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REPARO DO TECIDO ÓSSEO IRRADIADO APÓS UTILIZAÇÃO DE BETA FOSFATO TRICÁLCICO ASSOCIADO A HIDROXIAPATITA: ESTUDO EM RATOS

REPAIR OF IRRADIATED BONE TISSUE AFTER USE OF BETA TRICALCIUM PHOSPHATE ASSOCIATED WITH HYDROXYAPATITE: A STUDY WITH RATS

PORTO ALEGRE- RS
2012

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Tese apresentada à Faculdade de Odontologia da Pontifícia Universidade Católica do Rio Grande do Sul como parte dos requisitos para obtenção do título de Doutor em Odontologia – Área de Concentração em Estomatologia Clínica.

Orientador: Profa. Dra. Fernanda Gonçalves Salum

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BANCA EXAMINADORA

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"Que a inspiração chegue, não depende de mim. A única coisa que posso fazer é garantir que ela me encontre trabalhando".

(Pablo Picasso)

RESUMO

O biomaterial constituído por 60% de hidroxiapatita (HA) e 40% de beta fosfato tricálcico (β-TCP) (Bone Ceramic®, Straumann S. A. – Zurich, Suíça) é um enxerto ósseo sintético, biocompatível, cuja composição bifásica fornece capacidade de suportar a neoformação óssea e manter a estabilidade mecânica do tecido. No presente estudo foi avaliado o reparo em defeitos ósseos críticos, confeccionados em calvária de ratos, preenchidos com esse biomaterial, antes e após a terapia com radiação ionizante. A amostra foi constituída por 33 ratos Wistar distribuídos em dois grupos experimentais, que receberam 12 Gy de radiação em dose única, e um grupo-controle. No primeiro grupo experimental (n=12) a confecção e o preenchimento do defeito na calvária foram realizados duas semanas após a radioterapia (grupo pós-radioterapia). No segundo grupo (n=12) a confecção e o preenchimento do defeito ósseo ocorreram duas semanas antes da radioterapia (grupo pré-radioterapia). Os animais do grupo-controle (n=9) foram submetidos aos mesmos procedimentos cirúrgicos, no entanto, não receberam terapia com radiação ionizante. Os animais foram mortos 12 semanas após os procedimentos cirúrgicos. Em todos os grupos foi observado íntimo contato entre o tecido ósseo neoformado e os grânulos do biomaterial, bem como ausência de necrose. Na análise histomorfométrica não foram detectadas diferenças estatisticamente significativas entre os três grupos quanto ao percentual de tecido ósseo neoformado nos defeitos. Também não houve diferenças significativas entre os grupos quanto ao número de osteoblastos, osteoclastos e células inflamatórias. O percentual de imunodetecção do VEGF (fator de crescimento endotelial vascular) ($p<0,001$) e a densidade mineral na região dos defeitos ($p=0,020$) foram superiores

no grupo-controle. Pode-se concluir que o biomaterial constituído por HA e β-TCP promove osteocondução no tecido irradiado de forma semelhante a que ocorre no tecido não irradiado. Além disso, não há diferenças quanto ao momento do emprego da radiação ionizante, ou seja, se antes ou após a aplicação do biomaterial.

Palavras-chave: Radiação. Substitutos ósseos. Regeneração óssea. Ratos.

ABSTRACT

The biomaterial made up of 60% hydroxyapatite (HA) and 40% beta tricalcium phosphate (β-TCP) (Bone Ceramic®, Straumann S.A. – Zurich, Switzerland) is a synthetic bone graft, biocompatible, whose biphasic composition enables it to bear the bone newly formation and maintain the tissue mechanical stability. The present study has evaluated the repair in critical bone defects, made on rats calvaria, filled with that biomaterial before and after therapy with ionizing radiation. The sample was formed by 33 Wistar rats distributed into two experimental groups that received 12 Gy radiation (single dose), and a control group. In the first experimental group (N=12) the fabrication and filling of the calvaria defect were performed two weeks after radiotherapy (pos-radiotherapy group). In the second group (n=12) the fabrication and filling of the calvaria defect were performed two weeks before radiotherapy (pre-radiotherapy group). The animals in the control group (n=9) were submitted to the same surgical procedures but did not receive any ionizing radiation therapy. The animals were killed 12 weeks after surgical procedures. In all groups a close contact between the biomaterial granules and the newly formed bone tissue was observed, as well as absence of necrosis. No statistically relevant differences were detected in the histomorphological analysis of the three groups with regards to the percentage of newly formed bone tissue in the defects. There were also no differences among the groups with regards to the number of osteoblasts, osteoclasts and inflammatory cells. The VEGF (Vascular Endotelial Growth Factor) ($p<0.001$) immunodetection percentage, and the mineral density in the defect site ($p=0.020$) were higher in the control group. Thus, it can be concluded that the

biomaterial made up of HA and β-TCP promotes osteoconduction in the irradiated tissue similarly to what occurs in the non-irradiated tissue. Moreover, there is no difference regarding the moment the ionizing radiation is employed, that is, whether before or after applying the biomaterial.

Key Words: Radiation. Bone substitutes. Bone regeneration. Rats.

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LISTA DE ABREVIATURAS, SÍMBOLOS E SIGLAS

ADN – ácido desoxirribonucleico

Ca – cálcio

$Ca_{10}(PO_4)_6(OH)_2$ – fórmula química da hidroxiapatita

CFC – cimento de fosfato de cálcio

cm – centímetro

Co – cobalto

g – grama

Gy – Gray

H – hidrogênio

HA – hidroxiapatita

Kg – quilograma

mg – miligrama

mL – mililitro

mm – milímetro

O – oxigênio

OH – radical hidroxila

P – fósforo

Pb - chumbo

TCP – fosfato tricálcico

TGF- β1 – fator de transformação do crescimento tecidual beta um

Ti - titânio

VEGF – fator de crescimento endotelial vascular

β-$Ca_3(PO_4)_2$ – fórmula química do beta fosfato tricálcico

β-TCP – beta fosfato tricálcico

µm – micrometro

® - marca registrada

% - porcentagem

SUMÁRIO

1 INTRODUÇÃO

As neoplasias malignas da região de cabeça e pescoço são tratadas, com frequência, por ressecções cirúrgicas associadas à radioterapia, procedimento que gera efeitos adversos nos tecidos normais (EDWARDS; JOHNSON, 1999; MALARD et al., 2005). A radiação ionizante causa danos aos elementos envolvidos no reparo tecidual, reduzindo a função dos leucócitos e a atividade fagocítica, além de diminuir a expressão de colágeno durante a fase proliferativa (SHULTZE-MOSGAU et al., 2001; JEGOUX et al., 2010). No tecido ósseo, a radiação suprime a proliferação normal de osteoblastos, promove decréscimo do número de osteócitos e redução da vascularização tecidual. Os mecanismos moleculares que norteiam esses achados histológicos ainda não são completamente compreendidos (DUDZIAK et al., 2000; SZYMCZYK; SHAPIRO; ADAMS, 2004).

Os danos estéticos e funcionais decorrentes da ablação cirúrgica de tumores na região bucomaxilofacial geram necessidade de procedimentos corretivos (SZYMCZYK; SHAPIRO; ADAMS, 2004; INYANG et al., 2010). No entanto, há risco elevado de complicações após a realização de procedimentos cirúrgicos no tecido ósseo irradiado tais como fraturas tardias e osteorradionecrose, pois este tecido tem sua capacidade reparadora reduzida devido ao decréscimo celular e vascular (EVANS; BROWN; HURST, 1991; DUDZIAK et al., 2000; SZYMCZYK; SHAPIRO; ADAMS, 2004). A osteorradionecrose pode ser definida como ulceração ou necrose dos tecidos moles de revestimento, com exposição óssea por mais de três meses, na ausência de doença metastática ou recorrente. Essa enfermidade pode estar associada com infecção local e causar fratura patológica do tecido ósseo acometido (EPSTEIN et al., 1997; WONG et al., 2009).

O enxerto autógeno tem sido considerado pela literatura o melhor e mais bem aceito material para tratamento dos defeitos ósseos gerados pela ablação de tumores. Contudo, a necessidade de cirurgias adicionais para acesso em área doadora aumenta a morbidade do procedimento. Além disso, em muitos casos, a quantidade de enxerto disponível não é suficiente para o preenchimento da área receptora. As desvantagens ou limitações do enxerto autógeno estimulam a busca por materiais alternativos tais como os enxertos aloplásticos ou sintéticos (MACEDO et al., 2004; MATSUSHIMA et al., 2009).

Os enxertos aloplásticos possuem propriedades osteocondutoras, fornecendo o suporte que guiará a formação óssea. Esses materiais devem ser bioinertes ou bioativos, biocompatíveis, não alergênicos, não carcinogênicos, resistentes à deformação e podem ou não ser resistentes à reabsorção, conforme a indicação desejada. Além disso, sua forma e dimensões precisam favorecer o crescimento ósseo pelo interior do material, uma vez que a deposição óssea deve ocorrer por substituição (SANTOS, 2002; VALERIO et al., 2004; SHIRATORI et al., 2005). Entre as vantagens encontradas nos enxertos aloplásticos destacam-se a disponibilidade comercial, fácil manipulação, além da possibilidade de integração física e/ou química ao meio inserido. A hidroxiapatita é um material aloplástico formados por cálcio (Ca) e fósforo (P) em proporções similares às do osso. O fosfato tricálcico (TCP) e a hidroxiapatita (HA) são biocerâmicas com ampla aplicação na área biomédica, sendo destacadas sua biocompatibilidade e bioatividade, que permitem a osteocondução (NASR; AICHELMANN-REIDY; YUKNA, 1999). Além disso, cimentos de fosfato de cálcio podem ser preparados durante o ato cirúrgico (SANTOS, 2002), pois são de fácil manipulação e adaptam-

se totalmente à forma da cavidade, apresentando íntimo contato com suas paredes desde os primeiros estágios da implantação (DRIESSENS et al., 1997).

O grânulo beta fosfato tricálcico (β-TCP), que se apresenta quimicamente pela fórmula β-$Ca_3(PO_4)_2$, combina as propriedades de solubilidade com osteocondutividade e seu processo de absorção favorece a neoformação óssea (DRIESSENS et al., 1998; SANTOS, 2002). A hidroxiapatita (HA) é um fosfato de cálcio hidratado, componente majoritário da fase mineral dos ossos e dentes humanos, cuja fórmula química é representada por $Ca_{10}(PO_4)_6(OH)_2$. A adição de β-TCP à HA visa aumentar os níveis de solubilidade e biodegradação do material (CHOW, 1998), elevar a capacidade de manter o volume de defeitos ósseos, devido a sua maior permanência nos tecidos (GRANDI et al., 2011), e promover neoformação óssea a partir de células indiferenciadas (MATSUSHIMA et al., 2009).

O biomaterial composto por 60% de HA e 40% de β-TCP (Bone Ceramic®, Straumann S. A., Suíça) é um enxerto totalmente sintético, empregado como substituto ósseo. Além da elevada biocompatibilidade, sua composição bifásica fornece capacidade de suportar a neoformação óssea e manter a estabilidade mecânica (JENSEN et al., 2007; FROUM et al., 2008). Cordaro et al. (2008) constataram que o Bone Ceramic® apresenta propriedades semelhantes às do osso bovino liofilizado (Bio Oss®, Osteohealth S. A., USA), na quantidade e qualidade de tecido ósseo neoformado. Schwarz et al. (2009) observaram reação antigênica positiva à osteocalcina em estudo clínico no qual utilizaram o biomaterial composto por HA e β-TCP em defeitos ósseos. Os autores verificaram também que a formação óssea ocorreu a partir da degradação do enxerto sintético. Ao compararem o Bone Ceramic® e o Bio Oss® associados a células mesenquimais

provenientes da medula óssea, Khojasteh, Eslaminejad e Nazarian (2008) verificaram, por histomorfometria, maior formação óssea no primeiro grupo.

Apesar dos riscos de necrose após procedimentos cirúrgicos reparadores no tecido ósseo irradiado, o uso de fosfatos de cálcio tem sido descrito em experimentos com animais e parece apresentar resultados satisfatórios (MALARD et al., 2005; LEROUXEL et al., 2006; ESPITALIER et al., 2009). Os estudos diferem quanto ao melhor momento da implantação dos biomateriais em relação à radioterapia, ou seja, se eles devem ser utilizados antes ou após o tratamento com radiação ionizante (JEGOUX et al., 2010). Andrade et al. (2008) concluíram não haver diferença nos resultados de cirurgias reconstrutivas realizadas antes e após a radioterapia, com índices de complicações similares entre ambas. Kudo et al. (2001) afirmaram que a irradiação cinco dias após a implantação intra-óssea de hidroxiapatita inibiu o contato direto entre o tecido ósseo e o material. Por outro lado, o contato obtido com irradiação prévia foi minimamente afetado.

O material constituído por HA e β-TCP tem apresentado resultados satisfatórios em procedimentos empregados para neoformação óssea, entretanto, não há relatos acerca do seu emprego em osso irradiado. O presente estudo objetiva avaliar o reparo em defeitos ósseos críticos confeccionados em calotas cranianas de ratos, preenchidos com esse biomaterial antes e após a terapia com radiação ionizante.

2 PROPOSIÇÃO

2.1 Objetivo Geral

Avaliar o reparo e a área de neoformação óssea em defeitos críticos, confeccionados na calota craniana de ratos, preenchidos com o biomaterial constituído por HA e β-TCP (Bone Ceramic®, Straumann S. A., Suíça), antes e após a terapia com radiação ionizante.

2.2 Objetivos Específicos

Avaliar, em defeitos ósseos críticos confeccionados na calota craniana de ratos e preenchidos com β-TCP associado a HA (Bone Ceramic®, Straumann S. A., Suíça), antes e após terapia com radiação ionizante:

- a neoformação óssea por meio de análise descritiva e histomorfométrica;
- a densidade óssea mineral;
- o número de células ósseas e inflamatórias;
- a imunodetecção do fator de crescimento endotelial vascular (VEGF).

3 ARTIGO DE REVISÃO DE LITERATURA

EFFECTS OF RADIOTHERAPY IN HEAD AND NECK REGION ON BONE TISSUE: ALTERNATIVES FOR CORRECTIVE SURGICAL PROCEDURES

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EFFECTS OF HEAD AND NECK RADIOTHERAPY ON BONE TISSUE: ALTERNATIVES FOR CORRECTIVE SURGICAL PROCEDURES

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Abstract

Introduction: Therapy with ionizing radiation produces adverse effects that vary depending on its anatomic origin, dose used and structure of tissue involved. In bone tissue, it induces hypoxia, hypocelullarity and hypovascularization, resulting in complications such as osteoradionecrosis and pathogenic fractures. In the head and neck region, the mandible is the bone most often affected by the adverse effects of radiotherapy, due to its poor vascular supply and to its thin cover of soft tissues. The surgical ablation of tumors in this location causes visible facial defects, creating the need for reconstruction, which is generally performed at the same time as the removal of the tumor. However, the failure of primary grafts can lead to indication of secondary bone reconstructions. **Purpose:** The aim of this review was to evaluate the effects of the radiation on bone tissue, specifically the mandible bone, as well as different forms of primary and secondary reconstructions. **Results:** The various techniques and materials are described, with the majority showing satisfactory results. Still, the risks of complications, mainly osteoradionecrosis, are taken into consideration, where it is mandatory to use techniques that address the nutritional deficiencies of the tissue.

KEY WORDS: radiation, surgical reconstructive procedures, bone substitutes.

Introduction

Radiotherapy is often the treatment of choice or coadjuvant to other procedures in the malignant neoplasms managements of the head and neck region. Therapy with ionizing radiation produces irreversible effects in normal tissues, because it causes damage to the components directly involved in tissue repair, reducing the function of the leukocytes and phagocytic activity common in the inflammatory process [1-3]. In bone tissue, it suppresses the normal proliferation of osteoblasts, decreases the number of osteocytes and reduces tissue vascularization, which is associated with a significant degree of osteoradionecrosis. The molecular mechanisms that underlie these histological findings are still not completely understood [4-6].

Due to its reduced repair capacity, there is elevated risk of complications after surgical procedures in the irradiated bone tissue such as delayed fracture, with low repair rates, and osteoradionecrosis [6]. Compared to the other bones of the face, the mandibular bone is the most affect by osteoradionecrosis due to its poor vascular supply and gingival covering, considered a thin anatomic plane. The pathogenesis of this complication is attributed to factors induced by the radiation such as hypoxia and hypocellularity of the bone tissue [7].

Primary or immediate bone reconstruction is considered the gold standard after ablative surgery of the tumor. However, the loss of primary grafts can lead to an indication of secondary bone reconstructions or corrections in the irradiated tissue. The techniques vary, but all should take into account the risks in procedures performed in the irradiated tissue, since its behavior differs from that of normal tissue [4]. Andrade et al. [8] compared the results of primary and secondary

reconstructive surgeries, concluding that there was no difference in results between the two, with similar complication rates.

The literature addresses different corrective procedures in the irradiated mandible. Case reports in humans and experiments in animals try to uncover effective ways of reconstructing the lost bone structure, before as well as after radiotherapy. This review will discuss the alterations in mandibular bone tissue as a result of radiotherapy and will review the different techniques and the materials utilized in surgical procedures involving this irradiated tissue.

Review of the Literature

The biological effect of radiation on the tissues is influenced by three factors: type of radiation, that is, its atomic origin and level of energy used, the structure of the tissue to be irradiated, and the form such as the radiation is emitted. Tissues with greater regenerative capacity, with a high response rate, should receive smaller doses of radiation distributed in larger spaces of time. Those that respond in a slower way, such as bone tissue, suffer less collateral effects when treated with small fractionated doses [9].

Effects of the radiotherapy on bone physiology

Bone tissue shows different degrees of sensitivity to radiation, because it is composed of different components. Its mineral part is not considered radiosensitive, but the molecular composition of the hydroxyapatite of the bone surface undergoes alterations that cause changes in the mechanical properties of this tissue in the long term [10]. The effect of radiation on the bone marrow causes, in the short term,

reduction of the hematopoietic components by apoptosis and, consequently, inhibition of the differentiation of the mesenchymal cells to osteoprogenitors [11, 12]. According to Wong et al. [13], ionizing radiation interferes with the differentiation of osteoblasts, diminishes the production of alkaline phosphatase in the tissue, and causes destruction of the osteocytes, disorganization of tissue architecture and interruption of the process of homeostasis. According to Andreassen et al. [14], osteoblasts can appear dysfunctional, but are not necessarily killed by radioactive effects.

The periosteum is also affected by the reduction in celullarity and vascularization as well as decrease of the formation of the osteoid component [15]. Bone remodeling is altered by radiation, but the changes occur as a reversible phenomenon, secondary to quantitative and qualitative cellular alterations [16].

Radiation induces modifications not only in the osteogenic process, but also in the inflammatory process. In the initial phase of the post-irradiation inflammatory process, myofibroblasts appear which persist during the fibrotic phase. Other characteristics of this specific inflammatory process are: increase in vascular permeability, formation of edema, dysfunction of endothelial cells and microvascular thrombosis, leading to the necrosis of small vessels and local ischemia, with increase in fibroblastic activity [16]. Many types of cytokines play an important role in the tissue damage caused by radiation. TGF-β1 (transforming growth factor-β1) is the principal cytokine involved in this process, also interfering with the induction of fibroblast proliferation [17-20].

Radiotherapy in the head and neck region is associated with a significant extent of osteoradionecrosis, depending on the dosimetry utilized [13]. The mandibular bone is the most affected compared to the other bones of the face. About

10% of patients with neoplasms of the head and neck, treated with low doses of radiation, develop osteoradionecrosis. This percentage increases when the dose exceeds 60 Gy, and when the irradiated site suffers some type of trauma. The pathogenesis of this complication is attributed to factors induced by radiation such as hypoxia, hypocellularity and hypovascularization of the bone tissue [7]. The results of radiation damage in the bone tissue involve a picture of pain and functional deficits, which require debridement and subsequent reconstructions [14, 21].

Corrective procedures of the mandibular bone prior to radiotherapy

Reconstruction of the structure lost is generally done soon after or at the moment of surgical ablation of the tumor, and radiotherapy is indicated after these procedures. Vascularized bone grafts are a viable option for this type of reconstruction, although some degree of reabsorption can develop, especially when inserted in critical defects. The stability of vascularized grafts leads to better aesthetic results and greater predictability in rehabilitation with dental implants. However, surgical procedures of greater morbidity are required, which can be contra-indicated in patients of advanced age. Another disadvantage is the difficulty in modeling the graft in a similar shape as the lost structure [18, 19, 22], although there are digitally simulated templates that facilitate modeling of the graft during surgery [20].

Another possibility for the reconstruction of mandibular defects prior to radiotherapy is the use of non-vascularized grafts of the iliac crest. Since radiotherapy reduces their success rate, these grafts can only be indicated if the bone defect is not larger than 5.0 or 6.0 cm and if the recipient bed is surrounded by healthy soft tissues [19].

The use of plates and screws made of titanium (Ti) alloy maintain the distance between the remaining bone after surgical resection of the tumor. The surgical procedure is relatively simple, but the aesthetic results are not satisfactory, besides causing difficulties for dental rehabilitation. Furthermore, radiotherapy can cause the exposure of synthetic material [21,22].

Osteogenic distraction is an alternative and can be proposed for the repair of mandibular defects that require subsequent radiotherapy, which can substitute for invasive surgical procedures. This technique requires more studies, since even though demonstrating bone growth in the region of the defect, there is risk of the development of osteoradionecrosis, besides recurrence of the tumor because of the induction of angiogenesis [23]. According to González-Garcia et al. [24], the effects of radiotherapy performed after osteogenic distraction remains uncertain.

Corrective procedures of the mandibular bone after radiotherapy

The literature describes various forms of secondary reconstruction of the mandible after radiotherapy. In these cases, it is important to consider the characteristics of the tissue irradiated, which appears hypovascularized. Grafts of vascularized tissues are generally utilized in cases of bone discontinuity defects to reestablish the morphologic and functional normality and to guarantee blood supply to the region, when very compromised. Their disadvantages encourage research into alternative materials and techniques [25].

Osteogenic distraction can be used in these cases provided that a protocol optimizing the technique is established. Girod et al. [22] found that radiotherapy prior to osteogenic distraction does not impede mineralization or consolidation of the mandibular bone tissue in sheep. However, the osteoid surface of the

neoformed bone was significantly less in the group that received radiation. González-Garcia et al. [24] suggested that osteogenic distraction performed after radiotherapy be combined with hyperbaric oxygen therapy.

Studies such as that of Papadas et al. [26] report that the use of myocutaneous flaps combined with reconstructive plates of titanium in the mandible give acceptable results from aesthetic and functional point of view. An alternative that has been studied in animals is the utilization of biphasic calcium phosphate combined with mesenchymal cells recovered from bone marrow. The results reveal that this combination can induce angiogenesis and counter-balance the local effects of radiotherapy.

Table 1 describes samples of studies that examined different forms of corrections of bone defects pre- and post-radiotherapy. The surgical techniques and filling materials vary, and the analysis of their results should take into consideration factors that interfere with tissue repair.

Table 1. Samples of studies that describe different forms of bone reconstruction pre- and post-radiotherapy.

Authors	Sample	Treatment proposed	Results
Evans et al. [27]	Mandibles of 10 beagles: -5 irradiated -5 not irradiated (4080 cGy/day, for 4 weeks)	Rib graft prior to radiotherapy. Right side: vascularized, left side: not vascularized.	Graft vascularized, showed greater quantity of osteocytes, greater contact with recipient bed and better structure of periosteum and endosteum.
Greene et al. [28]	12 patients: -6 submitted to radiotherapy prior to reconstruction -6 irradiated after reconstruction	Non-vascularized graft of fibula combined with alloplastic condylar prosthesis and plate reconstruction.	5 patients without complications: 2 previously irradiated and 3 later irradiated. Complications: plate exposure, recurrence of tumor, osteoradionecrosis.
Girod et al. [22]	Mandibles of 16 sheep: -8 irradiated	Osteogenic distraction prior to radiotherapy.	Radiotherapy did not impede mineralization or consolidation of the tissue.

	-8 not irradiated (45 Gy fractionated over 15 sessions, for 35 days)		
Malard et al. [2]	Tibia and femur of 6 beagles (2 Gy/day, for 3 weeks)	Bone marrow cells combined with calcium phosphate after irradiation.	Presence of bone neoformation within pores of the implanted material.
González-Garcia et al. [24]	6 patients: ablation of tumors in mandible followed by radiotherapy	Osteogenic distraction after radiotherapy.	Bone exposure and loss of the distractor in 1 case. Results considered: - acceptable - 1 case - good - 2 cases - excellent - 2 cases
Torroni et al. [29]	3 patients: ablation of tumors in mandible followed by radiotherapy	Vascularized graft of iliac crest and fibula, followed by orthognathic surgery, after radiotherapy.	Iliac crest showed better results than the fibula.
Kashiwa et al. [30]	4 patients: ablation of tumors in mandible followed by radiotherapy	Vascularized bone graft followed by osteogenic distraction, after radiotherapy.	At 5 sites, there was formation of satisfactory quantity of bone tissue; at 1 site, fracture after distraction; at 1 site, formation of fibrous calum.
Espitalier et al. [31]	Tibia and femur of 23 rats (single dose of 20 Gy)	Use of bone marrow and mesenchymal cells combined with calcium phosphate cement, after radiotherapy.	Significant bone growth around the graft.
Leonhardt et al. [4]	5 patients: ablation of tumors in mandible followed by radiotherapy	Microvascularized graft of forearm after radiotherapy.	No case of graft loss; 2 cases required reintervention.
Inyang et al. [23]	Mandibles of 10 rats: -5 irradiated -5 not irradiated (36 Gy fractionated over 10 sessions)	Osteogenic distraction after radiotherapy.	Reduction of osteocytes; quantity of mineralized mature tissue similar in both groups (with and without radiotherapy).
Handschel et al. [19]	Retrospective study in 84 patients with surgical resection of tumor in mandible.	Non-vascularized graft of iliac crest prior to radiotherapy.	Success in 75% of patients.

Conclusions

For primary as well as secondary reconstruction of the irradiated mandibular bone, the literature does not question the superiority of the vascularized graft. What varies between the different authors is the choice of donor site [4, 19, 27, 29]. In view of the disadvantages of vascularized grafts, such as the necessity of access to a donor site and limited quantity of graft, alternatives are considered, such as osteogenic distraction and non-vascularized grafts.

Irradiation of tissue after osteogenic distraction is still not an extensively described protocol and requires more investigations to establish consistent results [23, 24]. On the other hand, osteogenic distraction has shown promising results when performed after irradiation of the bone tissue. This treatment needs longer time of use of the distractor due to hypocelullarity and hypovascularization, which results in delay of the maturation of the osteoid tissue formed between the edges of the defect [22, 23, 30].

The success of the procedures utilizing non-vascularized grafts in the irradiated mandible appears to depend on the extent of the defect and on the presence of bone continuity in its interior, that is, whether or not the extremities of the defect remain united by a bridge of remaining bone tissue. There are descriptions of the combination of these grafts with biomaterials, for the purpose of optimizing their contact with the recipient bed, besides providing a greater quantity of material for its filling [2, 19, 27].

The use of calcium phosphate for filling defects in irradiated bone is little described. Experiments in animals combining this biomaterial with progenitor cells have presented satisfactory results, with the absence of necrosis and bone

neoformation at the site [31]. Discussion on the best form of mandibular reconstruction, besides taking into account the techniques and materials, should also consider factors such as age of the patient, type of radiation emitted and time of treatment, as well as the extent of the area to be reconstructed [16]. The analysis of this set of factors is important to guide the surgeon with regard to the best approach and treatment of the patient.

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4 ARTIGO DE PESQUISA

REPAIR OF IRRADIATED BONE TISSUE AFTER USE OF BETA TRICALCIUM PHOSPHATE ASSOCIATED WITH HYDROXYAPATITE: A STUDY WITH RATS

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REPAIR OF IRRADIATED BONE TISSUE AFTER USE OF BETA TRICALCIUM PHOSPHATE ASSOCIATED WITH HYDROXYAPATITE: A STUDY WITH RATS

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Abstract

Background: In this study we assessed bone repair in bone critical defects carried out on rats calvaria and filled with phosphate beta tricalcium biomaterial associated with hydroxyapatite before and after radiotherapy. **Methods:** 33 Wistar rats were distributed into three groups. Experimental groups were irradiated (12Gy), two weeks before or after surgical procedures. The control was submitted to the same procedures but was not irradiated. **Results:** There was no necrosis and the newly formed bone presented close contact with the material. There was not significant difference between groups regarding the percentage of newly formed bone or number of osteoblats, osteoclasts and inflammatory cells. In the control group, the VEGF (p=0.001) immunodetection and mineral bone density were increased (p=0.020). **Conclusion:** The biomaterial promotes osteoconduction in the irradiated tissue similar to the one observed in the non-irradiated tissue. There are no differences regarding the moment radiation was used, that is, whether before or after the application of that material.

KEY WORDS: radiation, microscopy, bone substitutes, rats.

Introduction

Radiotherapy promotes the suppression of osteoblasts normal proliferation, decrease in the number of osteocytes, and reduction of the vascular input in the bone tissue. The molecular mechanisms that guide such histological findings have not been thoroughly understood.[1-3] There is high risk of complications after surgical procedures that have been performed in the irradiated bone, such as late fractures, with low repair rates, and osteoradionecrosis. However, esthetic and functional damages due to surgical ablation of tumors in the oral and maxillofacial region generate the need for corrective procedures.[2]

The autogenous graft has been considered the best and most accepted material for bone defect treatment. However, the need for additional surgeries increases the morbidity of the procedure and, in many cases, the amount of grafting available is not sufficient for filling the receiving site. These shortcomings or limitations foster the search for alternative materials, such as alloplastic or synthetic grafts.[4] Among the advantages found in the alloplastic grafts, the commercial availability, easy manipulation, besides the physical and/or chemical possibility of integration with the medium inserted,[5,6] can be pointed out. Due to the biocompatibility and bioactivity characteristics of the calcium phosphate materials and, in spite of necrosis risks, studies in animals have assessed those biomaterials as bone substitute in irradiated tissues.[3,7-9]

The beta tricalcium phosphate (β-TCP) granule, which is chemically represented by the formula β-$Ca_3(PO_4)_2$, combines properties of solubility with osteoconductivity, once its absorption process favors bone newly formation.[5,10] The addition of β-TCP to hydroxyapatite (HA) aims at increasing the material

biodegradation and solubility levels,[11] promoting the bone newly formation from undifferentiated cells,[12] as well as increasing its capacity to maintain the bone defect volume.[13] The biomaterial, composed of 60% HA and 40% β-TCP (Bone Ceramic®, Straumann S.A. – Zurich, Switzerland), is a total synthetic graft with high biocompatibility and biphasic composition that gives it the capacity to support bone newly formation as well as keep tissue mechanical stability.[5] The present study aimed to evaluate the repair in critical bone defects carried out on the rats calvaria, filled with β-TCP associated with HA, before and after the therapy with ionizing radiation.

Materials and Methods

The present study was carried out after being approved by the Ethics Committee for the Use of Animals (CEUA, register 10/00149) of the Pontifical Catholic University of Rio Grande do Sul, Brazil. Procedures were carried out according to the institutional guidelines for the care and use of experimental animals.

The sample was formed by 33 six-week-old adult male Wistar rats, weighing from 240 to 300 g at the beginning of the experiment. They were distributed randomly into two experimental groups, submitted to ionizing radiation therapy, and a control group. In the pos-radiotherapy experimental group (n=12), the preparation and the filling of the calvaria defect were carried out two weeks after radiotherapy. In the pre-radiotherapy experimental group (n=12) the preparation and the filling of the bone defect occurred two weeks before radiotherapy. The control group (n=9) was submitted to the same surgical procedure as the

experimental groups, but not irradiated. The methodological procedures adopted in this study are described in the items that follow and are represented in Figure 1.

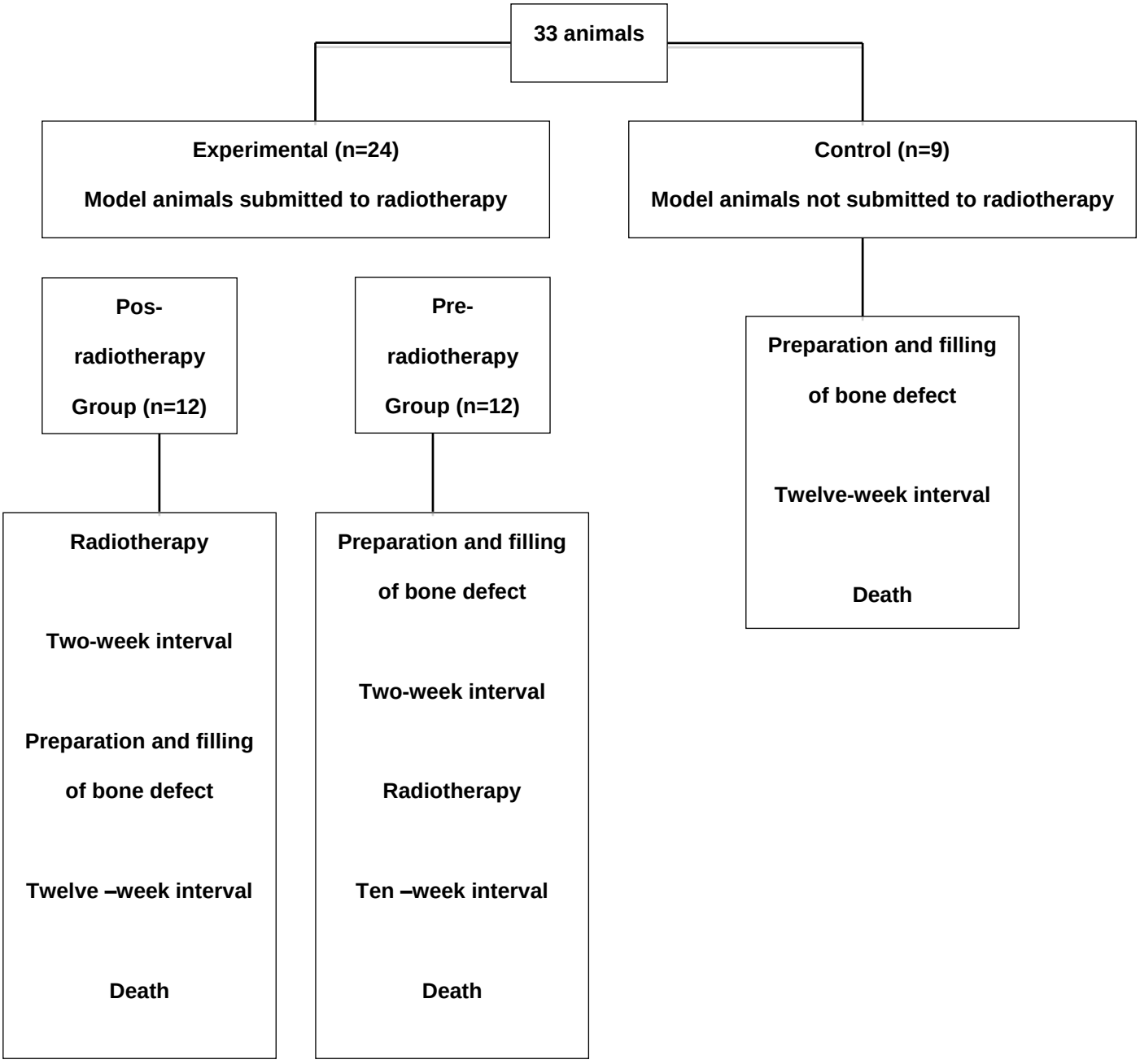

Figure 1. Chart showing the samples distribution and study procedures.

Surgical Procedure

The animals were anesthetized with ketamine hydrochloride (Dopalen®, 50 mg/Kg, 0.05 ml/100g) and xylazine hydrochloride (Anasedan®, 5 mg/Kg, 0.025 ml/100g) intra-peritonealy. In addition, subcutaneous infiltration, on the incision site, was carried out with lidocaine hydrochloride at 2% and norepinephrine 1:50.000 (Probem®, Lidostesim 2%) for hemostasis and analgesia.

Surgical access to the calvaria was through coronal linear incision in the area between the animal ears. Sagital suture was used as a guide, the critical defect was made in the center of the calvaria using an 8 mm-diameter trephine drill, lightly pressed with intermittent up and down movements until breaking the internal cortex of the cranial bone without damaging the meninges.[14-18] The literature describes more than one defect size as critical in rat cranial bone.[14-16] This study used an 8 mm measurement, which is considered the highest.[16]

The β-TCP associated with HA (Bone Ceramic®, Straumann S. A. – Zurich, Switzerland) was used according to manufacturer's indication and inserted into the bone cavity untill it was full (Figure 2). The head soft tissues were sutured using mononylon suture 5.0 and the area was cleaned using a gauze dampened in saline solution.

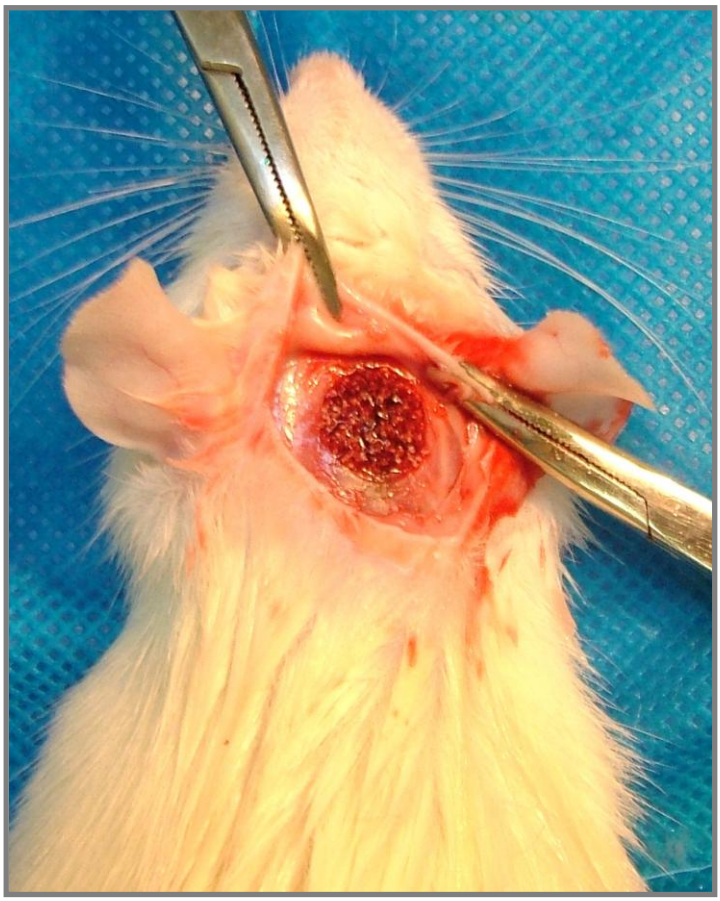

Figure 2. Defect filled with HA associated with β-TCP.

Radiotherapy

A 1.25 MeV power cobalt (Co) teletherapy irradiator was used for external radiation (Philips®, model XK5101, Eindohoven, Canada). The animals were sedated by isoflurane inhalation (Forane®, 240 ml, Abbot Laboratories of Brazil) and immobilized in individual plastic container. A radiation dose of 12 Gy was used, applied on a 1 cm-diameter circular area, delimited by lead collimator on the cranial bone. In order to obtain such a dose it was necessary a time of about 8 minutes, with a 65 cm distance between the source and the irradiated area.

This procedure was performed in both experimental groups; in the pos-radiotherapy group it was carried out two weeks before the surgical procedure, and in the pre-radiotherapy group, two weeks after the surgical procedure .

Obtaining the pieces

All the animals were killed by inhalation in isoflurane chamber 12 weeks after the surgical procedure. The soft tissue of the calvaria was removed and the osteotomy for the removal of the cranial bone was performed with the help of diamond disk in low rotation and under constant irrigation, at a 4 mm minimum distance from the defect rim. The pieces were macroscopically analyzed, submersed in a neuter formaldehyde solution buffered at 10% for fixation and conservation.

Densitometric analysis

For the mineral bone densitometry the pieces were positioned on an acrylic plate, images were obtained through Discovery equipment (Hologic®, WI Italy) and analyzed in the Apex software (version 2.3.1) in standardized fields and position covering all the cranial bone and the defect area.

Preparing the samples and histological analysis

The specimens were dehydrated in successive ethanol concentrations, and decalcified under constant agitation, in ethylene-diamine-tetra-acetic acid (EDTA) at 17% (pH 7.0) at 44°C temperature. The pieces were sectioned longitudinally; each cranial bone was equally divided. They were embedded in paraffin so that the defect longer diameter was exposed to the microtome cut. From each specimen three 6 µm wide sections were obtained and stained with hematoxylin and eosin (HE).

Histological analysis was performed in an optical microscope (Olympus®, BX 50, USA) by just one examiner, previously calibrated and blinded regarding the group to which the slides belonged. Calibration was carried out in triplicate assessment of 10 slides with a one-week interval between each analysis.

The images of the defects were captured by means of a digital system (Media Cybernetics®, model Cool SNAP-Procf, USA) attached to an optical microscope. The whole image of each defect was obtained by mounting individual images, captured in 200X magnification. The histomorphometric analysis was performed with the software Image Pro Plus (Adobe Inc.®, USA), version 6.2. The whole area of the defect was measured, the area of the neoformed bone in its edges and its center, as well as the remaining calcium phosphate area. The values were calculated as percentages in relation to the defect total area.

For the inflammatory infiltrate cells, osteoblasts and osteoclasts count, five fields in equidistant points of each defect were obtained through a 400X magnification.

Immunehistochemistry

Kit LSA + System-HRP (Dako Cytomation®, Denmark) were used for the immunehistochemical reaction. The cuts were kept in an incubator at 30ºC temperature for 12 hours. After that, they were deparaffined in xylene baths and hydrated in different alcohol concentrations. Soon after that, the slides were rinsed and placed into a humid chamber. Antigenic recuperation happened through enzymatic digestion by means of trypsine (Sigma®, St Louis, MO, USA). Once endogenous peroxidase blockade was done using the kit solution, the inhibition of the unspecific links occurred with BSA (bovine serum albumin) at 2% in PBS (buffer

phosphate solution), incubated for 12 hours in a humid chamber at 4ºC. The primary antibodies of the Vascular Endotelial Growth Factor (VEGF (C-1): sc-7269 – Santa Cruz Biotechnology®, USA) were applied at the 1:100 and 1:50 dilutions respectively, in PBS/BSA at 2%, also remaining for 12 hours at 4ºC. The secondary antibody was applied combined with biotin binder incubated at 30ºC for 25 minutes, and the streptadivin-peroxidase complex was used under same conditions. The development of staining was done using diaminobenzidin (DAB) and counterstaining with hematoxylin.

For the VEGF immunedetection the semi-automatic segmentation method was employed using Image J 1.41u program (National Institute of Health, USA). Five equidistant fields of each slide were captured at 400X magnification and the images were saved in JPEG format. One of the images was selected on which, ten points were marked corresponding to the positive immunoreactivity. This marking served as a pattern for the automatic selection of the immunoreactive areas from the other images. The average percentage of immunodetection was calculated in relation to the image total area.

Results

Densitometric analysis

Control group obtained significantly higher values of bone density in the defect region when compared with the pre-radiotherapy group (p=0.02). The pos-radiotherapy group did not differ from the others with respect to that variable. The total bone density of the cranial bone did not differ among the three groups (p=0.251) (Table 1).

Table 1. Mineral bone density (g/cm^2) in the defect area and in the cranial bone.

Bone density	Groups			P
	Control (n=9)	Pos-radiotherapy (n=12)	Pre-radiotherapy (n=12)	
Cranial bone	0.107±0.005	0.099±0.002	0.101±0.002	0.251*
Defect area	0.132±0.010a	0.104±0.002ab	0.110±0.003b	0.020*

Results presented in mean form and mean standard error.
* Variance Analysis Test (One Way) – Tukey Test Post Hoc, where the means followed by different letters differ statistically at a 5% level significance.

Histological analysis

In most of the slides analyzed, regardless of group, neoformed bone tissue was observed, whether on the edges or in the center of the defect (around the material). There was fibrous connective tissue in between the calcium phosphate granules. The neoformed bone tissue presented close contact with the bone tissue of the defect edges, as well as with the surface of the granules (Figures 3 and 4).

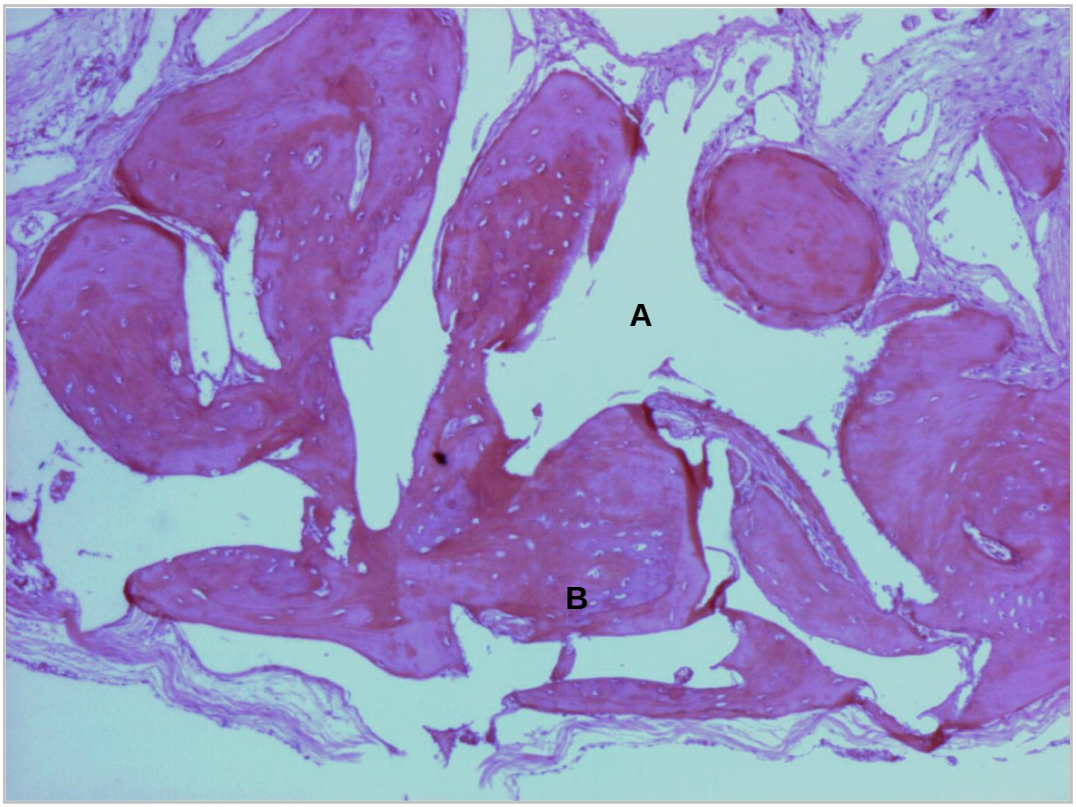

Figure 3. Bone newly formation around the biomaterial granules (A- biomaterial granule, B- newly formed bone) – HE staining – 200X magnification.

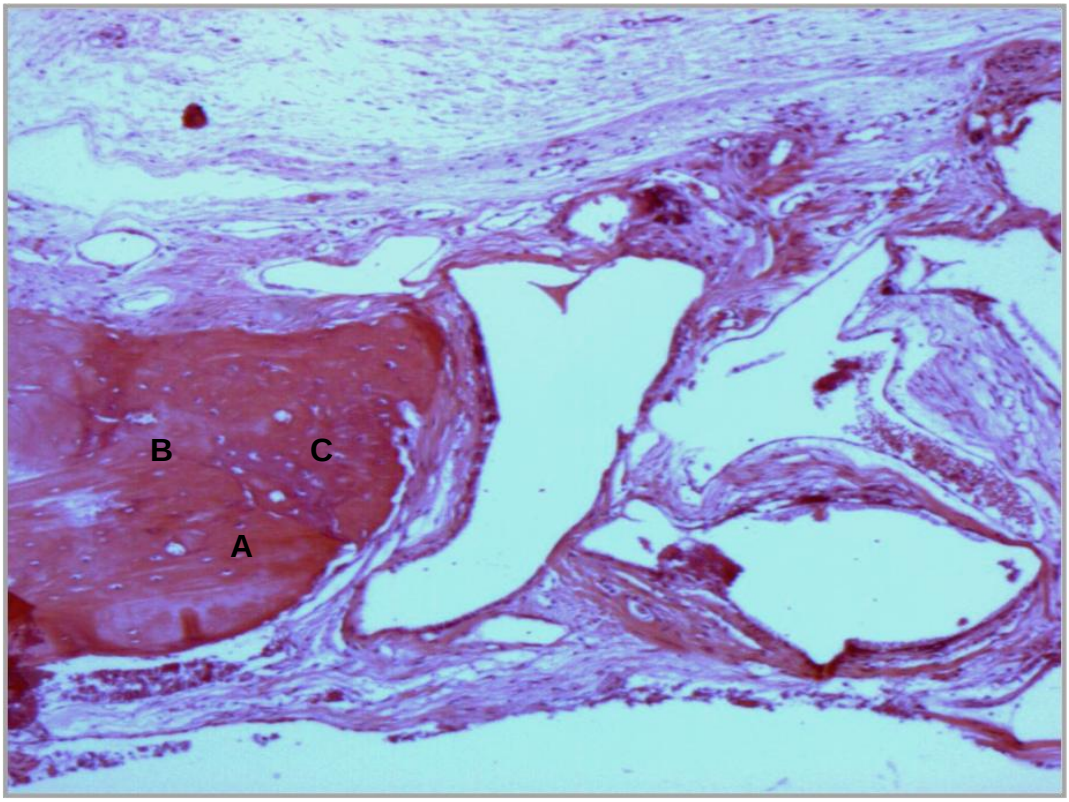

Figure 4. Bone newly formation on the defect edges (A- defect edge, B- reversal line, C- newly formed bone) – HE staining – 200X magnification.

Comparing the pre and pos-radiotherapy, and control groups, there were no significant differences with regards to the total percentage of neoformed bone tissue as well as percentage of bone tissue on the edges and in the center of the defect or regarding the remaining calcium phosphate area (Table 2).

Table 2. Percentage of newly formed bone tissue (on the edge, in the center and all over the defect) in relation to the defect total area, and percentage of remaining ceramic.

Variables	Groups			P
	Control (n=9)	Pos-radiotherapy (n=12)	Pre-radiotherapy (n=12)	
Bone tissue on the edge of the defect (%)	6.0±1.0	4.4±0.9	8.6±1.9	0.107*
Bone tissue in the center of the defect (%)	5.3±0.8	7.2±1.5	7.9±1.5	0.384*
Total neoformed bone tissue (%)	11.3±1.1	11.6±2.0	16.5±2.9	0.172#
Remaining biomaterial	30.9±2.2	28.4±3.4	24.9±1.2	0.235#

Results presented in means form and standard error mean.
* Variance Analysis Test (*One Way*)
# Variance Analysis Test (*One Way*) with *Brown-Forsythe* correction.

When quantifying the osteoblasts, osteoclasts and inflammatory cells, especially lymphocytes and plasmocytes, there was no significant difference among the groups (Table 3).

Table 3. Inflammatory cells, osteoblasts and osteoclasts, present in the selected fields on the histological slides.

Variables (cells)	Groups			P
	Control (n=9)	Pos-radiotherapy (n=12)	Pre-radiotherapy (n=12)	
Inflammatory	213.2±47.1	155.0±13.5	131.3±10.8	0.143*
Osteoblasts	184.9±29.9	200.6±18.6	213.4±29.0	0.747#
Osteoclasts	8.4±1.9	6.0±1.5	4.9±1.5	0.308*

Results presented in the form of mean and standard error mean.
* Variance Analysis Test (*One Way*)
# Variance Analysis Test (*One Way*) with *Brown-Forsythe* correction.

The average percentage of the VEGF immunodetection was significantly higher in the control group when compared with the pre and pos-radiotherapy experimental groups ($p<0.0001$). The experimental groups have not differed significantly between themselves regarding that variable (Table 4).

Table 4. VEGF (Vascular Endotelial Growth Factor) immunoreactivity in the samples.

	Groups			P
	Control (n=9)	Pos-radiotherapy (n=12)	Pre-radiotherapy (n=12)	
VEGF (%)	28.6±3.5a	9.3±4.3b	10.4±7.6b	<0.001*

Results presented in the form of mean and standard error mean.
* Variance Analysis Test (*One Way*) with Brown-Forsythe correction – Tamhane test Post Hoc where the means followed by different letters, in the lines, statistically differ at a 5% significance level.

Discussion

The favorable results of the biomaterial composed of β-TCP and HA as synthetic bone graft justify investigating its effects on the irradiated bone tissue repair. In the present study, both in the irradiated rats and in the controls, bone newly formation was observed in the center of the defects (around the material granules) and on its edges, which can be explained by the ceramic osteogenic capacity of the material, forming a calcification center around its granules[19]. The ceramics presented a direct continuity with the bone crystals, that is, collagen fibers have not been observed on the newly formed tissue/material interface neither chemical nor mechanical adhesion between them, corroborating with Ohura et al.[20] findings. Furthermore, necrosis in the irradiated tissues has not been found.

Since the literature describes that ionizing radiation suppresses the normal proliferation of osteoblasts, promotes a decrease in the number of osteocytes and the reduction of bone tissue vascularization[21], it was expected to find in this study a superior bone repair in the non-irradiated group. However, the histomorphometric analysis has shown that the percentage of the newly formed bone tissue has not significantly differed between the irradiated and non-irradiated groups. This result can be justified by the osteoconduction properties characteristic of the β-TCP and HA. On the other hand, the characteristics of the site that received the interventions must also be taken into account. Cranial bone might not have reproduced the same results as the intervention in the jaw, structure directly related with the oral cavity, a site that presents complex microbial flora and is constantly subjected to traumatic injuries[16].

The percentage of newly formed bone has not differed between the irradiated groups either, that is, the use of the biomaterial before or after ionizing radiation did not influence the results. Andrade et al.[22] have compared the results of reconstructive surgeries before and after radiotherapy, getting to the conclusion that there is no difference in both results, with similar complication rates. According to Kudo et al.[7] and Jegoux et al.[23], radiotherapy alters the cellular pattern of the bone tissue when applied during the healing stage, result not observed in the present study.

The values of the densitometric analysis in the defect area were higher in the control group, with a significant difference when compared with the pre-radiotherapy experimental group. This result might be associated with the greater maturation and, consequently, higher mineralization of the newly formed bone.[23-25] Another reason for obtaining a denser image in the defects of the control group could be a greater amount of material remaining inside the defects. Nevertheless, the percentage of remaining ceramic inside the defects did not differ between the groups. This remaining material has been explained in the literature by Chow[11] and Li et al.[26] on demonstrating that HA presents low solubility levels, property that guarantees in keeping the cavity volume.[13]

The angiogenesis is essential for increasing the oxygen and nutrients input required for tissue repair, and the VEGF expression by the osteoblasts, influences this process.[25] The expression of that protein was significantly higher in the control group, which has not received any radiation. This finding corroborates the literature as hypoxia and hyper-vascularization are characteristics of the irradiated tissue[28].

Despite literature demonstrating that ionizing radiation decreases the bone tissue cellularity[24], the present study has not found any significant differences

between the groups regarding the number of osteoblasts or osteoclasts. Since the histomorphometric analysis have shown that there is a similar amount of neo-formed bone in the three groups, it is justifiable that the osteoblasts and osteoclasts count have not differed either. However, it is worth considering that the control group tended to present increased inflammatory cells count. This finding can be explained by the action of the ionizing radiation in suppressing the blood supply to the tissue, thus reducing the number of those inflammatory cells in the experimental groups.[25] As VEGF imunodetection was increased, it is possible that the control group received a higher vascular volume, determining the presence of more inflammatory cells during the repair process.

Schmitz and Hollinger[16] and Kurashina et al.[29] determined the 12-week observation period as sufficient for studies on the repair of bone defects in rat calvaria. This was the period between the surgical procedure, the filling of the bone defect and the death of the animals in both experimental groups and control group. The radiation was given using a 1.0 cm-diameter collimator coupled to teletherapy unit and one fraction of 12 Gy was applied. Nussenbaum et al.[31] suggest that this dose is equivalent of multi-fractioned 60 Gy, according to the routine for the treatment of squamous cell carcinoma of the upper digestive tract in humans. This theory, associated with a greater methodology facility, guided the choice for using the 12 Gy only dose in the present research. Carrying out radiotherapy in two different moments, that is, before and after surgical procedures, tried to simulate what occurs in the radiotherapic treatment associated with human surgical resection. Reconstructive surgery can be performed at the moment of the tumor surgical ablation and radiation be applied later. In other cases, the surgical ablation

is performed without immediate reconstruction, the patient is submitted to radiotherapy and then is referred for reconstruction.[3,31]

According to the methodology used in this study, it was concluded that the material made up of β-TCP and HA promotes osteoconduction in the irradiated tissue similarly to what occurs in the non-irradiated tissue. Besides, there are no differences with respect to the moment of radiation application, that is, whether before or after the biomaterial insertion in the defect. This can be explained by the absence of necrosis and by the close contact between the biomaterial granules and the neo-formed bone tissue. Thus, β-TCP associated with HA can be used in irradiated bone tissue, representing an alternative to autogenous grafts. Further studies are necessary in order to verify the osteoconduction potentiality of the biomaterial in irradiated bone when applied in the oral cavity.

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5 DISCUSSÃO GERAL

Há risco elevado de complicações após a realização de procedimentos cirúrgicos no tecido ósseo irradiado (DUDZIAK et al., 2000; SZYMCZYK; SHAPIRO; ADAMS, 2004; MALARD et al., 2005). Entretanto, os danos estéticos e funcionais decorrentes da ressecção de tumores na região bucomaxilofacial geram necessidade de procedimentos corretivos e de buscarem-se técnicas e materiais adequados para esta finalidade (SZYMCZYK; SHAPIRO; ADAMS, 2004). Estudos em modelos animais têm demonstrado resultados satisfatórios com o uso de fosfatos de cálcio em tecido ósseo irradiado (MALARD et al., 2005; LEROUXEL et al., 2006; ESPITALIER et al., 2009). A cerâmica constituída por 60% de HA e 40% de β-TCP apresenta elevada biocompatibilidade, capacidade de suportar a neoformação óssea e manter a estabilidade mecânica do tecido (JENSEN et al., 2007; CORDARO et al., 2008; FROUM et al., 2008; SCHWARZ et al., 2009). Os resultados favoráveis desse biomaterial como enxerto sintético justificam investigar sua utilização em tecido ósseo irradiado.

No presente estudo houve neoformação óssea na calota craniana dos animais, tanto nas bordas dos defeitos, quanto em seu centro, em torno dos grânulos do material. A literatura descreve que o β-TCP possui habilidade osteogênica e contribui para a formação de um centro de calcificação em torno de seus grânulos (SHIRATORI et al, 2005). Estas características foram observadas na calvária dos modelos animais, independente do grupo ao qual pertenciam, ou seja, a propriedade osteogênica do material foi mantida mesmo no tecido irradiado. Além disso, não houve necrose e não foram observadas fibras colágenas na interface material/tecido neoformado, corroborando aos achados de Ohura et al. (1996).

A literatura descreve que a radiação ionizante suprime a proliferação normal de osteoblastos, promove decréscimo do número de osteócitos e redução da vascularização do tecido ósseo (LEONHARDT et al., 2009). Entretanto, a análise histomorfométrica demonstrou que o percentual de tecido ósseo neoformado não diferiu significativamente entre os grupos experimentais e controle. A neoformação óssea semelhante nos ratos irradiados e não irradiados pode ser justificada pelas propriedades de osteocondução inerentes ao composto de β-TCP e HA. Por outro lado, deve-se considerar que as intervenções cirúrgicas e a radioterapia foram realizadas na calvária, sítio que apresenta características distintas às da mandíbula e maxila. O tecido ósseo da maxila e, principalmente, da mandíbula possui risco elevado de complicações após radioterapia, pois se relaciona diretamente com a cavidade bucal, um sítio que apresenta flora microbiana complexa e é constantemente submetido a injúrias traumáticas (SCHMITZ; HOLLINGER, 1986).

O percentual de neoformação óssea também não diferiu entre os dois grupos experimentais, ou seja, quando da realização da radioterapia antes ou após os procedimentos cirúrgicos. Andrade et al. (2008) compararam os resultados de cirurgias reconstrutivas realizadas antes e após a radioterapia, concluindo não haver diferença nos resultados de ambas, com índices de complicações similares. Segundo Kudo et al. (2001) e Jegoux et al. (2010), a radioterapia altera o padrão celular do tecido ósseo quando aplicada durante a fase de cicatrização, resultado não observado no presente estudo.

No interior dos defeitos havia tecido conjuntivo fibroso, tecido ósseo neoformado e grânulos de material, ou seja, estruturas com diferentes densidades minerais. Para análise densitométrica da região do defeito, essas estruturas foram

calculadas de forma conjunta, ou seja, não foi possível quantificar especificamente a densidade do tecido ósseo neoformado. A densidade mineral nos defeitos ósseos foi superior no grupo-controle, o que pode estar associado a maior maturação do tecido ósseo neoformado. O biomaterial composto de β-TCP e HA apresenta baixos níveis de solubilidade e sua permanência nos tecidos é um resultado esperado, pois mantém o volume da cavidade (CHOW, 1998; LI; WENG; TAM, 2007; GRANDI et al., 2011). Entretanto, quando o percentual de material remanescente foi comparado, não foi observada diferença significativa entre os grupos.

A angiogênese é essencial para o aumento de aporte de oxigênio e nutrientes requeridos no processo de reparo do tecido ósseo. Uma vez que o VEGF é considerado um regulador chave no processo de neovascularização, induzindo a proliferação, migração e sobrevida de células endoteliais (STEINBRECH et al., 2000), a imunorreatividade deste marcador foi analisada neste estudo. Mesmo doze semanas após a realização dos procedimentos cirúrgicos foi detectada imunopositividade para o VEGF, indicando haver proliferação de osteoblastos e de tecido vascular. Quando a imunorreatividade deste marcador foi quantificada, observou-se que os grupos irradiados apresentaram percentuais significativamente inferiores em comparação ao grupo não irradiado. Este resultado corrobora a literatura que descreve que a radioterapia pode alterar a expressão do VEGF (ZHANG et al., 2011), além disso hipóxia e hipovascularização são características do tecido irradiado (GAL et al., 2000).

Apesar de a literatura demonstrar que a radiação suprime a proliferação de osteoblastos e promove decréscimo do número de osteócitos (SHULTZE-MOSGAU et al., 2001), no presente estudo não foram encontradas diferenças significativas

entre os grupos quanto ao número de osteoblastos e osteoclastos. Embora não tenha havido diferença significativa, o grupo-controle apresentou número mais elevado de células inflamatórias. Como neste grupo a imunodetecção do VEGF foi superior, é possível que tenha recebido maior aporte vascular, determinando a presença de mais células inflamatórias durante o processo de reparo (WONG et al., 2009).

A análise histomorfométrica permitiu quantificar a proporção de tecido ósseo neformado no interior e nas bordas dos defeitos confeccionados na calvária dos modelos animais. Marzouk et al. (2007) e Eski et al. (2007) relatam que a análise quantitativa é essencial para estudos que objetivam avaliar a efetividade de novas modalidades terapêuticas na neoformação óssea. A densitometria óssea e a imunodetecção de VEGF foram avaliações coadjuvantes, realizadas com o intuito de acrescentar informações à análise histomorfométrica. Enquanto na análise histomorfométrica não foram evidenciadas diferenças no percentual de neoformação óssea entre os grupos, a avaliação densitométrica e a imunodetecção do VEGF revelaram valores superiores nos animais não irradiados. Apesar de a imunodetecção do VEGF ter sido significativamente superior no grupo não irradiado, este resultado não se traduziu em percentual mais elevado de neoformação óssea no grupo-controle. A densidade mineral na região do defeito apenas diferiu significativamente entre o grupo-controle e o grupo experimental pré-radioterapia. Este resultado pode estar associado à maior maturação do osso neoformado e não necessariamente a um percentual mais elevado de neoformação óssea nos animais-controle (SHULTZE-MOSGAU et al., 2001; WONG et al., 2009; JEGOUX et al., 2010).

Kurashina et al (1997) revelaram que, quanto ao período, não houve diferença na quantidade de osso neoformado entre análises de 12 e 24 semanas, a não ser no seu grau de maturação do tecido. Kurashina et al. (1997) e Schmitz e Hollinger (1986) determinaram como suficiente o período de observação de 12 semanas para estudos da cicatrização dos defeitos ósseos em calota craniana de ratos. Nos três grupos do presente estudo este foi o período decorrente entre o procedimento cirúrgico de confecção e preenchimento do defeito ósseo e a morte dos animais.

A dose de radiação foi administrada com colimador de 1,0 cm de diâmetro acoplado a unidade de teleterapia, utilizando fração única de 12 Gy. Nussenbaum, Rutherford e Krebsbach (2005) sugerem que esta seja equivalente à dose multifracionada de 60 Gy, empregada no tratamento do carcinoma espinocelular do trato digestivo superior em humanos. Essa teoria, associada à maior facilidade metodológica, norteou a escolha pela utilização da dose única de 12 Gy na presente pesquisa. A realização da radioterapia em dois momentos, ou seja, antes e após os procedimentos cirúrgicos buscou simular o que ocorre no tratamento radioterápico associado à ressecção cirúrgica em humanos. A cirurgia reconstrutiva pode ser realizada no momento da ablação cirúrgica do tumor e a irradiação ser aplicada posteriormente. Em outros casos, a ablação cirúrgica é realizada sem reconstrução imediata, o paciente é submetido à radioterapia e, após, é encaminhado para a reconstrução (GIROD et al., 2005; MALARD et al., 2005).

Uma vez que não há estudos prévios da utilização do composto de β-TCP e HA no tecido ósseo irradiado, optou-se por realizar esta investigação no osso da calvária. Algumas vantagens para as pesquisas de enxertos ósseos em calvária de ratos são o baixo custo e a facilidade do procedimento cirúrgico para inserção do

biomaterial no defeito. O osso da calvária é um componente do complexo craniofacial, apresenta duas corticais com medular entre elas e é recoberto por um plano tecidual, podendo ser comparado estruturalmente à mandíbula. A confecção dos defeitos neste sítio impede que os animais contaminem a área por meio da boca e das patas e permite que o material permaneça na cavidade sem movimentação, uma vez que esse osso é imóvel e sem ação muscular ativa (SCHMITZ; HOLLINGER, 1986). No entanto, esse sítio apresenta a desvantagem de não reproduzir fielmente a situação intra-bucal, conforme discutido previamente.

Os resultados dessa pesquisa demonstraram que o biomaterial avaliado apresenta propriedades que encorajam seu uso em osso irradiado. No entanto o estudo ainda deve embasar novas pesquisas direcionadas a verificar seu uso na cavidade bucal, além de utilizar diferentes dosimetrias de radiação ionizante.

6 CONCLUSÕES

Os resultados deste estudo demonstram que o biomaterial constituído por β-TCP e HA (Bone Ceramic®, Straumann S. A., Suíça), ao ser empregado em tecido irradiado, apresenta-se como um substituto ósseo que não resulta em necrose, mantém o volume do defeito, não gera reação inflamatória exacerbada e permite a osteocondução.

O composto de β-TCP associado a HA promove osteocondução no tecido irradiado de forma semelhante ao que ocorre no tecido não irradiado, independente de a radiação ionizante ser aplicada antes ou após a inserção do biomaterial.

A densidade óssea é alterada quando esse tecido sofre irradiação, provavelmente devido a um retardo em sua maturação causado pela terapia. A expressão do VEGF também é diminuída nos tecidos irradiados.

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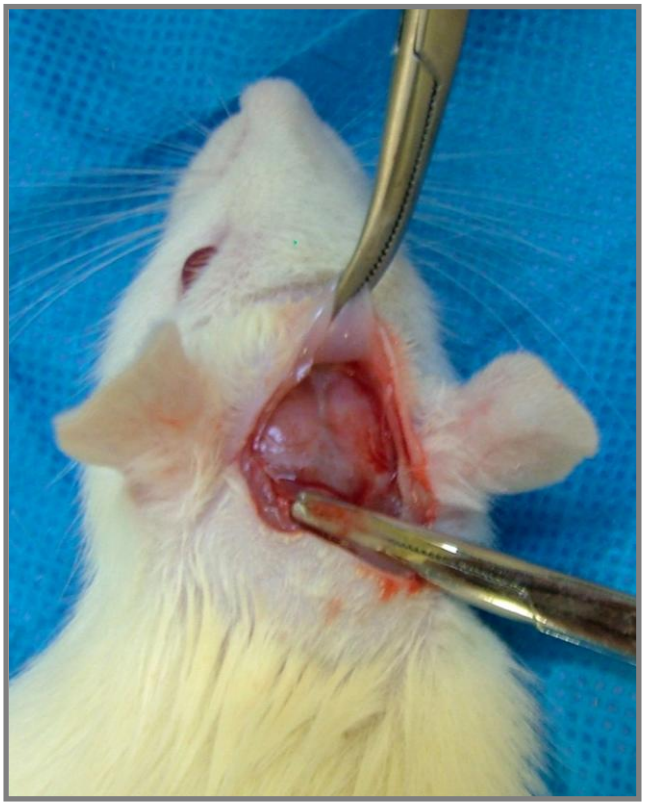
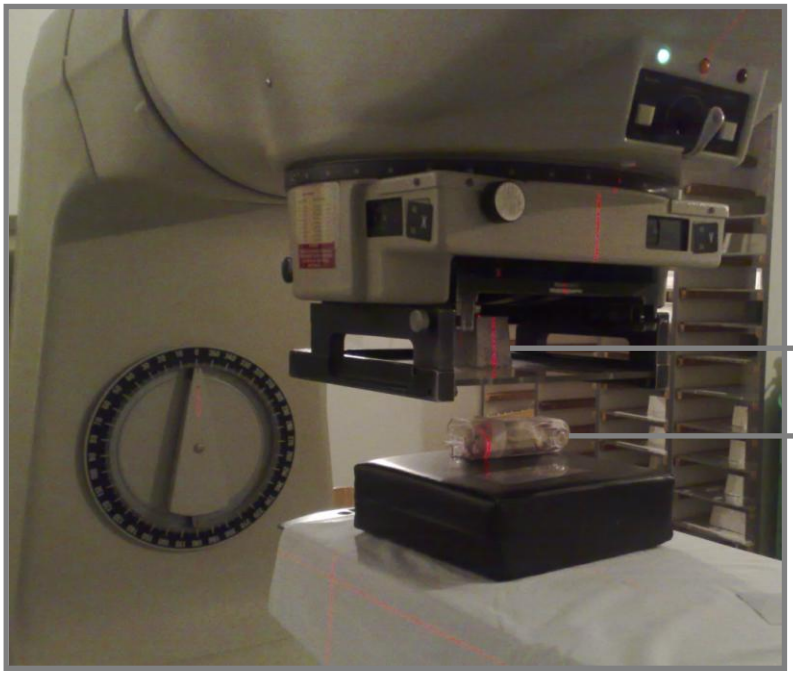
APÊNDICES

APÊNDICE I

IMAGENS DA METODOLOGIA APLICADA NA PESQUISA

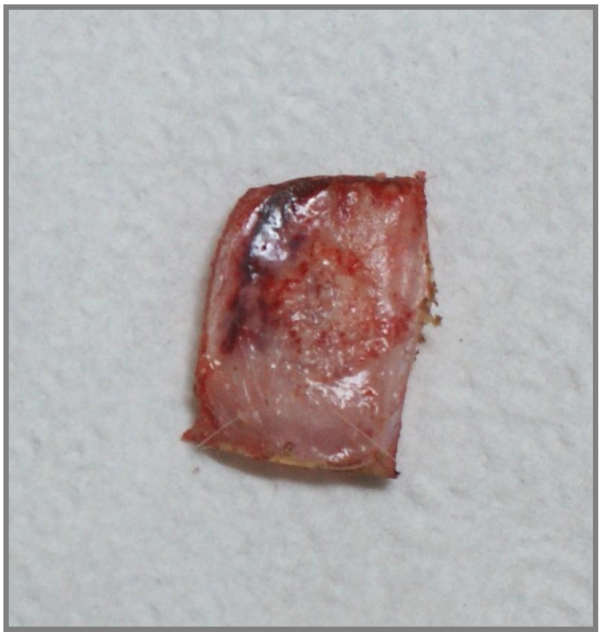


Área da calota craniana exposta para confecção do defeito crítico.

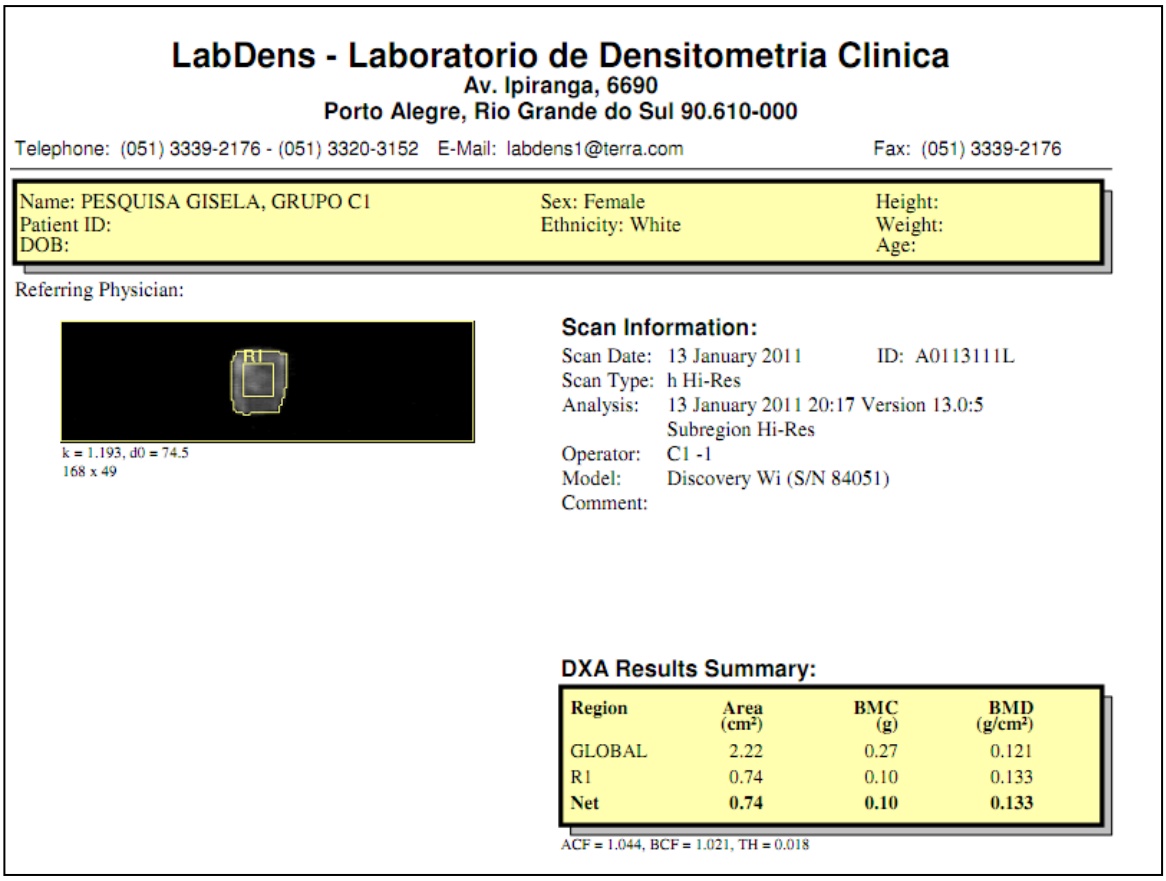


Animal imobilizado em caixa adaptada para receber radioterapia

Exposição da calota craniana 12 semanas após procedimento de confecção e preenchimento do defeito.



Calota craniana removida para análises.



LabDens - Laboratorio de Densitometria Clinica
Av. Ipiranga, 6690
Porto Alegre, Rio Grande do Sul 90.610-000

Telephone: (051) 3339-2176 - (051) 3320-3152 E-Mail: labdens1@terra.com Fax: (051) 3339-2176

Name: PESQUISA GISELA, GRUPO C1	Sex: Female	Height:
Patient ID:	Ethnicity: White	Weight:
DOB:		Age:

Referring Physician:

k = 1.193, d0 = 74.5
168 x 49

Scan Information:

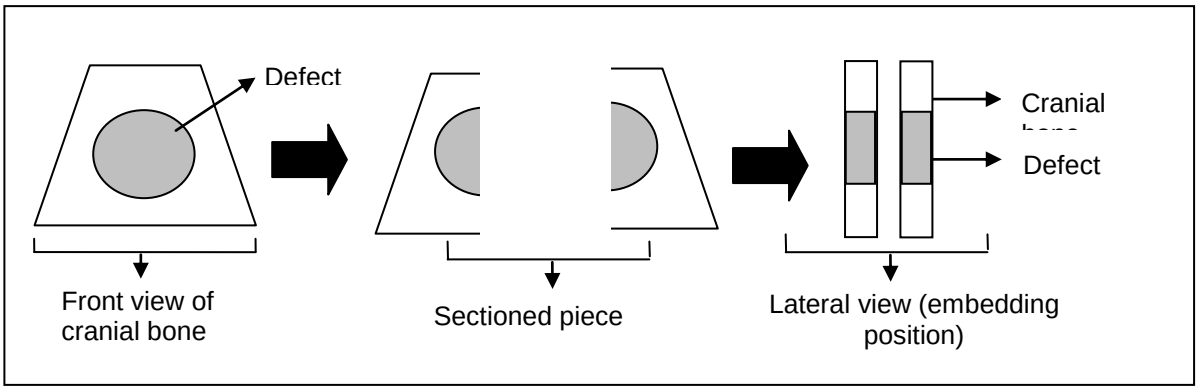
Scan Date: 13 January 2011 ID: A0113111L
Scan Type: h Hi-Res
Analysis: 13 January 2011 20:17 Version 13.0:5
Subregion Hi-Res
Operator: C1 -1
Model: Discovery Wi (S/N 84051)
Comment:

DXA Results Summary:

Region	Area (cm²)	BMC (g)	BMD (g/cm²)
GLOBAL	2.22	0.27	0.121
R1	0.74	0.10	0.133
Net	**0.74**	**0.10**	**0.133**

ACF = 1.044, BCF = 1.021, TH = 0.018

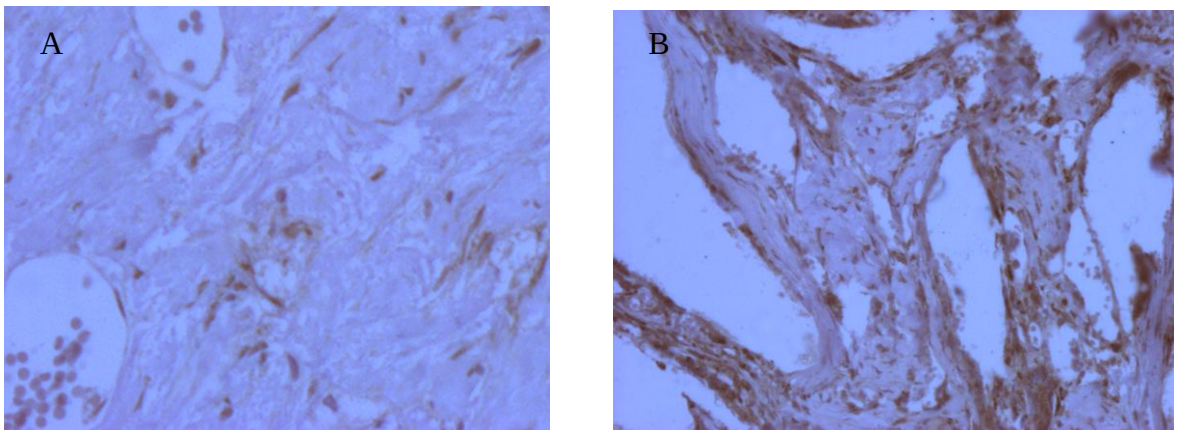
Imagem digitalizada do exame densitométrico acompanhada dos dados numéricos.



Desenho esquemático do preparo da peça para inclusão no bloco de parafina e corte por micrótomo.

Esquema gráfico para demonstração das áreas capturadas para contagem das células inflamatórias.

Fotomicrografia da área e bordas do defeito ósseo preenchido com β-TCP associado a HA.



Fotomicrografias demonstrando a imunodetecção do VEGF.

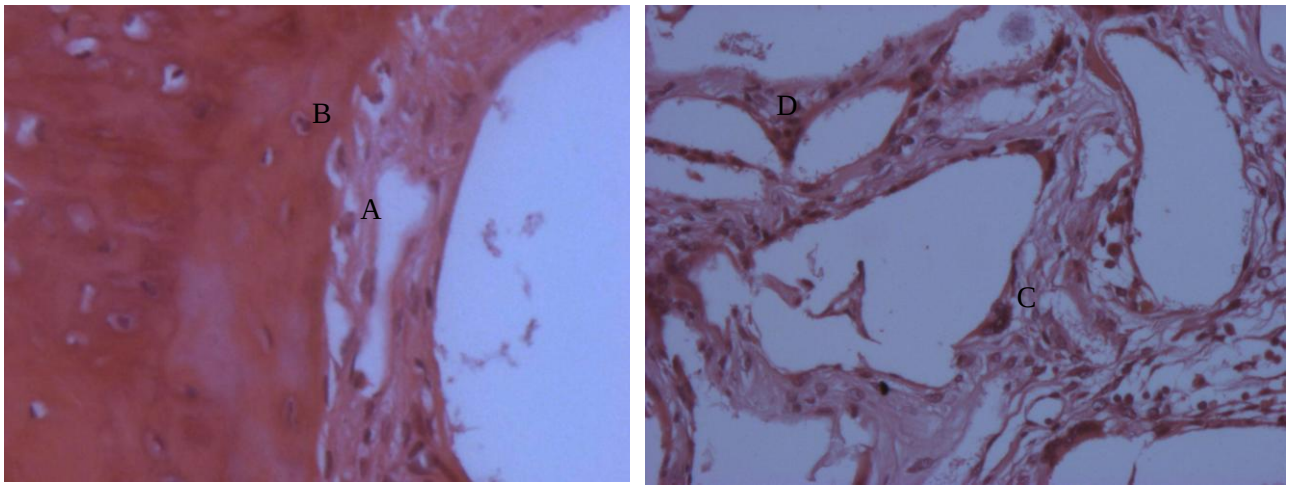


Imagem histológica – identificação de células ósseas e inflamatórias. A – osteoblasto, B – osteócito, C – osteoclasto, D – células do infiltrado inflamatório crônico.

APÊNDICE II

FICHA DE AVALIAÇÃO MACROSCÓPICA, DENSITOMÉTRICA E HISTOLÓGICA

Peça Nº: ..... Grupo: .....

Avaliação macroscópica (descritiva)

_____

Avaliação Densitométrica

	Densidade da massa total da calota	Densidade óssea mineral (valor padrão)
GLOBAL		
R1		

Avaliação Histológica (descritiva)

_____

Histomorfometria

Variável	Percentual
Área de neoformação óssea nas margens do defeito	
Área de neoformação óssea no centro do defeito	
Área total de neoformação óssea	
Área de cimento no interior do defeito	

Contagem de células (HE)

CAMPO	CELS INFLAMATÓRIAS	OSTEOBLASTOS	OSTEOCLASTOS
1			
2			
3			
4			
5			

Percentual de Imunodetecção do VEGF

CAMPO	PORCENTAGEM
1	
2	
3	
4	
5	

ANEXOS

ANEXO I

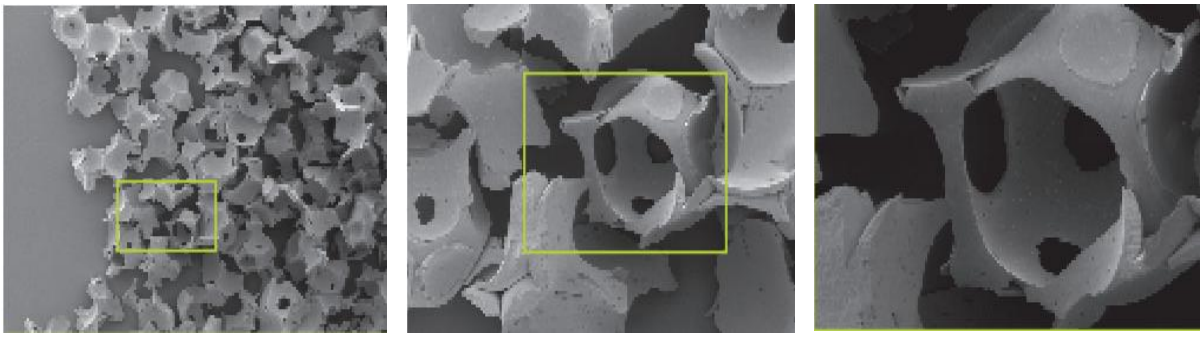
INFORMAÇÕES REFERENTES AO BIOMATERIAL BONE CERAMIC®

ORIENTAÇÕES

- Embalado em blister duplo e estéril 2 selos de blister e compartimento interno protegem os grânulos.
- Rápida absorção dos fluidos, formando uma massa granular húmida.
- As partículas devem ser misturadas com sangue/osso autógeno ou com soro fisiológico estéril.
- A forma triangular do blister facilita o manuseio e a remoção dos grânulos.
- Os grânulos umedecidos aderem ao instrumental.

Fonte. Instruções de utilização do Bone Ceramic®- bula, Straumann S.A.

IMAGEM ILUSTRATIVA DA MORFOLOGIA DO BIOMATERIAL



Fonte. Informações sobre o poduto Bone Ceramic® – bula, Straumann S.A.

ANEXO II

SUBMISSÃO DO ARTIGO DE REVISÃO NO PERIÓDICO *JOURNAL OF MATERIALS SCIENCE*

Dear Dr GISELA GRANDI,

Thank you for submitting your manuscript, EFFECTS OF RADIOTHERAPY IN HEAD AND NECK REGION ON BONE TISSUE: ALTERNATIVES FOR CORRECTIVE SURGICAL PROCEDURES, to Journal of Materials Science.

During the review process, you can keep track of the status of your manuscript by accessing the following web site:

http://jmsc.edmgr.com/

Your username is: GISELA GRANDI
Your password is: grandi548

Should you require any further assistance please feel free to e-mail the Editorial Office by clicking on "Contact Us" in the menu bar at the top of the screen.

Alternatively, please call us at +91 44 42197752 anytime between 9.00 - 17.00 hrs IST/5.00 - 13.00 hrs CET.

With kind regards,
Springer Journals Editorial Office
Journal of Materials Science

ANEXO III

SUBMISSÃO DO ARTIGO DE PESQUISA NO PERIÓDICO *HEAD & NECK*

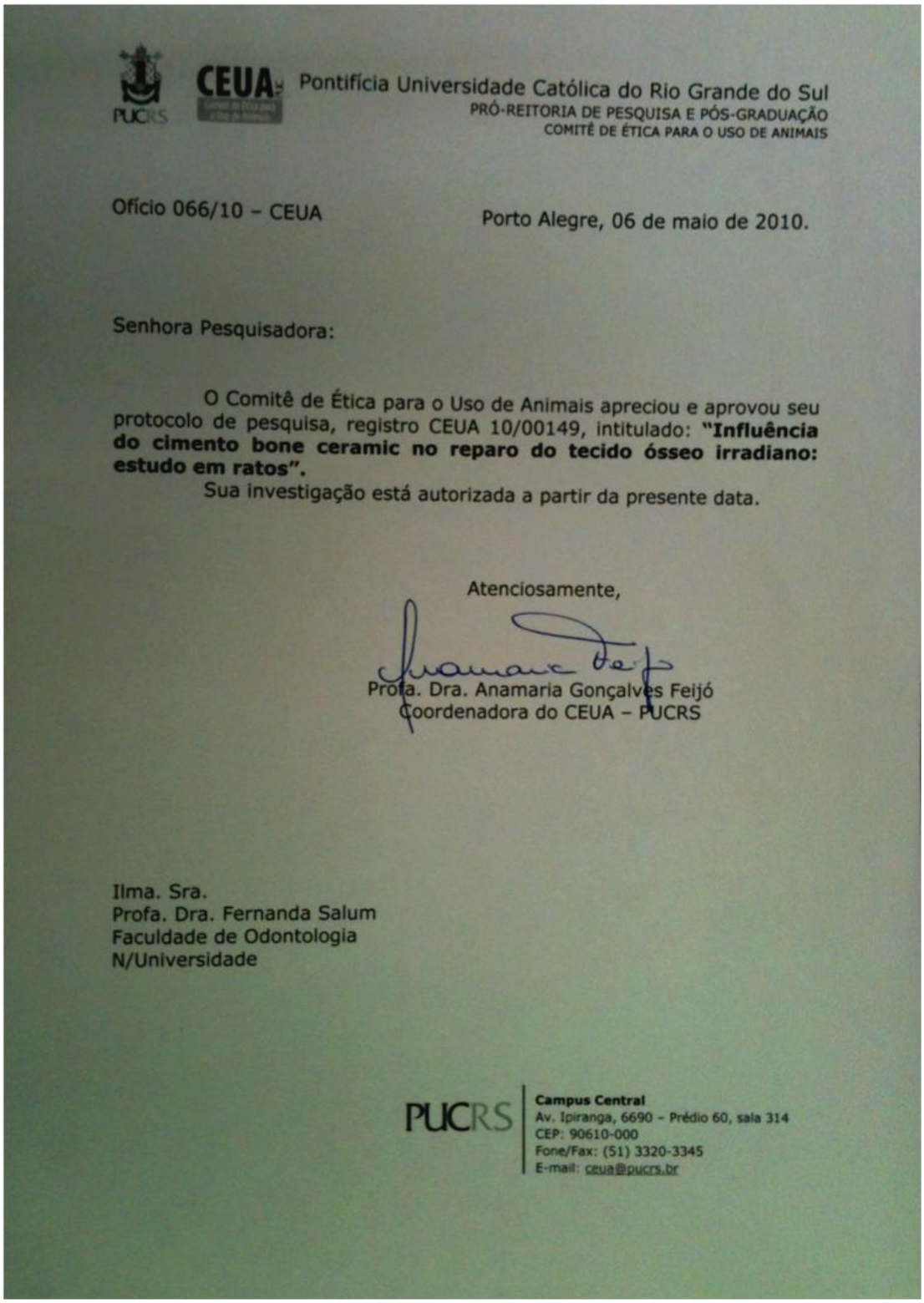
Dr. Grandi, thank you for submitting your manuscript, **REPAIR OF IRRADIATED BONE TISSUE AFTER USE OF BETA TRICALCIUM PHOSPHATE CEMENT ASSOCIATED WITH HYDROXYAPATITE: A STUDY WITH RATS** to **Head & Neck**

Edited By: Ehab Y. Hanna, MD

Regards,
Head and Neck.
http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1097-0347

ANEXO IV

APROVAÇÃO DO PROJETO PELA COMISSÃO DE ÉTICA NO USO DE ANIMAIS - PUCRS



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

Ofício 066/10 – CEUA

Porto Alegre, 06 de maio de 2010.

Senhora Pesquisadora:

O Comitê de Ética para o Uso de Animais apreciou e aprovou seu protocolo de pesquisa, registro CEUA 10/00149, intitulado: **"Influência do cimento bone ceramic no reparo do tecido ósseo irradiano: estudo em ratos".**

Sua investigação está autorizada a partir da presente data.

Atenciosamente,

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

Ilma. Sra.
Profa. Dra. Fernanda Salum
Faculdade de Odontologia
N/Universidade

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