the BV/TV ratio seemed to diminish between 4 and 12 weeks. At 12 weeks, the empty group (19.85%, SD 4.45%) showed a relatively greater ratio ($p<0.05$) than the autograft group (15.2%, SD 3.87%). Although the autograft is considered a gold standard, the displacement/instability of the bone fragments at the defect site might be the reason for the failure of bone regeneration. In contrast, the 3D-BCP showed a significantly higher ($p<0.05$) BV/TV ratio at both time points 4 weeks (57%, SD 16.9%) and 12 weeks (45.27%, SD 8.11%) compared with the empty and autograft groups.

Histology

Calvaria: The 4 µm thick longitudinal sections were characterized for bone healing at the defect site. No sign of inflammation was observed in any of the three groups (Figure 5). In the empty group, there was only a fibrous cap covering the defect at 4 and 12 weeks. A bridge-like bone front at the defect margins and a few bone islands with loose connective tissue at the center of the defect were observed both at 4 and 12 weeks. However, at 12 weeks, the number of bone islands was relatively higher at the center. In an autograft group, at 4 weeks osseous tissue of good quality was observed in which the density was higher at 12 weeks. In the 3D-BCP group, at 4 weeks BCP mesh and the loose connective tissue occupied the space at the center of the defect and osseous tissue of good quality was found around the ceramic mesh. At 12 weeks, the quantity of new bone tissue was increased. A thin layer of inflammatory cells was found around the mesh of 3D-BCP signaling its ongoing resorption. These results were concordant with the results of micro-CT.

Mandible: On the mandibular site (Figure 6), the same aspect in the autograft and empty groups was observed, reticulated osteogenesis of low density with numerous areas of fibro-adipose tissue was found at 4 weeks. At 12 weeks, the density remained low, and the areas of fibro-adipose tissue persisted, creating a fibrous pseudo-callus. Frequent and relatively large areas of apical periodontitis were found. These were related to the dental trauma during the osteotomy and impacted the quality of healing. In the 3D-BCP group, similar results were observed to those observed at the calvarial site: osseous tissue of good quality associated with a thin layer of inflammatory cells around the ceramic mesh. Apical periodontitis was only rarely found. The absence of fibro-adipose deposition was concordant with that found by micro-CT.

Discussion

Of the studies that investigated bone scaffolds in rabbit models, none evaluated scaffolds in the load-bearing mandible area and the non-load-bearing calvaria for bone regeneration in one rabbit simultaneously [10–21]. A model of co-existing calvarial and mandibular CSD in one rabbit can avoid the influence of interindividual variability and evaluation bias on the comparison.

Validation of a new animal model of bone regeneration often requires back translation from clinical applications to animal models, the comparison of the "gold-standard" bone autograft [22–24] with a non-treated defect (empty) will fulfil the criteria for new model validation. In addition, we have also grafted a custom-made (via 3D ceramic printing technique) BCP implant into the created defect. As BCP is common in clinical practice [25] and also has a good potential as a scaffold due to its osteoconductive, and biodegradable properties [19,26].

At the calvarial CSD, the obtained results matched the expected ones. The absence of bone graft (in the sham) led to a non-complete bone defect repair; hence the defect was of critical size. The bone autograft offered satisfactory bone healing, as expected from the clinical gold standard of bone reconstruction by increasing the BV/TV ratio of new bone tissue with time. This implies the good clinical relevance of this rabbit calvaria CSD to the human bone defect. The 3D-BCP used in the study showed bone regenerative potential at the calvarial CSD, while the percentage of new bone formation was higher than the sham group but still lower than the autograft group. Nevertheless, the results with naked 3D-BCP are encouraging, since biofunctionalization of the 3D-BCP with pro-osteogenic biological factors may further improve its efficacy. Furthermore, histological analysis revealed that in the sham group bone formation particularly occurred from

the margins of the defect showing the wedge-shaped bone fronts; while in autograft and 3D-BCP, the new bone formation within the defects relied on the presence of preexisting material structure to act as a lattice for osteoconduction. These may explain why autograft and 3D-BCP show better bone repair efficacy than a sham.

At the mandibular level, the autograft group did not perform better than the sham group. This failure hampers the validation of this design of defect model, and brings out again the fact that the environment (notably biomechanical factor) for mandible defects is different from calvarial defects. Since the defect site was filled by the harvested fragmented bone (autograft), although musculo-aponevrotic planes opposite the graft were carefully sutured, the mechanical strain due to mastication may have mobilized the bone chips between themselves and against the mandible itself. In our study, the proof of the instability of autograft fragments was found by both micro-CT and histology; 3D-BCP was stable and better-integrated at the defect site than autograft. Graft instability is a major factor of failure, as is well known in human clinical experience [24,27,28]. At the calvarial level, however, there is no mechanical strain, and this may explain the success of the autograft bone fragments at this spot, despite similar handling. In any case, a modified surgical procedure would be necessary to circumvent this problem of graft stability, such as titanium mesh coverage.

Another hypothesis for the failure of autograft in the mandibular defect could be linked with dental lesion and nerve injury (odontogenic infections). The rabbit dental roots are rather long, nearly reaching the basilar edge and the inferior alveolar nerve, which is responsible for the sensitive innervations of the mandible, teeth, lips and chin, is directly under the dental apexes and therefore at the basilar level [29,30]. When causing CSD in the corpus, dental lesion is nearly inevitable and nerve injury is very frequent. Published models of mandibular rectangular defects at the basilar

edge of the horizontal branch anterior to the angular notch [11,18,19,21]. Despite no mention of dental or nerve lesion, nor of infectious complications, given the height of the defects, dental apexes and inferior alveolar nerve damage could not be avoided. From their 12-week postoperative radiography, we have clearly observed rhizalysis facing the defect and even periapical radiotransparent lesions, despite the lifelong continuous dental growth of rabbits [19,29]. Such complications bonding with odontogenic infection also occurred in our study, i.e., frequent and relatively large apical periodontitis related to the dental trauma during the osteotomy, shown by micro-CT and histology.

The development of co-existing calvarial and mandibular CSD in one rabbit benefit the study in approximating clinical conditions while minimizing the animal number. But given the failure to validate the mandible defect in this study, further studies with modifications to the design of mandibular CSD should be pursued. After all, the good survival rate demonstrated that rabbits have a high tolerance for the double-site CSD model studied here. In order to improve the future study of mandibular CSD, the teeth located in the defect may need to be extracted [10]. Two-step surgical procedures, as done in human patients, would therefore be necessary in creating mandibular CSD: dental extraction first, and, after gingival healing, performing the bone CSD.

But a two-step surgical protocol like this with an interval in which gingival healing can take place would be very time-consuming. Moreover, extracting long-root molars brings an added level of technical difficulty. A 10-mm “partial-thickness” cylindrical defect [15] removing only the lateral bony cortex (without damaging tooth roots), could circumvent the problem of dental extraction. Moreover, in a “partial-thickness” defect model, an intrabony defect with the intact tooth roots and lingual cortical plate, stabilizes the bone fragments or biomaterial granules at the defect site. Given the advantages of this model, future research is a worthwhile topic to investigate.

To conclude, this study demonstrated the feasibility of a double-site CSD model in rabbits, comprising the non-load-bearing calvaria and load-bearing mandible defects in one rabbit. From a clinical perspective, the distinct regenerative cell population and environment present in the calvaria, and mandible make the evaluation of the bone repair material more comprehensive after testing at both sites. As such, the development of this model has a wide application in approximating clinical conditions for both calvarial and mandibular reconstruction in the same individual while minimizing the heterogeneity between the studies and animal number. The calvarial defect design has been validated, however, the design of the mandibular defect has not yet been validated, due to the particularities of the mandible, i.e., high mechanical stress and risk of infection. This reinforces that it is worth bearing in mind just how distinct the two defect sites are from one another other, both functionally and structurally. Nevertheless, this study showed for the first time that rabbits have a high tolerance for the double-site critical-sized defect model investigated here. And the encouraging results of naked 3D-BCP scaffold suggest that it is an appropriate model for testing the efficacy of bone substitute materials. Further studies are required to determine the design modification in mandibular CSD.

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Conflicts of interest:

The authors declare that they have no conflicts of interest. All authors have viewed and approved the draft submitted.

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Figure legends:

Figure 1: Creation of rabbit mandibular defect with a diameter of 11 × 6 mm.

A) and B) showing day 0 defect with EM (empty), AG (autograft), and 3D-BCP;

C) and D) at 12 weeks, showing the integration and bone healing of the of EM (empty), AG (autograft), and 3D-BCP. The dotted line at D, E and F denote the defect area.

Figure 2: Calvarial critical-size defect in rabbits with a diameter of 10 mm.

A) and B) showing day 0 defect with 3D-BCP, EM (empty), and AG (autograft);

C) and D) at 12 weeks, showing the integration and bone healing of the of 3D-BCP, EM (empty), AG (autograft).

Figure 3: Micro-CT axial cross sections showing a representative of 4- and 12-week defect healing.

A), B), C), D), E) and F) showing the calvarial defect of EM (empty), AG (autograft) and 3D-BCP;

G), H), I), J),K) and L) showing mandibular defect of EM (empty), AG (autograft) and 3D-BCP.

Figure 4: Quantitative analysis of micro-CT using BV/TV ratio for (a) calvarial and (b) mandibular defect at 4 and 12 weeks. Error bars indicate the means ± standard deviation. (*) indicates the statistically significant difference ($p<0.05$) in percentage of bone fill between the groups, EM (empty), AG (autograft) and 3D-BCP.

Figure 5: Calvaria sections stained with H & E showing the representative of tissue repair at A) 4 weeks of EM (empty), AG (autograft) and 3D-BCP; B) 12 weeks EM (empty), AG (autograft) and

3D-BCP. The dotted line denotes the defect area. The magnified images of the rectangular dotted line show the NB, CB and BM. The scale bar indicates 1000 μm. Abbreviations: CB: collagen bundles; NB: new bone; BM: bone marrow; BCP: Biphasic calcium phosphate scaffold.

Figure 6: Mandible sections stained with H & E showing the representative of tissue repair at:

A) 4 weeks of EM (empty), AG (autograft) and 3D BCP; Empty, 4 weeks: abundant fibrous tissue in the bone tissue (black arrow) and apical periodontitis (yellow arrow); Autograft, 4 weeks, abundant fibrous tissue in the bone tissue (black arrow); 3D-BCP, 4 weeks, bone tissue touching the 3D-BCP with areas of fibrous tissue (black arrow).

B) 12 weeks EM (empty), AG (autograft), and 3D-BCP. Autograft, 12 weeks, fibrous tissue in the bone tissue (black arrow); Empty, 12 weeks, fibro-adipose tissue in the bone tissue (black arrow); 3D-BCP, 12 weeks, bone tissue touching the 3D-BCP filling the defect with areas of osteoid tissue (black arrow). The scale bar indicates 200 μm. Abbreviations: CB: collagen bundles; NB: new bone; BM: bone marrow; BCP: Biphasic calcium phosphate scaffold; CN: connective tissue.