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WÂNEZA DIAS BORGES HIRSCH

ANÁLISE DA BIOCOMPATIBILIDADE, CITOTOXICIDADE E OSTEOCONDUÇÃO DO POLICAPROLACTONA – ESTUDO EM RATOS

Prof. Dr. Claiton Heitz
Orientador

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Tese apresentada como parte dos requisitos exigidos para a obtenção do título de Doutor em Odontologia pelo Programa de Pós-Graduação da Faculdade de Odontologia da Pontifícia Universidade Católica do Rio Grande do Sul, com área de concentração em Cirurgia e Traumatologia Bucomaxilofacial.

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Waner e Valdereza,
Viviane e Vinícius.

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RESUMO

Polímeros biorreabsorvíveis vêm sendo utilizados como *scaffolds* na engenharia tecidual, destacando-se como alternativa para reconstrução de lesões e perdas teciduais. Neste estudo, avaliou-se o desempenho *in vivo* de *scaffolds* tridimensionais de polímero policaprolactona (PCL), através do implante do PCL nos tecidos subcutâneos do dorso e na calvária, bem como da reação dos órgãos rins, pulmões e fígado de ratos. A análise histológica qualitativa do processo de reparo ósseo nas calvárias mostrou neoformação óssea e que o osso neoformado cresceu em direção ao centro de defeitos. Nos tecidos adjacentes ao *scaffold* implantado no dorso, percebeu-se que em todos os animais houve formação de cápsula fibrosa fina, com fibras colágenas organizadas envolvendo o implante. Com relação aos eventos ocorridos nos rins, fígado e pulmões dos animais, não houve alterações teciduais danosas aos órgãos, tampouco a presença de processo inflamatório, hiperplasia, metaplasia, displasia ou hemorragia. A análise quantitativa do processo de reparo ósseo foi realizada através de histomorfometria e tomografia computadorizada de feixe cônico (TCFC). Após análise estatística, a área total de neoformação óssea em mm^2 foi maior nos defeitos experimentais aos 21, 60 e 120 dias, com diferença estatisticamente significativa. Na análise tomográfica, percebeu-se uma tendência de maior neoformação óssea nos defeitos experimentais, mas sem diferença estatisticamente significativa. Considerando-se a análise tomográfica como uma nova metodologia para avaliação de neoformação óssea, os dados obtidos através dessa avaliação não puderam ser correlacionados com aqueles obtidos na análise histomorfométrica. Portanto, conclui-se que os *scaffolds* de PCL produzidos na plataforma experimental de manufatura aditiva são biocompatíveis, não citotóxicos, biorreabsorvíveis e promovem osteocondução. O PCL apresentou grande potencial de aplicação clínica nos defeitos onde se espera aumentar a área óssea e parece adequado como um biomaterial de escolha para outros estudos que elucidem as questões pertinentes. A TCFC não parece ser uma ferramenta útil na avaliação da neoformação óssea em calvária de ratos, de modo que a análise histomorfométrica permanece como método mais adequado.

Palavras-chave: Engenharia de tecido ósseo. Policaprolactona. Biocompatibilidade. Osteocondução. *scaffold*.


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ABSTRACT

Bioresorbable polymers have been used as scaffolds in tissue engineering, thus representing an important alternative for reconstruction of lesions and tissue losses. This study aimed to evaluate the *in vivo* performance of three-dimensional scaffolds made of polycaprolactone (PCL), by means of through a PCL implant on the subcutaneous tissues of rats' back and calvaria, as well as the reaction of their kidneys, lungs and liver. The histological analysis of the bone repair process in calvaria showed the presence of newly formed bone growing towards the center of the defects. The formation of a thin fibrous capsule was observed in the tissues adjacent to the scaffold implanted on the back of all animals, with collagenous fibers involving the implant. As for events occurring in animals' kidneys, lungs and liver, there were no harmful tissue alterations in these organs nor the presence of inflammatory process, hyperplasia, metaplasia, dysplasia or hemorrhage. A quantitative analysis of the bone repair process was performed using histomorphometry and cone beam computed tomography (CBCT). Results showed that the newly formed bone grew towards the center of the defects. Statistical analysis revealed that the total area of new bone formation was greater in experimental defects at 21, 60 and 120 days, showing a statistically significant difference. In tomographic analysis found that new bone formation is more likely to occur in experimental defects, but with no statistically significant difference. Considering tomographic analysis as a new method for the assessment of new bone formation, the data obtained from this assessment could not be correlated with those obtained from histomorphometric analysis. Therefore, it can be concluded that PCL scaffolds produced on an additive manufacturing machine are biocompatible, non-cytotoxic and bioresorbable products that promote osteoconduction. PCL showed great potential for clinical use in the treatment of bone defects by increasing bone área and seems to be an appropriate biomaterial to be used in other studies aiming to elucidate issues related to this topic. Additionally, CBCT does not seem to be a useful tool in the evaluation of new bone formation of rat calvaria, which means that histomorphometric analysis is still the most appropriate method.

Keywords: Bone tissue engineering. Polycaprolactone. Biocompatibility. osteoconduction. *scaffold*.

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LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

ANOVA- Análise de Variância
BIOFABRIS - Instituto Nacional de Biofabricação
CAPES - Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CBCT - tomografia cone beam
cm – centímetro
DF - degrees of freedom
g – grama
GPa - giga pascal
h – hora
HE - Hematoxilina e Eosina
Kg – quilograma
Km – quilômetro
Ltda. – limitada
msd - minimum significant difference
mg – miligrama
mL– mililitro
mm– milímetro
MSQ - mean of squares
no.- número
OM - overall mean
PCL – policaprolactona
PGA - poli(ácido glicólico)
PLA - poli(ácido láctico)
PLGA - poli(ácido láctico-co-ácido-glicólico)
PUCRS – Pontifícia Universidade Católica do Rio Grande do Sul
Sig. - Significance
SP – São Paulo
SPSS - Statistic Packet of Social Science
SSQ - sum of squares
SV - source of variation
TIC - Terminal Intermodal de Cargas
3D – tridimensionais
µm - micrômetro
® – marca registrada
% – por cento
%CV - percentage of coefficient of variation
°C – graus Celsius
x – vezes

SUMÁRIO


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1 INTRODUÇÃO

Perdas de tecido ósseo em decorrência de anormalidades congênitas (fendas palatinas) ou adquiridas dos ossos faciais (traumatismo facial, patologias, infecções, sequelas de tratamentos cirúrgicos) podem resultar em grandes defeitos ósseos na face dos pacientes (PETERSON et al., 2005; EAP et al., 2012; LOHFELD et al., 2012).

A capacidade de influenciar ou estimular o crescimento ósseo no local onde ocorreram perdas ósseas tornou-se mais previsível nos últimos anos. Os materiais para aumento do volume ósseo podem ser incorporados com o intuito de estimular o crescimento em áreas onde houve perda desse tecido (GRANDI et al., 2011; LOHFELD et al., 2012).

Os biomateriais para substituição do tecido ósseo podem ser classificados de acordo com seu modo de ação em osteocondutores ou osteoindutores. Uma grande vantagem dos substitutos ósseos é não produzir um trauma adicional ao paciente, o que ocorre na obtenção do enxerto autógeno - o único com propriedades osteogênicas, isto é, o crescimento ósseo derivado das células viáveis transferidas dentro do enxerto (MISCH, 2006; MARZOUK, 2007).

O material osteocondutor é aquele que promove o crescimento ósseo por meio da aposição do osso circunjacente, ocorrendo, portanto, na presença de osso ou células mesenquimais diferenciadas. Sua estrutura serve de arcabouço estrutural favorável para a migração celular e deposição óssea. (URIST, 2002). São biocompatíveis e não possuem capacidade de induzir a citodiferenciação de osteoblastos, embora preencham a falha orientando as novas células originadas por proliferação de células osteoprogenitoras das bordas do defeito a promoverem a neoformação de tecido ósseo (COOK; RUEGER, 1996; MISCH, 2006).

Os materiais osteoindutores promovem a formação de osso novo a partir de células osteoprogenitoras derivadas das células mesenquimais primitivas, sob a influência de um ou mais agentes indutores que emanam da matriz óssea. Eles contribuem mais para a formação óssea durante o processo de remodelagem (COOK; RUEGER, 1996; MISCH, 2006).

A osteogênese refere-se ao crescimento ósseo das células viáveis e sua forma mais eficaz é o osso esponjoso, que fornece a maior concentração de células ósseas. O osso neoformado é regenerado pelos osteoblastos e pelas células que se

originam na medula, transferidas com o enxerto. O enxerto autógeno, o único com tais propriedades, possui um crescimento ósseo de três fases. A fase um refere-se à proliferação e formação de um produto osteóide, está associado ao número de células transplantadas e determina a quantidade de osso novo que se formará, além da dimensão original. A fase dois reabsorverá e substituirá o osso da fase um, na proporção de um para um. A fase três se dá quando o osso novo se forma por meio da substituição por deformação (MISCH, 2006).

Os substitutos ósseos devem apresentar características como biocompatibilidade, atoxicidade e resistência à deformação, para que sejam utilizados no organismo. A resistência ou não à reabsorção depende da aplicação desejada e caso sejam reabsorvíveis, devem ser metabolizados pelo organismo ou excretados por uma via normal fisiológica. Além disso, eles não devem ser alergênicos nem carcinogênicos (SANTOS, 2002; VALERIO et al., 2004).

Biomateriais como as biocerâmicas (hidroxiapatita ou corais), os polímeros naturais (colágeno, quitosana) ou os sintéticos (PGA poli(ácido glicólico), PLA poli(ácido láctico), PLGA poli(ácido láctico-co-ácido-glicólico) e PCL poli(ε-caprolactona) vêm sendo considerados de excelência para a remodelação e reconstrução de defeitos ósseos (FONTES, 2010). Dentre os polímeros bioabsorvíveis utilizados como *Scaffolds* (suporte, arcabouço) para a cultura de células na engenharia tecidual, o polímero PCL apresenta grande potencial de uso, pois apresenta características mecânicas semelhantes aos dos materiais biológicos (PIETRZAC; SARVER; VERSTYNEN, 1997; BARBANTI, 2005; BÁRTOLO et al., 2008; BARBANTI et al., 2011; SENEDESE, 2011).

O PCL é um termoplástico sintético, denso e poroso, preparado com características precisas, que permitem o crescimento, a proliferação celular e a formação de um novo tecido. É descrito como um material biodegradável e biorreabsorvível (SENEDESE, 2011; EAP et al., 2012; GANESH et al., 2012).

Biodegradável é a denominação utilizada para polímeros e dispositivos sólidos que, devido à degradação macromolecular, sofrem dispersão *in vivo*, mas sem a eliminação dos produtos e subprodutos pelo organismo. Biorreabsorvível significa um material polimérico e dispositivo sólido que apresenta degradação através da diminuição de tamanho, e é reabsorvido *in vivo*, isto é, é eliminado totalmente sem efeitos colaterais residuais (PIETRZAC; SARVER; VERSTYNEN, 1997; BARBANTI, 2005; BARBANTI et al., 2011).

Originalmente, o PCL foi utilizado para a confecção de fios de sutura reabsorvíveis, mas, atualmente, pode ser utilizado em reconstituição nervosa periférica, sistemas de liberação controlada de drogas ou, como substituto ósseo temporário, sendo esta a aplicação mais recente e em fase de pesquisas (CHOONG et al., 2006; CHEN et al., 2011; GANESH et al., 2012; LOHFELD et al., 2012).

O PCL possui temperatura de fusão entre 58 e 63 graus Celsius (°C), módulo de elasticidade de 0,4 giga pascal (GPa) e seu tempo de reabsorção varia de 24 a 36 meses[22]. Destaca-se, ainda, que é biocompatível em vários ensaios e surge como alternativa ao autoenxerto, demonstrando, assim, sua eficiência, melhorando qualitativa e quantitativamente a regeneração periférica (MIDDLETON; TIPTON, 2000; WOODRUFF; HUTMACHER, 2010; SENEDESE, 2011).

A presente tese é composta por dois trabalhos apresentados sob a forma de artigos científicos. O primeiro teve por objetivo apresentar a biocompatibilidade, a citotoxicidade e a osteocondução de *scaffolds* tridimensionais (3D) de PCL estruturados por meio da plataforma experimental de manufatura aditiva Fab@CTI, através de um estudo *in vivo*. O segundo descreve outro experimento *in vivo*, cujo objetivo foi realizar uma análise tomográfica, através de TCFC, e histomorfométrica de *scaffolds* de PCL no reparo ósseo em calvárias de ratos.

2 ARTIGO 1

O artigo a seguir intitula-se Analysis of biocompatibility, cytotixicity and bone conductivity of polycaprolactone: an in vivo study e foi formatado e submetido de acordo com as normas do periódico International Journal of Oral and Maxillofacial Surgery (Anexo A).

ANALYSIS OF BIOCOMPATIBILITY, CYTOTIXICITY AND BONE CONDUCTIVITY OF POLYCAPROLACTONE: AN IN VIVO STUDY

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Running head: PCL biocompatibility and conductivity

ABSTRACT

Bioresorbable polymers have been used as scaffolds in tissue engineering, thus representing an important alternative for the treatment of lesions and tissue losses. This study aimed to evaluate the *in vivo* performance of three-dimensional scaffolds made of polycaprolactone (PCL), by means of through a PCL implant on the subcutaneous tissues of rats' back and calvaria, as well as the reaction of their kidneys, lungs and liver. The histological analysis of the bone repair process in calvaria showed the presence of newly formed bone growing towards the center of the defects. The formation of a thin fibrous capsule was observed in the tissues adjacent to the scaffold implanted on the back of all animals, with collagenous fibers involving the implant. As for events occurring in animals' kidneys, lungs and liver, there were no harmful tissue alterations in these organs nor the presence of inflammatory process, hyperplasia, metaplasia, dysplasia or hemorrhage. Therefore, in view of the results obtained, it can be concluded that PCL scaffolds produced on an additive manufacturing machine are biocompatible, non-cytotoxic and bioresorbable products that promote osteoconduction. Thus, PCL seems to be an appropriate biomaterial to be used in other studies aiming to elucidate issues related to this topic.

INTRODUCTION

Bioresorbable polymers have been used as scaffolds (support) for cell cultures in tissue engineering, thus representing an important alternative for the treatment of lesions and tissue losses[1]. The polymer named polycaprolactone (PCL), a dense and porous type of support, is prepared with specific characteristics that allow for cell growth and proliferation, as well as the formation of new tissue. It is described as a biodegradable and bioresorbable material with very well established indications[2-4], having a melting point between 58 and 63 degrees Celsius (°C) and elastic modulus of 0.4 gigapascal (GPa). Additionally, its time of degradation ranges from 24 to 36 months[2,5,6].

Furthermore, biomaterials like PCL have properties that are of great interest for tissue engineering, such as time of degradation, porosity, biocompatibility, and mechanical resistance. Scaffolds from these materials may be made with a variety of shapes and sizes[4,7,8].

The processes of biodegradation and bioresorption have a complex mechanism of cellular and biochemical events. With the implantation of a synthetic material, the organism promotes an inflammatory reaction to the foreign body. The influence of bioresorbable polymers on the degradation due to the presence of peroxides, enzymes, and phagocytic cells represents an important focus of research on bioresorbable polymers[2,9].

This study used PCL to structure three-dimensional scaffolds by means of an experimental platform made on the Fab@CTI additive manufacturing machine, which has an interchangeable extrusion head designed to allow the material to be inserted

as a filament. From then on, scaffolds may be prototyped in different shapes and sizes[10].

Bioabsorbable polymers, such as PCL, are alternative materials for the treatment of lesions and tissue losses. They have great potential of use, in addition to presenting mechanical characteristics similar to those of biologic materials. These polymers allow for cell growth and proliferation, as well as for the formation of new tissue[3,8,11,12].

In order to contribute to the study on bone substitutes, this paper aimed to observe their biocompatibility by analyzing the reactions between prototyped PCL scaffolds and subcutaneous tissues of rats' back. It also aimed to assess systemic toxicity by analyzing animals' liver, lungs and kidneys 60 days after surgery by microscopic analysis, as well as 7, 21, 60, 90 and 120 days after surgery in animals that received a calvarial implant.

MATERIALS AND METHODS

The present study was approved by the institution where it was conducted (protocol no. 10/00204), and animal care was in accordance with institution guidelines. Thirteen 120 days-old male Wistar rats weighting between 250 and 300g were used.

During the entire experiment, all animals were given water and Nuvital® (Nuvital Nutrientes S/A, Curitiba, Brazil) chow *ad libitum* and were housed in a vivarium in ventilated shelves equipped with input and output air filters (Alesco Ltda., Monte Mor, Brazil), at a controlled temperature (22 $\pm$ 1ºC) and a dark-bright cycle of 12h (lights are turned on at 7 a.m. and turned out at 7 p.m.). Rats were kept in

standard cages filled with pine wood chips, which were changed three times a week, and properly identified according to the group animals belonged to, and containing at most six animals per cage.

Rats were randomly distributed into two groups, one with five animals (group 1) and another with six animals (groups 2). In group 1, systemic toxicity was evaluated by analyzing their organs according to the time when animals were euthanized: 7, 21, 60, 90 and 120 days after surgery, with PCL being inserted into the bone defect of each animal's calvarium.

In group 2, biocompatibility and systemic toxicity were assessed 60 days after surgery for PCL scaffold implantation on rats' back by observing animals' tissue responses to the implanted biomaterial and by analyzing their organs. PCL implants were subcutaneously inserted into animals' back with the preparation of surgical cavities in the subcutaneous connective tissue. The left (experimental) cavity was filled with PCL, while the right (control) cavity did not receive any material, because it acted as a control cavity for wound repair.

In the control group, which included two animals, PCL was not implanted, so their organs were used for the sake of comparison to evaluate tissue alterations in the organs of animals that received the implants.

After being weighed on a precision scale, animals were anesthetized by an intraperitoneal injection of a mixture of ketamine hydrochloride (ketamin®, Cristália Produtos Químicos Farmacêuticos Ltda., Itapira, Brazil) (100mg/kg) and xylazine hydrochloride (calmiun®, Agener União, São Paulo, Brazil) (10mg/kg). Once anesthesia was induced, hairs were removed from the upper region of the head located between external ears, in animals of group 1, and from the back, in animals of group 2, using an electric hair trimmer (Panasonic® ER389K mustache and beard

trimmer, Osaka, Japan) Subsequently, the hairless region and the surrounding coat underwent antisepsis with 2% chlorhexidine digluconate. Next, animals received local anesthesia by subcutaneous anesthetic infiltration with 2% lidocaine chlorhydrate and 1:50.000 norepinephrine (Lidostesim 2%, Probem®, Catanduva, Brazil), in order to achieve hemostasis and additional analgesia during surgery, besides controlling pain at the immediate postoperative period.

After anesthetic infiltration, animals from group 1 received a coronal linear incision between the two ears, which was made with a scalpel blade no. 15 (Solidor, São Paulo, Brazil) mounted on a Bard Parker scalpel handle no.3 (Schobell Industrial Ltda., Rio Claro, Brazil) and measuring around 1.5 cm in size, always supported by a bone base. After this procedure, soft tissues of the head were retracted using two Farabeuf retractors (Schobell Industrial Ltda. Rio Claro, Brazil), providing good visualization of the periosteum, which was incised, divulsed by a Molt retractor and retracted along with the remaining tissues, thus exposing the external surface of the calvarium. Subsequently, the region was irrigated with 0.9% saline using a 20-ml disposable syringe and then dried with sterile gauze.

Two bone defects were prepared using an electric motor rotating at low speed and bone trephine measuring 5 mm in diameter, which corresponded to the size of the bone defects created during surgery (Figure 1). After being prepared, cavities were abundantly irrigated with saline to remove the residues produced in the process of defect preparation and dried with sterile gauze. PCL was inserted into the cavities located on the left side of calvaria using Adson Brown forceps (Schobell Industrial Ltda., Rio Claro, Brazil). Control cavities were prepared on the right side of calvaria and filled with blood cloth (Figure 1).

After anesthetic infiltration, animals from group 2 received two midline incisions that were equidistant from tail and head insertions and located 7 cm apart from each other. Incisions measured approximately 8 mm in length and were made using a scalpel blade no. 15 (Solidor, São Paulo, Brazil) mounted on a Bard Parker scalpel handle no. 3 (Schobell Industrial Ltda., Rio Claro, Brazil). The subcutaneous tissue was laterally divulsed with rounded point scissors in order to form surgical cavities with approximately 18 mm in depth. Subsequently, each PCL implant was inserted into the experimental cavity until reaching its entire depth using Adson Brown forceps (Schobell Industrial Ltda., Rio Claro, Brazil). Special care was taken not to perforate or lacerate rats' tissues. Implants were carefully inserted in a non-parallel fashion to the incision line, with the purpose of preventing their expulsion or mobility (Figure 2).

The PCL (CAPA® 6505 polycaprolactone) used in this research, whose chemical formula is (C6H10O2), was synthesized by Solvay Interox Limited, Warrington, UK. According to manufacturer's recommendations, this material can be used to produce several products, including adhesives, films, fixation agents, and blocks.

Soft tissues were then repositioned so that the periosteum covered bone cavities, and incision edges were sutured with a suture thread mononylon 5-0 (Johnson & Johnson, Sorocaba, Brazil) doing single interrupted stitches using a Mayo Hegar needle holder and Adson Brown forceps (Schobell Industrial Ltda., Rio Claro, Brazil). Afterwards, the surgical area was cleaned with gauze dampened with saline to remove blood residues, and animals were placed in the prone position in their corresponding cages to recover from anesthesia.

Postoperative pain was controlled with paracetamol (Tylenol® JANSSEN-CILAG Farmacêutica, São Paulo, Brazil) (80 mg/kg) given orally immediately after the procedure and after 12 hours. All animals were given a single intramuscular dose of penicillin G benzathine (Benzetacil, Eurofarma Laboratórios Ltda., São Paulo, Brazil) (20000 units/kg) immediately after the end of the procedure.

After the end of the postoperative observation period proposed for each group, animals were euthanized by isoflurane inhalation. Hairs from the regions of interest were removed using an electric hair trimmer (Panasonic® ER389K mustache and beard trimmer, Osaka, Japan) and then these areas underwent antisepsis with 0.12% chlorhexidine digluconate.

Specimens from animals of group 1 were obtained through an incision in the most posterior region of soft tissues of the head using a scalpel blade no. 15 mounted in a Bard-Parker scalpel handle no. 3 (Schobell Industrial Ltda., Rio Claro, Brazil). The soft tissue overlying the calvarium was removed using Metzenbaum scissors and Adson Brown (Schobell Industrial Ltda., Rio Claro, Brazil), which made it possible to achieve a great visualization of the calvarium, including parietal bones. Subsequently, the calvarium was removed by osteotomy using a conical stem multilaminated drill no. 701 rotating at low speed and under constant irrigation with 0.9% saline. Four osteotomy lines were drawn around bone defects and the calvarium was removed using a straight chisel and Adson Brown forceps (Schobell Industrial Ltda., Rio Claro, Brazil). In order to evaluate systemic toxicity, animals' liver, lung and kidneys were removed through an abdominal incision for histological analysis.

Specimens from animals in group 2 were obtained through excision biopsy of the implant area, after the implant was located by palpation. This biopsy was

performed with a safety margin of 1 cm and began with an incision using a scalpel blade no. 15 mounted in a Bard-Parker scalpel handle no. 3 (Schobell Industrial Ltda., Rio Claro, Brazil). The dorsal subcutaneous tissue was divulsed using Metzenbaum scissors and Adson Brown forceps (Schobell Industrial Ltda., Rio Claro, Brazil), which made it possible to achieve a great visualization of the calvarium, including the PCL implant and an enough amount of normal adjacent tissue. In order to evaluate systemic toxicity, animals' liver, lungs and kidneys were removed for histological analysis. After local macroscopic examination, specimens were immediately stored in identified plastic containers and immersed in 10% neutral buffered formalin for tissue fixation and conservation, in order to prevent post-mortem alterations in the tissues.

After specimens were fixed in formaldehyde for more than 24 hours and less than 72 hours, another stage of the research started: the preparation and analysis of histological slides. Specimens from group 1 were decalcified in 5% nitric acid solution (10 ml) for approximately 72 hours and defects were separated between themselves and divided in half. Specimens from the back, belonging to group 2, and from organs used to evaluate systemic toxicity did not require decalcification. Subsequently, standard procedures for staining with hematoxylin and eosin (HE) were performed, as well as the routine histological processing for the preparation of slides, which included paraffin embedding, the performance of four semi-serial sections of approximately 6 µm in thickness in each block – with a distance of 15 µm between each section, measured on a microtome (Jung RM 2055 microtome, Leica Biosystems, Wetzlar, Germany) –, HE staining, and examination of the slides on a light optical microscope (BX 50 microscope, Olympus, Melville, NY, USA). Slides

were codified in such a way that the observer was unaware of which group they belonged to.

Evaluation was performed by the same previously calibrated examiner. Histological analyses were carried out using a light microscope at 40, 100 and 400x magnifications, distributed into fields scanning all the area containing PCL.

Analysis and description of the slides were based on the criteria established next. Calvaria containing PCL were assessed for new bone formation originating from the margins of the bone defect or from the center of the bone defect, or located on the edges of the biomaterial, as well as for the presence of absence of material resorption.

Back containing PCL were microscopically evaluated for cellular and tissue reactions, the presence of fibrous capsule adjacent to the material that had been implanted and its thickness, the presence of inflammatory infiltrate and of inflammatory multinucleated giant cells, vascular alterations, and the formation of granulation tissue. The fibrous capsule was defined as thin or thick; the granulation tissue as young or mature; fibrosis as organized or disorganized; finally, vasodilatation, hyperemia and edema were defined as mild, moderate and severe. Moreover, the inflammatory infiltrate located close to the material under analysis was defined as absent when the percentage of inflammatory cells was up to 10%; moderately present if the presence of inflammatory cells was observed, but they did not dominate the histological field in analysis, with a percentage ranging from 10 to 50%; and severely present when cells form an infiltrate around the bone portion to be observed, with a percentage higher than 50%[13].

According to Souza et al.[14], experimental materials are considered biocompatible if the intensity of the inflammatory reaction in the connective tissue

decreases over time. Therefore, after microscopic evaluation of specimens for 60 days, the material under investigation was considered biocompatible when the sample has a thin layer of fibrous capsule around the implant and there was no evidence of inflammatory reaction, macrophages or inflammatory multinucleated giant cells. On the other hand, it was considered non-biocompatible when there was a persistent inflammatory reaction related to macrophages and giant cells, as well as the development of a thick fibrous capsule.

Additionally, each animal was assessed for systemic toxicity by investigating liver, kidney and lung changes, such as the presence of cellular or inflammatory infiltration and tissue alterations like hyperplasia, metaplasia and/or dysplasia. No statistical tests were applied, since it was a qualitative study.

RESULTS

In group 1, specimens from calvaria containing PCL implants were investigated through histological analysis, and systemic toxicity was observed through the analysis of animals' organs. It was found that there was new bone formation after 21 days of postoperative follow-up, which means that the area of newly formed bone gradually increased over 60, 90 and 120 days (Figure 3). In all animals, new bone formation originated from the margins of the bone defect. New bone formation in the borders of the biomaterial and PCL resorption were also observed.

An analysis of the events occurring in the kidneys, liver and lungs from animals of group 1 showed that there were no tissue alterations that could damage these organs.

No presence of inflammatory process, hyperplasia, metaplasia, dysplasia or hemorrhage was observed in rats' kidneys. There were no cases of tubular necrosis.

The only alterations found in these animals were mild glomerular hypercellularity, vascular congestions, and foci of capillary aggregates, which also appeared in control animals.

There were no signs of inflammatory process, hyperplasia, metaplasia, dysplasia or hemorrhage in animals' liver as well. In addition, no microvesicular steatosis, necrosis or apoptosis were observed. There were only very few cells with macrovesicular steatosis or vascular and sinusoidal congestions, events that were also observed in control animals.

No presence of inflammatory process, hyperplasia, metaplasia, dysplasia or hemorrhage was found in animals' lungs. The only significant finding was the presence of peribronchial lymphoid aggregates, alveolar septal thickening, and vascular congestion, events that were also observed in control animals (Figure 4).

In animals from group 2, specimens from rats' back containing a PCL implant were investigated through histological analysis, and systemic toxicity was observed through the analysis of animals' organs.

When tissues adjacent to the disc implanted on animals’ back were observed after 60 days, the formation of a thin fibrous capsule was found in all animals, with organized collagenous fibers involving the implant (Figure 5). There were no signs of inflammatory infiltrate, granulation tissue, vasodilation, hyperemia, edema or abscess 60 days after discs were implanted.

When it comes to events occurring in the kidneys, lungs or liver of animals from group 2, no harmful tissues alterations were reported. No inflammatory process, hyperplasia, metaplasia, dysplasia or hemorrhage were observed in animals' kidneys, lungs and liver. Their kidneys did not present with tubular necrosis, and only cases of mild glomerular hypercellularity, vascular congestion, and foci of capillary

aggregates were found. Their liver did not develop microvesicular steatosis, necrosis or apoptosis. There were only a few isolated cells with macrovesicular steatosis and vascular and sinusoidal congestion. Rats' lungs showed peribronchial lymphoid aggregates, mild punctual alveolar septal thickening, and vascular congestion, events that were also observed in the two control animals (Figure 6).

DISCUSSION

The use of materials to improve or repair the body dates back to antiquity, when natural materials such as wood were used in an attempt to structurally replace tissues lost to trauma or disease[15]. Since the 20th century, these natural materials began to be replaced with polymers, which provided better performance, functionality and reproducibility[15].

Currently, biomaterials are an increasingly important alternative source in bone regeneration. They should ideally be biocompatible and biodegradable, as well as having the appropriate porosity that allows for vascularization and ensures mechanical resistance. Additionally, its degradation products should be non-toxic[16-18].

PCL is a type of bioabsorbable polymer that has a great potential for use in bone repair, because it presents mechanical characteristics similar to that of biologic materials, allowing for cell growth and proliferation, as well as the formation of new tissue[3,8,11,12].

The preparation of an appropriate three-dimensional scaffold is essential to determine whether the material can be used as a bone substitute. An ideal scaffold should have pores able to provide enough space for a uniform cell distribution and an appropriate oxygen and nutrient reception, in addition to having good biocompatibility and osteoconductivity[19,20].

In the present study, PCL scaffolds were prototyped in an experimental platform of the Fab@CTI additive manufacturing machine, in order for the material to be initially transformed into filaments to be used on the machine. Therefore, it became necessary to observe the *in vivo* characteristics of PCL after all this process[10]. This study evaluated PCL biocompatibility through the histological analysis of tissue reaction to PCL scaffolds implanted on rats' back and calvarium, as well as their systemic toxicity through the analysis of animals' kidneys, lungs and liver.

The main advantages of producing scaffolds by additive manufacturing are precision in material deposition and process reproducibility, making it possible to obtain three-dimensional complex structures and to control internal morphology. Additionally, this process takes a short time and has a relatively low cost[10,21].

The methodology used in this study also allowed evaluating tissue reactions in animal models, which is an essential stage to complete the evaluation of this type of material. In areas where PCL scaffolds were implanted, this material became directly in contact with tissue, including bone tissue, similarly to what would occur if the biomaterial was clinically applied[22].

Our histological analysis made it possible to assess the presence of newly formed bone on calvaria, showing that new bone formation occurred towards the center of the defects, as well as to qualitatively assess the presence of remaining portions of the PCL disc[19,23,24]. The results obtained from this analysis showed that new bone formation occurred after 21 days post-implantation, with the formation of a bone bridge from one margin of the defect to another (Figure 3 E) but not the total replacement of the biomaterial with bone tissue[25]. Thus, this evaluation made it possible to investigate the beginning of the osteoconduction process, as well as the slow biomaterial resorption and the replacement of PCL with bone.[24] The histological

analysis of tissues from animals' back at 60 days allowed observing the formation of a thin fibrous capsule in all animals, with organized collagenous fibers involving the PCL implant, which confirmed findings from other studies[26,27].

With regard to the events occurring in animals' organs, histological analysis did not reveal tissue alterations that could damage their organs, since no signs of inflammatory process, hyperplasia, metaplasia, dysplasia or hemorrhage were observed in rats' kidneys, lungs and liver.

Some punctual isolated alterations were found, such as mild glomerular hypercellularity and vascular congestion in the kidneys; isolated cells with macrovesicular steatosis and vascular and sinusoidal congestion in the liver; and mild alveolar septal thickening and vascular congestion in the lungs. However, these events were also observed in control animals, which did not receive any type of treatment.

Thus, the characteristics observed in the PCL used in the present study corroborate those conceptually necessary for the material to be appropriate for use in tissue repair, since it did not produce an exacerbated inflammatory reaction, was not rejected by the body, and allowed for osteoconduction[19,28-30].

Therefore, in view of the results obtained, it is possible to conclude that PCL scaffolds produced on the Fab@CTI additive manufacturing machine are biocompatible, non-cytotoxic and bioresorbable products that promote osteoconduction. Hence, PCL seems to be an appropriate biomaterial to be used in other studies aiming to elucidate issues related to this topic and in future clinical trials.

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Conflict of interest

None to declare

Role of the funding source

None

Statement of authorship

All authors have read and approved the manuscript as submitted, are qualified for authorship, believe the submission represents honest work and take full responsibility for the reported findings.

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CAPTIONS TO ILUSTRATIONS

Figure 1. A- Incision in rat's calvarium. B- Bone defects prepared with bone trephine. C- Experimental bone defect filled with polycaprolactone disc and empty control defect.

Figure 2. Incisions at midline on rat's back. B- Insertion of a polycaprolactone disc into surgical cavity. C- Suture of dorsal tissues.

Figure 3. Histologic images of new formed bone in defects containing biomaterial at 7 days (A), 21 days (B), 60 days (C), 90 days (D), and 120 days, showing the formation of a bone bridge (E). Areas of new bone formation (arrow).

Figure 4. Histologic images of animals' organs. Kidney with mild glomerular hypercellularity (A), kidney with vascular congestion and foci of capillary aggregates

(B), liver with vascular and sinusoidal congestion (C), liver with cells presenting with macrovesicular steatosis (arrow) (D), lung with peribronchial lymphoid aggregates (E), and lung with mild alveolar septal thickening and vascular congestion (F).

Figure 5. Histologic images of tissues adjacent to the disc implanted on animals' back at 60 days. Formation of a thin fibrous capsule involving the implant (A), detail of the fibrous capsule, with organized collagen fibers involving the implant (B and C).

Figure 6. Histologic images of animals' organs. Kidney with mild glomerular hypercellularity and vascular congestion (A), liver with vascular and sinusoidal congestion and cell presenting with macrovesicular steatosis (arrow) (B), and lung with peribronchial lymphoid agglomerates, mild alveolar septal thickening, and vascular congestion (C).

FIGURES

Figure 1

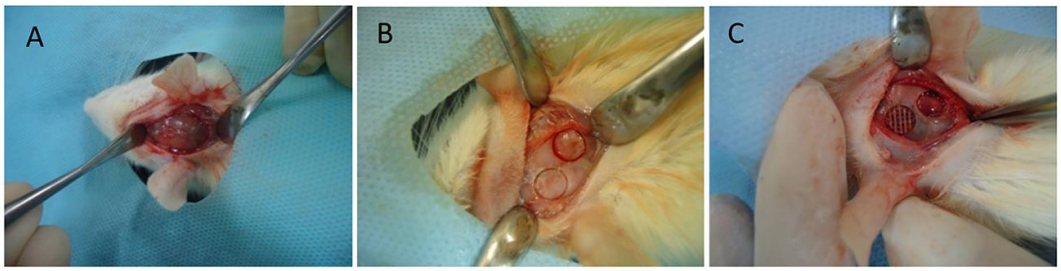


Figure 2

Figure 3

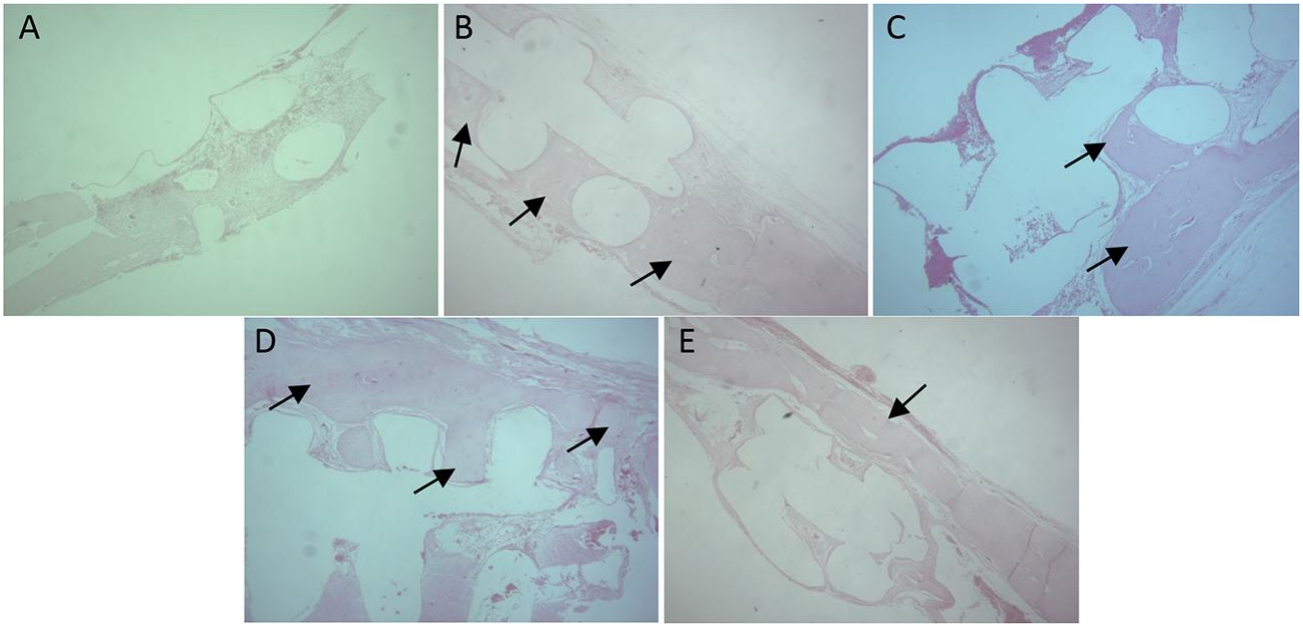


Figure 4

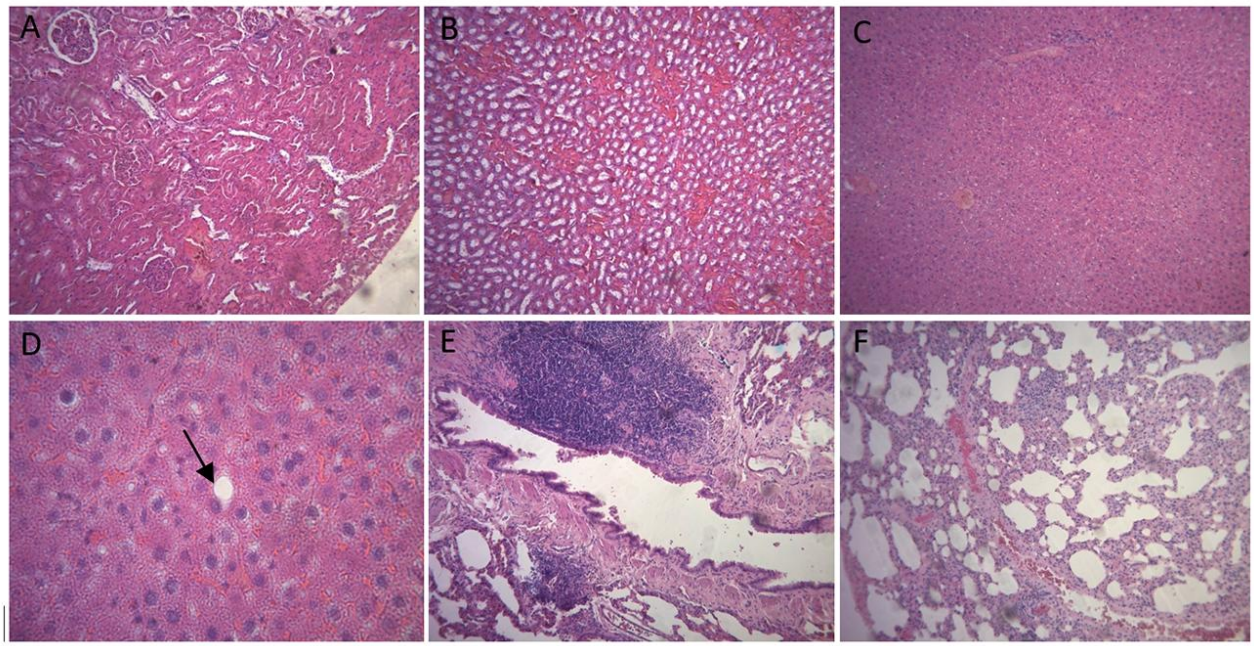


Figure 5

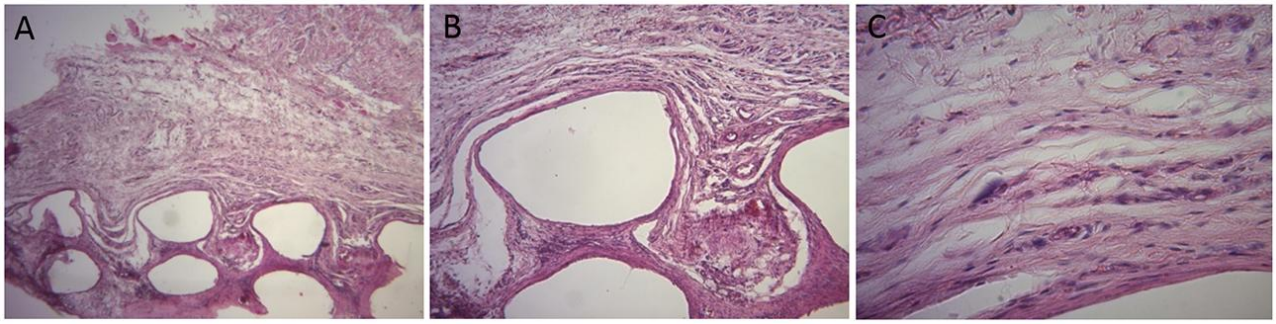
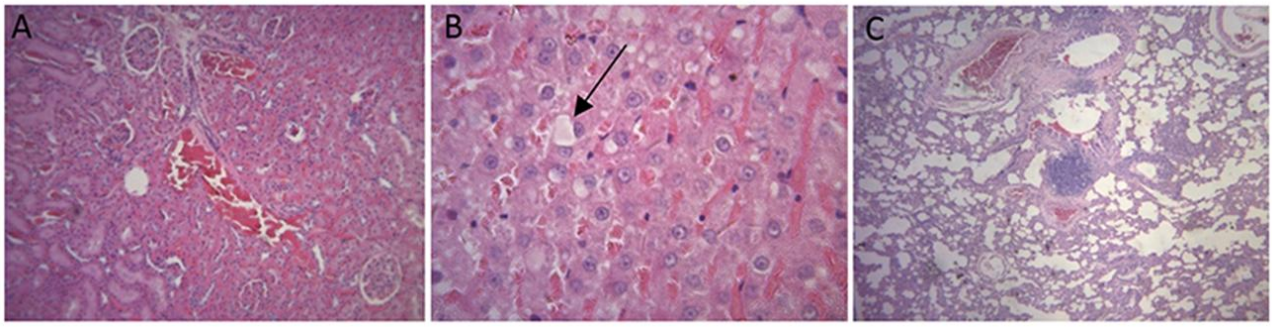


Figure 6



3 ARTIGO 2

O artigo a seguir intitula-se Tomographic and histomorphometric analysis of polycaprolactone scaffolds in bone repair – an in vivo study e foi formatado e submetido de acordo com as normas do periódico Biomaterials (Anexo B).

TOMOGRAPHIC AND HISTOMORPHOMETRIC ANALYSIS OF POLYCAPROLACTONE SCAFFOLDS IN BONE REPAIR – AN IN VIVO STUDY

Abbreviated title: Bone repair vs. polycaprolactone – a study in rats

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Abstract

Tissue engineering has been studying several biomaterials for bone tissue replacement. The present study evaluated the *in vivo* performance of polycaprolactone (PCL) scaffolds in bone repair of rat calvarial defects. A quantitative analysis of the bone repair process was performed using histomorphometry and cone beam computed tomography (CBCT). Results showed that the newly formed bone grew towards the center of the defects. Statistical analysis revealed that the total area of new bone formation was greater in experimental defects at 21, 60 and 120 days, showing a statistically significant difference. However, a tomographic analysis found that new bone formation is more likely to occur in experimental defects, but with no statistically significant difference. Thus, considering tomographic analysis as a new method for the assessment of new bone formation, the data obtained from this assessment could not be correlated with those obtained from histomorphometric analysis. Therefore, PCL showed great potential for clinical use in the treatment of bone defects by increasing bone area, due to the fact that it promoted osteoconduction. Additionally, CBCT does not seem to be a useful tool in the evaluation of new bone formation of rat calvaria, which means that histomorphometric analysis is still the most appropriate method.

Keywords: bone tissue engineering; histomorphometry; scaffold; polycaprolactone

Impact statement: Results of studies with bone substitutes are promising and have several uses in the biomedical field. Every day, new materials and techniques to manufacture scaffolds are developed with the purpose of providing biomaterials with increasingly improved physical and chemical characteristics. Thus, PCL scaffolds, prototyped through bioextrusion on a Fab@CTI manufacturing machine, need to undergo preclinical laboratory tests, in order to study their behavior during bone repair. The results obtained showed the potential of PCL scaffolds for clinical use in bone repair.

1. Introduction

Surgical procedures to improve facial and body esthetics have gained significant importance in several fields of health sciences. Many patients with loss of bone tissue seek for oral and maxillofacial surgery, whether it was caused by congenital anomalies (cleft lip or palate) or by acquired facial bone anomalies (facial trauma, pathologies, infections, surgical sequelae) [1, 2].

These anomalies leading to bone loss may result both from small defects, such as alveolar clefts, and from defects leading to the loss of great portions of the maxilla (e.g., after mandibulectomy) and of its associated structures, which may not be completely repaired, resulting in a defect that may cause partial or total loss of functioning of the injured structure, in addition to leaving several valuable structures unprotected [3].The reconstruction of these bone losses usually requires extensive treatment and multiple surgeries to restore patient's function and esthetics as properly as possible [4, 5].

The rehabilitation of bone defects to improve functional and esthetic appearance may be performed in many different ways and using a variety of bone

substitutes, such as autogenous graft (which is the gold standard), allogeneic graft, xenogeneic graft, the combination of these grafts, and alloplastic or synthetic grafts [5].

Bioabsorbable polymers, such as polycaprolactone (PCL), are alternative materials for the treatment of lesions and tissue losses. They show great potential to be used as support for cell culture in tissue engineering, in addition to presenting mechanical characteristics similar to those of biological materials. These polymers allow for cell growth and proliferation, as well as the formation of new tissue [2, 6-8].

PCL is a biodegradable and bioresorbable material that provides a dense and porous support for the newly formed bone [1, 8]. It has a melting point between 58 and 63 degrees Celsius (°C) and elastic modulus of 0.4 gigapascal (GPa), and its time of degradation ranges from 24 to 36 months [9, 10].

In view of the foregoing, the aim of this study was to observe the *in vivo* performance of PCL at 7, 21, 60, 90 and 120 days after graft implant surgery by undertaking a tomographic and histomorphometric analysis of bone repair in rats with critical calvarial defects.

2. Materials and Methods

2.1. Study design

The present investigation was developed following a traditional quantitative paradigm and was characterized as a true experimental study. The tree-dimensional PCL scaffolds used in this study were prototyped by means of bioextrusion using on the platform of an additive Fab@Home manufacturing machine at the Information Technology Center of Centro de Tecnologia da Informação Renato Archer

(Campinas, Brazil), contained 0.5 mm micropores, and measured 5 mm in diameter and 1 mm in thickness. Afterwards, they were inserted into critical bone defects of rat calvaria, with the purpose of evaluating new bone formation.

2.2. Animal model

The present study was approved by the Animal Research Ethics Committee of Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), and all research procedures complied with guidelines for animal care established by PUCRS. The sample comprised 30 male Wistar rats (Rattus norvegicus) from the vivarium at Universidade Federal de Pelotas with a mean age of 120 days and mean weight of 250 g. Animals were individually identified in their tails and housed in plastic cages filled with pine wood chips (six rats per cage) and placed in ventilated shelves (Alesco, Monte Mor, Brazil) at a temperature of 22°C and with a bright/dark cycle of 12 hours (lights were turned on at 7.00 a.m. and turned off at 7.00 p.m.). During the experiments, rats were given a standard diet consisting of chow (Nuvilab, Colombo, Brazil) and filtered water ad libitum. Cages were cleaned and changed three times a week. Experimental procedures were not performed at the same place where animals were kept, in order to avoid any type of stress. Animals were randomly divided into five groups of six animals each according to the time when animals were euthanized: 7, 21, 60, 90 and 120 days after surgery.

Two cavities were prepared on each rat's calvarium. The left (experimental) cavity was filled with PCL, while the right (control) cavity was filled with autologous blood clot.

Sample size (N=6 per group, total N=30) was defined using literature data [3, 11]; thus, we decided to work with the minimum number that would not compromise the results.

2.3. Surgical procedures

Surgical procedures were carried out at the Laboratory of Applied Pharmacology, room 148, block C, of the School of Pharmacy of PUCRS and complied with all principles of biosecurity and infection control.

After being weighed, animals were anesthetized by an intraperitoneal injection of a mixture of ketamine hydrochloride (ketamin®) (100mg/kg) (Cristália Produtos Químicos Farmacêuticos Ltda., Itapira, Brazil) and xylazine hydrochloride (calmiun®) (10mg/kg) (Agener União, São Paulo, Brazil). Subsequently, hair was removed and antisepsis was performed with 2% chlorhexidine digluconate (Clorhexidina s, Digluconato de clorexidina 2%, FGM Produtos Odontológicos Ltda., Joinvile, Brazil).

Next, surgery was performed using a sterile fenestrated surgical drape. Animals received local anesthesia by subcutaneous anesthetic infiltration with 2% lidocaine hydrochloride and 1:50.000 norepinephrine (Probem® – Lidostesim 2%, Catanduva, Brazil), in order to achieve hemostasis and additional analgesia during surgery. A coronal linear incision of nearly 1.5 cm in length was done between the two ears using a scalp and a blade no.15 (Solidor, São Paulo, Brazil). The soft tissues of the head were separated, providing good visualization of the periosteum, which was subsequently incised, divulsed and moved away along with the other tissues to expose the external surface of the calvarium. The region was irrigated with 0.9% saline and then dried with sterile gauze.

The sites where cavities should be prepared were delimited using an exploratory probe that preserved the median sagittal suture. Right and left cavities were distributed laterally to the median sagittal suture, at the parietal bones, with a distance of 2 mm between each other, as measured by a Quinelato® analogue surgical caliper (www.quinelato.com.br/odonto/imagens/compasso1.gif).

Bone defects were prepared using an electric motor rotating at low speed and bone trephine measuring 5 mm in diameter, which corresponded to the size of the bone defects created during surgery (Figure 1). Trephine was slightly pressed with intermittent movements in the superoinferior direction, making it possible to prepare the bone defect by disrupting external and internal cortical bones of the calvarium without damaging the meninges. Cavities were abundantly irrigated with saline to remove the residues produced in the process of defect preparation and dried with sterile gauze.

PCL was inserted into left (experimental) cavities, which were the using Adson Brown forceps (Figure 2). Control cavities were prepared on the right side of the calvarium, but they did not receive any material and were filled with clot. The PCL (CAPA® 6505 polycaprolactone) used in this research was synthesized by Solvay Interox Limited, Warrington, UK. Its chemical formula is ($C_6H_{10}O_2$). According to manufacturer's recommendations, this material can be used to produce several products, including adhesives, films, fixation agents, and blocks.

Subsequently, soft tissues were repositioned so that the periosteum recovered bone cavities, and incision edges were sutured with a suture thread mononylon 5-0 (Johnson & Johnson, Sorocaba, Brazil) doing simple interrupted stitches. The surgical area was cleaned with gauze dampened with saline to remove blood

residues, and animals were placed in the prone position in their corresponding cages to recover from anesthesia.

Postoperative pain was controlled with paracetamol (80 mg/kg) (Tylenol®, JANSSEN-CILAG Farmacêutica, São Paulo, Brazil) given orally immediately after the procedure and after 12 hours. All animals were given a single intramuscular dose of penicillin G benzathine (20000 units/kg) (Benzetacil, Eurofarma Laboratórios Ltda., São Paulo, Brazil) immediately after the end of the procedure.

After the end of the postoperative observation period proposed for each group, animals were euthanized by isoflurane inhalation. Hairs were removed and the area underwent antisepsis with 2% chlorhexidine digluconate (Clorhexidina s, Digluconato de clorexidina 2%, FGM Produtos Odontológicos Ltda., Joinvile, Brazil).

Specimens were obtained by an incision in the most posterior region of the soft tissues of the head with a scalp and a blade no. 15, and the soft tissue overlying the calvarium was removed using Metzenbaum scissors and Adson Brown forceps, which made it possible to achieve a great visualization of the calvarium, including parietal bones. Subsequently, the calvarium was removed by osteotomy using a conical stem multilaminated drill no. 701 rotating at low speed and under constant irrigation with 0.9% saline. The osteotomy line kept a distance of 4 to 5 mm of defective areas. Four osteotomy lines were drawn around bone defects and the calvarium was removed using a straight chisel and Adson Brown forceps. After local macroscopic examination, specimens were stored in identified containers with 10% neutral buffered formalin.

2.4. Histological process

After being fixed in formaldehyde, specimens were decalcified in 5% nitric acid solution and defects were separated between themselves and divided in half. Subsequently, standard procedures for staining with hematoxylin and eosin (HE) were performed, as well as the routine histological processing for the preparation of slides, which included paraffin embedding, the performance of four semi-serial sections of approximately 6µm in diameter in each block – with a distance of 15 µm between each section, measured on a microtome (Jung RM 2055 microtome, Leica Biosystems, Wetzlar, Germany) and based on the greatest diameter of the defect –, HE staining, and examination on a light optical microscope (BX 50 microscope, Olympus, Melville, NY, USA).

Histological assessment was performed by the same previously calibrated examiner. Fifteen slides were used for examiner's calibration and examined both by the examiner (evaluator A) and by an experienced pathologist (evaluator B). Errors were analyzed by comparing the results of evaluators A and B through the kappa test for interobserver agreement. The level of agreement for the results of the kappa test was quantified according to the percentage recommended by Landis and Koch [12], considering the following values: 0.61 to 0.80, representing significant agreement, and 0.81 to 1.0, representing almost complete agreement.

2.5. Histomorphometry

The histological images were captured from the microscope by the computer at 40X magnification. The slides were analyzed using the Image Pro Plus software, version 6.2® (Media Cybernetics, Bethesda, USA) (Figure 3), which allowed

measuring, in millimeters, the total area of each bone defect and the area of newly formed bone inside the defect.

Histological analyses included fields scanning all the area of the defect. Analysis and description of the slides were based on the area of newly formed bone from the edge of the bone defect and on the amount of material resorption. The percentage of newly formed bone and of remaining material was quantified. Values were recorded on a table specifically designed for data collection.

2. 6. Cone beam computed tomography (CBCT)

Tomographic images were obtained using a volumetric CBCT scanner (Kodak Cone Beam 3D System, Carestream Health Inc., Rochester, USA) and then analyzed using the Image J software (National Institute of Health, Bethesda, USA) (Figure 4), which allowed measuring the total area of each defect, the area of newly formed bone inside the defect, and the amount of material that remained inside the experimental defect, all of them measured in pixels and converted into millimeters.

Analyzes were performed by segmenting the images and applying masks to eliminate regions outside our regions of interest and to determine the region affected by bone defects (with and without biomaterial).

The percentage of newly formed bone and of remaining material were quantified in millimeters, and values were recorded on a data collection table.

2.7. Statistical analysis

Experimental vs. control cavities were compared with regard to the percentage of newly formed bone using descriptive statistical analysis (mean and standard

deviation) in the form of tables and graphs. The results obtained were assessed by means of analysis of variance (ANOVA) at 5% probability.

Data processing and analysis were performed using the Statistical Package for Social Sciences (SPSS)® software, version 17 (SPSS Inc., Chicago, USA), as well as Assistat software, version 7.6 (Departamento de Engenharia Agrícola do Centro de Ciências e Tecnologia da Universidade Federal da Paraíba, Campina Grande, Brazil), on the Microsoft Windows operating system.

3. Results

3.1. Histomorphometric analysis

Figure 5 shows the results for the comparison of the area of new bone formation between the two groups (with and without PCL [treatment]) at the respective times (7, 21, 60, 90 and 120 days [time blocks]), which revealed an interaction between treatments and time blocks.

Figure 6 describes the analysis of the interaction between the use of the biomaterial or not (treatment) and blocks of time simultaneously. Results show that there was a statistically significant difference of 5% in the groups analyzed at 21, 60 and 120 days after surgery (squares), in which there was a greater area of new bone formation in the defects filled with biomaterial. In the groups analyzed 7 and 90 days after surgery, although the area of new bone formation was statistically greater on the defect containing biomaterial, these values were not statistically significant.

3.2. Tomographic analysis

Table 1 shows that means for new bone formation were higher along the experimental period in the groups in which the defect was filled with biomaterial compared to those in which the defect was not filled with biomaterial.

Table 2 shows the analysis of mean areas of new bone formation for time blocks (7, 21, 60, 90 and 120 days), with new bone formation increasing over the study period.

Table 3 shows that, when time blocks were compared between themselves, there was a statistically significant difference, i.e., there was higher new bone formation over time. However, when the use of PCL or not to fill the cavities was compared over time, it did not show statistically significant difference.

3.3. Comparison between histomorphometric and tomographic analyses

A comparison of the total area of new bone formation in mm^2 obtained from histomorphometric and tomographic analyses revealed that histomorphometric analysis showed greater new bone formation in the groups in which the defects were filled with PCL, with a statistically significant difference in the groups analyzed 21, 60 and 120 days after surgery. In the tomographic analysis, it can be observed that there was a trend towards higher new bone formation in the groups that used PCL to fill the defects, although this difference was not statistically significant (Figures 7, 8 and 9).

4. Discussion

Tissue engineering has been studying several biomaterials to replace bone tissue that underwent total or partial losses, regardless if caused by pathological,

traumatic or congenital reasons. Autologous or allogeneic grafts are the most commonly used procedures to replace bone tissue. However, autologous grafts have the disadvantage of requiring an additional surgery on the donor area, in addition to the possibility of the amount of graft not being enough to fill the receptor area [3, 4]. Allogeneic grafts may lead to intense immune response, require the use of immunosuppressants, or transmit diseases [3, 4]. Thus, synthetic biomaterials are viable alternatives to replace bone tissue.

The ideal biomaterial should be biocompatible and biodegradable, as well as having the appropriate porosity that allows for vascularization and ensures mechanical resistance. Additionally, its degradation products should be non-toxic [13-15].

PCL was one of the first polymers to be synthesized and made commercially available in the 1930s by the Carothers group due to its ability of being degraded by microorganisms. However, although being initially the focus of many investigations, it was set aside with the emergence of other polymers that were more rapidly resorbed [16]. Thus, developments in tissue engineering have revived the interest in using PCL, because it has appropriate properties to be used as biomaterial for tissue reconstruction [10, 17-19]. The characteristics observed in the PCL used in the present study corroborate those conceptually necessary for the material to be appropriate for use in tissue repair, since it did not produce an exacerbated inflammatory reaction, was not rejected by the body, and allowed for osteoconduction [20-22].

Additionally, the analysis of the defects induced in this study showed that the volume of the defect containing PCL was maintained, which did not occur in the control defect filled with blood clot. This finding suggests that the portion of the PCL

disc which was not resorbed can serve as a scaffold for bone formation, corroborating the findings of Grandi et al. [3].

The histomorphometric analysis performed in this study allowed assessing the presence of PCL discs and of newly formed bone, as well as quantifying the area of new bone formation in mm^2. This type of assessment is reliable and important tool to quantitavely prove the effectiveness of biomaterials in promoting new bone formation [5, 23].

The results from this analysis showed that there was a statistically greater area of new bone formation in the defects that were filled with PCL at 21, 60 and 120 days. No new bone formation was observed at 7 days in any of the defects. There was a larger area of new bone formation in the defects filled with biomaterial at 90 days, although this difference was not statistically significant, possibly due to the fact that the number of animals per group was limited to six.

This assessment of new bone formation over time is important, because it allows establishing when the process of osteoconduction started and investigating the slow biomaterial resorption and the replacement of biomaterial with bone. Additionally, a close relationship was found between PCL and newly formed bone, as reported by several authors assessing bioceramics [24].

Tomographic analysis made it possible to evaluate the presence of newly formed bone, but the results obtained only showed that there was a trend of higher rates of new bone formation in the groups whose defects were filled with PCL. However, the difference between groups was not statistically significant, i.e., whether using the biomaterial or not, the defect presented a larger area of new bone formation over time. Moreover, PCL did not appear on tomographic images, which only showed the area of newly formed bone interspersed with the PCL disc.

Thus, the results of both analyses revealed that, in absolute values, animals that had their defects filled with PCL showed a larger area of new bone formation. However, after statistical analysis, it was observed that these values were not statistically significant in the tomographic analysis. Considering tomographic analysis as a new method to evaluate new bone formation in rat calvaria, data obtained with the evaluation of areas of newly formed bone using CBCT cannot be correlated with those obtained with histomorphometric analysis, which is considered the gold standard to evaluate new bone formation. This finding is corroborated by the study of Massotti et al. [25].

Grey levels on the region of new bone formation within the defects under investigation as assessed by CBCT lacked coherence with data obtained when the same defects were studied using histomorphometric analysis. Therefore, tomographic analysis does not seem to be a useful tool to evaluate new bone formation in rat calvaria [25].

In view of the results obtained and of the data found in the literature, it is believed that PCL has the characteristics required for clinical use, especially in defects in which an increase in bone area is expected, such as those leading to esthetic-functional impairment.

5. Conclusions

In view of the results obtained, it is possible to conclude that PCL promotes osteoconduction and is biocompatible; in addition, defects filled with PCL showed a larger area of new bone formation than that of defects filled with blood clot over time. However, the amount of newly formed bone did not fill all the volume of the bone defects at the time points analyzed in this study, in whether they were filled with PCL

or not. Furthermore, histomorphometry is still considered the most appropriate method to evaluate new bone formation in rat calvaria, since tomographic analysis using CBCT has been shown to be unsuitable for this type of assessment.

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Figure Captions

Fig. 1. Schematic representation of the computed tomography scan of a rat calvarium. Bone defects (experimental and control cavities).

Fig. 2. A- Defect preparation in a rat calvarium using bone trephine. B-Bone defects (experimental and control cavities). C- Experimental bone defect filled with PCL.

Fig. 3. Schematic representation of analysis using the Image Pro Plus software, version 6.2® (Media Cybernetics, Bethesda, USA). The total area of control (A) and

experimental (B) defects and the area of new bone formation were measured, as well as the amount of remaining biomaterial within the experimental defect.

Fig. 4. Schematic representation of analysis using the Image J software (National Institute of Health, Bethesda, USA). Three-dimensional original image (A). Application of a mask to eliminate regions external to the regions of interest. (B) Result of the application of the mask (C). Mask to determine the total area of the defect without the biomaterial (D) and with the biomaterial (E).

Fig. 5. Analysis of an experiment using a randomized block design with repetitions.

* Significant at 1% probability level ($p < 0.01$).

** Significant at 5% probability level ($0.01 \leq p < 0.05$).

*** Different letters indicate statistically significant differences.

SV = source of variation, DF = degrees of freedom, SSQ = sum of squares, MSQ = mean of squares, msd = minimum significant difference.

Fig. 6. Analysis of the interaction between treatment and times blocks

Different letters indicate statistically significant differences.

Tukey's test was performed

B1 = 7 days, B2 = 21 days, B3 = 60 days, B4 = 90 days, B5 = 120 days, T1 = control group, T2 = experimental group, msd = minimum significant difference, OM = overall mean, %CV = percentage of coefficient of variation.

Fig. 7. Comparison of the area of new bone formation in the different time blocks.

Fig. 8. Analysis of the area of new bone formation using the Image Pro Plus software, version 6.2® (Media Cybernetics, Bethesda, USA). Defect with biomaterial at 7 days (A), 21 days (B), 60 days (C), 90 days (D), and 120 days (E). Defect without biomaterial at 7 days (F), 21 days (G), 60 days (H), 90 days (I), and 120 days (J). Areas of new bone formation (arrow).

Fig. 9. Analysis of the area of new bone formation using the Image J software (National Institute of Health, Bethesda, USA). Defect with biomaterial at 7 days (A), 21 days (B), 60 days (C), 90 days (D), and 120 days (E). Defect without biomaterial at 7 days (F), 21 days (G), 60 days (H), 90 days (I), and 120 days (J). Areas of new bone formation (arrow).

FIGURES

Figure 1

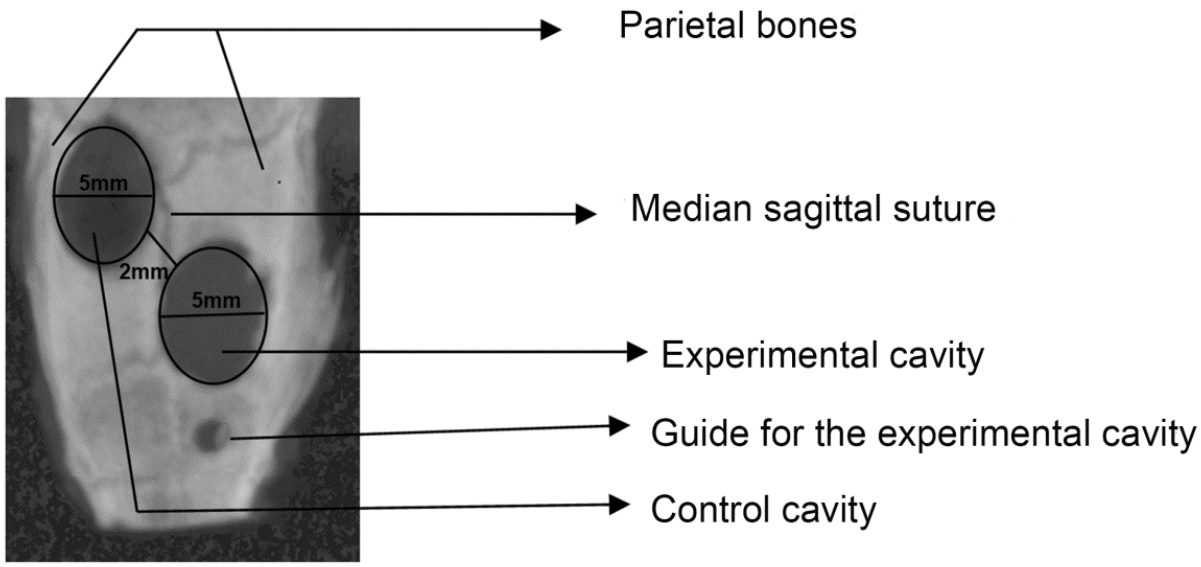


Figure 2

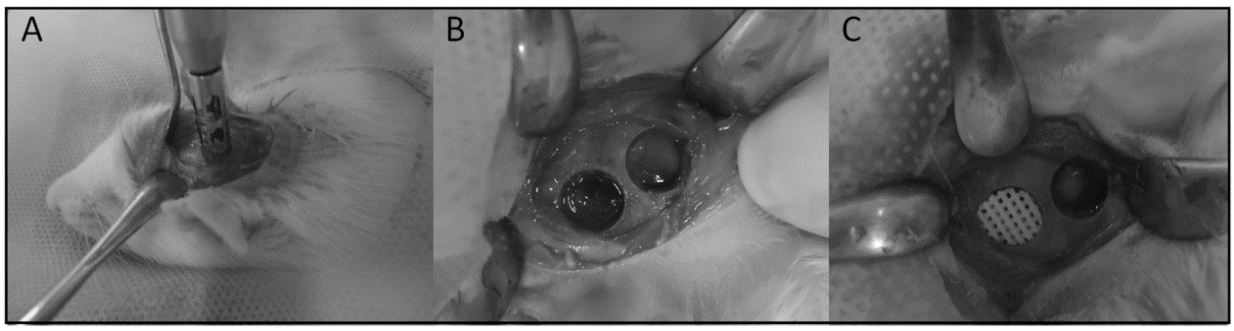


Figure 3

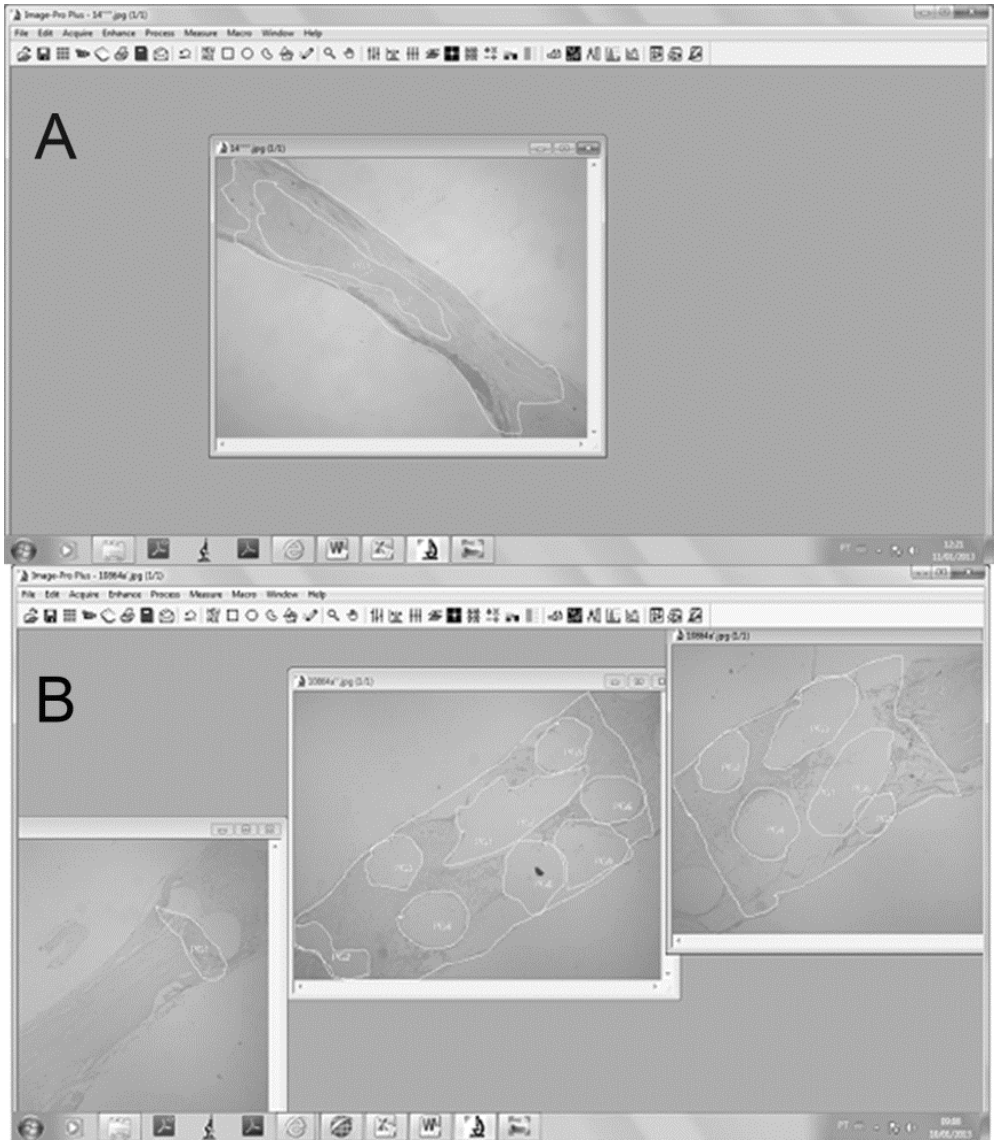


Figure 4

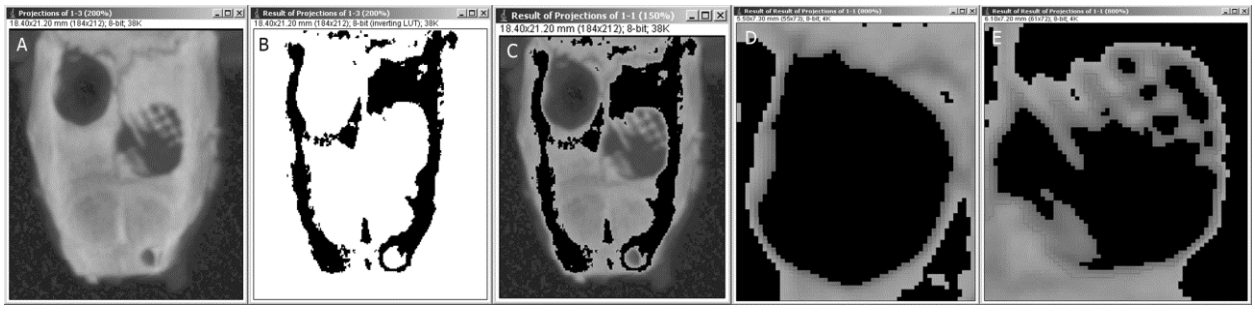


Figure 5

ANALYSIS CHART

SV	DF	SSQ	MSQ	F	
Treatments	1	31.84561	31.84561	34.2202	*
Blocks	4	56.30297	14.07574	15.1253	*
Treat vs. blocks	4	13.64535	3.41134	3.6657	**
Residuals	50	46.53043	0.93061		
Total	59	148.32436			

	DF	F-crit	F	*p*
1	50	7.1719	34.2202	<0.001
4	50	3.7207	15.1253	<0.001
4	50	2.5575	3.6657	0.0108

MEANS AND MEASURES

Means for treatment***

1	0.81828	b
2	2.27534	a

msd = 0.50055

Means for time blocks ***

1	0.00000	c
2	1.23531	b
3	1.72599	b
4	1.76977	b
5	3.00299	a

Figure 6

BLOCK

	B1	B2	B3	B4	B5
T1	0.0000 aB	0.4444 bAB	0.5338 bAB	1.3683 aAB	1.7448 bA
T2	0.0000 aC	2.0262 aB	2.9182 aAB	2.1712 aB	4.2611 aA

msd for columns = 1.1193 msd for lines = 1.5769

Classification with lower case letters

Classification with upper case letters

MG = 1.54681

CV% = 62.37

Midpoint = 3.38447

Figure 7

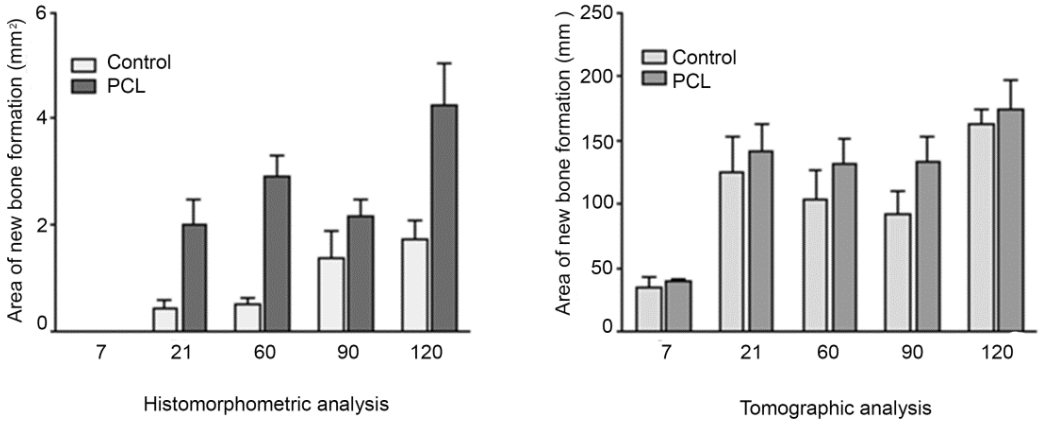


Figure 8

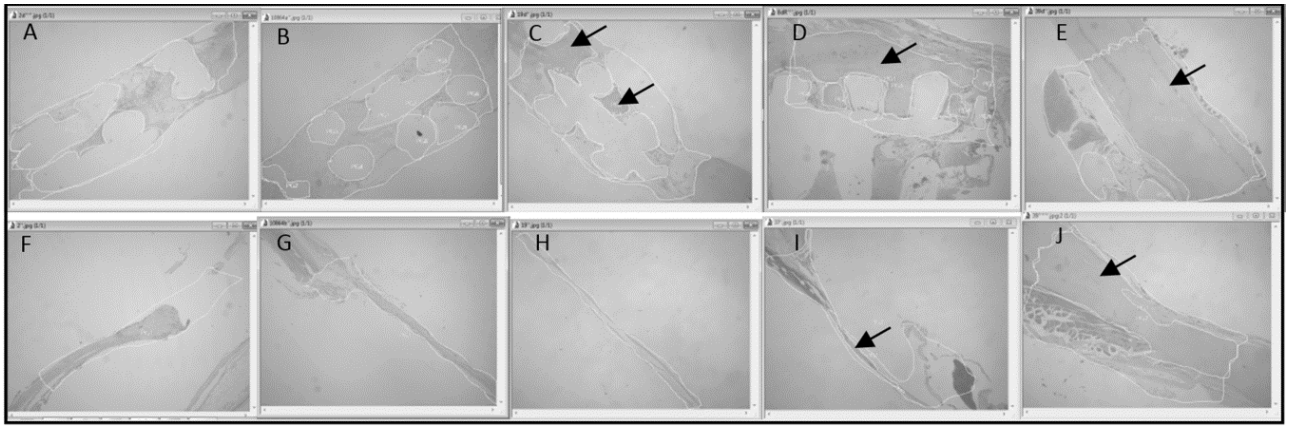
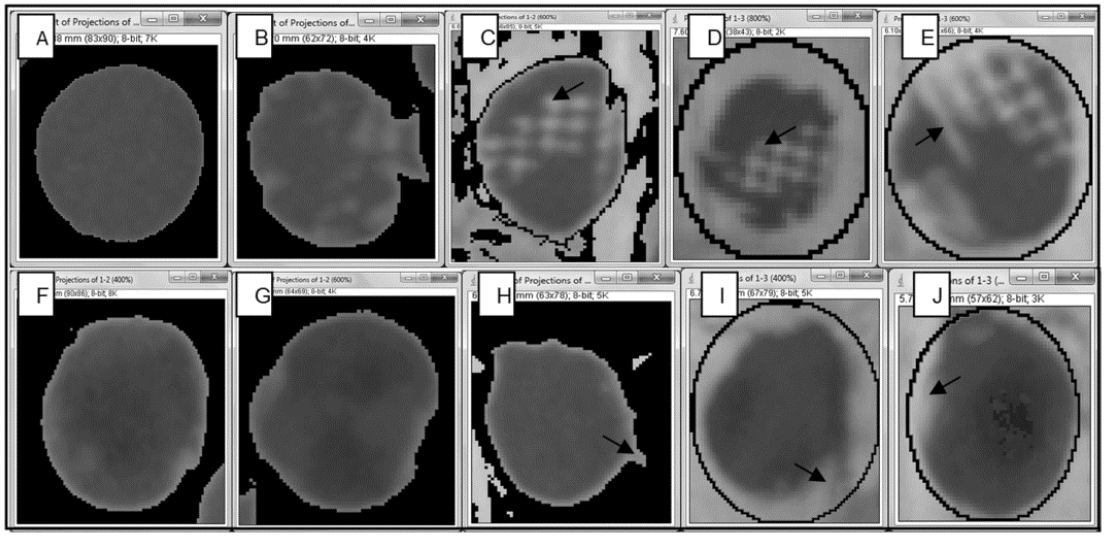


Figure 9



TABLES

Table 1. Descriptive statistics of the association between the use of biomaterial and new bone formation in the different time blocks.

Time (days)	Mean area of new bone formation (mm)	Standard deviation	N
7			
Without biomaterial	33.775	17.8248	4
With biomaterial	38.800	4.9538	4
Total	36.287	12.4056	8
21			
Without biomaterial	137.820	56.8020	5
With biomaterial	131.320	42.4382	5
Total	134.570	47.3937	10
60			
Without biomaterial	103.833	55.6606	6
With biomaterial	131.517	46.5824	6
Total	117.675	51.0251	12
90			
Without biomaterial	91.368	46.0256	6
With biomaterial	133.867	45.9040	6
Total	112.617	49.1251	12
120			

Without biomaterial	162.800	28.2921	6
With biomaterial	174.000	55.7309	6
Total	168.400	42.5422	12
Overall sample			
Without biomaterial	110.082	58.8650	27
With biomaterial	127.707	58.5231	27
Total	118.895	58.8145	54

Table 2. Mean area of new bone formation (mm) in the different time blocks

		Subset		
Time (days)	N	1*	2**	3***
7	8	36.287		
90	12		112.617	
60	12		117.675	117.675
21	10		134.570	134.570
120	12			168.400
Sig.		1.000	0.792	0.088

Tukey's honestly significant difference (HSD) test

Sig. = Significance

* Little or no bone formation.

** Greater bone formation.

*** Better bone formation.

Table 3. Tests of between-subjects effects

Source	Type III sum of squares	df	Mean square	F	Sig.
Time (days)	86948.734	4	21737.183	10.852	0.000*
BIOMATERIAL1no2yes	3360.566	1	3360.566	1.678	0.202
Time (days) * BIOMATERIAL1no2yes	4055.959	4	1013.990	0.506	0.731
Error	88136.297	44	2003.098		
Total	946675.282	54			
Corrected total	183334.903	53			

* R squared = 0.519 (adjusted R squared = 0.421).

df = degrees of freedom, Sig. = Significance.

4 DISCUSSÃO GERAL

A eficácia do uso clínico dos biomateriais para reparar, reconstruir, substituir ou regenerar áreas lesadas por perda óssea foi um dos fatores responsáveis pela ampla difusão do uso de substitutos ósseos nas últimas décadas. O uso de biomateriais sintéticos é uma alternativa frente a algumas das limitações do uso de enxerto autógeno para reparar estruturas ósseas perdidas, como a necessidade de uma cirurgia adicional em área doadora diferente da área receptora, bem como a questão da quantidade de enxerto disponível, que pode não ser suficiente para o preenchimento da área receptora (MACEDO et al., 2004; VALERIO et al.,2004; CARDOSO et al., 2006; GRANDI et al., 2011;).

A literatura comporta uma série de pesquisas que investigam o desempenho dos biomateriais, o que é imprescindível antes do uso clínico. Um biomaterial ideal deve ser biocompatível, biodegradável com produtos de degradação atóxicos, além de possuir porosidade adequada que permita vascularização e garanta resistência mecânica (OUSTERHOUT; STELNICKI, 1996; VALERIO et al., 2004; KNABE et al., 2005).

O PCL é descrito na literatura como um biomaterial que possui propriedades adequadas para ser utilizado na reconstrução tecidual, dentre elas, a lenta degradação, a biocompatibilidade e a resistência mecânica (VALERIO et al., 2004; KNABE et al., 2005; SENEDESE, 2011; FU et al., 2012). Além disso, pode ser utilizado para construir *scaffolds* com as mais variadas formas e dimensões (LIU et al., 2007; DOMINGOS et al., 2009; CHEN et al., 2011).

O processo de produzir *scaffolds* por manufatura aditiva apresenta vantagens como a precisão na deposição do material e a reprodutibilidade do processo, permitindo a obtenção de estruturas complexas 3D, bem como o controle da morfologia interna, o tempo e o custo relativamente baixos (RAYMOND, 2010; SENEDESE, 2011).

O presente estudo permitiu verificar, através de análise histológica, que os *scaffolds* de PCL utilizados nas calvárias de ratos permitem neoformação óssea, a qual ocorreu em direção ao centro dos defeitos (ESKI et al., 2007; MARZOUK et al., 2007; FU et al., 2012) a partir do 21.º dia, de modo que, aos 120 dias, houve a formação de uma ponte óssea de margem a margem do defeito. Não ocorreu a

substituição total do biomaterial por tecido ósseo, o que evidenciou a lenta degradação do PCL (ESKI et al., 2007).

A análise histológica dos tecidos subcutâneos do dorso dos animais, aos 60 dias, permitiu visualizar a formação de cápsula fibrosa fina em todos os espécimes, com fibras colágenas organizadas envolvendo o implante de PCL, o que indica biocompatibilidade do biomaterial (GIAVARESI et al., 2006; SOUZA et al., 2006; FOLLMANN, 2011).

Quanto à citotoxicidade sistêmica, avaliada através da análise dos eventos ocorridos nos rins, pulmões e fígado dos animais, mostrou que a implantação do PCL no dorso dos ratos não promove alterações teciduais danosas nestes órgãos. Não sendo observada a presença de processo inflamatório, hiperplasia, metaplasia, displasia ou hemorragia. Algumas alterações isoladas foram encontradas, pontualmente, nos órgãos, como uma hipercelularidade glomerular leve e congestão vascular nos rins; células isoladas com esteatose macrovesicular e congestão vascular e sinusoidal no fígado; assim como leve espessamento dos septos alveolares e congestão vascular. Entretanto, esses eventos também foram observados nos animais-controle, que não haviam recebido qualquer tipo de tratamento.

O reparo ósseo foi analisado quantitativamente, através de análise tomográfica, por TCFC, e histomorfométrica dos defeitos preenchidos com PCL ou coágulo nas calvárias dos animais.

Os resultados obtidos através da análise histomorfométrica mostram que houve uma área maior de neoformação óssea nos defeitos que foram preenchidos com PCL aos 21, 60 e 120 dias, sendo estatisticamente significantes, quando comparados aos defeitos que continham coágulo sanguíneo. Aos 7 dias, não ocorreu neoformação óssea nos defeitos; aos 90 dias, apesar de ter havido uma maior área de neoformação óssea no defeito preenchido com biomaterial, esse dado não foi estatisticamente significativo.

Observou-se que, na análise dos defeitos confeccionados, o volume do defeito contendo PCL foi mantido, o que não ocorreu no defeito controle, preenchido com coágulo, sugerindo que a porção do disco que não foi reabsorvida serve como arcabouço para a formação óssea.

Na análise por TCFC avaliou-se a presença de osso neoformado, entretanto os resultados obtidos mostram que houve uma tendência de maior neoformação

óssea nos defeitos que foram preenchidos com PCL, mas sem diferença estatisticamente significativa. Então, independentemente do uso do biomaterial, ao longo do tempo todos os defeitos apresentavam maior área de neoformação óssea.

Assim, em ambas as análises, os resultados revelaram que, em valores absolutos, os defeitos preenchidos com PCL apresentaram maior área de neoformação óssea. Entretanto, após análise estatística, esses valores não foram significativos para a análise tomográfica. Então, considerando-se a análise tomográfica como uma nova metodologia para avaliação de neoformação óssea em calotas cranianas de ratos, os dados obtidos através das avaliações de áreas ósseas neoformadas pela tomografia *cone beam* não puderam ser correlacionados com aqueles obtidos na análise histomorfométrica, considerada como padrão-ouro para avaliar neoformação óssea.

Neste estudo, os níveis de cinza da região de neoformação óssea dos defeitos avaliados na tomografia *cone beam* não demonstraram coerência com os dados obtidos através da análise histomorfométrica dos mesmos defeitos.

Frente aos resultados obtidos, pode-se concluir que os *scaffolds* de PCL produzidos na plataforma experimental de manufatura aditiva Fab@CTI são biocompatíveis, não citotóxicos, biorreabsorvíveis e preservam as condições de osteocondução. Novas pesquisas que investiguem o reparo ósseo frente ao uso do PCL, com tempo de acompanhamento mais longo, poderão corroborar tais achados. Os resultados são sugestivos de que o PCL possui características necessárias para uso clínico, como nos defeitos com comprometimento estético-funcional.

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Normally one or two, and no more than three, authors should appear on a short communication, technical note or interesting case/lesson learnt. Full length articles may contain as many authors as appropriate. Minor contributors and non-contributory clinicians who have allowed their patients to be used in the paper should be acknowledged at the end of the text and before the references.

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Text

Please ensure that the text of your paper conforms to the following structure: Introduction, Materials and Methods, Results, Discussion. There is no separate Conclusion section. There should be no mention of the institution where the work was carried out, especially in the Materials and Methods section.

Introduction

• Present first the nature and scope of the problem investigated

• Review briefly the pertinent literature
• State the rationale for the study
• Explain the purpose in writing the paper
• State the method of investigation and the reasons for the choice of a particular method
•; Should be written in the present tense

Materials and Methods
• Give the full details, limit references • Should be written in the past tense • Include exact technical specifications, quantities and generic names • Limit the number of subheadings, and use the same in the results section • Mention statistical method • Do not include results in this section

Results
• Do not describe methods
• Present results in the past tense
• Present representations rather than endlessly repetitive data
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• Discuss - do not recapitulate results • Point out exceptions and lack of correlations. Do not try to cover up or 'fudge' data • Show how results agree/contrast with previous work • Discuss the implications of your findings • State your conclusions very clearly

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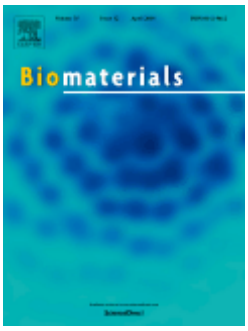

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2. Nancollas H. In vitro studies of calcium phosphate crystallisation. In: Mann S, Webb J, Williams RJP, editors. Biomineralization. Chemical and biochemical perspectives. New York: VCH, 1989. p. 157-182.
3. Brown W, Chow LC. Combinations of sparingly soluble calcium phosphates in slurries and paste as mineralizers and cements. US Patent No. 4612053, 1986.

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3. Keeney M, Lai JH, Yang F. Recent progress in cartilage tissue engineering. Curr Opin Biotechnol. 2011. Available from URL: http://www.ncbi.nlm.nih.gov/pubmed/21531126 (DOI: 10.1016/j.copbio.2011.04.003).

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Keywords">Keyword List

ANEXO C - Aprovação da Comissão Científica e de Ética da Faculdade de Odontologia da PUCRS

Comissão Científica e de Ética
Faculdade da Odontologia da PUCRS

Porto Alegre 16 de Setembro de 2010

O Projeto de: Tese

Protocolado sob nº:	0072/10
Intitulado:	Análise histomorfométrica da biocompatibilidade e condutibilidade óssea do policaprolactona - estudo em ratos
Pesquisador Responsável:	Prof. Dr. Cláiton Heitz
Pesquisadores Associados	Wâneza Dias Borges Hirsch; Daniela Nascimento Silva; Helena Willhelm de Oliveira; Gustavo Henrique de Lima Paschoal
Nível:	Tese / Doutorado

Foi ***aprovado*** pela Comissão Científica e de Ética da Faculdade de Odontologia da PUCRS em *16 de Setembro de 2010.*

Este projeto deverá ser imediatamente encaminhado ao CEUA/PUCRS

Profa. Dra. Ana Maria Spohr
Presidente da Comissão Científica e de Ética da
Faculdade de Odontologia da PUCRS

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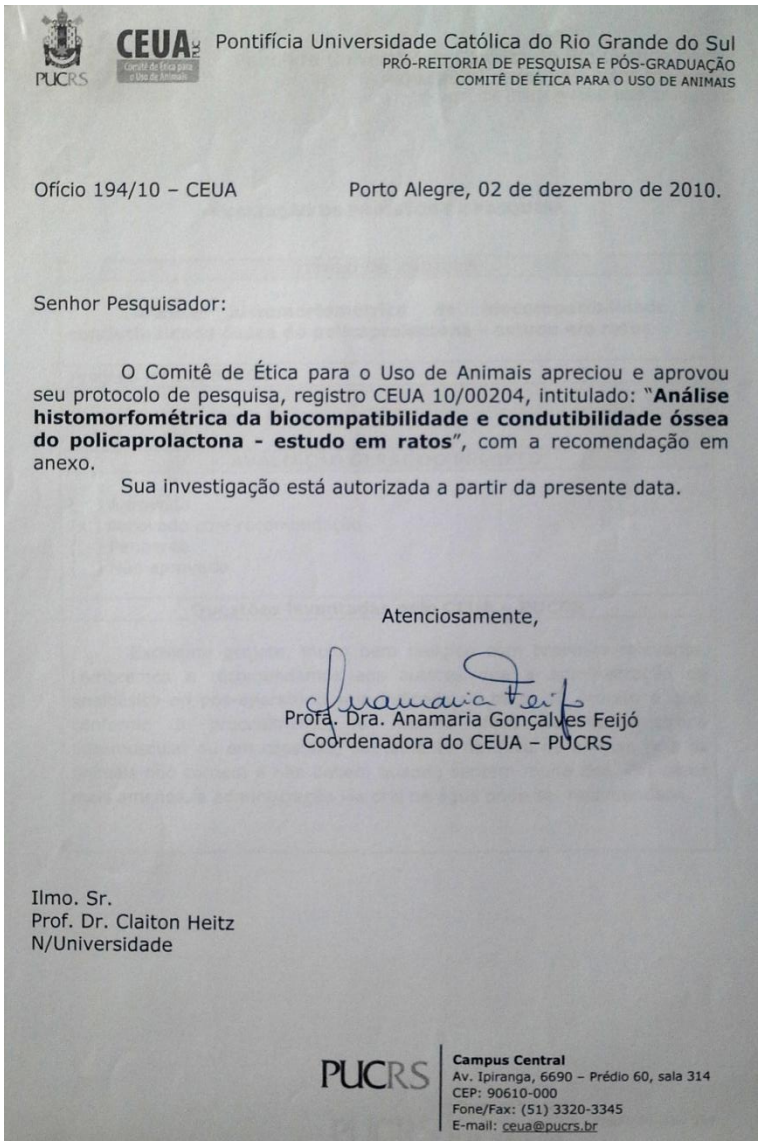
e-mail: odontologia-pg@pucrs.br

ANEXO D – Aprovação do Comitê de Ética para o uso de animais



PUCRS CEUA Comitê de Ética para o Uso de Animais

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMITÊ DE ÉTICA PARA O USO DE ANIMAIS

Ofício 194/10 – CEUA Porto Alegre, 02 de dezembro de 2010.

Senhor Pesquisador:

O Comitê de Ética para o Uso de Animais apreciou e aprovou seu protocolo de pesquisa, registro CEUA 10/00204, intitulado: "**Análise histomorfométrica da biocompatibilidade e condutibilidade óssea do policaprolactona - estudo em ratos**", com a recomendação em anexo.

Sua investigação está autorizada a partir da presente data.

Atenciosamente,

Profa. Dra. Anamaria Gonçalves Feijó
Coordenadora do CEUA – PUCRS

Ilmo. Sr.
Prof. Dr. Claiton Heitz
N/Universidade

PUCRS **Campus Central**
Av. Ipiranga, 6690 – Prédio 60, sala 314
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Fone/Fax: (51) 3320-3345
E-mail: ceua@pucrs.br

AVALIAÇÃO DE PROJETOS DE PESQUISA

TITULO DO PROJETO
Análise histomorfométrica da biocompatibilidade e condutibilidade óssea do policaprolactona - estudo em ratos
Projeto nº 10/00204
Pesquisador: Claiton Heitz
AVALIAÇÃO GERAL DO PROJETO
( ) Aprovado (x) Aprovado com recomendação ( ) Pendente ( ) Não aprovado
Questões levantadas pelo CEUA – PUCRS
Excelente projeto, muito bem redigido com proposta relevante. Lembramos e recomendamos aos autores que a administração do analgésico no pós-operatório seja indicada no texto do projeto e que, conforme o procedimento, o mesmo deve ser administrado intramuscular ou em capsulas, diretamente na boca do animal, pois os animais não comem e não bebem quando sentem muita dor. Em casos mais amenos, a administração via oral na água pode ser recomendada.

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