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Optical density evaluation around the zirconia granules used as graft in rat calvaria

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ABSTRACT. The aim of this study was to evaluate the optical density of the zirconia granules used as a graft in a rat calvarium model. Forty animals (Rattus Novergicus, albinus, Wistar) were divided into control and experimental groups and submitted to bone detects in the cranial cap using a trephine type burr 6 mm in diameter. The control group had the defect filled with blood coagulum, and the experimental group was filled with zirconia granules of 300 to 850 μm. The animals were sacrificed at intervals of 7, 14, 30, and 60 days. Digital images were obtained from the bone defect region, and optical density of the zirconia granules was measured using the Image Tool® 3.0. software (UTHSCSA, San Antonio, EUA). The histological analysis of the specimens was also evaluated. The values obtained were analyzed by statistical methods: Analysis of Variance, Welch's Anova, and F Test. It was verified that the mean optical density of the bone repair process differs in all analyzed regions, irrespective of time ($p < 0.0001$). In the region with the zirconia graft, the mean density was always higher than the other regions analyzed, and the control group showed the same behavior of the experimental groups, but for the region with zirconia graft, the average density is always greater.

Keywords: digital radiography, bone density, zirconia, biomaterials, histology.

Avaliação da densidade óptica ao redor dos grânulos de zircônia usados como enxerto na calvária de ratos

RESUMO. O objetivo deste estudo foi avaliar, comparativamente, a densidade óptica no processo de reparação óssea utilizando enxertos com grânulos de zircônia em calvária de ratos. Foram utilizados 40 animais (*Rattus Novergicus*, albinus, Wistar), divididos em grupo controle e experimental, os quais foram submetidos a defeitos ósseos na calota craniana com broca do tipo trefina de 6 mm de diâmetro. Os animais do grupo controle tiveram o defeito preenchido com coágulo e os do grupo experimental com grânulos de zircônia de 300 a 850 μm. Os animais foram sacrificados nos períodos de 7, 14, 30 e 60 dias. Foram obtidas imagens digitais da região do defeito ósseo e a densidade óptica foi mensurada no programa Image Tool® 3.0 (UTHSCSA, San Antonio, EUA). A análise histológica das amostras também foi avaliada. Os valores obtidos foram analisados pelos métodos estatísticos Análise de Variância Welch´s Anova e Teste F. Verificou-se que a média da densidade óptica do processo de reparação óssea difere-se em todas as regiões analisadas, independente do tempo, ou seja, apresenta diferença estatisticamente significante ($p < 0,0001$), entretanto para a região com o enxerto de zircônia a média da densidade é sempre maior que as demais regiões analisadas.

Palavras-chave: radiografia digital, densidade óssea, zircônia, biomateriais, histologia.

Introduction

With the advent of digital radiology and cone beam computerized tomography, there has been remarkable advancement in the development of Oral Radiology and Imaginology, especially the increase in availability of diagnostic tools. These new methods revolutionized and changed the diagnostic protocols for a significant number of diseases (OZEN et al., 2009). The main advantages are reduction in the dose of exposure, elimination of chemical processing of radiographic films, and the possibility of manipulating the images. Correction of contrast, brightness, and verification of optical density are feasible alternatives for the application of specific items of software for images treatment (TEWARY et al., 2011).

Osseointegrated implant placement depends almost exclusively on the quantity of bone available. The term "available bone" may be described as volume and density. Bone density is a variable and determinant factor in the planning and placement of osseointegrated implants and may be estimated by evaluating tomographs and conventional and digital dental radiographs, such as periapical and panoramic types (TURKYILMAZ; MCGLUMPHY, 2008).

Various grafts types were proposed and used to restore bone losses that occurred after tooth extraction, or in angular defects caused by periodontal disease, with the purpose of regenerating tissues lost as a result of this disease (ALMEIDA et al., 2000). According to the literature, the use of bone substitutes may promote or increase new bone formation (Al RUHAIMI, 2001; BANSAL et al., 2009).

The digital radiographic image is important as it makes it possible to evaluate bone density for local alterations observation and the existent bone repair process (BERTI et al., 2005).

Recent studies have pointed to the use of zirconium in the areas of health, promoting functional reestablishment with prosthetic parts in hip and femur prosthesis, dental implants, and coping (DENRY; KELLY, 2008; HISBERGUES et al., 2009). Zirconium is a highly biocompatible and bioinert biomaterial as it induces weak encapsulation by conjunctive tissue when it is implanted. Another important aspect of zirconia is its osteoconductive capacity, which, in contact with bone, allows bone growth (HISBERGUES et al., 2009; MURUGAN; RAMAKHRISHNA, 2005).

In view of the above-mentioned aspects, it was pertinent to conduct a comparative experimental study on optical density around the zirconia granules used as graft and compare them with the capacities of coagulum for 7, 14, 30, and 60 days using a direct digital radiography system.

Material and methods

This experiment was approved by the Ethics Committee on the use of Animals in Experiments of Maringá State University, UEM, according to report no. 079/2010.

This study used 40 adult *Wistar* rats (*Rattus Novergicus*, *variety albinus*), 90 days old, with body weight of 250 and 300 g, from UEM central vivarium.

The animals were submitted to intramuscularly general anesthesia, consisting of a mixture in the ratio of 1:1 of 10% Ketamine Chlorhydrate (KetaminaAgener 10%, Agener União, União Química Farmacêutica Nacional S/A, Embu–Guaçu, São Paulo State, Brazil) for muscle relaxation; and 2% Xylazine Chlorhydrate (Rompun, Bayer-S/A, São Paulo, São Paulo State, Brazil), a sedative for animal usage, at the dosage of 0.1 mL for each 100 g of body weight. After anesthesia, each animal was submitted to manual trichotomy of the frontopariental region of the animal's head, and after this, a transverse incision was made in the skin and subcutaneous tissue in the region of cranial cap to expose fully the local bone cortical. Next, a bone defect 6 mm in diameter was produced between the sutures of the occipital and frontal bones at the junction with the parietal bones passing through the entire thickness of the díploe, using a trephine type bur NEODENT® (Curitiba, Paraná State, Brazil) mounted in a straight Kavo® handpiece (Joinville, Santa Catarina State, Brazil) coupled to a Bränemarck System-Nobel Biocare® (YorbaLinda-Califórnia State, USA) surgical electric motor at a speed of 1500 rpm with abundant irrigation with sterile physiological solution. The dura-mater was kept intact.

In the control group, the defect was filled with coagulum from the animal itself whereas in the experimental group (Figure 1 - Bone cavity filled with zirconia granules). The bone defect was filled with approximately 0.155 g of ground zirconium with a granule size of 300 to 850 μm Zirkonzahn® Prettau (Gais-Sudtirolia, Italy), previously sterilized in an autoclave.

Figure 1. Bone cavity filled with zirconia granules.

The animals were divided into eight experimental groups:

Group 1. Rats with defects in the calvarium filled with coagulum and sacrificed after 7 days (control group) (n = 5);

Group 2. Rats with defects in the calvarium filled with coagulum and sacrificed after 14 days (control group) (n = 5);

Group 3. Rats with defects in the calvarium filled with coagulum and sacrificed after 30 days (control group) (n = 5);

Group 4. Rats with defects in the calvarium filled with coagulum and sacrificed after 60 days (control group) (n = 5);

Group 5. Rats with defects in the calvarium filled with zirconia granules and sacrificed after 7 days (experimental group) (n = 5);

Group 6. Rats with defects in the calvarium filled with zirconia granules and sacrificed after 14 days (experimental group) (n = 5);

Group 7. Rats with defects in the calvarium filled with zirconia granules and sacrificed after 30 days (Experimental Group) (n=5);

Group 8. Rats with defects in the calvarium filled with zirconia granules and sacrificed after 60 days (Experimental Group) (n = 5).

After filling the cavities, the flap was sutured with continuous stitches using Mononylon 4-0 Ethicon® (Somerville, New Jersey, USA) and the region received a topical alcoholic solution application containing polyvinyl pyrrolidone-iodine, as a local antiseptic measure.

The animals were observed in the initial period of anesthetic recovery and after they were kept in individual cages under appropriate environmental conditions, 12/12 hour light/dark cycle and temperature of 68ºF, with rations and water *ad libitum*.

The animals were sacrificed by means of infiltration of a triple anesthetic dose after the experimental time intervals of 7, 14, 30, and 60 days.

The calvarium of each animal was removed, fixed in 4% paraformoldehyde for 48h, and then sent for direct digital radiographic exam.

The specimens were radiographed in a Kodak X–ray appliance, manufactured by Eastman Kodak Company, Rochester, Nova York State, USA, model 2200 Dental X–Ray System, operating at 70 kVp, 7 mA, and exposure time of 0.010 s. To obtain digital radiographs, the Kodak Dental Systems (RVG 6100) digital radiographic system was used. A size 2 sensor with an external dimension of 45 x 32 mm and active area measuring 36 x 27 mm was used, which had an electrical and optical system with three juxtaposed plates, crystal scintillator, fiber optic, and CMOS (complementary metal–oxide–semiconductor), producing an electrical signal that generated an image with real resolution of 20 pL mm^{-1} and real resolution of the image capturer of 27.03 pL mm^{-1}. The bony parts were placed in the central regions of this sensor and the cylinder of the radiographic at a perpendicular focus/sensor distance of 40 cm, so that between one exposure and the next, only the bony part was replaced, in an endeavor to standardize the radiographic techniques.

The optical density study was conducted by digital radiography means, and the bony parts were simultaneously placed parallel to an aluminum scale (stepwedge) fabricated of a specific and internationally standardized alloy (aluminum alloy 2026, ABNT, Brazil), constituted of eight degrees, with a thickness of 1 mm in each increment, used as a densitometric reference. The aluminum stepwedge was fixed to the radiographic sensor on the longer side (BARBOSA et al., 2013; PEKKAN; OZCAN, 2012) (Figure 2A). Five radiographic images were obtained for each group (control and experimental), totaling 40 images. The digital radiographic images were saved in a file in TIFF format.

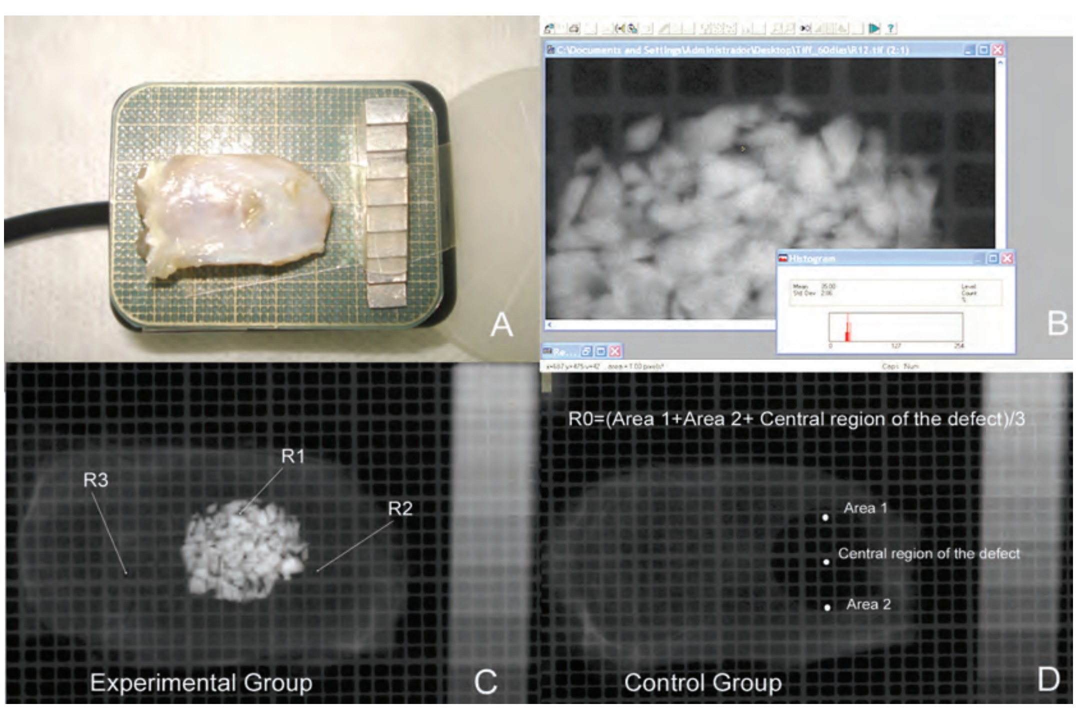

Figure 2. Determination of radiographic density using Image Tool® 3.0. software.

For image analysis and manipulation, Image Tool® version 3.0. (UTHSCSA, San Antonio, USA) software was used (Figure 2B, C and D). Among other resources, this software enables radiographic density to be determined (densitometric analysis); it is the conversion of radiopacity/radiolucency of a determined object into scale gray tones. After this, with the use of the histogram tool, areas of standardized sizes (01x01 pixels) were selected among the zirconia granules at a distance of 1 mm and 4 mm from the bone defect edge for the experimental group. In the control group, the same procedure used in the experimental group was followed but with variation of the analyzed region.

Two equidistant regions from the edge of the bone defect and center of it were selected, and afterwards the mean of these regions was calculated to obtain only one value of normal bone formed from the coagulum. To obtain more faithful values, the demarcations and optical density calculations of each region were completed three times, and a mean of the values obtained in the readouts was calculated.

The radiographic density value was converted into aluminum-equivalent millimeters (mmEq Al^{-1}), comparing the mean density of the selected region with the mean density of the aluminum stepwedge scale, thereby determining the aluminum–equivalence in millimeters of the selected region density. The values in mmEq Al^{-1} were submitted to statistical analysis for comparison of the regions analyzed in the different time intervals between the control and experimental groups.

After the radiographs, the specimens were fixed in 4% paraformaldehyde and decalcified in Morse's solution (50% formic acid and sodium 20% citrate) for 8 days. After decalcification, the specimens were routinely processed and embedded in paraffin. The sections were made of 7 μm and stained with hematoxylin and eosin (HE). The defect morphology was studied using a conventional optical microscope BX41 (Olympus®).

In the control group microscopic sections, the blood coagulum was gradually replaced with connective tissue. At 7 days, there was loose connective tissue, vascularized, rich in fibroblasts and a few macrophages (Figure 3A). In subsequent periods (14, 30, and 60 days), there was a gradual increase in the formation of dense connective tissue (Figure 3B). There was continuous bone growth at the margins of the defect (Figure 3A). The growth was complete in 60 days (Figure 3B).

In the experimental group, after 7 days, loose connective tissue formation was observed. The presence of a bone stump originated from the margin of the defect on both sides (Figure 4A). At 60 days, organized connective tissue around the granules appeared (Figure 4B).

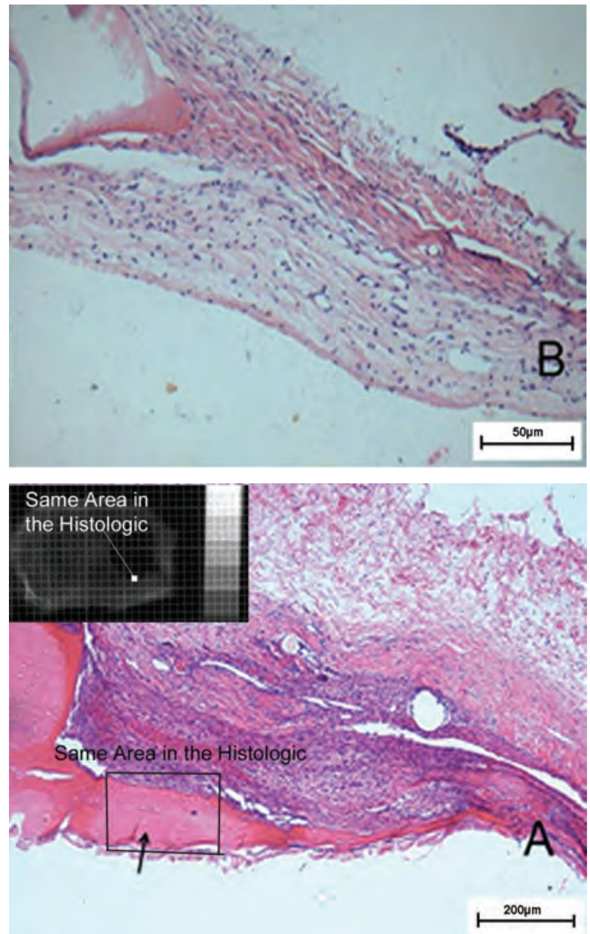

Figure 3. Defect in the skull filled with blood coagulum after 7 days (A) and 60 days (B). The arrow indicates the bone stump formed from the defect margins.

A gradual increase in granules involved in bone tissue was observed, as well as the amount of bone around the granules. During this period, other granules are surrounded by a thin capsule of dense connective tissue.

Results

The Image Tool® 3.0. software verified the mean differences in optical density of the bone repair process in mmEq Al^{-1} in the regions R0 (control), R1 (experimental region 1-among the granules), R2 (experimental region 2-distance of 1 mm from the edge of the bone defect), R3 (experimental region 3-distance of 4 mm from the edge of the bone defect) for 7, 14, 30, and 60 days using the following statistical methods: variance analysis, Welch's Anova, and F Test in the statistical software R. Application of this type of variance analysis revealed that the mean of the variable optical density of the bone repair process in mmEq Al^{-1} was not the same in all the groups; that is, it presented heterogeneity.

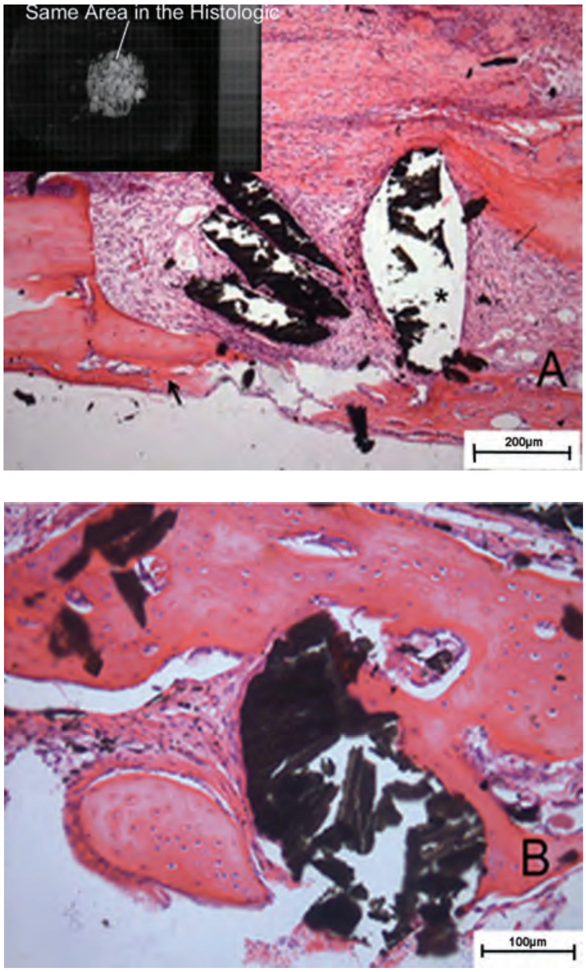

Figure 4. Defects in rat calvaria to 7 (A) and 60 (B) days after filling with zirconia. The figures indicate selected areas for optical density evaluation.

To evaluate the possible effects of the variables–region, time, and region X time-on the response, the analysis of variance was applied, considering the heterogeneity of variances. The analysis of variance indicated a significant effect of time (p–value < 0.0001) and absence of effect of region (p–value = 0.1125) and interaction between region and time (p-value = 0.5408), as may be seen in Table 1 (Analysis of variance for the variable mmEq Al^{-1} for the Image Tool® 3.0. software).

Table 1. Analysis of variance for the variable mmEq Al^{-1} for the Image Tool® 3.0. program.

Effect	Value of F Statistics	p-Value
Region	2.67	0.112
Time	70.69	<0.001
Region x Time	0.93	0.540

From the contrasts estimates (Table 2 - Contrasts for the variance mmEq Al^{-1} for the Image Tool® 3.0. software) it is observed that the mean optical density differs among all the regions, irrespective of time.

Table 2. Contrasts for the variance mmEq Al^{-1} for the Image Tool® 3.0. program.

Differences	Estimate	Standard-Error	Value of t Statistics	p-Value
(Control)–(Exp. Region 1)	-1.295	0.099	-13.09	<0.0001
(Control)–(Exp. Region 2)	-0.383	0.054	-7.11	<0.0001
(Control)–(Exp. Region 3)	-0.547	0.048	-11.40	<0.0001
(Exp. Region 1)-(Exp. Region 2)	0.912	0.099	9.19	<0.0001
(Exp. Region 1)-(Exp. Region 3)	0.748	0.096	7.77	<0.0001
(Exp. Region 2)-(Exp. Region 3)	-0.164	0.048	-3.38	0.0063

The graph (Figure 5 - Variable optical density of the bone repair process in mmEq Al^{-1} for the Image Tool® 3.0. software) for the variable optical density of the bone repair process in mmEq Al^{-1} for the Image Tool® 3.0. software clearly shows that the mean of the variable optical density differs among the regions, irrespective of time ($p < 0.0001$).

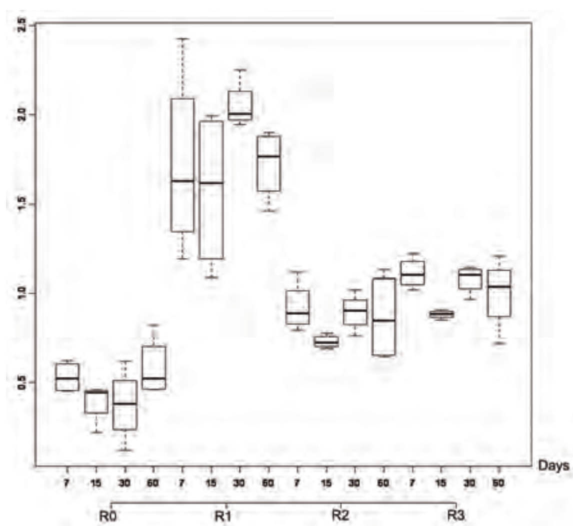

Figure 5. Graph variable optical density of the bone repair process in mmEq Al^{-1} for the Image Tool® 3.0. software

Discussion

Bone density evaluation using digital imaging is an important method in dentistry; it helps in the preparation of diagnosis of a local or systemic pathology, which is closer to the ideal, and in the identification of an existent bone repair process

(BERTI et al. 2005). This may be performed both with conventional digitized radiographs and by direct digital imaging (KOPARAL; AKDENICZ, 2001; MARTINS et al., 2005; SHROUT et al., 2003; SILVA et al., 2007).

To measure relative bone density, an aluminum stepwedge is normally used in research as a unit of reference for density measurement, which enables a correlation between the values of bone and metal. Density measured in pixels can be transformed into aluminum-equivalent millimeters (mmEq Al^{-1}), and thus become a known value (BERTI et al., 2005).

Berti et al. (2005), Koparal and Akdeniz (2001) and Shrout et al. (2003) used digitized ultra-speed films in an endeavor to determine bone density to correlate the bone density values obtained in pixels and in mmEq Al^{-1} by selecting areas of interest in bony regions and the steps of the aluminum stepwedge that showed similarity between the gray tones. Silva et al. (2007) used direct digital radiographic imaging, obtained by means of optical plate of Digora digital system, and as densitometric reference in radiographic techniques. They used the aluminum stepwedge, thus making it valid to use aluminum-equivalent millimeters as a known unit of reference in bone density studies (BERTI et al., 2005). Therefore, as in the above-mentioned studies, in the present research, the use of a direct digital system, Kodak Dental Systems RVG 6100, in conjunction with an aluminum scale was efficient for optical density evaluation of bone repair process resulting from grafts made with zirconia granules in bone defects in rat calvaria.

Bone density is a variable and determinant factor in the planning and placement of osseointegrated implants (TEWARY et al., 2011). The high success rate in Implant Dentistry is associated with the volume (quantity) and density (quality) of bone at the implantation site (ESPOSITO et al., 1998).

Grafts with the use of synthetic material have various benefits, such as availability, sterilization, abundance of donors, and reduced morbidity. This eliminates many of the deficiencies of autogenous grafts (SUR et al., 2011). Synthetic materials for these purposes may be metals, polymers, composites, ceramics, and glass. Zirconia is a frequently used synthetic material at present, particularly in the fields of medicine and dentistry, promoting functional re-establishment with prosthetic parts in hip and femur prostheses, dental implants, and copings (AL RUHAIMI, 2001; SUR et al., 2011) as it is a highly biocompatible and inert biomaterial, like ceramics (HISBERGUES et al., 2009).

There are various zirconia types, but the most frequently used type in dentistry is Yttria-stabilized polycrystalline tetragonal zirconia (3Y-TZP) (DENRY; KELLY, 2008). Many authors classified it as being a ceramic, but this material is a metal, with some special properties which in some aspects make it behave like a ceramic in the oral environment due its optimal biocompatibility, chemical properties, and translucency. Nevertheless, other factors hint at its metallic origin, such as its mechanical properties, high resistance to wear and fracture, and hardness and thermal expansion coefficient similar to steel (TORCASIO et al., 2008).

Another important aspect of zirconia is its osteoconductive capacity (HISBERGUES et al., 2009; MURUGAN; RAMAKRISHNA, 2005), defined as an agent that acts as a structure on which the host bone might grow, and in some cases, is progressively replaced by bone (MURUGAN; RAMAKRISHNA, 2005) that is, bone in contact with zirconia facilitates the induction of osteogenesis (KOPARAL; AKADENICZ, 2001).

Zirconia represents a new biomaterial in clinical dentistry, and though it is not yet routinely used, it may eventually represent a good option among biomaterials used in prosthetic rehabilitation in dentistry because of its properties. Generally speaking, it is used in the form of single parts, such as implants and copings, but there are no studies about its use as a bone substitute in granulated form or about its response. In addition to its advantages and biological properties, these were the factors that motivated this research to evaluate zirconia in granulated form as an aid in the bone repair process of a defect in a rat calvarium model by means of radiographic optical density to obtain a material that enables anatomic and functional recovery of a given region.

The physico-chemical characteristics of the zirconia granules produced in the present study were based on the reference to inorganic particulate bovine bone graft Bio-oss® (Osteohealth Co.), which presents a vast scientific literature as it is considered an efficient bone graft biomaterial (TADIC; EPPLE, 2004).

Therefore, when analyzing Figure 5, a bone repair pattern is shown in all the groups in the time interval of 14 days after the graft, with a drop in values occurring in mmEq Al^{-1} corresponding to the radiopacity values when compared with 7 days after the graft. And as the 30- and 60-day periods that follow, these radiopacity values increase. The reduction in optical density values 14 days after the graft may be related to tissue necrosis that precedes bone repair (ALMEIDA et al., 2000). The same bone repair pattern among the groups was an important datum because the tested material had similar characteristics to those of the control group (R0).

The result in Figure 5 showed the radiographic density of experimental region 1 (R1), in which the area analyzed comprised the region between the zirconia granules, which always presented greater radiographic density when compared with the control group, irrespective of time. The same occurred when comparing it with the other regions analyzed. This allows us to infer that the presence of the biomaterial allowed greater bone tissue formation compared to the control group, reaffirming the zirconia osteoconductive potential. However, when analyzing these values by applying the F Test, on average, they varied from 1.3 to 2 mmEq Al^{-1}, which corresponds to discrete radiopacity and may evidence low bone repair. The control group shows that a bone defect is not critical, requiring complete bone repair. When comparing the test group to the control, the test group compared areas of bone tissue formation. The higher optical density is in the test group. The tissue formed likely has more bone density.

In the literature, there are no reports in which granulated zirconia was used for grafts, but Sá et al. (2007) study on dog femurs using zirconia composite implants, hydroxyapatite (Z4H6 and Z6H4), as a bone substitute, observed a discrete radiopacity (50%) radiographically in the cortical region around the implant. In contrast, a radiolucent area also appears in the medullary region close to the implant. Absence of radiopacity around the implant may be attributed to invasion of the implant site by conjunctive tissue associated with the bone repair process (SÁ et al., 2007).

Comparing the result in Figure 5, the mean radiographic density was always lower in the control group than in the other regions, irrespective of time. Experimental regions 2 and 3 presented almost the same density. These observations are in agreement with the results of Almeida et al. (2000), who showed repair of bone defects produced in rat mandibles occurring from bone tissue at defect margins, with neoformation possible from remaining bone splinters. As the mean of the three analyzed regions was calculated in the control group, this may have influenced the result.

Development and delay in the repair process as well as radiographic evaluation are performed in a subjective manner, but optical density allows the professional to obtain numerical data for objective analysis of bone repair development, offering good conditions for radiographic interpretation (ESPOSITO et al., 1998). As there are few studies about this type of biomaterial, further research is necessary to gain a better understanding and to allow greater success in the methodology of the material's clinical applicability.

Based on the methodology and results obtained, the zirconia graft was shown to behave in a manner similar to blood coagulum graft, from a qualitative clinical aspect, with regard to the bone repair sequence. Nevertheless, the mean optical density values were always higher, and there were statistically significant differences between all the regions, irrespective of time.

After the radiographic examinations, a morphological evaluation was carried out by optical microscopy to observe the tissue response to zirconia in the rat calvaria.

The radiographic images (Figure 2) revealed that zirconia granules between 300 and 850 μm in diameter had irregular surfaces consisting of aggregated particles of varying sizes and separated by spaces.

Yamashita et al. (2009) demonstrated the effect of surface roughness on the initial connection of osteoblasts-like cells in mice of yttria-stabilized zirconia; the yttria produced good cell linkage and a greater number of cells bound to a surface with zirconia prepared roughly compared to a smooth surface.

In this research, zirconia showed excellent osteoconductive capacity, and the new bone was deposited directly on the granules. The zirconia is a bioinert material, so the fibrosis development around the granules was fairly unimportant, restricted to a thin connective capsule formation that was rich in fibroblasts for all time periods (Figure 4).

Conclusion

Based on the methodology and the results, from a qualitative clinical aspect and with regard to the bone repair sequence, zirconia graft behaves in a manner similar to blood coagulum graft. Nevertheless, the mean optical density values were always higher, and there were statistically significant differences between all the regions, irrespective of time.

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commercial or associate interest that represents a conflict of interest in connection with the submitted manuscript.

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