



Histologic and histomorphometric study of bone repair around short dental implants inserted in rabbit tibia, associated with tricalcium phosphate graft bone

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ABSTRACT. The use of short dental implants represents one way to overcome this limitation, in association with bone grafting procedures. Tricalcium phosphate-based grafts are among those widely used. The purpose of this study was to assess the biocompatibility of this biomaterial in the coverage of bone defects around short dental implants. Ten New Zealand rabbits were used in this study, each animal received 4 implants, two were placed in the right tibia region (control group) and two in the left tibia region (test group). Forty implants were used, with 4mm diameter and 6mm length. For the control group, holes 6 mm deep were made, and the implants were then inserted at the level of bone tissue. In the control group, the implants of 6 mm in length were inserted to its full length, while in the test group, the same implant was inserted up to 4 mm and left exposed 2 mm. These 2 mm exposed were coated with a bone substitute of tricalcium phosphate and a collagen membrane. After three months, the animals were prepared for histomorphometric analysis, which showed that the control group had a higher number of osteoblasts per μm^2 than the test group ($p < 0.001$). It was concluded that under these experimental conditions, tricalcium phosphate showed tissue biocompatibility and osteoconductive potential.

Keywords: dental implants, biocompatible materials, bone grafts.

Estudo histológico e histomorfométrico do reparo ósseo ao redor de implantes dentários curtos instalados em tíbias de coelhos associado ao uso do enxerto de fosfato tricálcio

RESUMO. Para a instalação de implantes em pacientes que possuem perdas ósseas, surgiram os implantes curtos e enxertos ósseos como uma opção de tratamento. Um enxerto que vem sendo utilizado é o biomaterial à base de fosfato tricálcio. O objetivo deste trabalho foi avaliar microscopicamente a neoformação óssea ao redor de implantes curtos. Para este estudo foram necessários dez coelhos da raça Nova Zelândia, sendo que cada animal recebeu quatro implantes, instalados dois na região da tíbia direita (controle) e dois na esquerda (teste). Foram utilizados 40 implantes com diâmetro de 4 mm e comprimento de 6 mm. No grupo controle, implantes de 6 mm foram inseridos no seu comprimento total, enquanto no grupo teste, o mesmo implante de 6 mm foi inserido apenas 4 mm, deixando expostos 2 mm. Esses 2 mm expostos foram cobertos com o enxerto de fosfato tricálcio e uma membrana de colágeno. Após três meses, os animais foram sacrificados para realizar os testes de resistência e a análise histomorfométrica. Na análise histomorfométrica, o grupo controle apresentou maior número de osteoblastos por μm^2 do que o grupo teste (p -valor $< 0,001$). Concluímos que enxertos à base de fosfato tricálcio possuem biocompatibilidade tecidual e potencial osteocondutivo na neoformação óssea ao redor dos implantes.

Palavras-chave: implantes dentários, materiais biocompatíveis, enxerto ósseo.

Introduction

Osseointegrated dental implants are an effective alternative in the rehabilitation of partially or completely edentulous patients and this technique provide an improvement in the patients' quality of life in terms of comfort, function and esthetics (STELLINGSMA et al., 2005). One of the

primordial factors in their placement is bone anchorage for their functional stability (MITRI et al., 2005). However implants placement can be limited due to situations of either reduced bone height or presence of anatomical structures in the extensive maxillary sinus pneumatization and mandibular canal in proximity to tooth sockets (ARLIN, 2006; CHIZOLINI et al., 2011; RENOARD; NISAND, 2006).

With the purpose of allowing implant placement in these cases, and simultaneously simplifying treatment, short dental implants are available for use as an option in treatment planning (ARLIN, 2006; CHIZOLINI et al., 2011; RENOUEAU; NISAND, 2006). The use of short dental implants has long been associated with low survival rates (ANITUA; ORIVE, 2010; BERNARD et al., 2003; TELLEMAN et al., 2011), and their use has also been discouraged from a biomechanical point of view, when combined with poor bone quality and high occlusal loads. However, at present, by means of new studies that have been conducted, authors have shown high success rates with their placement (MENCHERO-CANTALEJO et al., 2009; YOUNG et al., 2011). This survival rate may be reduced in patients who smoke, have oral parafunctional habits, systemic alterations, or in cases in which there is poor bone quality (CHIZOLINI et al., 2011; TELLEMAN et al., 2011).

Renouard and Nisand (2006) concluded that implant length has no significant influence on the success rate. Factors that really interfere with this rate are the following: primary stability, and implant surface in conjunction with the quality of the patient's bone. Primary implant stability can be achieved by performing the surgical technique adequately. Bernard et al. (2003) showed that short dental implants with treated surfaces have shown a high success rate. Because bone tissue quality is individual, some patients may require the use of bone substitutes. In addition, during implant placement, bone loss may occur around it. Many bone grafts, such as autogenous, allogeneic grafts or synthetic biomaterials may be used to restore this bone loss, and thus cover the exposed implant surface (BOIX et al., 2004; PIATTELLI et al., 1996).

Contemporary studies have pointed out that the use of synthetic bone grafts, such as tricalcium phosphate, have promoted an increase in bone regeneration around implants (MITRI et al., 2005; BOIX et al., 2004; PIATTELLI et al., 1996; SVANBORG et al., 2011; YANG, 2001). Piattelli et al. (1996) mentioned the use of tricalcium phosphate, and by means of histological studies, proved that the particles of this biomaterial provided bone repair around implants. This synthetic material is biocompatible and has osteoconductive activity (PIATTELLI et al., 1996; YANG, 2001). According to Schropp et al. (2003), the addition of these osteoconductive materials in the implant surface provides an increase in osseointegration.

Therefore, the purpose of this study was to assess the biocompatibility of the tricalcium phosphate

graft in covering bone defects around short dental implants, by evaluating the number of osteocytes per μm^2 existent in the test and control groups.

Material and methods

The methodology used in this study was approved by the Animal Research Ethics Committee of the State University of Maringá, protocol no. 020/2008.

In this study 40 Morse Taper WS (Neodent[®], Curitiba, Paraná State, Brazil) implants, 4 mm in diameter and 6 mm long were used. Tricalcium phosphate (Bone Ceramic[®], Institut Straumann AG, Basel, Switzerland) and absorbable Collagen type I biologic membrane (30 mm) (Consulmat[®], São Carlos, São Paulo State, Brazil) were evaluated for use as a bone substitute.

Ten female New Zealand albino rabbits, between six and eight months old, weighing 3.5 - 4.0 kg were used. Each animal received four implants, two being placed in the right tibia region and two in the left tibia region.

Animals were kept in cages, at room temperature, for eight days before performing the surgery, so that they could adapt to the environment, and remained under these conditions throughout the experimental period. They were fed a solid diet and water *ad libitum*.

Before surgery, all the animals were submitted to trichotomy on the internal face of the leg, the rabbits were weighed and calculations were made with reference to the anesthetic volume for each animal, in the proportion of 0.1 ml per each 200 g live weight of the general anesthetic mixture of Ketamine (Francotar[®]; Virbac do Brasil Industria e Comércio LTDA, Roseira, São Paulo State, Brazil) and aqueous solution of 2% Xylazine (Rompun[®] - Bayer HealthCare S.A, São Paulo, São Paulo State, Brazil) (MASSONE, 2008) in equal parts, given by deep intramuscular injection. Ten minutes before anesthetic induction, atropine was administered subcutaneously in a dose of 0.08 mg kg^{-1} , with the purpose of preventing possible bradycardia caused by the general anesthetics.

Animals were fasted for six hours before surgery, and feeding was resumed two hours afterwards.

The study consisted of two groups, divided as follows:

A - Placement of Neodent 4.0 x 6.0 mm implants without graft (control group – right tibia);

B - Placement of Neodent 4.0 x 6.0 mm implants associated with graft (test group – left tibia).

The period for implant osseointegration was three months. Experimental surgical procedures were

performed at the surgical center of the Animal Farm of the State University of Maringá. The operative technique and medication were based on a protocol similar to that described by Johansson et al. (1991), carefully performed according to the sequence described below.

After performing the incisions, holes were drilled in the bone using series of surgical burs, under constant irrigation with 0.9% sodium chloride physiological solution. A speed of 1000 rpm and torque of 36 Ncm were applied, according to the manufacturer's recommendations. A standardized minimum distance of 10 mm between implants was considered.

For the control group, holes 6 mm deep were made, which were measured with the use of positioners with millimetric markings, and the implants were then inserted at the level of bone tissue (Figure 1). In the test group, holes 4 mm deep were made and 6 mm implants were inserted. As 2 mm of the implant surfaces were exposed (Figure 2), these regions were covered with tricalcium phosphate graft and membrane (Figures 3 and 4). The mucoperiosteal flaps were sutured with non-absorbable Nylon Suture thread (Ethicon® 4.0- Johnson & Johnson, São Paulo, São Paulo State, Brazil).



Figure 1. Implant placement in the Control Group at the bone level.



Figure 2. Implant in the Test Group with exposure of 2 mm.



Figure 3. Graft placement (Bone Ceramic – Straumann®).

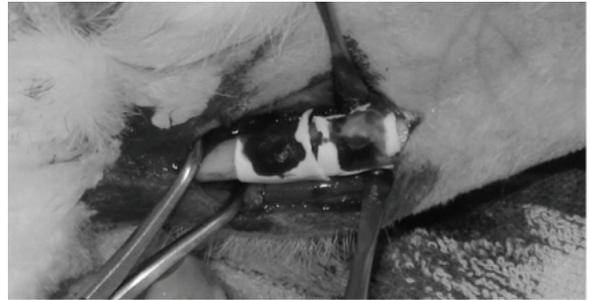


Figure 4. Resorbable membrane placement (Consulmat, Brazil).

Animals remained under observation during the anesthetic recovery period and then taken to their cages and kept confined until the time they were sacrificed. Immediate antibiotic (Baytril® - 5 mg kg⁻¹ – Bayer, São Paulo, São Paulo State, Brazil) was administered to prevent possible infection and analgesic (Dipyrone - 1 mL kg⁻¹) in the post-operative time to prevent any type of pain.

After three post-operative months animals were sacrificed by infiltration of a triple dose of the anesthetic mixture containing Ketamine and Rompun. After this, bone blocks containing the implants were removed. Osteotomies were performed with a safety margin of 5 mm distance from the implant, to avoid thermal trauma to the adjacent bone.

Bone segments were immediately desiccated, preserved in 10% formaldehyde and then taken for microscopic analyses.

Samples were subjected to histological study to examine the peri-implant bone tissue morphology. These bone blocks were immersed in 10% formaldehyde solution to be decalcified afterwards. At the end of this process, each bone fragment containing the implants was sectioned longitudinally to the implant, using a razor blade (Gillette®, Manaus, Amazonas State, Brazil).

By means of a longitudinal section made in the bone block, each implant was delicately removed

from its respective site, and the stages of laboratory processing were performed. Serial cuts 6 μm thick were made longitudinally to the implant bed and then stained with Carazzi Hematoxylin and Eosin-Lison, for later visualization under a common optical microscope and for histomorphometric analysis (SLAETS et al., 2009).

After preparing the slides, images were captured under an optical microscope at 40x magnification, coupled to a computer equipped with a high-resolution monitor, in which the images were processed and digitized. Histomorphometric analysis was performed using the program Image-Pro Plus 4.5 (Media Cybernetics, Inc., Silver Spring, USA), being counted the number of osteoblasts that surrounded the region of the implants corresponding to 2 mm of graft, for both the control and test groups.

To check possible differences between the ratio of osteocytes per μm^2 of the control and test group, a paired Student's T-test (0.001) was applied. To perform this procedure the T-test command of the Software R was used.

Results

Among the ten animals used in the study, none presented post-operative complications, and therefore, no losses occurred. The histological analysis evidenced a significant difference for the number of osteocytes between the control and test groups. Nevertheless, the test group presented osteocytes, indicating bone neoformation in the evaluated tissue.

In the 12 weeks of study, a very similar coefficient of variation was observed in the control (15.78%) and test (16.43%) groups (Table 1). The control and test group presented different behaviors with regard to the number of osteocytes per μm^2 , with the control group presenting a higher ratio of these cells (Figure 5).

When the paired Student's T-test was applied to the data obtained, the p-value (p-value > 0.001) found proved that the control group was statistically superior to the test group (Table 2).

Table 1. Description of the ratio of osteocytes per μm^2 for the Control and Test Groups.

Group	Mean	Standard Deviation	Coefficient of Variation	Minimum	Median	Maximum
Control	0.000102	0.0000162	15.78%	0.0000667	0.000101	0.000141
Test	0.00009880	0.0000162	16.43%	0.00005770	0.0000978	0.000142

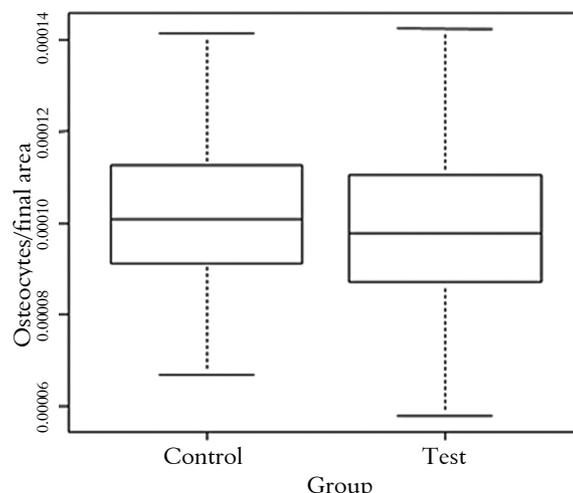


Figure 5. Box plot of the ratio of osteocytes per μm^2 for the Control and Test Groups.

Table 2. Results of the paired Student's-t test for the comparison of the means of the ratio of osteocytes per μm^2 in the Control and Test Groups.

t Statistics	Degrees of Freedom	p-Value
3.9029	511	<0.001

Discussion

The use of implants for the aim of rehabilitation has undergone various changes over time. Based on various questions that have arisen in this period, there was the need to do complementary experimental studies in an attempt to improve implant placement techniques and the quality of material used.

Cases in which there is limited bone height or anatomic structures are very close, short dental implants are indicated (ANITUA; ORIVE, 2010; PIATELLI et al., 1996; YOUNG et al., 2011). Short implants are available for use as an option in treatment planning (ARLIN, 2006; CHIZOLINI et al., 2011; RENOARD; NISAND, 2006). This survival rate may be reduced in patients who smoke, have oral parafunctional habits, systemic alterations, or in cases in which there is poor bone quality (CHIZOLINI et al., 2011; TELLEMAN et al., 2011).

With the development of treated surface and improvement of surgical technique, studies have shown that short dental implants present high success rates and are an excellent treatment alternative (ARLIN, 2006; RENOARD; NISAND, 2006; STELLINGSMA et al., 2005).

Other important allies in the planning and treatment of numerous clinical cases are bone substitutes. Because bone tissue quality is individual and some patients may require the use of bone substitutes during implant placement, bone loss may

occur around it. An example of these is tricalcium phosphate, which has bone regeneration capacity and is a biocompatible material (MITRI et al., 2005).

According to various authors (ARISAN et al., 2008; BOIX et al., 2004; KIM et al., 2011; MITRI et al., 2005; PIATELLI et al., 1996; STELLINGSMA et al., 2005; YANG 2001), tricalcium phosphate-based bone substitutes are capable of promoting new bone tissue formation around bone defects in implants. This bone formation occurs due to the presence of a local increase in calcium and phosphate ion concentrations, with subsequent hydroxyapatite crystal precipitation. As this graft has an osteoconductive potential, hydroxyapatite maintains a scaffold enabling cell migration and consequent bone tissue formation.

New Zealand albino rabbits were used in this study. This animal was chosen because it is easy to manipulate and maintain in captivity. Generally speaking, these are not aggressive animals and have a bone quality similar to that of human bone (Type II bone) (KESMAS et al., 2010). The tibia region was chosen for implant placement because it is a region to which there is easy and direct access, it presents sufficient tissue for covering the implants, and it is similar to the region of the mandible, since it has two corticals and one medullary bone. The duration of the experimental period was three months, once this period is sufficient to allow observing a high degree of bone maturation in rabbits (SVANBORG et al., 2011).

Regarding the histomorphometric study of this research, the results evidenced a statistically significant difference between the control and test group, with number of osteoblasts per μ^2 in the control group (Figures 6 and 7). These results corroborate those found by Mitri et al., (2005), who conducted a study with tricalcium phosphate around implants and found that two months was not sufficient to complete bone maturation around the graft. However, in the study of Piattelli et al. (1996) this same graft around implants showed complete bone maturation in the period of six months. Therefore, the lower number of cells found in the test group could be related to the experimental period of the present study, three months, which could have been insufficient for complete healing of the graft.

A recent study by Slaets et al. (2009) also evaluated bone healing around implants in rabbits for later histological study, and verified the presence of osteogenic cells in the process of new bone formation around implants. The present research is in agreement with the findings of Slaets et al. (2009) because both the control and the test groups

presented good bone healing around implants and presence of osteogenic cells. Thus, the results of this study demonstrated that the tricalcium phosphate-based graft showed tissue biocompatibility and osteoconductive properties. This biocompatibility is noticed by the absence of inflammatory reactions in the tissue, corroborating the study of Mitri et al. (2005) and Kim et al. (2011). The osteoconductive potential is demonstrated by the presence of osteocytes in the area corresponding to the graft (Figure 7).

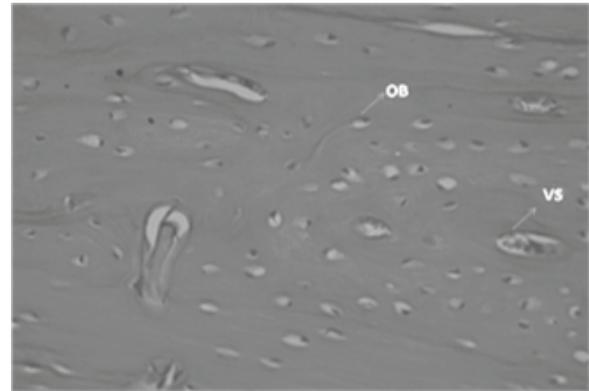


Figure 6. Control Group, HE 40 X. Bone/ implant interface region. Presence of osteoblasts (OB) and blood vessels (VS).

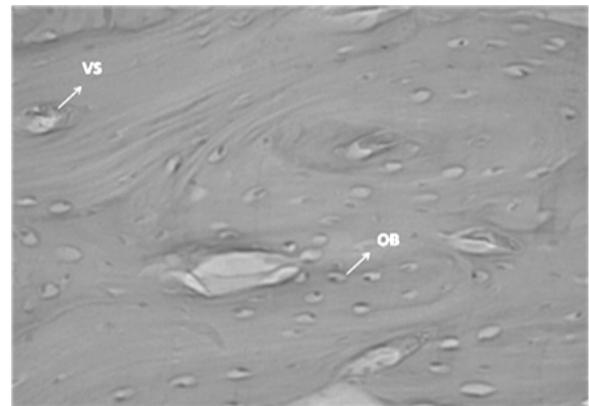


Figure 7. Test Group, HE 40 X. Bone/ implant interface region. Presence of osteoblasts (OB) and blood vessels (VS).

The use of membranes associated with a tricalcium phosphate graft around implants was also employed by Piattelli et al. (1996), Mitri et al. (2005) and Kesmas et al. (2010). These studies applied collagen membranes derived from bovine cartilage around implants and found that they maintained the graft position during the healing period, and therefore, prevented the interference of epithelial tissue (Figure 8). Membranes had dimensions 5 x 30 x 0.1 mm. The results of this research validate those of Piattelli et al. (1996) and Mitri et al., (2005), as the use of membrane guaranteed the formation of

bone tissue without the interference of epithelial tissue during healing.

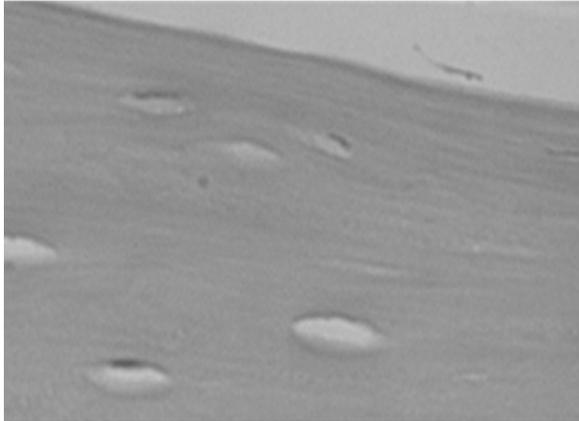


Figure 8. Test Group - Bone/implant interface region. Absence of conjunctive tissue in healing.

Another topic to discuss in the present study is that in the test group (graft) a higher coefficient of variation (16.43% - Table 1) was observed; that is, the values found in the graft group showed a greater variability. This fact may be associated with the processes of healing that occur in different animals, since each individual has a unique response to the healing process (FRAME, 1980). The group associated with the tricalcium phosphate graft became more susceptible to the actions of the healing process than the control group, because, according to Yang (2001), all biomaterial used requires the action of the blood stream and cellular activity, and these actions are individual to each organism.

Conclusion

From the results of this study, it could be inferred that the tricalcium phosphate-based graft is an important auxiliary in the planning and treatment of clinical cases that require the use of bone substitutes around implants. However, it will be necessary to conduct further studies to confirm this fact. Within the experimental conditions used in the study, it was possible to conclude that tricalcium phosphate-based bone grafts present tissue biocompatibility and osteoconductive potential.

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