UNIVERSIDADE ESTADUAL DE CAMPINAS

ROSANE VIEIRA DA SILVA

RECONSTRUÇÃO DE DEFEITOS ÓSSEOS COM CERÂMICA DE FOSFATO DE CÁLCIO OU LASERTERAPIA DE BAIXA ENERGIA ASSOCIADO A PROCEDIMENTO DE ENXERTIA

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RESUMO

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Procedimentos que permitem a reconstrução e acelerem o reparo de fraturas ósseas são de grande importância clínica. Sendo assim, considerando as vantagens oferecidas pelos biomateriais como substitutos aos enxertos ósseos autógenos e os efeitos positivos do laser de baixa energia no processo de regeneração óssea, as propostas do presente trabalho foram estudar a contribuição do laser de baixa energia no processo de reconstrução de falhas ósseas tratadas com enxerto ósseo autógeno, assim como, analisar "in vitro" e "in vivo" a eficácia da cerâmica de fosfato de cálcio como possível substituta aos enxertos ósseos autógenos. O resultado da laserterapia na dosagem estabelecida em nosso trabalho demonstrou que a mesma acelerou a osteogênese na área de enxertia somente nos primeiros períodos do tratamento e esse efeito foi dose dependente. Enquanto isso, o estudo "in vivo" da biocerâmica demonstrou que o implante foi tão eficiente quanto o enxerto ósseo autógeno no processo de reconstrução da falha óssea. No estudo "in vitro", os osteoblastos humanos apresentaram boa interação com a cerâmica, apresentando maior preferência pelas superfícies mais irregulares do material.

ABSTRACT

ABSTRACT

Procedures that allow the reconstruction and speed up the bone repair are of great clinical importance. Being thus, considering the advantages offered for the biomaterials as substitute to the autogenous bone graft and the positive effect of the low-power-laser in the process bone regeneration, the proposals of the present work had been to study the contribution of the low-power-laser in the process of reconstruction of bone defect treated with autogenous bone graft, as well as, to analyze "in vitro" and "in vivo" the efficiency of calcium phosphate ceramic as possible substitute to the autogenous bone graft. The result demonstrated that laser irradiation at the grafted site stimulated osteogenesis during the initial stages of the healing process in na skull defect and this effect was dose dependent. While this, the “in vivo” study of the bioceramic demonstrated that the implantation was so efficient how much autogenous graft in the bone reconstruction process. In the study "in vitro", the human osteoblast had presented good interaction with ceramics, presenting bigger preference for the surfaces most irregular of the material.

INTRODUÇÃO

INTRODUÇÃO

Acidentes de trabalho e automobilísticos são os causadores mais freqüentes de fraturas. Freqüentemente, esses acidentes envolvem pessoas jovens e em fase bastante produtiva[1]. Sendo assim, procedimentos que permitem a reconstrução e acelerem o reparo da fratura possibilitando a reintegração desses indivíduos mais rapidamente ao mercado de trabalho são de grande importância clínica.

Atualmente, defeitos com perda de substância óssea provocados por infecção, ressecção tumoral e principalmente por traumas mecânicos têm sido predominantemente tratados com enxertos ósseos autógenos obtidos da costela, crista ilíaca e tíbia. Esse tipo de enxerto não apresenta reação imunológica e ainda oferece células osteogênicas e fatores de crescimento que contribuem para o reparo dos defeitos ósseos. Dependendo da área doadora, esses enxertos podem ser do tipo esponjoso ou compacto. Enxertos esponjosos são mais vascularizados e oferecem maior quantidade de células osteogênicas e fatores de crescimento, os quais contribuem no processo de regeneração óssea. No entanto, o fato de possuir maior porosidade faz com que esses enxertos sejam mais facilmente reabsorvidos. A rápida reabsorção do enxerto pode não garantir tempo suficiente para que o organismo sintetize a quantidade desejada de tecido ósseo para a regeneração do defeito. Por outro lado, o enxerto ósseo compacto apresenta uma matriz com maior dificuldade de reabsorção, essa característica faz com que o mesmo persiste por mais tempo no local da implantação podendo comprometer o processo de remodelação da área lesada.[2-4].

Outra desvantagem é a obtenção dos enxertos ósseos, uma vez que os mesmos requerem intervenção cirúrgica adicional que pode apresentar complicações como

hemorragias, pneumotórax, infecção, dor crônica e distúrbios no desenvolvimento ósseo quando o paciente é ainda jovem[5]. Além disso, a quantidade de osso autógeno utilizada para o reparo nem sempre é suficiente para preencher toda a falha, havendo a necessidade do uso de enxertos homólogos e heterólogos. Porém, enxertos homólogos e heterólogos exigem tratamentos prévios do material e podem induzir reações imunológicas, havendo a necessidade de um tempo mais prolongado para a recuperação do paciente[6,7].

Dessa forma, materiais alternativos como as cerâmicas de fosfato de cálcio[8-12] têm sido pesquisados e usados como substitutos aos enxertos ósseos autógenos, pois dispensam intervenções cirúrgicas adicionais, podem ser sintetizados em grande quantidade e no formato desejado e ainda são osteocondutoras e biocompatíveis[11, 13-16]. Acrescentando-se a isso, essas cerâmicas podem ter sua porosidade controlada para adequar-se a área receptora. Assim como os enxertos, quanto mais porosa for a cerâmica maior será a facilidade de infiltração de vasos e tecido ósseo para o seu interior, garantindo a osteointegração do material a área receptora. No entanto, até que essa osteointegração ocorra, o material terá uma menor resistência mecânica. Sendo assim, é de extrema importância à compreensão da interação entre cerâmica e o tecido ósseo tanto "in vivo" como "in vitro" para garantir o sucesso da restauração da área lesada.

Outro procedimento que pode contribuir para a reconstrução de defeitos ósseos é a laserterapia. Desde o descobrimento do laser em 1.960, por Maiman, o uso dessa forma de energia tem atraído à atenção de vários pesquisadores em diferentes áreas da medicina. A utilização do laser no processo de reparação tecidual foi publicada pela primeira vez por Mester et al. em 1968, posteriormente, diversos pesquisadores passaram a demonstrar os efeitos da radiação a laser sobre os tecidos biológicos tais como: estimulação da

microcirculação local[17,18], indução da atividade das células epiteliais[19], aumento da síntese de colágeno [20-26] e bioestimulação celular na osteogênese[27-33].

A irradiação a laser de baixa energia promove nas células efeitos bioenergéticos, bioelétricos, bioquímicos e bioestimulantes. Os fotorreceptores da cadeia respiratória são ativados aumentando a produção de ATP necessária para as reações interestruturais dos ciclos metabólicos de grande consumo de oxigênio, o que facilita os processos de reparos celulares. Os fotorreceptores da membrana celular absorvem o laser normalizando o potencial iônico da membrana, assim, vitalizando a célula para as suas funções. A fotoestimulação aumenta a síntese de ATPsintetase promovendo a produção de ácido nucléico e, conseqüentemente, a aceleração da divisão celular [27,34,35]. Mediante a esses efeitos, a estimulação a laser além de promover a proliferação e diferenciação de osteoblastos, estimula o aumento de mRNA para a síntese de colágeno tipo I, o qual corresponde a principal porção protéica da matriz óssea. O aumento da produção de colágeno favorece a formação de tecido ósseo e, conseqüentemente, da regeneração da falha óssea [21,24,32,33].

Sendo assim, a associação de materiais reparadores como os enxertos ósseos autógenos e as biocerâmicas com procedimentos aceleradores da regeneração óssea têm grande importância clínica, pois juntos podem acelerar a recuperação do indivíduo, diminuindo o impacto tanto social como econômico causado para essas pessoas.

OBJETIVOS

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Considerando os efeitos positivos da laserterapia no reparo de lesões ósseas e as vantagens oferecidas pelos biomateriais como possível substituto aos enxertos ósseos autógenos, as propostas do presente trabalho foram:

Analisar a contribuição da laserterapia como acelerador na reconstrução de falhas ósseas tratadas com enxerto autógeno.

Contribuir para o desenvolvimento de uma cerâmica favorável ao crescimento de tecido ósseo, hidrolisável no pH fisiológico, biocompatível, moldável na sua forma polimérica inicial e ainda de baixo custo de produção. Essa cerâmica tem sido desenvolvida juntamente com o Instituto de Química da UNICAMP.

Analisar tanto “in vivo” como “in vitro” a cerâmica como material alternativo aos enxertos ósseos autógenos.

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Experimental Study

Repair of Bone Defects Treated with Autogenous Bone Graft and Low-Power Laser

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Because bone healing at the graft site is similar to a fracture repair, the purpose of the present study was to evaluate the effects of low-power laser irradiation on the repair of rat skull defects treated with autogenous bone graft. A defect measuring 3 mm in diameter was produced in the left parietal bone and filled with an autogenous bone graft obtained from the right parietal bone. The animals were divided into 3 groups of 20 rats each: nonirradiated control, irradiated with 5.1 J/cm^2, and irradiated with 10.2 J/cm^2. The laser (2.4 mW, 735 nm, 3.4 $\times$ 10^{-2} W/cm^2, 3-mm spot size) was applied three times per week for 4 weeks. Greater volume of newly formed bone was observed in the irradiated group with 10.2 J/cm^2. In both irradiated groups, a greater volume of newly formed bone occurred only in the first 2 weeks. The results demonstrated that laser irradiation at the grafted site stimulated osteogenesis during the initial stages of the healing process in a skull defect of the rat and that this effect was dose dependent.

Key Words: Autogenous bone graft, laser irradiation, bone regeneration

Automobile and work accidents are the most frequent causes of fractures. Several of these fractures can be accompanied by great loss of bone mass in the skull. Because these accidents mainly affect young people, who are in their productive phase of life, procedures that permit bone reconstruction or accelerate the bone repair process are of great clinical importance.[1,2] Autogenous bone grafts have currently been the material of choice in reconstructive surgeries because of advantages such as mechanical stability, which permits implant placement, and lack of immunogenicity, and also because they are a source of osteogenic cells and osteoinductive substances.[3]

Low-power laser therapy has become common both experimentally and clinically as a noninvasive method for the stimulation of osteogenesis and to reduce the time of fracture consolidation.[4–6] Laser irradiation exerts bioenergetic, bioelectrical, biochemical, and biostimulatory effects on cells. Regarding the bioenergetic effect, photoreceptors of the respiratory chain are activated, increasing the production of adenosine triphosphate (ATP) necessary for interstructural reactions in metabolic cycles; this consumes large amounts of oxygen, thus, facilitating cell repair processes. The bioelectrical effect normalizes the membrane ionic potential, thus, vitalizing cell functions. In biostimulation, photostimulation increases the synthesis of ATP synthetase, promoting the production of nucleic acid, which accelerates cell division.[7–9]

In addition to inducing the proliferation and differentiation of osteoblasts, laser stimulation increases the amount of messenger RNA for the synthesis of collagen type I, the main protein portion of the bone matrix. An increased production of collagen favors the formation of bone tissue and the regeneration of bone defects.[5,10,11]

According to Glowacki et al.[12] and Soballe[13], the mechanisms involved in the bone repair process that occurs at the site of biomaterial implantation are similar to those observed during fracture healing. Based on the principle that bone healing at the graft site is similar to a fracture repair, the objective of the present study was to determine the tissue response of the rat skull defects treated with autogenous bone graft and submitted to low-power laser irradiation.

Materials and Methods

Surgical Procedure

Sixty-one male Wistar rats were used. The animals were anesthetized with a 1:1 mixture of

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xylazine hydrochloride (2%) and ketamine at a dose of 0.15 mL/100 g, intramuscularly. The head of each rat was shaved and sterilized. A longitudinal incision was made in the cranial skin and the periosteum was separated from the parietal bone to expose its surface. Using a surgical trephine, a full-thickness bone defect measuring 3 mm in diameter was produced in both parietal bones. The circular bone fragment obtained from the right parietal bone was used as autogenous bone graft to fill the defect in the left parietal bone. The periosteum and skin were then repositioned. All animals received 875 mg/kg of dipyrone added to water (ad libitum) for 4 days postoperatively.

The study was conducted in accordance with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation and was approved by the Institutional Research Ethics Committee, protocol No. 157-1 (State University of Campinas).

Laser Irradiation Procedure

The laser (Opto Eletrônica S.A.) used was a gallium arsenide (GA-AS) laser, 735 nm in wavelength, and up to 2.4 mW intensity. A continuous irradiation was applied to a spot size, 3 mm in diameter, with a power density of 3.4×10^{-2} W/cm^2. The laser was applied three times per week for 4 weeks, with each application lasting 152 seconds (5.1 J/cm^2) or 304 seconds (10.2 J/cm^2). The first application was performed immediately after surgery.

The animals were divided into three groups of 20 rats each: a nonirradiated group, a group irradiated with 5.1 J/cm^2, and a group irradiated with 10.2 J/cm^2. Each group was divided into four subgroups of five animals and killed at weeks 1, 2, 4, and 24 after surgery, respectively. The subgroups irradiated with 5.1 J/cm^2 received at weeks 1, 2, 4, and 24 after surgery, the respective cumulative total doses of 1.08 J, 2.16 J, 3.24 J, and 3.24 J. The subgroup irradiated with 10.2 J/cm^2 received at weeks 1, 2, 4, and 24 after surgery, the respective cumulative total doses of 2.16 J, 4.32 J, 6.48 J, and 6.48 J.

Radiographic and Histologic Analysis

The calvaria of all rats were x-rayed using a Rigaku RU-200 apparatus with a focal point of 0.8×0.8 mm. The samples were fixed in 10% formalin, decalcified, and embedded in paraffin. Cross-sections (7-μm thick) were stained with hematoxylin and eosin (HE). One of the animals was killed immediately after surgery, x-rayed, and used to establish comparative parameters for radiographic analysis among the experimental periods.

Morphometric Analysis

For morphometric analysis, images of histologic sections from all samples were obtained. The autogenous graft was not considered in the morphometric analysis. The volume fraction of the newly formed bone was estimated by point volumometry following the Delesse theorem.[14] All statistical comparisons were performed using analysis of variance (ANOVA), with $P < 0.05$ considered significant.

RESULTS

Radiographic Findings

Immediately after surgery, the autogenous bone graft was radiopaque and there was a radiolucent area around it. This area around the graft corresponds to bone abrasion caused by the wall of the trephine drill used in the production of the defects (Fig 1A).

One week after surgery, there was no evidence of margin resorption at the implantation sites in the group irradiated with 10.2 J/cm^2, whereas intense resorption was observed in the control group and in the group irradiated with 5.1 J/cm^2; a thicker radiolucent area was observed around the grafts. In the group irradiated with 10.2 J/cm^2, small radiopaque points of newly formed bone tissue were observed in the margin around the graft, indicating bone tissue deposits.

In the second week after surgery, radiopaque areas of newly formed bone were observed around the graft in the control and irradiated groups. In the fourth week after surgery, radiopaque areas connecting the graft to the border of the defect were observed in the irradiated and control groups. Differences in radiopacity/radiolucency were noted in the bone grafts of the three groups, indicating bone graft absorption activity.

At 24 weeks after surgery, radiolucent areas could still be observed in the groups irradiated with 5.1 J/cm^2 and 10.2 J/cm^2, despite various radiopaque points around the grafts. However, very little of these areas were observed in the group irradiated with 10.2 J/cm^2 (Fig 1B–D).

Histologic Findings

At one week after surgery, periosteal cells between the margin of the graft and the border of the defect

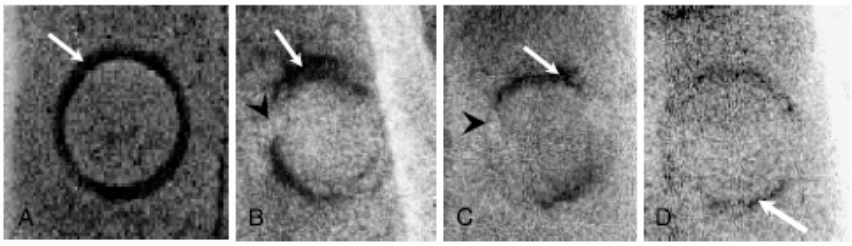

Fig 1 Radiographic aspects of the defects filled with autogenous graft. (A) Immediately after surgery (original magnification, ×3). Note the radiolucent area (arrow) around the graft (*). (B) Control group 24 weeks after surgery. Note the radiolucent area (arrow) around the graft and the newly formed bone union between the graft and the margin of the defect (arrowhead). (C) Group irradiated with 5.1 J/cm^2, 24 weeks after surgery. Observe the smaller radiolucent area (arrow) around the graft and the newly formed bone (arrowhead). (D) Group irradiated with 10.2 J/cm^2, 24 weeks after surgery. Note that the defects were almost completely closed, with few radiolucent areas present (arrow).

and a small deposit of osteoid tissue were observed in control animals. In the groups irradiated with 5.1 J/cm^2 and 10.2 J/cm^2, undifferentiated cells proliferated around the grafts, with deposits of osteoid tissue being observed on the graft surface and close to the border of the defects (Fig 2A–C).

In the second week after surgery, bone deposits were observed on the graft surface and close to the border of the nonirradiated defect. At some sites, this new bone formation promoted the union between the graft and parietal bone. Bone deposits promoting the union between the graft and parietal bone were also observed at graft sites in the groups irradiated with 5.1 J/cm^2 and 10.2 J/cm^2. Larger amounts of newly formed bone tissue were evident in the group irradiated with 10.2 J/cm^2 (Fig 2D–F).

In the fourth week after surgery, alterations in shape and a reduction in graft volume were observed

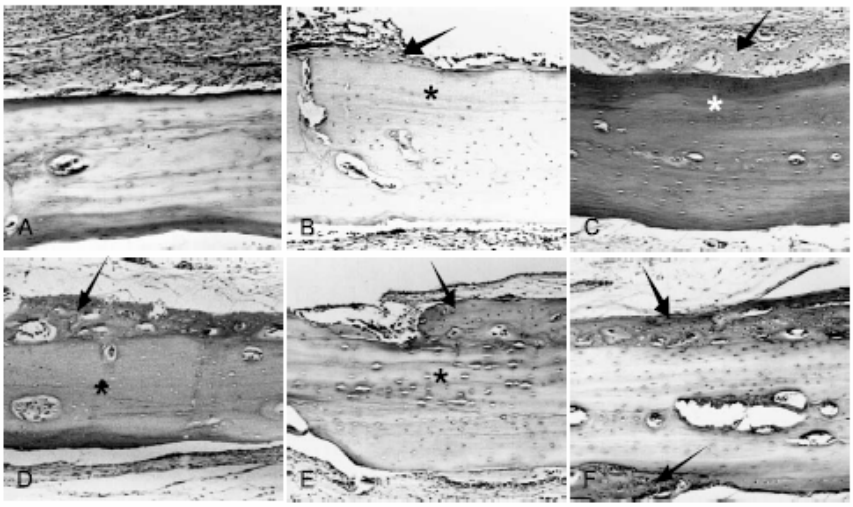

Fig 2 Cross-sections of a parietal bone defect filled with autogenous graft. (A) Control group 1 week after surgery (HE; original magnification, ×600). (B) Group irradiated with 5.1 J/cm^2 1 week after surgery (HE; original magnification, ×600). Observe the newly formed bone (arrow) on the graft surface (*). (C) Group irradiated with 10.2 J/cm^2 1 week after surgery (HE; original magnification, ×600). Note the more evident new bone (arrow) on the graft surface (*). (D) Control group 2 weeks after surgery (HE; original magnification, ×600). Note the newly formed bone (arrow) on the graft surface (*). (E) Group irradiated with 5.1 J/cm^2 2 weeks after surgery (HE; original magnification, ×600). Note that the volume of newly formed bone (arrow) on the graft surface (*) is similar to that observed for the control group. (F) Group irradiated with 10.2 J/cm^2 2 weeks after surgery (HE, original magnification, ×600). Note the greater bone volume (arrows) on the graft surface.

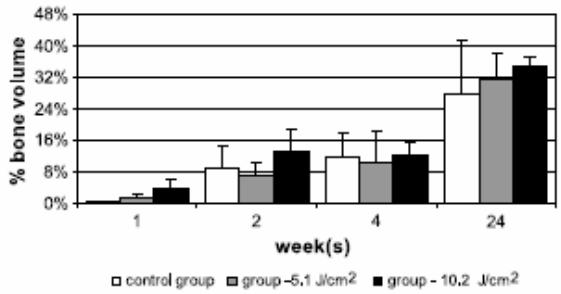

Fig 3 Percent volume of newly formed bone in the defect reconstructed with autogenous bone graft and submitted to laser irradiation. Values are expressed as mean ± SD (N = 6). There was statistical significance at 1 and 2 weeks (*P < 0.05 between control group and treated groups; **P < 0.05 between treated groups).

in the three groups. In addition, continuous deposition of bone tissue promoted the union between the grafts and their respective implantation sites.

At 24 weeks after surgery, a reduction in graft volume and alterations in shape could still be observed in the three groups studied.

Morphometric Analysis

A significant difference ($P < 0.05$) in the volume of newly formed bone was observed between the group irradiated with 5.1 J/cm^2 (1.4 ± 0.011%) and the control group (0.2 ± 0.0%) in the first week after surgery. During the same period, the bone volume was greater at the implantation sites irradiated with 10.2 J/cm^2 (3.75 ± 0.0241%) compared with the control group (0.2 ± 0.0%) and the group irradiated with 5.1 J/cm^2 (1.4 ± 0.011%), with these differences being significant ($P < 0.05$). In the second week after surgery, the bone volume was similar in the group irradiated with 5.1 J/cm^2 (6.9 ± 0.0378%) compared with the control group (8.7 ± 0.0593%). During the same period, the bone volume was greater when used 10.2 J/cm^2 (13.48 ± 0.0560%) compared with both the control group (8.7 ± 0.0593%) and the group irradiated with 5.1 J/cm^2 (6.9 ± 0.0378%). These differences were significant ($P < 0.05$).

At the 4th week after surgery, no significant difference in bone volume at the implantation sites was observed between the irradiated and control groups (Fig 3).

Discussion

Bone formation at the graft site is fundamental for the success of bone defect reconstruction. Therefore, noninvasive procedures that positively interfere with this process, such as hyperbaric oxygenation[15], electrical stimulation[16], ultrasound[17], and low-power laser irradiation[4], are of great clinical importance. With respect to low-power laser irradiation, the present results demonstrated that, within the physical parameters defined for the experiment, laser therapy significantly increased the volume of newly formed bone at the graft site only during the first 2 weeks of irradiation.

This stimulatory action of laser irradiation, only during the initial healing stages, has also been reported in studies on the regeneration of palatine sutures during palatal expansion in rats. Positive effects were only observed when the laser was applied during the early repair period (days 0–2), whereas laser therapy had no effects when applied during later periods (days 4–6).[12] In vitro studies regarding laser application to osteoblasts at different stages of development have demonstrated that the stimulatory action of laser irradiation only occurs during the stages of proliferation and differentiation of immature precursors; no effects were observed during later cell stages.[5] Based on these findings, we think that, in the present study, the laser therapy exerted a stimulatory action on osteogenic cells of the graft and of the defect during the first 2 weeks. This action resulted in cell proliferation and differentiation, increasing the volume of newly formed bone. Laser irradiation had no effect on the proliferate and osteogenic activity of cells after the 15th day because these cells were already in advanced stages of differentiation.

There is evidence that the bioenergetics, bioelectrical, and biostimulatory effects of laser therapy increase the production of ATP to facilitate cell repair processes. In addition, these effects lead to a normalization of the cell membrane ionic potential, revitalizing cell functions, and promoting the synthesis of ATP synthetase for the production of nucleic acid and the acceleration of cell division.[7–9] Because these effects manifest during the early period after trauma, this evidence is in agreement with the present results.

We also observed that the effect of laser therapy during the first 2 weeks after surgery is dose dependent, because the energy density of 10.2 J/cm^2 stimulated higher bone tissue deposition than the energy density of 5.1 J/cm^2. A study using a helium-neon laser applied to tibia fractures in rats at doses of 3.15, 31.5, and 94.7 J/cm^{-2} demonstrated a marked bone formation in animals treated with 31.5 and 94.7 J/cm^{-2}; the effect was greater at the higher dose.[18] In a study analyzing laser therapy in the regeneration of palatine sutures during palatal

expansion, Saito and Shimizu[12] also showed that the effect of low-power laser treatment is dose dependent because prolongation of exposure to the laser from 3 to 10 min/d resulted in an increase in bone volume.

Postoperative laser therapy has been used not only to stimulate the regeneration of fractures but also to promote the osteointegration of orthopedic implants. This combination yielded successful results because laser therapy improved the osteointegration of implants, such as hydroxyapatite and inorganic bovine bone.[4,19] A study associating laser therapy with organic bovine bone (Bio-Oss) for the reconstruction of bone defects in the alveolar process of rats found positive results on increase bone formation during the bone regeneration process.[20] On the other hand, no studies are available associating laser therapy as a stimulator for the osteointegration of autogenous bone grafts at the site of implantation. Based on the principle that the mechanisms involved in bone graft site healing are similar to those observed during fracture repair, the present results suggest that laser therapy applied to autogenous grafts during the early postoperative stages accelerates cell proliferation and increases the volume of newly formed bone, thus, helping the integration of the graft to the recipient area.

In conclusion, our results demonstrated that laser irradiation in the rat skull defects treated with bone graft stimulated osteogenesis during the initial stages of healing process, and this effect was dose dependent.

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REPAIR OF CRANIAL BONE DEFECTS WITH CALCIUM PHOSPHATE CERAMIC IMPLANT OR AUTOGENOUS BONE GRAFT

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(ACEITO)

REPAIR OF CRANIAL BONE DEFECTS WITH CALCIUM PHOSPHATE CERAMIC IMPLANT OR AUTOGENOUS BONE GRAFT

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ABSTRACT: Autogenous bone grafts have frequently been used in the treatment of bone defects; however, this procedure can cause clinical complications after surgery. Besides, the amount of available bone is sometimes insufficient. Therefore, synthetic biomaterials have been researched as an alternative to autogenous bone graft implants. The objective of this study was to evaluate the repair of bone defects treated with compact autogenous bone graft or porous calcium phosphate ceramics. Three defects of 3-mm in diameter were produced in the skull of 21 rats. One the defects was produced in the frontal bone, which remained empty, while the others were produced in the right and left parietal bones, which were filled respectively with ceramics and autogenous bone graft. The animals were sacrificed1, 2, 4 and 24 weeks after surgery and analyzed by light microscopy and radiography. In the twenty-fourth week, the defects filled with autogenous bone graft and ceramics had similar volumes of newly formed bone tissue. The ceramics offered favorable conditions to bone tissue growth. Thus, we concluded that the calcium phosphate ceramic implant proved to be effective in repairing defects produced in the skull of rats.

KEY WORDS: biomaterial, bone regeneration, ceramics, graft.

INTRODUCTION

Defects with bone loss have frequently been treated with autogenous bone grafts, either spongeous or compact, obtained from the rib, iliac crest, jaw and tibia[1-3]. This type of treatment has been widely employed because it offers osteogenic and undifferentiated cells from bone marrow, which contribute to the regeneration process of the bone. However, incorporation of the bone graft responds differently depending on the type of bone tissue used. The cancellous bone graft has trabeculae that are more easily reabsorbed, what prevents the maintenance of graft volume, while the compact bone graft volume can be maintained for years. This characteristic is an advantage because the compact bone graft provides a scaffold for bone remodeling. In addition, it provides the host site with good mechanical resistance, which is necessary in some cases. The procedure for obtaining both compact and cancellous bone graft involves additional surgeries, which may cause postoperative complications and sometimes provide an insufficient quantity of bone to the repair of defect[4-6].

Therefore, alternative biomaterials such as calcium phosphate ceramics have been used as substitutes for autogenous bone grafts because they are biocompatible, can be obtained in large quantities and do not require additional surgical procedure[7-12]. Since they present basically the same composition as the mineral phase of bone, calcium phosphates can take part in the ionic equilibrium with the biological fluid and be absorbed. Because absorption is a process caused by physical-chemical dissolution, it depends on the solubility product of the material and on the pH of the physiological medium, and can be accelerated by a reduction of crystallinity and an increase in the surface area of the material. Thus, the

microstructure of the bioceramics can be controlled to promote the formation of pores that can allow the migration of blood vessels and bone tissue into the material[13].

However, although these pores increase the osteconductive capacity of the implant, they also reduce its mechanical resistance, what limits its clinical application[9, 14]. Despite this disadvantage, porous ceramics can be used for filling bone defects where a strong mechanical resistance is unnecessary, as it is the case in the reconstruction of some oral and maxillofacial defects.

Considering the advantages offered by biomaterials as substitutes for autogenous bone grafts, the objective of the present work was to evaluate the repair of bone defects produced in the skull of rats treated with porous calcium phosphate ceramics or autogenous bone graft formed mainly by compact tissue. The calcium phosphate ceramics used in this experiment was not a commercial product, but a material developed to be favorable to bone growth at a low cost.

MATERIALS AND METHODS

Production and characterization of the ceramics

For the production of the calcium phosphate ceramics, around 25g of potassium dihydrogen phosphate was melted at 950^0C and kept at this temperature for about 4 hours. After that, the material was poured onto a graphite tablet, resulting in a glass whose composition was predominantly sodium polyphosphate (Equation 1). The sodium polyphosphate was dissolved in 25 ml of water, forming a gel to which 200 ml of $CaCl_2$ 3

mol L^{-1} was added under intense agitation. This mixture resulted in a precipitate. After filtration, the precipitate originated a moldable material with plastic consistency.

The plastic material was molded into small spheres of about 1 cm in diameter and heated up to 150^0C at a heating rate of 5^0C/minute. After reaching 150^0C, the material was rapidly heated to around 300^0C (20^0C/minute) suffering a large expansion and transforming into a porous ceramics.

The formation of the calcium polyphosphate can be briefly outlined by the following equations:

$$n\ NaH_2PO_4^- \rightarrow Na_n\ [-\ O_2P\text{-}O\text{-}PO_2\text{-}O\text{-}PO_2\text{-}O\ ...]\ +\ nH_2O \quad (1)$$

$$Na_n\ [-\ O2P\text{-}O\text{-}PO2\text{-}O - PO2\text{-}O\ ...]^{n-}\ +\ y\ Ca^{2+} \rightarrow$$

$$Na_{(n-2y)}[-\ O2P\text{-}O\text{-}PO2\text{-}O\text{-}PO2\text{-}O]Ca^{2+}_y\ +\ 2y\ Na^+ \quad (2)$$

The porous mass was cut into discs of around 3mm in diameter with a surgical trephine.

The ceramic discs were characterized by SEM (Scanning Electron Microscopy) and EDS (Energy-Dispersive Spectroscopy).

Surgical procedure and analysis methods

Twenty-one male Wistar rats at the age of eight weeks were used in this experiment. The animals were anesthetized with a solution of 1:1 xylazine clorohydrate and ketamine at a 9mg/0.1Kg dose, by intramuscular injection. After the cranial skin had been shaved, a longitudinal incision was made in the skull and the periosteum was removed to expose the

surface of parietal bones and frontal bone. With a surgical trephine, a full-thickness bone defect 3mm in diameter was produced in each parietal bone and another in the frontal bone. The defect in the right parietal bone was filled with a calcium phosphate ceramic disc of approximately the same diameter as the lesion, while the defect in the left parietal bone was filled with autogenous bone graft obtained from the right parietal bone of the same animal. The defect in the frontal bone remained empty. Then, the periosteum and the skin were returned and sutured.

The rats were divided into 4 groups of 5 animals each and sacrificed 1, 2, 4 and 24 weeks after surgery. The calvaria of all rats were X-rayed using a Rigaku RU-200 with a focal spot of 0.8 x 0.8 mm. One of the 21 rats was sacrificed immediately after surgery and then X-rayed, to be used as a parameter for the groups. For histological analysis, samples were fixed in Formol 10%, decalcified in EDTA and embedded in paraffin. Seven-micrometer thick transverse sections were stained with hematoxylin and eosin.

For morphometric analyses, 5 areas of each animal were selected for quantification of newly formed bone and osteoid tissue. The volume fraction of the bone was estimated using point volumetry according to the Delesse Principle [14]. After obtaining bone volume, significance analysis was conducted through “t student” test.

Animal experimentation was conducted in accordance with the Ethical Principles for Animal Research established by the Brazilian Association for Laboratory Animal Science (COBEA) and was approved by the Institutional Committee for Ethics Research – protocol n^{o} 157-1 (State University of Campinas – UNICAMP).

RESULTS

SEM and EDS observations:

Figure 1 shows that the material presents two porosity groups. Figure 1-A has pores between 10 and 40 μm in diameter while Figure 1-B shows the material is formed from crystal agglomerates of around 1μm. These crystal agglomerates originate a microporous structure with pores smaller than 1μm in diameter.

The composition of the material was analyzed through EDS/SEM analysis. In the EDS spectrum, elements Ca, P and Na are shown to be predominant in the composition of the material. The ratio between EDS peak intensities shows that Ca/P ratio is around 1/3 (Fig. 2). The Ca/P ratio of the material is much smaller than that of hydroxyapatite and other calcium phosphates frequently used in implants, and this value, as indicated by reactions (1) and (2), shows that sodium ions exchange for calcium ions is not complete.

$$n\ NaH_2PO_4^- \rightarrow Na_n\ [-\ O_2P\text{-}O\text{-}PO_2\text{-}O - PO_2\text{-}O\ ...]\ +\ nH_2O \quad (1)$$

$$Na_n\ [-\ O2P\text{-}O\text{-}PO2\text{-}O - PO2\text{-}O\ ..\ ]^{n-}\ +\ y\ Ca^{2+} \rightarrow$$

$$Na_{(n-2y)}[-\ O2P\text{-}O\text{-}PO2\text{-}O - PO2\text{-}O]Ca^{2+}_y\ +\ 2y\ Na^+ \quad (2)$$

Radiographic observations

Immediately after surgery, the defect in the frontal bone that remained empty was completely radiolucent. In parietal bone defects, both the calcium phosphate ceramics and the bone graft were radiopaque. A small radiolucent margin was observed around the

ceramic disc and the autogenous bone graft. This area around the ceramics and the graft corresponds to bone abrasion caused by the wall of the trephine drill used in the production of the defects (Fig. 3-A). One week after surgery, in the defect filled with ceramics, radiopaque areas of newly formed tissue were observed in the margin around the implant. The same radiopaque areas of newly formed bone tissue were observed around the bone graft. Small radiopaque areas were present in the interior of the defect in the frontal bone on the second postoperative week. In the same period, the radiolucent contour around the ceramics and that around the graft were thinner when compared to the first week. In some areas, radiopaque points of bone tissue connected the ceramics and the bone graft to the border of their respective parietal bone defects. In the fourth postoperative week, differences of radiopacity/radiolucent in the bone graft and the ceramics indicated bone tissue formation and degradation. Twenty-four weeks after surgery, the defects in frontal bones presented radiopaque areas of newly formed bone, what reduced the size of the defect. In parietal bone defects, both left and right, there were still small radiolucent areas between the margin of the defects and the border of implanted materials (Fig. 3-B).

Microscopic Observations:

At one week after surgery, periosteal cells infiltrated to the defect of the frontal bone. In the defect filled with autogenous bone graft there were periosteal cells between the border of the graft and the margin of the lesion. The ceramics presented fragmentation and periosteal cells were found among the ceramic fragments and the surface of the implanted material. In the frontal bone defect there was no formation of osteoid tissue, while in the

defects containing ceramics or bone graft this tissue was present, mainly from the margin of the defects.

In the second postoperative week, newly formed bone tissue and ossification centers were observed in the interior and from the margin of the defect in the frontal bone (Fig.4-A). There were bone tissue deposits on the graft surface and along the edge of the defect. In some areas this tissue united the graft with the left parietal bone (Fig. 4-C). In the defect filled with ceramics there was bone tissue forming from the wall of the lesion, as well as ossification centers among the ceramic fragments. In some areas, the bone tissue formed from the margin of the defect promoting the union between the implant and parietal bone (Fig. 4-E). Blood vessels were found in the interior of the three defects, even among the ceramic fragments.

In the fourth postoperative week, the degradation process of the ceramics enlarged the spaces, allowing larger osteogenic cell infiltration and bone tissue deposits. The bone graft disc presented alterations in shape and volume due to bone resorption. In addition, continous depositions of bone tissue promote the union between the graft and their respective implant sites.

In the twenty-fourth postoperative week there was bone tissue with a lamellar aspect in the center and from the margin of the frontal bone defect, not in enough quantity to completely fill the lesion (Fig. 4-B). There was a resorption of the bone graft, whose modificated its original size and volume (Fig. 4-D). In both parietal defects, the newly formed bone was not enough to fill up the whole defect area, although there were few areas not filled with bone tissue (Fig.4-D and F). In the ceramics-filled defect there were bone tissue deposits with a lamellar aspect from the margin of the defect and among the fragments of the ceramics (Fig. 4-F e G).

Quantitative analysis

In the first week after surgery there was no significant difference in the volume of new bone between the frontal defects (0.0%) and those filled with ceramics (0.6%; p=0.085) and bone graft (0.2%; p=0.19). This no significance (p=0.17) also was found between the groups treated with ceramics (0.6%) and bone graft (0.2%).

The same lack of difference occurred in the other weeks. However, when comparing the neoformed bone volume in the 24th week between the defect filled with ceramics (35.5%) and frontal defect (18.2%), this significance was p= 0.06 (Fig. 5).

DISCUSSION

In experimental studies on the efficiency of treatments for bone defect reconstruction it is primarily important to determine the critical size of the defect so that there is no spontaneous closure of the lesion. Some authors have noticed that defects measuring 2mm in diameter for parietal bone of 12 weeks-old Wistar rats and defects measuring 4mm in diameter for 28 days-old Wistar rats persist for up to six moth after surgery[16-17]. In our work, a defect with 3mm in diameter was produced in the frontal bone, which did not receive any treatment. This defect was not completely closed until the twenty-fourth postoperative week, what indicates that the chosen diameter was appropriate for assessing the performance of the ceramic implant and the autogenous bone graft in bone defect repair.

In the present work, through radiographic analysis of the calvaria, radiopaque points were observed in the radiolucent contour around the implants after two postoperative weeks.

This aspect reveals the presence of newly formed bone, which permitted a union of the ceramics and bone graft with their respectively parietals bone. Some authors have also noticed some radiopaque areas around and inside hydroxyapatite implants in the radiographs of patients submitted to orthognathic surgery [14, 18,19].

In our study, after the fourth postoperative week, there were some radiolucent points both at the border of grafted bone and in the hydroxyapatite implants inside bone defects. This indicates the absorption of the autogenous bone graft and the degradation of the calcium phosphate ceramics. Even though this processes caused the shape and volume alteration of the bone graft and the ceramic implants, in the twenty-fourth postoperative week both the ceramics and bone graft were still present in the defect.

Histologic findings confirmed the radiographic results, because new bone formation was also found around the autogenous graft and the ceramics. The bone tissue formed from the margin of the defects united to the edge of materials promoting the osteointegration of the materials implanted in the defects. Throughout the several experimental periods in this work, histologic observations also indicated that the ceramics had its original shape altered. This shows that the ceramics suffered degradation when in contact with tissular liquids. This degradation created spaces that, together with the porosity of the material allowed bone growth inwards the ceramics.

In the twenty-fourth postoperative week of our experiment, we found a larger volume of newly formed bone in the defect filled with ceramics than in the frontal bone defect, which did not receive any treatment for bone reconstruction. We therefore concluded that the ceramic implant offered favorable conditions to bone tissue growth. Apart from this characteristic, the quantity of newly formed bone found in the ceramics-filled defect was

similar to that found in the defect filled with autogenous bone graft. Ohbayashi et. al (2000)[8] showed that hydroxyapatite is not only osteoconductive, but also osteoinductive, since it promoted the formation of ectopic bone tissue around the carotid artery of dogs.

Up to the twenty-fourth postoperative week, neither the quantity of bone present in the defects filled with ceramics nor the quantity present in the ones filled with autogenous bone graft was enough to completely fill the defects. There were some small areas occupied with connective tissue in the defects. In spite of that, the integration of both implanted materials, that is, calcium phosphates granules and the reminiscent compact tissue of the autogenous bone graft with the newly formed bone in both defects, allowed the reestablishing of the bone architecture of the skull. In view of these results, we concluded that the calcium phosphate ceramics and the autogenous graft with compact bone used in this experiment proved to be effective in repairing defects produced in the skull of rats.

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FIGURES

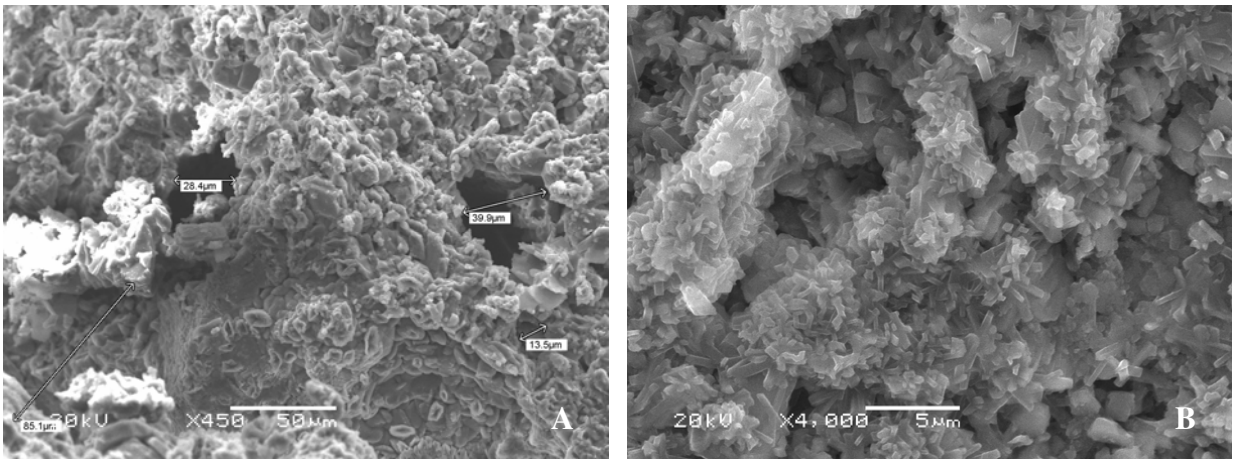

Fig. 1: Electronic scanning microscopy of ceramics. A: Area of the ceramics with pores between 10 and 40 µm in diameter, 450X. B: Area of the ceramics with pores smaller than 1µm in diameter. Observe the crystal agglomerates with about 1µm, 4.000X.

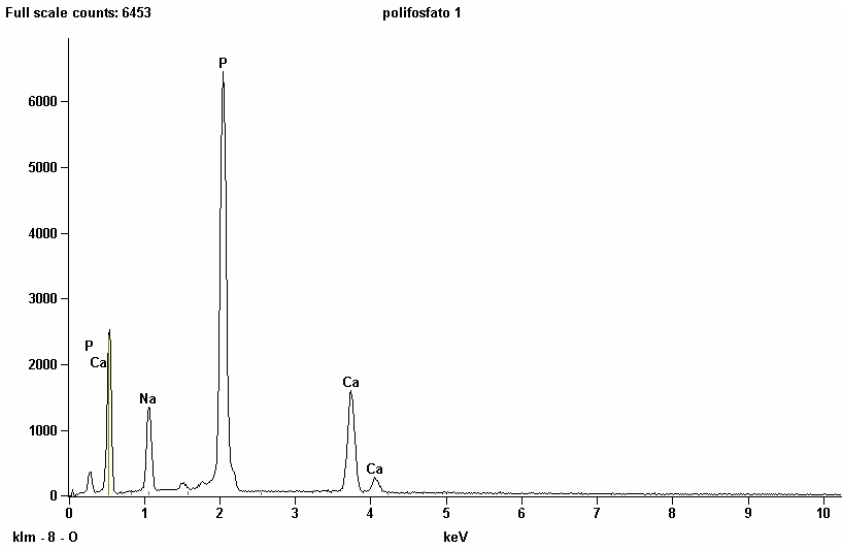

Fig. 2: EDS spectrum of the ceramics. Notice that the elements Ca, P and Na predominate in the composition of the material.

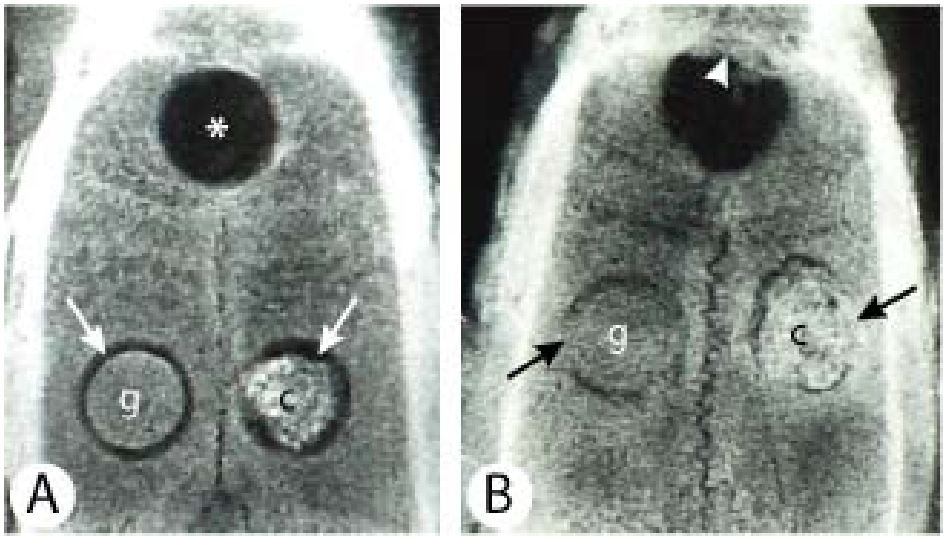

Fig. 3: Radiographic aspects of the defects of the calvarium. A- Immediately after surgery, 3X. Note the radiolucent contour (arrows) around the ceramics (c) and the autogenous graft (g). The defect in the frontal bone appears completely radiopaque(*). B- Twenty-four weeks after surgery, 3X. Observe that the edges of the autogenous graft (g) and of the ceramics (c) are united with the margin of their respective defects through the newly formed bone tissue (arrows). A small quantity of bone tissue (arrowhead) can be identified in the frontal bone defect.

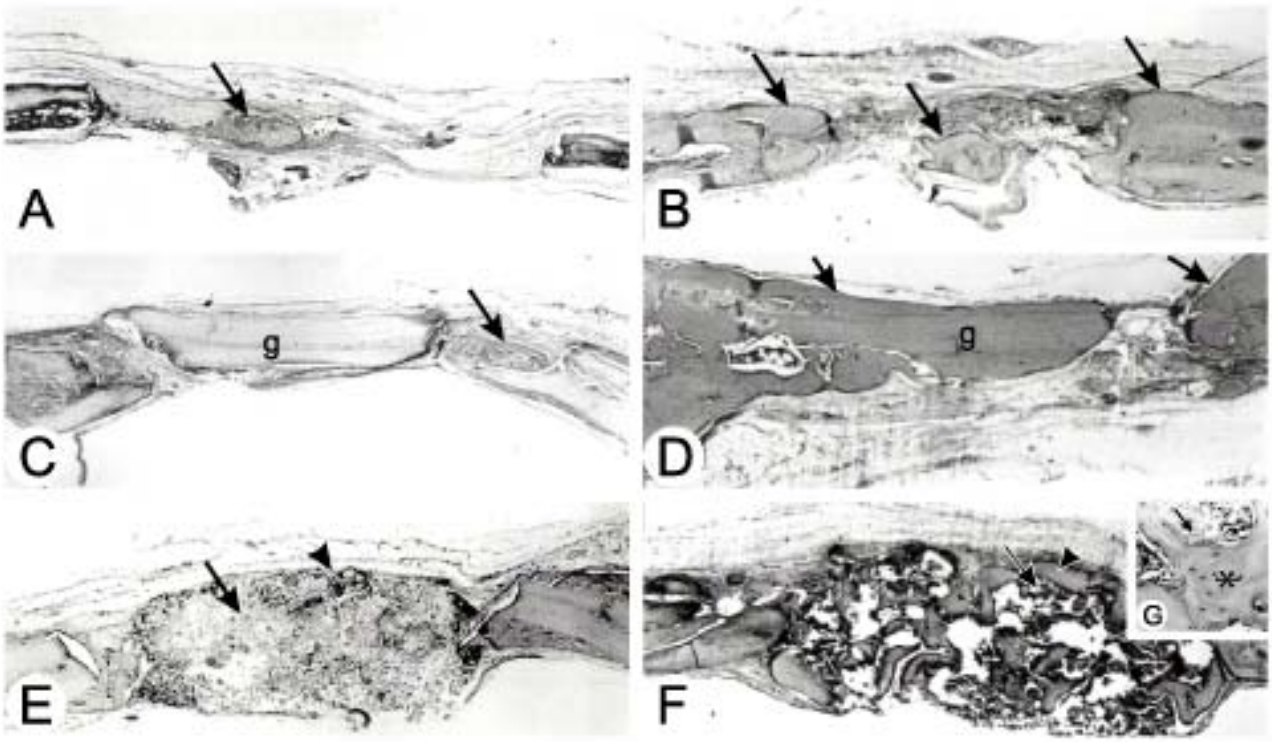

Fig. 4: Transverse sections of the skull after surgery. A- Frontal bone defect at two postoperative weeks, H.E-25X. Notice the newly formed tissue (arrow) in the center of the defect. B- Frontal bone defect at 24 postoperative weeks, H.E-25X. Notice that the newly formed bone tissue (arrows) was not enough to close the defect. C- Defect filled with autogenous graft (g) 2 weeks after surgery, H.E-25x. Observe the newly formed bone (arrow) from the margin of the defect. D- Defect filled with autogenous graft (g) 24 postoperative weeks, H.E-25x. Observe the newly formed bone tissue (arrows) near the graft (g) and from the margin of the defect (*). E- Defect filled with ceramics two weeks after surgery, H.E-25x. Note the ceramics (arrow) and the bone tissue (arrowhead) infiltrating inwards the material. F- Defect filled with ceramics twenty-four weeks after surgery, H.E-25x. Note the bon e tissue (arrowhead) among the ceramics granules (arrow). G- Defect filled with ceramics, H.E-150x. Note the bone tissue with a mature aspect (*) among the ceramics granules (arrow).

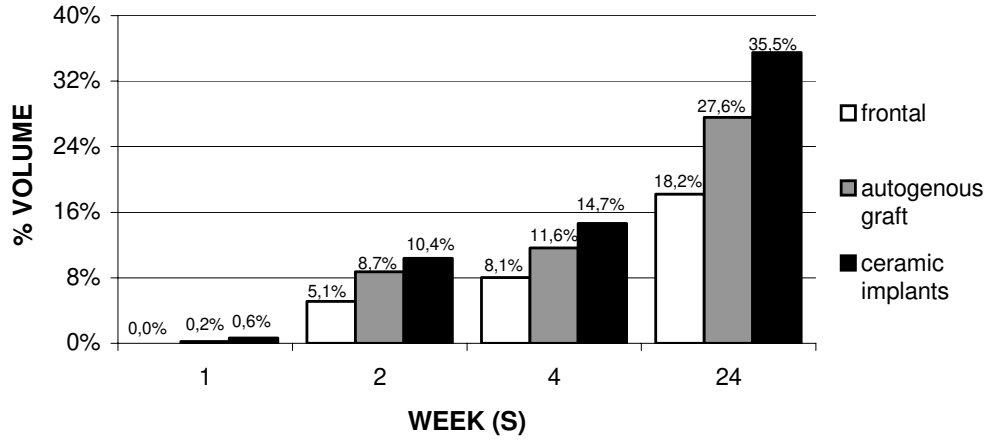

Fig.5 - Volume percentage of neoformed bone into cranial defects.

OSTEOBLAST INTERACTION ON CALCIUM PHOSPHATE CERAMIC

Silva R.V., Bertran C.A, Camilli J.A.

OSTEOBLAST INTERACTION ON CALCIUM PHOSPHATE CERAMICS

Silva RV, Bertran CA, Camilli JA

ABSTRACT: The study on human osteoblasts and bioceramics interaction contributed to understanding the possible effects of this material on the functionality and differentiation of these cells, which enables a better adjustment on the bioceramics composition and microstructure. In such case, this study aims at analyzing *in vitro* human osteoblast and calcium polyphosphate interaction. Therefore, scanning electronic microscopy and immunolabeling for osteocalcin and type 1 collagen was used. Results *in vitro* showed that human osteoblasts had good interaction with the ceramics for maintaining their typical characteristics. Physiologically, they presented osteocalcin and type 1 collagen synthesis, as they were found morphologically flattened and with numerous phyllopodia, presenting a major preference for the irregular surfaces of the ceramic.

INTRODUCTION

Both in medical and odontological areas, the use of bioceramic materials have been studied for treatment and restoration of bone losses. The search for alternatives that avoid risks and inconvenience in the use of autogenous bone grafts[1-3] led to a research for alloplastic materials which would be able to replace it. Bioceramic materials are biocompatible; they exempt additional surgical interventions, offer osteoconductivity and can be synthesized in large amounts[4-7].

Tests *in vivo* and *in vitro* have been applied in order to check the cytotoxicity and biofunctionality of several alloplastic materials[5, 8-11]. Understanding the interactions between

cells and biomaterial is extremely important, as well as the material influence on the functions performed by these cells. This information can be explored to develop materials that mimicry the bone matrix, optimizing, thus, the biological response to the implant.

Therefore, the aim of this work was analyzed *in vitro* the interaction of human osteoblasts with the calcium polyphosphate ceramics. This ceramic is a product non-commercial, is porous and present similar mineral composition of the bone. It has an elevated surface area and it can be molded in its initial polymeric shape.

MATERIALS AND METHODS

Ceramics production and characterization

To produce the ceramics, about 25g of sodium dihydrogen phosphate were fused at 950°C and kept at this temperature for about 4 hours. After fusion, the material was poured on a black lead plate, resulting on a glass which composition was predominantly of sodium polyphosphate (equation 1). The sodium polyphosphate was dissolved in 25ml of water, forming a substance similar to a gel. To this substance, 200ml of solution $CaCl_2$ 3 mol L^{-1} were added under intense agitation, producing a precipitate which, after filtration, originated a material with a molding and plastic consistency.

The plastic material was molded into the shapes of about 1cm-diameter small spheres and heated up to 150ºC with a heat rate of 5ºC/minute. After reaching the temperature of 150ºC, the material was quickly heated up to about 300ºC (20ºC/minute), suffering an accentuated expansion, which resulted in porous ceramics. The sodium polyphosphate formation can be represented briefly by the equations:

$n\ NaH_2PO_4^- \rightarrow Na_n\ [-\ O_2P\text{-}O\text{-}PO_2\text{-}O - PO_2\text{-}O...] + nH_2O$ (1)

$Na_n\ [-\ O2P\text{-}O\text{-}PO2\text{-}O - PO2\text{-}O...]^{n-} + yCa^{2+} \rightarrow$

$Na_{(n-2y)}[-\ O2P\text{-}O\text{-}PO2\text{-}O - PO2\text{-}O]Ca^{2+}{}_y + 2y\ Na^+$ (2)

With the use of a trephine drill, the porous substance was cut in 3mm-diameter discs.

Scanning Electronic Microscopy and EDS (Energy Dispersive Spectroscopy) were used to characterize the ceramics.

Cellular Culture

The 1.19 hFOB (Human Fetal Osteoblastic cells) were obtained at the American Culture Collection (ATCC – University Boulevard, Manassas, VA, USA) and kept at the Department of Cellular biology at UNICAMP in HAM F-12 medium (Sigma Chemical Co., St. Louis, MO – USA), supplemented with 10% of fetal Bovine Serum (SFB, Nutricell, Campinas, SP – Brazil) at 37ºC.

Scanning Electron Microscopy

After a week of osteoblast cultivation (density of $1x10^5$ cells/cm^2) over calcium phosphate ceramics, glass coverslips (positive control), and silicone (negative control), the samples were fixed with glutaraldehyde 2.5% in phosphate-buffered saline (PBS) 0.1M, pH 7.4) for 2 hours. After washing in PBS, the osmium tetroxide (Sigma) was applied and the samples were dehydrated in a series of increasing alcohol concentrations. Afterwards, the

samples were dried at critical point (Balzers CPD030) and covered with gold in “Sputtering” (Balzers SCD050). The material analysis was made using the microscope JEOL JSM-5800.

Osteocalcin activity

The cells were inoculated in the ceramics and glass coverslips (background control) at density of $5x10^4$ cells/ml in HAM F-12 medium (Sigma) supplemented with 10% of fetal Bovine Serum (Nutricel) and cultivated for 7 days. After washing them in cold PBS, the non-specific sites were blocked with PBS+BSA at 1% for 60 minutes at 37ºC. Afterwards, the samples were washed in PBS at 4ºC and hatched with monoclonal anti-osteocalcin (G-12) (Sigma) dissolved in PBS (1:200) for 30 min. After being washed for 20 min., the samples were hatched with FICT-conjugate anti-mouse IgG secondary antibodies (Sigma). As negative control, the conjugate secondary antibody was directly used.

Subsequently, the samples were washed in PBS, fully set on glass in fluorescent medium (DABCO – Sigma). As positive control, DAPI (Sigma), a nuclear DNA marker, was added to the setting medium, which enabled the visualization of the cells nuclei.

Type 1 collagen activity

The cells were inoculated at density of $5x10^4$ cells/ml in HAM F-12 medium (Sigma) supplemented with 10% of fetal Bovine Serum (Nutricel) and cultivated on ceramics and glass coverslips (background control). After the 7^{th} day of cultivation, the samples were washed in PBS at 37ºC and sequentially fixed in paraformaldehyde at 4% for 20 minutes. After that the samples was washed one more time in PBS at 37ºC. The non-

specific sites were blocked with BSA at 1% in PBS and then, the samples were gently washed in PBS at 37ºC. Next, they were treated with 100µl of type 1 anti-collagen primary monoclonal antibodies (Sigma: clone COL 1, dissolution 1:500) and hatched for 30 min. at 4ºC. After being washed, 100µl of anti-mouse IgG secondary antibodies FICT-conjugate were added (Sigma, dissolution 1:200).

As negative control, the conjugate secondary antibody was directly used. Subsequently, the samples were washed in PBS and fully set in glass coverslips with fluorescent medium (DABCO-Sigma + DAPI-Sigma). The DAPI, a nuclear DNA marker was used as positive control.

RESULTS

Ceramic characterization

Figure 1 shows the material micrographics taken by Scanning Electron Microscopy. Figures show that the material presents two groups of porosity. In Figure 1-A, it is possible to see pores with 10 and 40µm diameter while Figure 1-B shows that the material is made of crystals agglomerates, which have about 1µm. Crystal's agglomerates generate a microporose structure with pores that are smaller than 1µm of diameter.

The material composition can be assessed by EDS spectrum carried out in conjunction to SEM. In the EDS spectrum, it is observed that the elements Ca, P e Na prevail in the material composition. The ratio of EDS peak intensities shows that the material Ca/P ratio is from 1/3 order (Fig. 2). Material Ca/P ratio is a lot smaller than the hydroxyapatite and other phosphate calcium ratios, which are frequently used in implants,

and this value, as indicated by reactions (1) and (2), shows that the exchange of sodium ions to calcium ions is not complete.

$$n\ NaH_2PO_4^- \rightarrow Na_n\ [-\ O_2P\text{-}O\text{-}PO_2\text{-}O - PO_2\text{-}O...] + nH_2O \quad (1)$$

$$Na_n\ [-\ O2P\text{-}O\text{-}PO2\text{-}O - PO2\text{-}O...]^{n-} + y\ Ca^{2+} \rightarrow$$

$$Na_{(n\ -\ 2y)}[-O2P\text{-}O\text{-}PO2\text{-}O - PO2\text{-}O]Ca^{2+}{}_y + 2yNa^+ \quad (2)$$

Scanning Electron Microscopy

After the 7th incubation day, cells cultivated on a glass coverslips (positive control) presented good spreading over the surface. Its shape was flat, extended and had numerous and thin cytoplasmatic prolongations, suggesting the typical characteristic from osteoblasts. Numerous vesicles were observed in dorsal surface of several osteoblasts, such as structures similar to microvillus (Fig. 3-A and B). Some osteoblasts connected to each other through phyllopodia (Fig. 3-C).

The osteoblasts upon the calcium phosphate ceramics presented less extended, showing shorter cytoplasmatic prolongations than the positive control. Vesicles were also observed upon the dorsal surface of the osteoblast, suggesting synthesis activity of theses cells (Fig. 3-D). The cells that grew in ceramic areas with roughness surface were more dispersed and irregular, while those that grew upon a smoother surface presented fewer numbers of cytoplasmatic prolongations and grew in a way to obliterate the space between them (Fig. 3-D and E). Some rounded cells implying in mitotic process were found (Fig. 3-F).

In negative control there was a fewer number of osteoblasts upon silicone, that is, the few present cells were found highly retracted suggesting cell death (Fig.3-G).

Type 1 collagen and osteocalcin activity:

After the 7th day of incubation, the osteoblasts cultivated above ceramics showed type I collagen and osteocalcin activity similar to its respective positive control – glass coverslip (Fig.4). There were human osteoblasts on surface, as well as inside the ceramics pores in both immunolabeling of type I collagen and osteocalcin.

DISCUSSION

Analyzing interaction *in vitro* of human osteoblasts with calcium phosphate ceramics through scanning electronic microscopy, it was verified that these cells presented normal morphological characteristics conserving the mitotic process. It is believed that due to the inequality in ceramic porosity, osteoblasts showed different behavior according to surface type where they were cultivated. In more porous and irregular areas of ceramics, these cells showed longer phyllopodia and in increased number, whereas in less irregular areas these cytoplasmatic prolongations were smaller and less numerous, occurring osteoblasts growth in order to obliterate the gaps between them. Probably, as a consequence of the ceramics' surface irregularity, osteoblasts developed longer and numerous phyllopodia so that these cells could be able to adhere to each other and adapt to the pores' size and shape.

Studying the osteoblasts behavior on various materials, some authors verified that the materials' chemical and structural composition interfere on osteoblasts activity, allowing the promotion of a larger or smaller proliferation and cell differentiation [12-14].

Studying the interaction of human osteoblasts in different surfaces of hydroxyapatite implants, they noticed that these cells presented better cellular activity in roughness surfaces[12,15,16]. Studies have shown that porosity promotes a "contact orientation", where direction of cellular movement is influenced by substrate's morphology, which makes the material more osteoconductive[13,17].

In our study, the cultivated osteoblasts upon ceramics showed type I collagen and osteocalcin activity similar to the control. Thus, the ceramics/osteoblast interaction did not interfere in the functionality of this cell, once it presented production of biochemical markers related to osteoblasts differentiation. The osteocalcin production, osteoblastic differentiation marker involved in bone remodeling process, confirms that these cells did not lose their typical characteristic, when in contact with ceramics.

Thus, results *in vitro* indicated that osteoblasts presented good interaction with the ceramics because they mantained their differentiation and functionality process, showing larger preference for more irregular surfaces of the ceramics.

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FIGURES

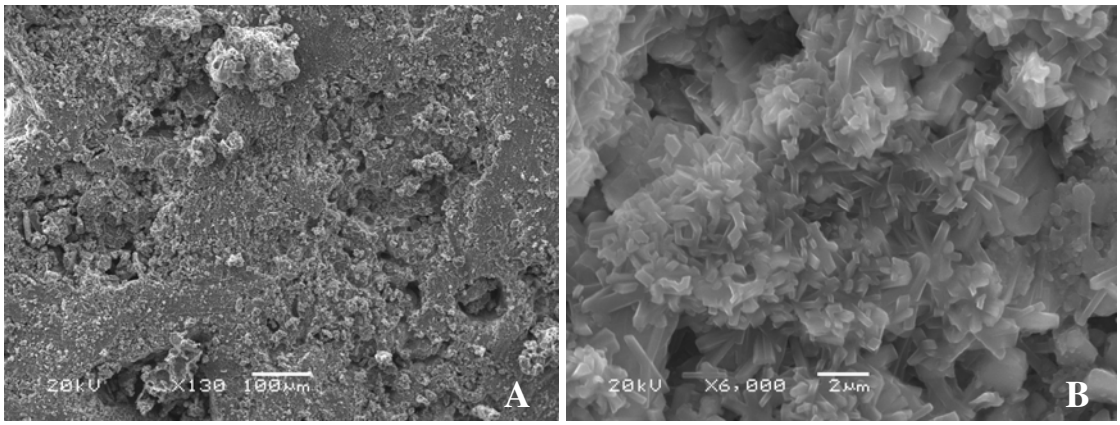

Fig. 1: Scanning Electron microscopy of ceramics. A: Area of the ceramics with pores between 10 and 40 µm in diameter, 130X. B: Area of the ceramics with pores smaller than 1µm in diameter. Observe the crystal agglomerates with about 1µm, 6,000X.

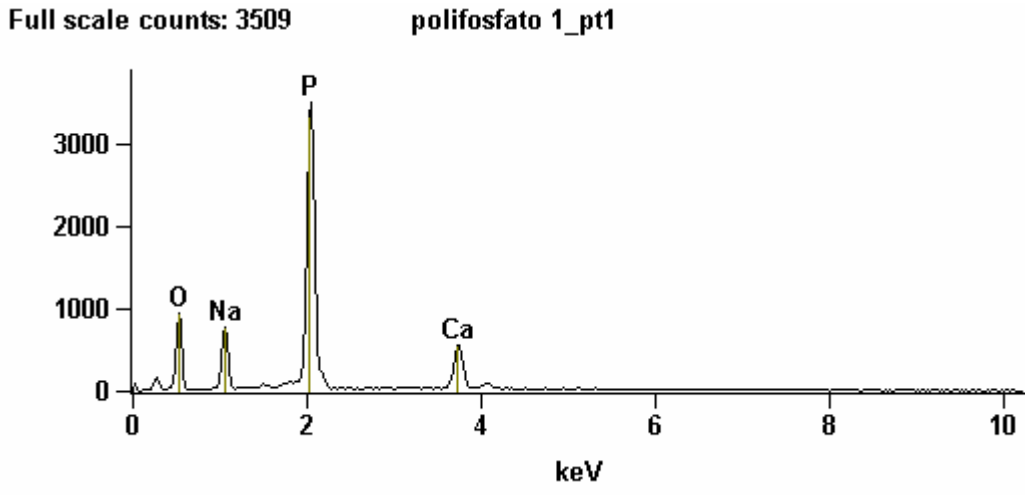

Fig. 2: EDS spectrum of the ceramics. Notice that the elements Ca, P and Na predominate in the composition of the material.

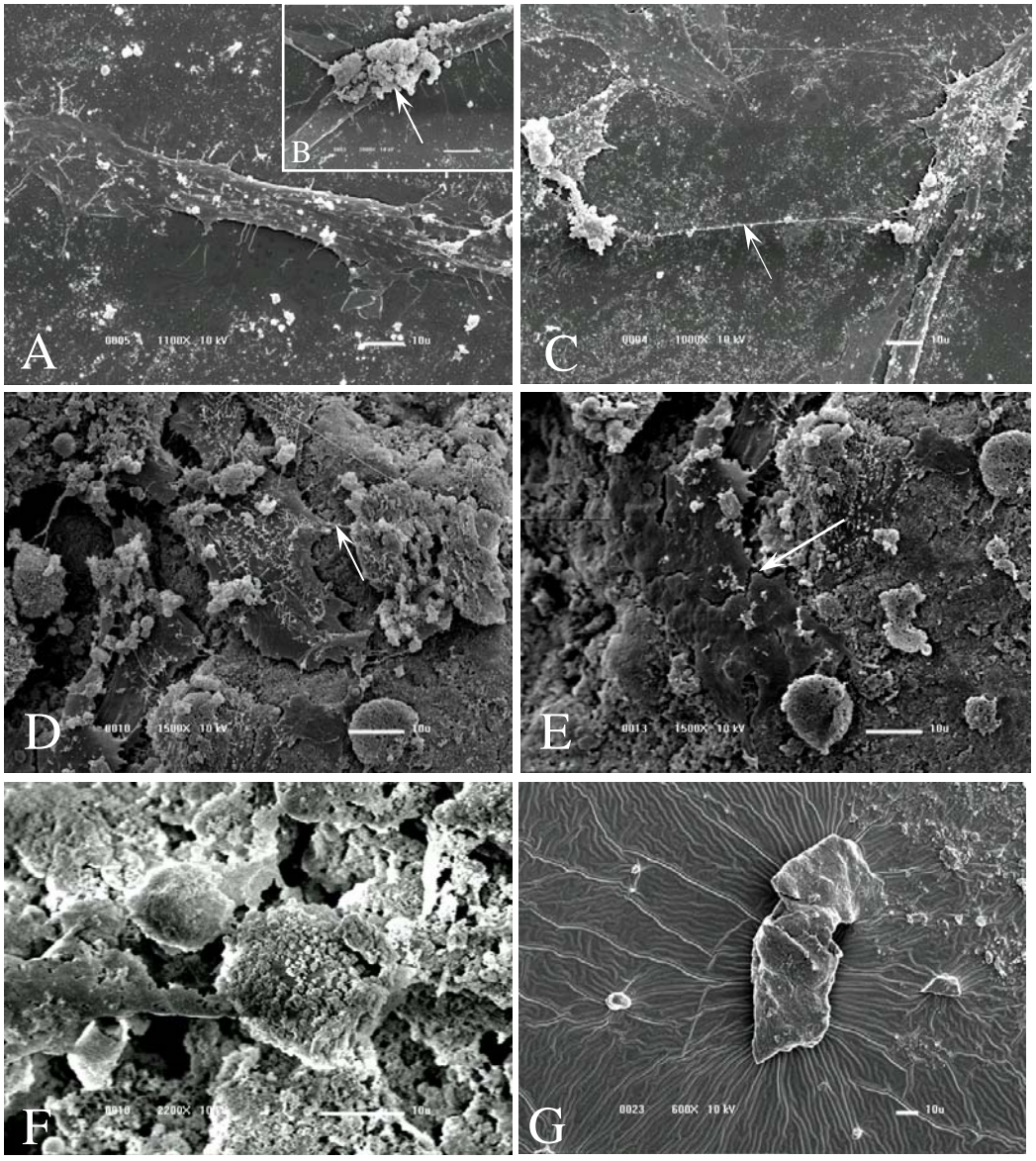

Fig. 3: (A) Human osteoblasts scanning eletron microscopy (SEM) upon glass coverslips (positive control), 1100x. Observe the cell´s flat and extended morphology, as well as the numerous phyllopodia (arrow). (B) Human osteoblasts SEM upon glass coverslips (positive control), 2000x. Notice the vesicles (arrow) on osteoblast dorsal surface. (C) Human osteoblasts SEM upon glass coverslips (positive control), 1000x. Observe osteoblasts connecting to each other through phyllopodia (arrow). (D) Human osteoblasts SEM upon more irregular areas of calcium phosphate ceramics, 1500x. Observe phyllopodia (arrow) that are less numerous and shorter than the positive control, as well as the presence of vesicles on osteoblast´s dorsal surface. (E) Human osteoblasts SEM upon less irregular areas of calcium phosphate ceramics, 1500x. Observe the shorter and in less number cytoplasmatic prolongations, as well as cell growth as to somehow obliterate the space between them (arrow). (F) Human osteoblasts SEM upon calcium phosphate ceramics, 2200x. Observe rounded osteoblasts (arrow) suggesting mitotic process. (G) Human osteoblasts SEM upon silicone (negative control), 600x. Observe cell retraction, suggesting cell death.

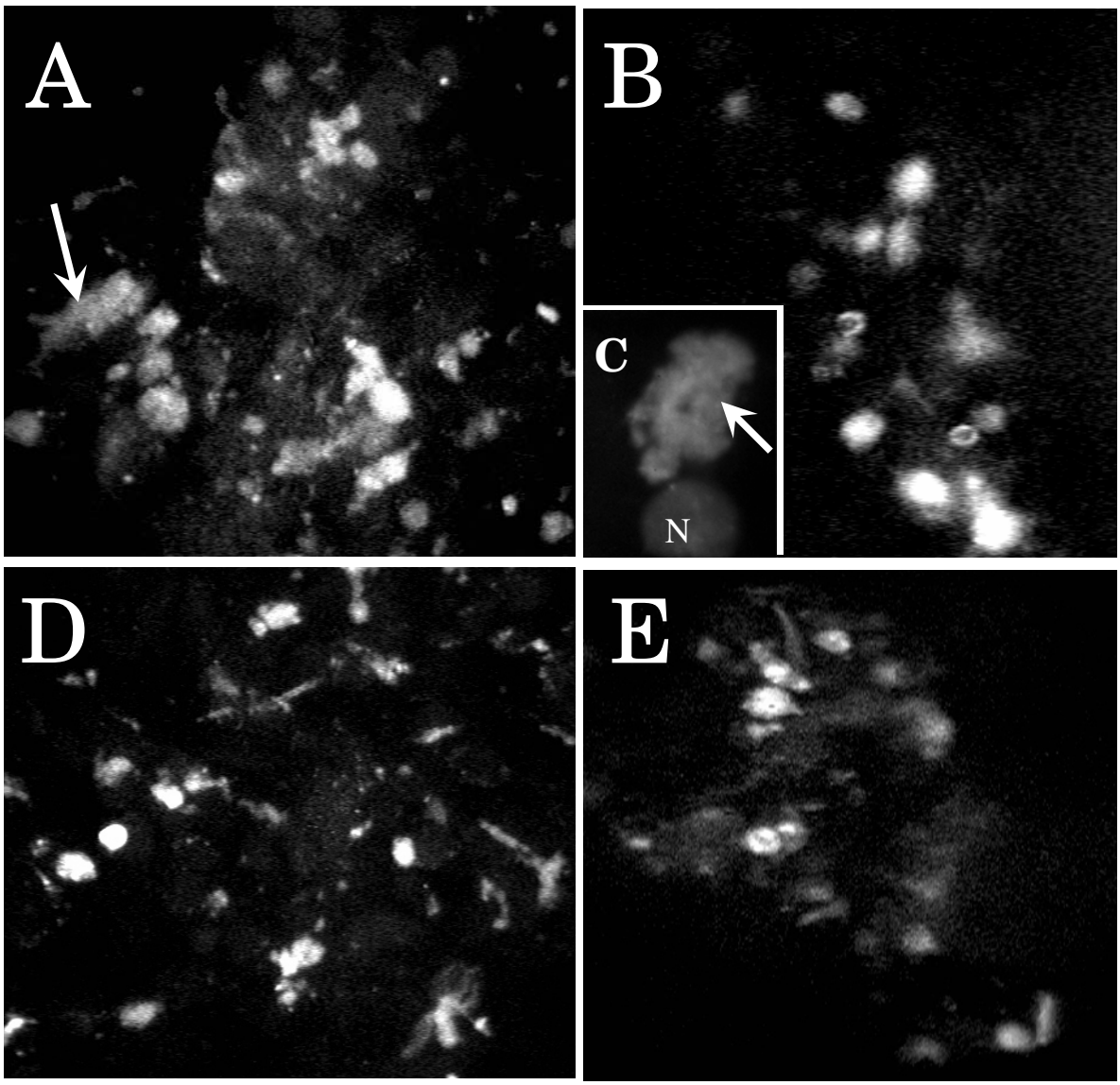

Fig.4: A- Type I collagen activity upon glass coverslips (positive control), 200x. Observe the type I collagen activity (arrow). B- Type I collagen activity on ceramics, 200x. C- Osteoblast upon ceramics, 1000x. Observe the nucleus (N) and type I collagen activity (arrow). D- Osteocalcin activity in osteoblasts upon glass coverslips (positive control), 200x. E- Osteocalcin activity in osteoblasts upon ceramics, 200x.

CONCLUSÕES GERAIS

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- A laserterapia estimulou a osteogênese em defeitos ósseos no crânio de ratos durante os estágios iniciais do tratamento e esse processo foi dose dependente.
- A cerâmica ofereceu condições favoráveis ao crescimento ósseo, apresentando-se tão eficiente quanto o enxerto ósseo autógeno no reparo de defeitos produzidos na calota de ratos.
- Os osteoblastos humanos tiveram boa interação com a cerâmica, pois mantiveram suas características típicas. Essas células apresentaram maior preferência pelas superfícies mais irregulares da cerâmica.

TRABALHOS PARALELOS

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The use of hydroxyapatite and autogenous cancellous bone grafts to repair bone defects in rats.

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Research and Emerging Technologies
Osteobiology

The use of hydroxyapatite and autogenous cancellous bone grafts to repair bone defects in rats

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R. V. Silva, J. A. Camilli, C. A. Bertran, N. H. Moreira: The use of hydroxyapatite and autogenous cancellous bone grafts to repair bone defects in rats. Int. J. Oral Maxillofac. Surg. 2005; 34: 178–184.

Abstract. Bone grafts are frequently used in the treatment of bone defects. Bone harvesting can cause postoperative complications and sometimes does not provide a sufficient quantity of bone. Therefore, synthetic biomaterials have been investigated as an alternative to autogenous bone grafts. The objective of this study was to evaluate the repair of bone defects by autogenous cancellous bone grafts or porous bioceramic discs of hydroxyapatite/phosphate cement mixture. Two 5-mm diameter defects were made in the skulls of rats and filled with the bioceramic material or cancellous bone. The rats were sacrificed 2, 4, 8 and 24 weeks after surgery and tissue samples were analyzed by radiography and histology. By the 24th week, the defects filled with autogenous cancellous bone grafts or bioceramic material showed similar volumes of bone tissue within the defect. However, defects treated with bioceramic material were almost completely closed as a result of the joining of ceramic fragments and the neoformed bone tissue, while those filled with autogenous grafts showed several areas filled with connective tissue. These results indicated that the osteointegration of bioceramic fragments allowed the reconstruction of parietal bone defects without the need for a bone graft.

Key words: autogenous graft; bone; hydroxyapatite; implant.

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The repair of congenital cleft palate and of cranial defects that involve a loss of bone substance caused by tumor resection or infection is of fundamental importance in restoring the shape and function of the skull. Autogenous cancellous and compact bone grafts have been used in the reconstruction of these defects because they do not cause immunogenic reactions, and because they have osteogenic cells' release growth factors that contribute to repair of the defects[10,15,20].

Cancellous bone grafts show faster revascularization but their trabeculae are more easily reabsorbed, thus impairing the maintenance of the shape and volume of the graft. On the other hand, reabsorption allows earlier bone substitution compared to compact bone grafts that persist for many years and show viable and necrotic regions[2]. The donor sites most frequently used to obtain compact and cancellous bone grafts are the iliac crest, mandible, maxillary tuber, rib, tibia and fibula[3,12,24]. Despite the advantages offered by autogenous bone grafts, these grafts require a longer surgical time, they double the number of surgical sites involved, and they sometimes provide an insufficient quantity of bone. In addition, there can be postoperative complications[7,9].

For these reasons, alternative biomaterials to bone have been investigated[17,19]. Synthetic hydroxyapatite has been the most frequently used material because its chemical composition is similar to human bone, it is no toxic,

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has a high chemical stability, and does not cause inflammatory or antigenic reactions[1,5,17,19,21]. Another important property of synthetic hydroxyapatite is that its microstructure can be controlled to promote the formation of pores that can allow the migration of blood vessels and bone tissue into the material. However, although these pores increase the osteoconductive capacity to the implant, they also reduce its mechanical resistance and limit its clinical use[4,19]. Despite this disadvantage, porous hydroxyapatite ceramic implants can be used at sites where a strong mechanical resistance is unnecessary, as in the reconstruction of some craniofacial defects[10,15,20].

Considering the usefulness of porous hydroxyapatite as a substitute for autogenous bone grafts in the reconstruction of bone defects, in this study we compared the behavior of porous bioceramic discs prepared with hydroxyapatite in combination with a phosphate cement to that of autogenous cancellous bone grafts in the repair of surgically-produced bone defects in rat parietal bone.

Materials and methods

Production of ceramic discs

Porous ceramic discs were made by molding the white, sludge-like material obtained by mixing hydroxyapatite powder with cement. This cement was prepared by dissolving $CaHPO_4$ in a 50% (v/v) water/phosphoric acid solution and by the drop-wise addition of concentrated ammonium hydroxide. The resulting white, a gel-like suspension of amorphous particles that was filtered and washed with cold water.

The hydroxyapatite powder was added while the cement was still wet. The consistency of the resulting white sludge depended on the proportion of powder and cement used on the quantity of water present in the gel-like material. The discs were prepared by pouring the sludge into metal molds 5 mm in diameter and 1 mm thick, followed by drying at room temperature for 24 h and heating at 325 °C for 5 h. There was no evidence of any chemical change in the material during this final heating, which was necessary to prevent the cement from fragmenting upon contact with water.

The final products were porous rigid discs composed of hydroxyapatite crystals dispersed in a dense amorphous phosphate matrix. The porosity of the material was determined by mercury intrusion porosimetry (Porosizer from Micromeritics – model 9320), which revealed pores with diameters of 4–20 μm.

Surgical procedure and analytical methods

Twenty-nine 8-week-old male Wistar rats were used. The rats were anesthetized with a 1:1 mixture of xylazine hydrochloride (2%) and ketamine at a dose of 0.15 ml/100 g, I.M. After shaving the cranial skin, a longitudinal incision was made in the skull and the periosteum was separated to expose the surface of the parietal bones. Using a surgical trephine, a full-thickness bone defect 5 mm in diameter was produced in each parietal bone. The defect in the right parietal bone was filled with the porous ceramic disc, while the defect in the left parietal bone was filled with cancellous bone taken from the tibia of the same rat. The volume of the autogenous bone graft taken from the tibia was sufficient to completely fill the defect. The periosteum and skin were subsequently repositioned.

Two rats with 8 and 24 weeks after surgery were sacrificed for macroscopic observation of the final reconstruction of the defects. The calvarium of these four rats was removed, placed in running water for four days and then in 10 vol. of hydrogen peroxide to clear the bones. The samples were photographed with a Zenit 12XL camera. Of the remaining 25 rats, one was sacrificed immediately after surgery and was X-rayed to be used as a control. The other rats were divided into four groups of six animals each and sacrificed for radiographic and microscopic studies 2, 4, 8, and 24 weeks after disc or graft implantation. The calvaria of all the rats were X-rayed using a Rigaku RU-200 apparatus with a focal point of 0.8 mm × 0.8 mm. Samples for histological analysis were then fixed in 10% formalin, decalcified in a mixture formic acid and sodium citrate solution, and embedded in paraffin. Seven micrometer thick transverse sections were stained with hematoxylin and eosin.

For morphometric analysis, images of histological sections from all samples were obtained with a camera coupled to the microscope and then analyzed by computer using the software Sigma Scan (Jandel Corporation). In the left parietal defect, the combined volume of neoformed bone and of the autogenous graft was considered, whereas in the defect in the right parietal only the volume of neoformed bone was considered. The biomaterial was not considered in the morphometric analysis. The volume fraction of the bone was estimated using point volumometry according to the Delesse theorem[13]. All statistical comparisons were done using Student's *t*-test, with a value of $P < 0.05$ indicating significance.

The animal experiments were done in accordance with the ethical principles for animal research established by the Brazilian College for Animal Experimentation (COBEA) and were approved by the institutional Committee for Ethics in Animal Research – protocol no. 349-2 (State University of Campinas – UNICAMP).

Results

Macroscopic observations of macerated skulls

Eight weeks after implantation, the bioceramic discs were joined to the parietal bone by bone tissue formed around the borders of the defect. The defect in the parietal bone was closed, and only a few sites around the margin did not have bone. The defect filled with the cancellous bone graft contained neoformed bone, especially close to the border of the defect. However, this neoformed bone was not sufficient to fill the entire defect (Fig. 1A). Twenty-four weeks after surgery, the defects filled with bioceramic discs were almost closed, whereas those filled with cancellous bone grafts still showed areas without bone (Fig. 1B).

Radiographic analysis

Immediately after surgery, the porous bioceramic disc showed a radiopaque circular image no radiolucent contour. The defect that was filled with a cancellous bone graft was slightly radiopaque (Fig. 2A). Four weeks after surgery, a thin radiolucent area was seen between the opaque ceramic disc border and the margin of the bone defect. A radiograph of the same area in the rat sacrificed after 2 weeks showed that the radiolucent area was larger than that seen after 4 weeks. With the cancellous bone graft, there was a continuous increase in the radiolucent areas up until 8 weeks after surgery, suggesting the occurrence of graft absorption. However, between 8

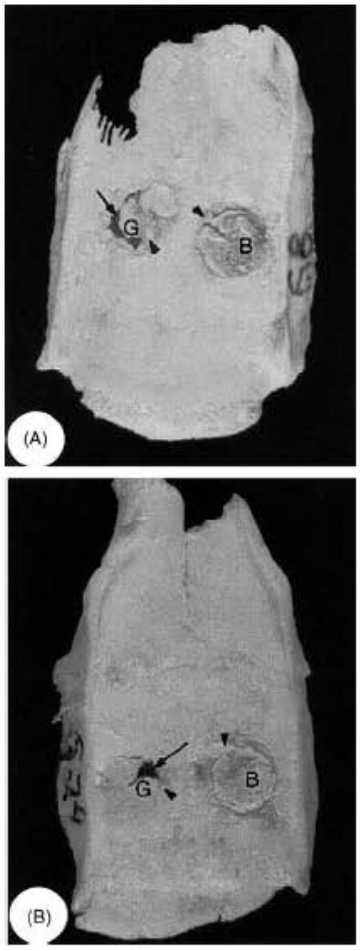

Fig. 1. Macroscopic aspect of defects filled with a bioceramic disc (B) or with a cancellous bone graft (G). (A) Eight weeks after surgery, 3×. (B) Twenty-four weeks after surgery, 3×. Note the neoformed bone tissue (arrowhead) bridging the ceramic disc and the bone graft at the border of the respective defects. Areas with no bone tissue (arrows) can be seen in the defect filled with the autogenous graft.

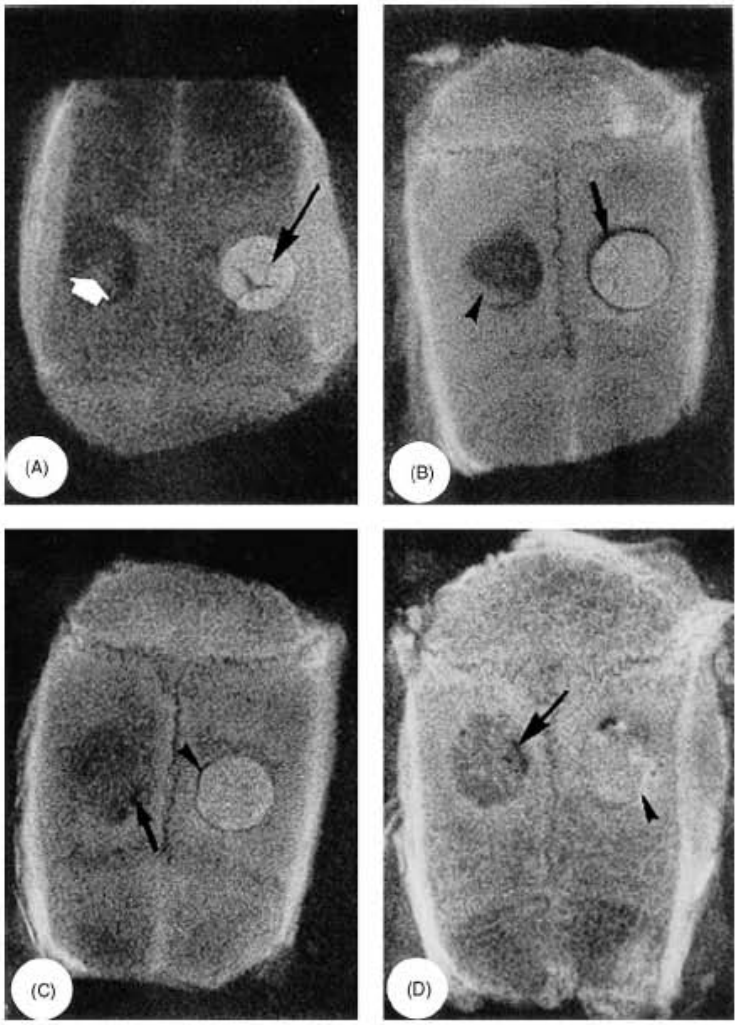

Fig. 2. Radiographic aspects of the defects of the calvarium. (A) Immediately after surgery, 3×. Note the radiopacity of the bioceramic disc (black arrow) and the cancellous bone graft (white arrow). (B) Four weeks after surgery, 3×. Note the radiolucent border (arrow) around the bioceramic disc and the radiopaque area indicating bone formation (arrowhead) in the defect on the left. (C) Eight weeks after surgery, 3×. Note the thinner radiolucent border (arrowhead) of the ceramic disc and the radiolucent area (arrow) of the defect on the left indicating the absence of bone. (D) Twenty-four weeks after surgery, 3×. Note the radiolucent area (arrowhead) in the defect filled with the autogenous graft and the edge of the bioceramic disc bridging the edge of the defect (arrowhead).

and 24 weeks, the cancellous bone graft showed increased radiopacity, indicative of new bone formation, although there we still some small radiolucent areas with no new bone growth.

The radiolucent area between the ceramic disc and bone became thinner after 8 and 24 weeks, indicating progressive osteointegration. Fig. 2D shows a small radiolucent area caused by remodeling at the margin of the bone defect 24 weeks after the ceramic implant.

Microscopic observations

Two weeks after surgery, there was cell infiltration into the space between the margin of the porous bioceramic disc and the border of the defect. Cells were seen between fragments of the ceramic disc, especially those located on the surface of the disc. A small amount of neoformed bone was present in the peripherial region of the ceramic disc (Figs 3A and 4A). Four weeks after surgery, there was more evidence of neoformed bone tissue, blood vessels and undifferentiated cells along the surface, mainly between the ceramic disc fragments. These findings showed that the formation of new bone and blood vessels

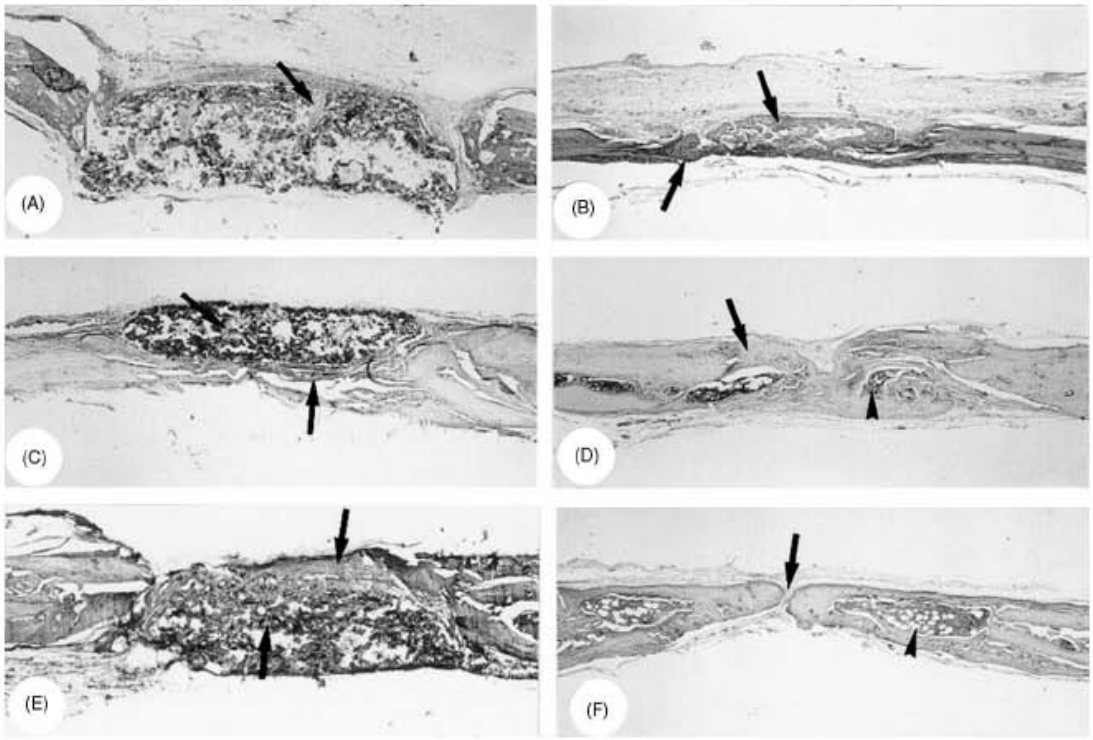

Fig. 3. Transverse sections of the right and left parietal bones. (A) Defect filled with hydroxyapatite 2 weeks after surgery, HE – 60×. Note the infiltration of cells (arrow) into the space between the fragments of the bioceramic disc. (B) Defect filled with cancellous bone graft 2 weeks after surgery, HE – 55×. Note the neoformed bone tissue (arrows) within and along the edge of the parietal bone defect. (C) Defect filled with hydroxyapatite 8 weeks after surgery, HE – 60×. Note the bone (arrows) in central region and along the surface of the bioceramic disc. (D) Defect filled with cancellous bone graft 8 weeks after surgery, HE – 60×. Note the neoformed bone (arrow) at the edge of the defect and the bone-marrow cells (arrowhead). (E) Defect filled with hydroxyapatite 24 weeks after surgery, HE – 60×. Note the bone (arrows) inside and along of the surface of the ceramic disc. (F) Defect filled with cancellous bone graft 24 weeks after surgery, HE – 60×. Note the bone with a mature aspect, the bone-marrow cells (arrowhead) and the area without bone (arrow).

occurred simultaneously with fragmentation of the ceramic disc. This fragmentation increased the initial porous structure (Fig. 4A and B). By the eighth week after surgery, disc fragmentation allowed the bone to grow from the border of the defect into the space around the disc fragments. Cells were also seen along the disc surface and around fragments located in the central region of the implant (Figs 3C and 4C). Finally, at 24 weeks after surgery, with the integration between the neoformed bone tissue and the fragments of the implant, the defects were almost completely closed, with few cells between the fragments of the ceramic disc (Figs 3E and 4D).

Bone tissue growth was seen in the second week after surgery in the defect filled with cancellous bone. Trabeculae of the grafted bone and some osteoclasts were seen within the defect (Fig. 3B). At 4 weeks after surgery, there was neoformed bone tissue in the central region and at the edge of the defect. The grafted bone did not maintain its original volume. By the eighth week, the neoformed bone at the border of the defect had increased. There was also new bone in the central area of the defect (Fig. 3D). By 24th week after surgery, it the neoformed bone had a mature appearance, with bone-marrow cells and fibrous tissue occurring in the area without neoformed bone (Fig. 3F).

Quantitative analysis

In the left parietal bone defect, the volume of neoformed bone tissue plus cancellous bone grafts was 45.5 ± 0.1% 2 weeks after surgery. However, this bone volume decreased from the fourth (27.9 ± 0.2%) through to the eighth (21.7 ± 0.2%) postoperative week. At 24 weeks, the amount of bone increased to 42.8 ± 0.2%.

Two weeks after surgery, the defects filled with ceramic showed a bone volume of 31.8 ± 0.1%. The amount of material deposited increased from 34.9 ± 0.1% in the eighth postoperative week to 46.4 ± 0.2% in the 24th week after surgery.

There was no significant difference in the volume of bone between the defects filled with bioceramic discs and those filled with autogenous bone grafts, except at 2 weeks after surgery, when the volume in defects filled with bone grafts (45.5 ± 0.1%) has significantly greater ($P = 0.0026$) than in defects filled with ceramic discs (31.8 ± 0.1%) (Fig. 5).

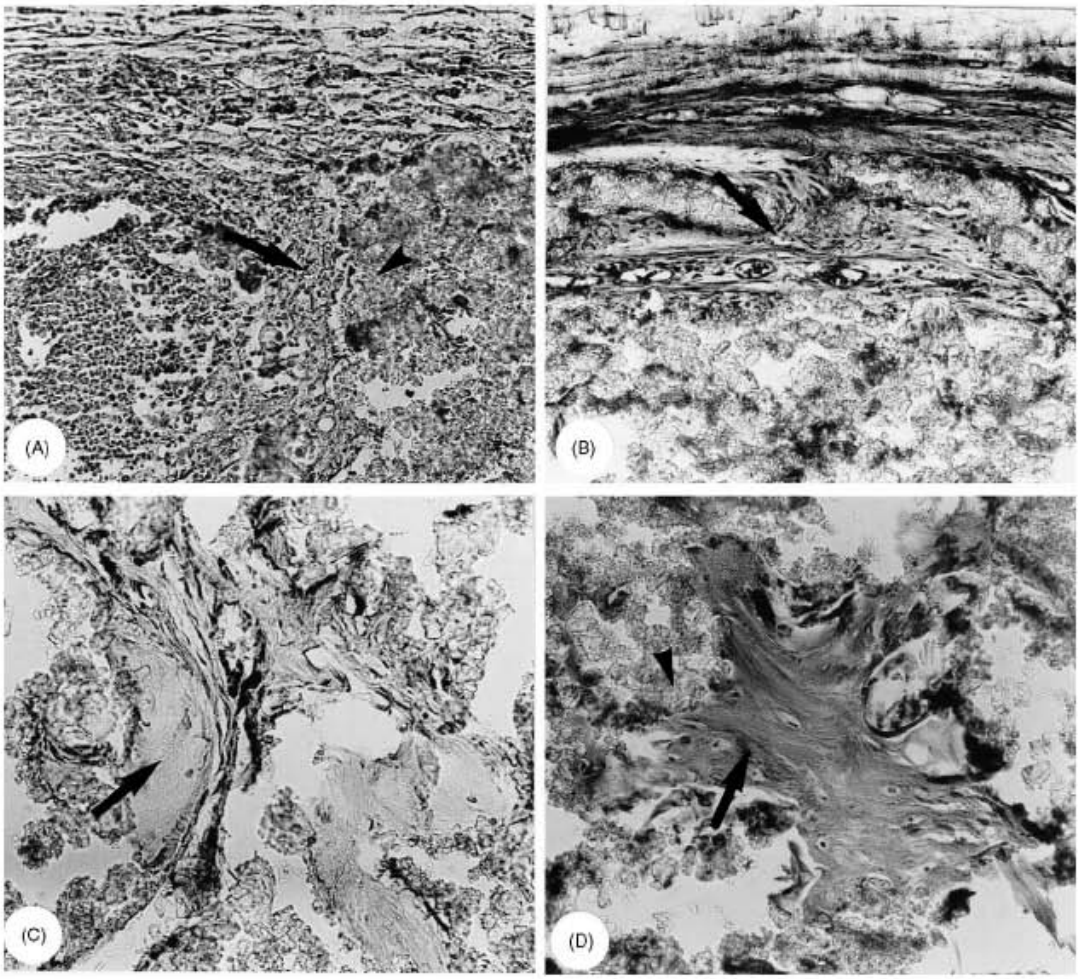

Fig. 4. Transverse sections of the right and left parietal bones. (A) Defect filled with hydroxyapatite 2 weeks after surgery, HE – 600×. Note the cells (arrow) between the fragments (arrowhead) of the bioceramic located on the surface of the disc. (B) Defect filled with hydroxyapatite 4 weeks after surgery, HE – 600×. Note the infiltration of cells (arrow) between the fragments of the ceramic on the surface of the disc. (C) Defect filled with hydroxyapatite 8 weeks after surgery, HE – 800×. Note the bone (arrow) in the central region of the ceramic disc. (D) Defect filled with hydroxyapatite 24 weeks after surgery, HE – 800×. Note the bone (arrow) between the fragments (arrowhead) of the bioceramic disc.

Discussion

In experimental studies of the regeneration and reconstruction of cranial bone defects, determination of an ideal defect size based on the biological model chosen is of great importance since spontaneous healing of the lesion can, compromise the results. Defects measuring 2 mm in diameter in the parietal bone of 12-week-old Wistar rats and 4 mm in 28-day-old Wistar rats have been shown to persist for up to six months after surgery[6,16]. In the present study, we produced a 5-mm defect to avoid spontaneous closure of the parietal bone, thus guaranteeing the reliability of our conclusions regarding the efficiency of autogenous grafts and porous bioceramic discs in bone defect repair.

The defects treated with cancellous bone grafts showed a bone volume of 45.5 ± 0.1% at 2 weeks after surgery, but this volume decreased to 21.7 ± 0.2% by the eighth week. Since both grafted bone and neoformed bone tissue were considered in the quantitative analysis, this decrease reflected the reabsorption of the grafted bone, which did not maintain its original volume. Other authors have also observed that cancellous bone grafts failed to maintain their volume, whereas compact bone grafts maintain their initial volumes for a long time[2,8,23]. This difference probably reflects the micro-architecture of cancellous bone grafts whose delicate trabeculae are reabsorbed faster[8,23,25]. On the other hand, cancellous bone grafts, in addition to releasing growth factors *in situ*, have osteogenic cells derived from the endosteum and bone marrow, that can contribute to the reconstruction of bone defects[22].

The micro-architecture of biomaterials also interferes with the tissue response at the site of implantation[14,21]. Studies on the porosity of hydroxyapatite

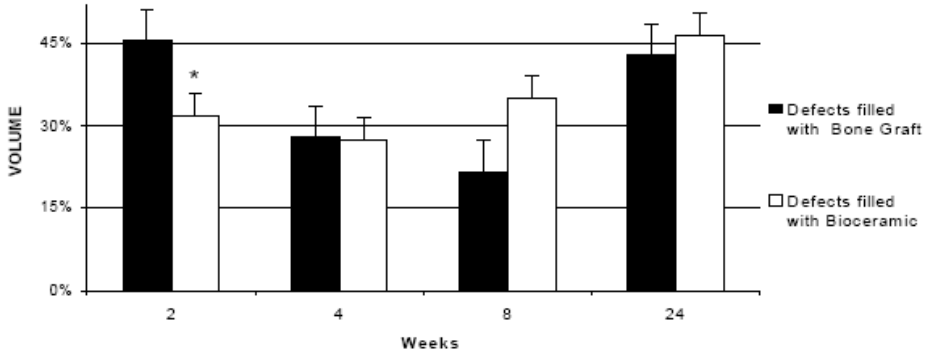

Fig. 5. Volume of neoformed bone in defects reconstructed with bioceramic disc and the volume of bone graft and neoformed bone in defects reconstructed with autogenous bone graft. The columns represent the mean ± 1SE ($n = 6$). *Statistical significance at 2 weeks: $P < 0.003$.

implants have revealed that dense implants can impair the ingrowth of bone tissue because of the physical barrier, which limits the proliferation of blood vessels essential for bone repair. Pores smaller than 50 µm are too small to allow the growth of calcified tissue, whereas implants with pores 50–300 µm in diameter show bone ingrowth[11,18,19]. Although the latter pores can increase the osteoconduction capacity of the implant, they also make the implant fragile by reducing its mechanical resistance[4,19].

The bioceramic discs used here had pores of 4–20 µm and were sufficiently resistance to allow handling during surgical procedures. Although the pores were too small to allow bone growth, fragmentation of the bioceramic disc created spaces that allowed bone growth into the defect. The fragmentation of the bioceramic disc reduced the mechanical resistance of the site of implantation, however, the newly grown bone between the disc fragments counterbalanced this effect by helping to maintain the resistance. These findings agreed with those of Orr et al.,[20] who observed a significant increased in the elasticity and compressive strength of defects treated with synthetic hydroxyapatite. These properties reflected the greater density of bone filling the interstices of the defects and bridging the particles.

The volume of bone tissue found in bone defects with bioceramic discs (46.4 ± 0.2%) and in the defects with cancellous bone graft (42.8 ± 0.2%) 24 weeks after surgery was similar. Nevertheless, because of the integration between the implant and the neoformed bone tissue, the defects filled with bioceramic were almost completely closed, while those filled with a cancellous bone graft showed several regions with no bone formation. Thus, our results indicated that bioceramic disc fragmentation enhanced bone growth and that osteointegration of the bioceramic fragments promoted the reconstruction of the parietal bone defects in the rat without the need to use an autogenous bone graft.

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