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Reliable critical sized defect rodent model for cleft palate research

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ABSTRACT

Background: Suitable animal models are necessary to test the efficacy of new bone grafting therapies in cleft palate surgery. Rodent models of cleft palate are available but have limitations. This study compared and modified mid-palate cleft (MPC) and alveolar cleft (AC) models to determine the most reliable and reproducible model for bone grafting studies.

Methods: Published MPC model ($9 \times 5 \times 3$ mm^3) lacked sufficient information for tested rats. Our initial studies utilizing AC model ($7 \times 4 \times 3$ mm^3) in 8 and 16 weeks old Sprague Dawley (SD) rats revealed injury to adjacent structures. After comparing anteroposterior and transverse maxillary dimensions in 16 weeks old SD and Wistar rats, virtual planning was performed to modify MPC and AC defects dimensions, taking the adjacent structures into consideration. Modified MPC ($7 \times 2.5 \times 1$ mm^3) and AC ($5 \times 2.5 \times 1$ mm^3) defects were employed in 16 weeks old Wistar rats and healing was monitored by micro-computed tomography and histology.

Results: Maxillary dimensions in SD and Wistar rats were not significantly different. Preoperative virtual planning enhanced postoperative surgical outcomes. Bone healing occurred at defect margin leaving central bone void confirming the critical size nature of the modified MPC and AC defects.

Conclusions: Presented modifications for MPC and AC models created clinically relevant and reproducible defects.

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1. Introduction

Repair of alveolar clefts remains a challenging surgical procedure with a limited number of treatment options for cleft patients including gingivoperiosteoplasty and secondary bone grafting. The gingivoperiosteoplasty technique is utilized to permit bone healing, by creating a periosteal tunnel between the cleft alveolar segments. Together with orthodontics, the alveolar gap is reduced and adequate alveolar bone regeneration has been reported in ~60% of patients (Santiago et al., 1998). The use of autologous bone, commonly obtained from the iliac crest, is an alternative treatment modality. However, donor site morbidity, persistent pain, infection, fracture, scarring, and paraesthesia may hamper this method (De Riu et al., 2008). Therefore, developing innovative grafting therapies, based on artificial bone grafts, would have a tremendous impact on cleft palate reconstruction. The main benefits of these grafts would contribute to a reduction in hospital stay, reduced donor site morbidity for the patient and an improvement in the overall cost for the procedure to the patient and health care system.

In order to obtain the ideal bone graft alternative, an appropriate animal model is essential for testing new bone grafting therapies. Currently, the animal models of cleft palate, which are available for experimental testing, include both congenital and surgically-created clefts. Teratogenic and transgenic mouse cleft models have also been established utilizing teratogenic pharmaceuticals (phenytoin and corticosteroids) and genetic mutations (Twirler gene (Tw/Tw) in mice) respectively, resulting in either unilateral or bilateral clefts (Melnick et al., 1981; Gong et al., 2000; Yamada et al., 2006; Juriloff and Harris, 2008). These models significantly enhanced our understanding of etiologic factors contributing to cleft development, but they were not utilized for development of new grafting therapies (Nguyen et al., 2009a). This could be due to the small maxilla size in mice models as well as the variability in cleft size and anatomical location. Therefore, surgical cleft models were considered more suitable for efficacy testing of new biomaterials for bone grafting as long as the created surgical

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defects are critically sized, preventing spontaneous healing without intervention (Spicer et al., 2012).

Surgical clefts have been created in primates (Boyne, 1974; Boyne et al., 1998), dogs (Mayer et al., 1996), goats (De Ruiter et al., 2011), and rabbits (Puumanen et al., 2005; Sawada et al., 2009). However, high husbandry and operational expenses limit the use of large animal models. Therefore, rodent models are the most commonly used animals in biomedical research for testing the efficacy of new therapies since they are readily available with low operational and husbandry cost. Two surgical rodent models of cleft palate are currently published in the literature: mid-palate cleft (MPC) model and the alveolar cleft (AC) model. One study (Mehrara et al., 2000) developed the MPC model ($9 \times 5 \times 3$ mm^3) and demonstrated complete bone healing by 12 weeks. However, this study did not report the strain, age, or weight for the tested rat model. It also provided semi-quantitative assessment of bone formation based on anteroposterior and mediolateral radiographs that are limited by: superimposition of structures, variable magnification, and distortion. This led to the development of the AC model ($7 \times 4 \times 3$ mm^3) in 8 weeks old Sprague Dawley (SD) rats (Nguyen et al., 2009a). Based on histology and micro-computed tomography (μCT), there were no significant difference in bone formation in the defect site between 4, 8 and 12 weeks, making it a suitable critical size defect. However, bone grafting using bone morphogenetic protein (BMP-2) based scaffolds in the established AC model (Nguyen et al., 2009a) did not reveal significant effects for BMP-2 on bone formation over 12 weeks (Nguyen et al., 2009b). Authors justified these results as being due to the burst release kinetics of BMP-2 from the collagen scaffold and incompletely sealed oral tissues resulting in local leakage of the protein away from the defect site. The μCT images of the created alveolar defects (Nguyen et al., 2009a), demonstrated communication between the surgical cleft defect and the surrounding anatomical structures including the nasal cavity and periodontal ligament space of the incisor teeth. This could result in substantial loss of BMP-2 and suboptimal BMP-2 concentration at the defect site.

Consequently, there is no reliable, reproducible and cost-effective animal model for testing new bone grafting alternatives, which could explain the delays for developing alternative therapies for secondary bone grafting. Therefore, our study critically assessed and compared the current rodent models of cleft palate (MPC and AC) to identify the most reliable model. This study also proposed alterations in the design of both models based on preoperative virtual planning to avoid damage to surrounding anatomical structures. Finally, bone healing in the modified models was thoroughly evaluated over an 8 week healing period, to confirm the critical size nature of the defects.

2. Material and methods

A series of successive experiments is presented in this article. In the pilot study, we attempted to reproduce the published dimensions for the AC model ($7 \times 4 \times 3$ mm^3) in 8 weeks old SD rats. This resulted in significant injury of the surrounding structures including the incisor teeth, nasal septum and palatine foramen. The same dimensions were then reproduced in 16 weeks old SD rats and damage to the same structures was again observed. We then compared the anteroposterior and transverse dimensions of the maxilla in 16 weeks old SD vs. Wistar rats ($n = 4$/group). Subsequently, virtual planning for the appropriate design for MPC and AC defects was performed in 16 weeks old Wistar rats ($n = 4$). Finally, we conducted a comparative study to assess bone healing following employing the modified defects based on virtual planning in 16 weeks old Wistar rats weighing 375–400 g ($n = 6$/group). Modified defects were designed to be at least 1 mm away from roots of the incisors, palatine foramen and zygomatic arch. Bone healing in the modified models was assessed by *in vivo* μCT (weeks 0, 4, and 8) and histology (week 8).

2.1. Animals and surgical procedures

The animal care committee at the University of Alberta approved the experimental protocol. Animals were housed in standard conditions (2 rats/cage at room temperature with 12 h of light/dark cycle). Soft diet and liquid gel packs were provided for the animals 2 days preoperative and 1 week postoperative, and then advanced gradually to a regular diet. Sample size calculation based on a power of 80% suggested that six animals/group were sufficient to provide reliable data with 95% of confidence level for statistical analysis.

Rats were anesthetized by intra-peritoneal injections of Ketamine (75 mg/kg) and Domitor (0.5 mg/kg). Additionally, 0.25 ml of 0.4% lidocaine was injected locally. For MPC group, V-shaped incision was performed at the maxillary midline and extended bilaterally to the zygomatic arches. Using Bien Air surgical hand piece, MPC defects of $7 \times 2.5 \times 1$ mm^3 were created in the premaxilla. For AC group, longitudinal incision was made at the alveolar crest and AC defects of $5 \times 2.5 \times 1$ mm^3 were created. Mucosal flaps were closed using 4-0 polyglactin absorbable sutures. Then, animals were recovered by intra-peritoneal injections of 1 mg/kg revertor (atipamezole hydrochloride). Postoperative pain control included subcutaneous administration of Metacam at 2 mg/kg (once/day) and Butorphanol at 0.2 mg/kg (twice/day) for 3 days. Rats were evaluated daily for two week postoperative for signs of pain (e.g. reduced activity, porphyrin staining, lethargy, loss of appetite or weight loss).

2.2. In vivo μCT assessment

Rat maxilla was scanned with μCT scan (SkyScan 1176 *in vivo* μCT, SkyScan NV, Kontich, Belgium). Isoflurane (2% in oxygen) was used for anaesthesia during μCT imaging. All μCT scans were conducted at 100 kV through 180° with 0.5° rotation step to produce serial cross-sectional images of isotropic 18 μm^3 voxels. Cross-sectional μCT images and 3D models were utilized for cleft defect size measurements as well as morphometric analysis.

2.3. Radiomorphometric analysis

Reconstructed 3D images were subjected to analysis using Mimics software (Materialise, Leuven, Belgium) to measure anteroposterior and transverse dimensions of the maxillae in non-operated 16 weeks old SD vs. Wistar rats ($n = 4$). Landmarks established by Gomes et al. (Gomes et al., 2012) were utilized. The distances from the infraorbital foramen to incisal point (IF-IP) were measured bilaterally to represent the anteroposterior dimension, while the distance between right and left IF was utilized for transverse measurements. Additionally, we used the same landmarks to compare maxillary growth in 16, 20 and 24 weeks old Wistar rats ($n = 4$).

Subsequently, 3D μCT reconstructions of non-operated Wistar rats (16 weeks old, $n = 4$) were utilized for preoperative surgical planning. For MPC defect model, distance between IP and a point located 1 mm anterior to the palatine foramen was measured to represent the maximum length (anteroposterior dimension) of the defect. The distance was measured between lines drawn 1 mm away from the right and left incisors and along the contour of palatine bone, representing the width of the defect. While, for the AC model, the defect was designed to be 1 mm away from zygomatic arch, incisor root and palatine foramen. Additionally, 2D image μCT were used to measure the thickness of the palatine bone that represents the maximum depth for both MPC and AC defects.

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