Alveolar osseous defect in rat for cell therapy. Preliminary report[1]

Defeito ósseo alveolar em ratos para terapia celular. Estudo preliminar

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ABSTRACT
Purpose: To study were to reproduce an alveolar bone defect model in Wistar rats to be used for testing the efficacy of stem cell therapies. Additionally, we also aimed to determine the osteogenesis process of this osseous defect in the 1 month period post-surgery. **Methods**: The animals were randomly divided into two groups of 7 animals each. A gingivobuccal incision was made, and a bone defect of 28 mm^2 of area was performed in the alveolar region. Animals were killed at 2 weeks after surgery (n=7) and 4 weeks after surgery (n=7). **Results**: The average area of the alveolar defect at time point of 2 weeks was 22.27 ± 1.31 mm^2 and the average area of alveolar defect at time point of 4 weeks was 9.03 ± 1.17 mm^2. The average amount of bone formation at time point of 2 weeks was 5.73 ± 1.31 mm^2 and the average amount of bone formation at time point of 4 weeks was 19 ± 1.17 mm^2. Statistically significant differences between the amount of bone formation at 2 weeks and 4 weeks after surgery were seen (p=0.003).**Conclusion**: The highest rate of ossification occurred mostly from 2 to 4 weeks after surgery. This observation suggests that 4 weeks after the bone defect creation should be a satisfactory timing to assess the potential of bone inductive stem cells to accelerate bone regeneration in Wistar rats.
Key words: Alveolar Bone Loss. Tissue Therapy. Rats.

RESUMO
Objetivo: Reproduzir um novo modelo de defeito ósseo alveolar em ratos Wistar que será utilizado para terapia genética e estudos com células tronco. Adicionalmente, outro objetivo do presente estudo foi determinar o pico de regeneração óssea do defeito criado na região alveolar do modelo experimental. **Métodos**: Os animais foram aleatoriamente divididos em dois grupos de sete animais. Através de uma incisão gengivobucal foi criado um defeito ósseo medindo 28 mm^2 de área na região alveolar dos ratos. Os ratos foram sacrificados após duas semanas (n=7) e quatro semanas (n=7) da cirurgia. **Resultados**: A área média do defeito alveolar após duas semanas de cirurgia foi de 22.27 ± 1.31 mm^2 e a área média do defeito alveolar após quatro semanas de cirurgia foi de 9.03 ± 1.17 mm^2. A taxa de formação óssea foi de 5.73 ± 1.31 mm^2 após duas semanas de cirurgia e de 19 ± 1.17 mm^2 após quatro semanas de cirurgia. Foi observada diferença estatisticamente significante na taxa de formação óssea entre o grupo dos animais sacrificados com duas e quatro semanas (p=0.003). **Conclusão**: Este estudo demonstrou que a maior taxa de regeneração óssea ocorreu no período entre duas e quatro semanas após a cirurgia de criação do defeito ósseo alveolar, portanto esta observação sugere que o período de tempo de quatro semanas será suficiente para avaliar a capacidade de células tronco em regenerar osso em ratos Wistar com defeito ósseo alveolar.
Descritores: Perda Óssea Alveolar. Terapia Celular. Ratos.

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Introduction

Cleft lip and palate (CLP) represent one of the most common congenital human malformations, corresponding to nearly 1/3 of all birth defects. They are very heterogeneous and etiologically complex. The phenotype can vary from simple lip scar or a cleft lip to a complex craniofacial pattern involving the palate, maxilla, zygoma, orbit and cranium. The incidence of the different forms vary: cleft lip with or without cleft palate (CLP) is the most frequent (~1:1000 live births) while craniofacial clefts are quite rare, with an estimate incidence of 1.43 to 4.84 per 100 000 live births[1-3].

Surgical correction of CLP cleft can be challenging. Strategies for closing the bone defect include autologous bone

grafts and soft tissue repair with local flaps[4]. An adequate bone stock in the cleft region is required to establish continuity of the craniofacial skeleton. In addition, satisfactory bone stock in the cleft region is required to facilitate tooth eruption and the closure of oronasal fistulas[5-10]. Older patients with cleft lip and/or cleft palate may experience slow wound healing, bone graft absorption or recurrent fistulas, resulting in failed tooth eruption[11]. Early treatment may avoid an unsatisfactory outcome.

An alternative promising technique to enhance bone regeneration in patients with CLP involves the use of tissue-engineered bone, which involves stem cells of various sources with osteogenic potential placed within a biocompatible scaffold[7,12,13].

An appropriate animal model is essential for testing tissue-engineered bone and new strategies for healing bone defects[12,14-16]. Alveolar maxillary bone defects have been described in primate, ovine and canine models[17-20], however, the use of large animals is expensive and requires a specific setting.

Rodent models have been used in the majority of studies, investigating new bone-inductive agents[13-16,21,22]. Mehrara *et al.*[15] created a rat model of gingivoperiosteoplasty in which a bone defect in the palatal bone was produced[15]. However, the lack of soft tissue coverage of rat palatal bone may be a limitation of this model with regard to the development of three dimensional biocompatible scaffolds seeded with cells for bone regeneration. Nguyen *et al.*[23] recently established a critical-sized alveolar defect in rats and determined by means of micro-computed tomography and histology the ratio of ossification at 4, 8 and 12 weeks after surgery. They did not observe statistically significant difference in bone formation among 4, 8 and 12 weeks, suggesting that 4 weeks would be a plateau of bone formation.

The purposes of the present study were to reproduce an alveolar bone defect model in Wistar rats to be used for testing the efficacy of stem cell therapies as well as to determine the rate of bone formation of this osseous defect within the first month post-surgery.

Methods

All experiments were performed in accordance with the guidelines set by the Standing Committee on Animal Research of the Institute of Bioscience, University of São Paulo. Adult male, 4-months-old, Wistar-rats, weighing 350g to 500g, were housed individually in a temperature-, light-,and humidity- controlled environment. Animals were fed a standard diet preoperatively. Postoperatively, animals were fed a soft diet for 1 week, after which the normal diet was re-established.

Surgical procedure

Animals were anesthetized with an intraperitoneal injection (0.3 mL/100 g body weight) of ketamine hydrochloride (5%) combined with xylazine (2%). Rats were placed in a lateral position for the surgical procedure. Lidocaine with adrenaline, (0.3 mL) was used for anesthesia of the oral mucosa. A 1.5-cm incision was made in the transitional zone of dry to wet oral mucosa. A tongue traction maneuver was performed to keep the airway open during the surgical procedure. The underlying muscle and maxillary periosteum were elevated. The maxilla and zygomatic bone were completely undermined. A fabricated methyl methacrylate rectangular mold was used to produce a standardized bone defect of 28 mm^2 of area (7 mm anteroposterior, 4 mm mediolateral and, 2 mm deep). A caliper was used to measure the bone defect. A high-speed burr with constant irrigation was used to create the bone defect. One rat aspirated water during surgery and died. This animal was replaced. Otherwise, all 14 rats tolerated the surgical procedure well. They were fed a standard soft diet for 1 week after surgery and seemed to eat without problem; average weight loss at 4 weeks was 25 g, corresponding to a 6% to 7 % loss of body weight. Animals were killed at 2 and 4 weeks (n=7 per time point) by carbon dioxide narcosis. The size of bone defect as well as measurement of bone formation at each time point were performed and compared (Figures 1 and 2).

FIGURE 1 - Wistar rats were placed in a lateral position. A 1.5-cm incision was made in the gingivobuccal sulcus. The muscles of the face were released, and the periosteum was retracted. A methyl methacrylate mold was placed into the appropriate position, and a bone defect was created with a high-speed burr. The tongue was pulled out to avoid water aspiration and to facilitate breathing

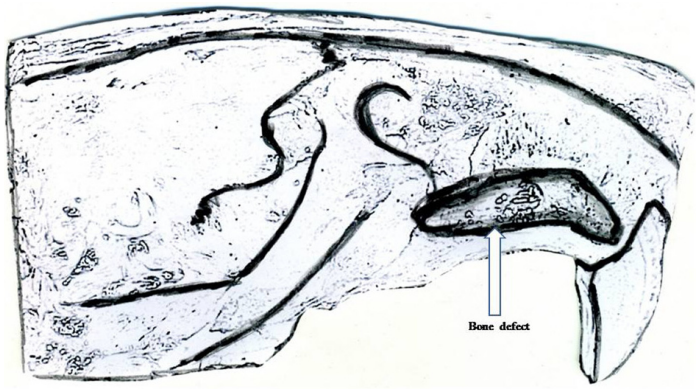

FIGURE 2 - Illustration of the bone defect compromising the maxillary and alveolar regions

Histologic preparation

Samples of the alveolar bone defect were prepared for histologic analysis. The studied tissue samples were fixed in 10% formalin for 24 hours, decalcified in 5 % formic acid for 48 hours, and paraffin-embedded. Sections measuring 5 µm were stained with hematoxylin and eosin and examined under a light microscope.

Radiographic analysis

Rats craniofacial skeleton were imaged immediately after surgery and postoperatively at 2 weeks (n=7) and 4 weeks (n=7) by computerized tomography. Scans were reconstructed as tree-dimensional isosurfaces using InVesalius biomedical software (DT3D-CTI-Brazil). Each tree-dimensional imaging was evaluated at a critical threshold toll, with the densest intensity interpreted as bone. The area of the bone defect was measured on a tree-dimensional images using Magics 13.0 (Materialise-Belgic, software CAD) at O, 2 and 4 weeks.

Measurement of bone formation

The amount of bone formation was measured by calculating the difference between the initial size of bone defect from the size of the bone defect at 4 weeks and 2 weeks after surgery.

Statistical analysis

The amount of bone formation at 2 weeks and 4 weeks after surgery were compared using a Mann-Whitney test. All data were expressed as mean± SEM. A value of $p<0.05$ was considered to be statistically significant.

Results

The average area of the alveolar defect at 2 weeks after surgery was 22.27 ± 1.31 mm^2 and the average area of alveolar defect at 4 weeks after surgery was 9.03 ± 1.17 mm^2. The average amount of bone formation at time point of 2 weeks was 5.73 ± 1.31 mm^2 and the average amount of bone formation at time point of 4 weeks was 19 ± 1.17 mm^2. Statistically significant differences between the amount of bone formation at 2 weeks and 4 weeks after surgery was seen (p=0.003). Our study showed that the highest rate of ossification occurred mostly from 2 to 4 weeks, being more than two times higher at 4weeks period in comparison of 2 weeks period (Figure 3).

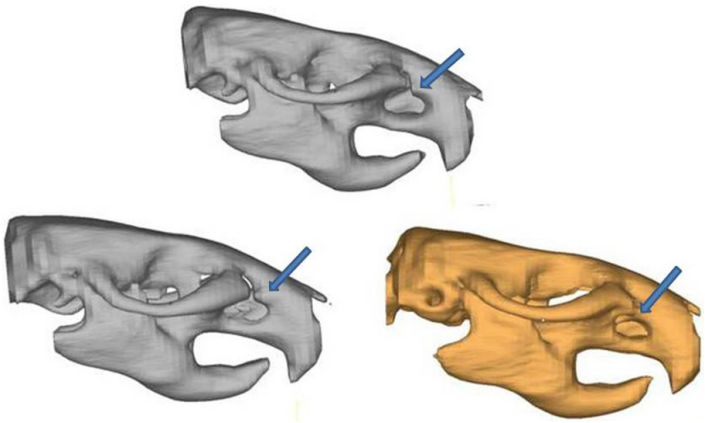

FIGURE 3 - Computerized tomography imaging showing the initial size of bone defect with 28 mm^2 of area (*above*) and at 2 weeks and 4 weeks after surgery (*below, left and below, right*). Note that the bone defect is demonstrated with arrows

Histologically, intense osteoblast activities with polymorphonuclear cells as well as woven bone layer was seen (Figures 4 and 5).

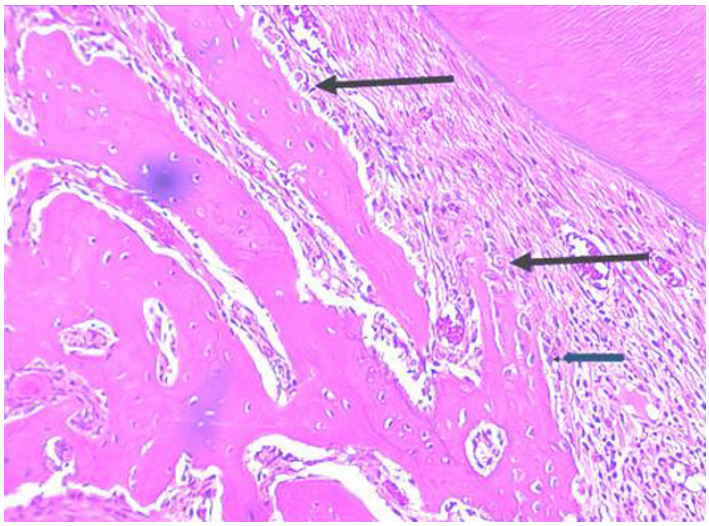

FIGURE 4 - Photomicrographs obtained for histologic evaluation (100X magnification). Sagittal sections of rats maxillary region, showing intense osteblastic activity (*large arrow*), woven bone within the alveolar gap (*small arrow*)

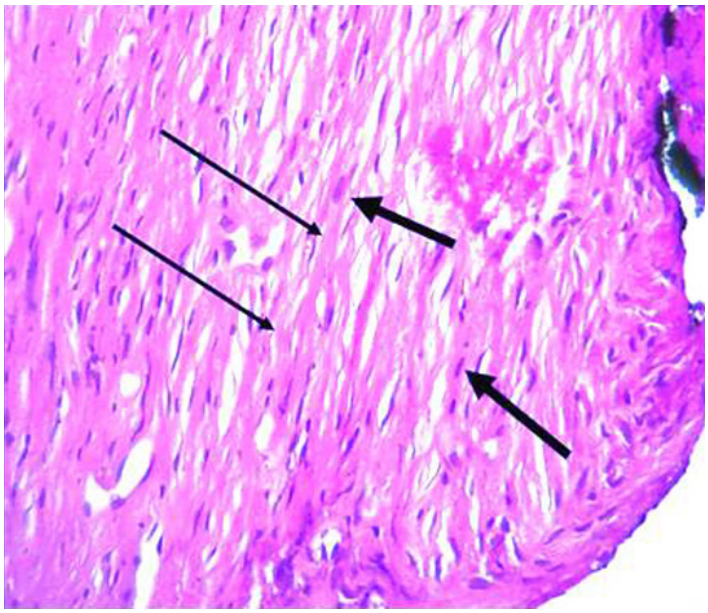

FIGURE 5 - Photomicrographs obtained for histologic evaluation (100X magnification). Sagittal sections of rats maxillary region, fibroblast (*large arrow*) and matrix collagen fibers (*small arrow*) are also seen in the alveolar bone defect

Discussion

Currently, CLP teams are focused to decrease the number of operations of their children during the process of rehabilitation. Attempts of early bone grafting with rib has brought several complications as well as high morbidity of donor sites[23].

Gingivoperiosteoplasty (GPP) was an alternative technique described in 1965 that aimed to regenerate bone on the

alveolar defect, without the potential complication of having a donor site at early age[24]. However, studies have described variable rates of success of bone regeneration on the alveolar defects and long term complications on facial growth[25,26].

Tissue engineering studies have identified alternative methods that may allow early rehabilitation and decreased average number of operations until adult age. Bone morphegenic protein (BMP-2) was used to reconstruct cranial defects[27]. Alonso *et al.*[29] have demonstrated in a clinical study that the reossification occurred in different distribution pattern and more slowly in the group of BMP in comparison with the group of iliac crest bone graft, that remains as gold standard therapy for alveolar cleft repair[28]. The potential of bone formation after placement of BMP was also showed in animal models with cranial defects[29]. Interestingly, BMP did not cause immunologic reaction after its use in a rat model. Stem cells with osteogenic potential has been another alternative, that may be used in further clinical trials to promote early rehabilitation of children with cleft lip and palate. Bone marrow stem cells seeded onto a resorbable sponge with satisfactory bone healing outcomes has shown several advantages regarding to donor site morbidity[7]. Ideally, patients would be benefited with autologous sources of stem cells, however it may not be promptly available. A novel source of osteogenic stem cell were isolated from the lip muscle of children born with CLP. Small fragments of lip muscle are commonly discarded after primary surgery of cleft lip repair at 3 months of age[30]. Bone inductive stem cells could be included in the alveolar pocket of patients with CLP at the time of palate repair, promoting bony growth and consequently early rehabilitation. Alternatively, a stem cells bank would lead widespread use for early rehabilitation of the growing population of cleft children. However, several issues need to be addressed before its use in clinical trials, such as; 1- amount of stem cells needed to regenerate sufficient bone in the alveolar gap. 2- The possibility of using heterologous sources of stem cells from stem cells bank. 3- Quality and amount of bone formed in the alveolar gap. For this reason an animal model, that allows some answers prior to clinical trials is primordial. In the present study, we reproduced an osseous defect in the maxillary alveolar region of Wistar rats. The present report confirms that after one month post-surgery the original defect of 28 mm^2 was still not healed. We also observed that the highest rate of ossification occurred mostly from 2 to 4 weeks. Histologically, osteoblast activities and polymorphonuclear cells as well as woven bone layers are seen, suggesting intense osteogenesis phenomenon, however, this process does not seem enough to totally regenerate the bone defect. Considering that a plateau of bone formation starting at 4 weeks after surgery was previously demonstrated[31], we suggest that evaluation at 4 weeks after the bone defect creation should be a satisfactory timing to assess the potential of bone inductive stem cells to accelerate bone regeneration in Wistar rats.

The model presented herein is an alveolar osseous defect, that is, on the other hand, reproducible and feasible, that simulates the tridimensional osseous defect in cleft lip/palate patients. Traditionally, bone calvarial defects are being used to study bone inductive agents, however satisfactory results achieved in the cranial bone would not be replicable to alveolar and maxillary bone, because of the role of dura mater and pericranium on the bone formation. This is a preliminary report constructed for further cell therapy testing. Results of this study suggests that 4 weeks after the bone defect creation should be a satisfactory timing to assess the potential of bone inductive agents, including stem cells to accelerate bone regeneration and answer the queries previously raised. Our results suggested that most of the osteogenesis phenomenon occurred from 0 to 4 weeks in this animal model. In summary, this model will allow studies with tissue-engineered bone associated with stem cells in order to evaluate the effectiveness of strategies to speed up the healing process of CLP alveolar defects, leading to early rehabilitation of these children.

Conclusion

Our study showed that the highest rate of ossification occurred mostly from 2 to 4 weeks after the alveolar bone defect creation. This observation together with the data on the literature suggests that 4 weeks after the bone defect creation should be a satisfactory timing to assess the potential of bone inductive stem cells to accelerate bone regeneration in Wistar rats.

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