Analysis of indicators of osteogenesis, cytotoxicity and genotoxicity of an experimental β-TCP compared to other bone substitutes

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ABSTRACT. The aim of this study was to evaluate the indicators of osteogenesis, cytotoxicity and genotoxicity of an experimental beta tri-calcium phosphate (experimental β-TCP) compared with two other bone substitutes: bovine hydroxyapatite (HA) (Bio-Oss® - Geistlich) and beta tri-calcium phosphate (β-TCP - Bionnovation). The cell viability and genotoxicity were measured by MTT and MNT assay, respectively. The indicators of osteogenesis were analyzed by alkaline phosphatase activity, total protein content, and calcium deposition. The MTT and MNT assay showed that none of the tested materials was cytotoxic nor genotoxic. Concerning the indicators of osteogenesis, it was observed that cells in contact with all the materials were able to induce the osteogenesis and this process was influenced by the period of the cell culture in contact with bone substitutes. Based on the results of this study, it was concluded that this experimental β-TCP appears to be a promising material as a bone substitute.

Keywords: Beta tri-calcium phosphate, bovine hydroxyapatite.

Análise dos indicadores da osteogênese, citotoxicidade e genotoxicidade de um β-TCP experimental comparado a outros substitutos ósseos

RESUMO. O objetivo deste estudo foi avaliar os indicadores da osteogênese, citotoxicidade e genotoxicidade de um beta-tricálcio fosfato (β-TCP experimental) comparado com dois outros substitutos ósseos : Hidroxiapatita Bovina (HA) (Bio-Oss® - Geistlich) e beta-tricálcio fosfato (β-TCP - Bionnovation). A viabilidade celular e genotoxicidade foram mensuradas pelos ensaios MTT e MNT, respectivamente. Os indicadores da osteogênese foram analisados pela atividade de fosfatase alcalina (ALP), conteúdo de proteína total, e deposição de cálcio. Os ensaios MTT e MNT mostraram que nenhum dos materiais testados foi citotóxico ou genotóxico. Em relação aos indicadores da osteogênese, foi observado que as células em contato com todos os materiais foram capazes de induzir a osteogênese, e que esse processo foi influenciado pelo período da cultura celular em contato com os substitutos ósseos. Baseado nos resultados desse estudo, conclui-se que este β-TCP experimental parece ser um material promissor para ser utilizado como substituto ósseo.

Palavras-chave: Beta-tricálcio fosfato, Hidroxiapatita Bovina.

Introduction

Bone substitutes are used in order to fill, rebuild or replace bone defects (Carrel et al., 2014; Draenert, Huetzen, Neff, & Mueller, 2014). There is an intense search by the medical and dental fields for materials that can replace lost bone tissue with consequent anatomical and functional recovery of the tissue or organ. Autogenous bone, despite being considered the gold standard for use in grafts, has limited use because it causes damage at the donor site and presents qualities related to age and general health of the individual, besides being exhaustible (Carrel et al., 2014).

The calcium phosphate materials have received significant attention in the last years due to their mechanical and chemical similarity with bone tissue (Al-Sanabani, Madfa, & Al-Sanabani, 2013). They may present natural or synthetic origin. Concerning the natural-origin materials, there is the hydroxyapatite mineral matrix derived from bovine bone (Accorsi-Mendonça et al., 2008; Liu et al., 2013) in addition to those of synthetic origin, such as hydroxyapatite (HA), beta tri-calcium phosphate

(β-TCP), and biphasic calcium phosphate (HA + β-TCP) (Liu et al., 2013; Yang et al., 2014), which are commonly used as bone substitutes.

The bovine hydroxyapatite (Bio-Oss®) has been commonly used as it is considered a biomaterial that has a predictable behavior due to its physicochemical properties. Bio-Oss® has a porous nature that corresponds to 75 to 80% of its total volume, which greatly increases the surface area of the material and contributes to osteogenesis (Sollazzo et al., 2010) This bone substitute allows bone repair, since it is potentially osteoconductive and characterized for not activating the immune system (Bassi & Carvalho, 2011). It has been used to compare with other materials once many studies of this material have been performed with positive results (Chaves et al., 2012; Kim et al., 2010; Sollazzo et al., 2010).

The tri-calcium phosphate (TCP) exists in several phases (α, β, γ, and super α), where phases α and β are used as biomaterials (Al-Sanabani et al., 2013). Some studies have indicated that α-TCP particles present cytotoxicity (Santos et al., 2002) and instability (Lew, Othman, Ishikawa, & Yeoh, 2012). On the other hand, β-TCP has been considered biocompatible, absorbable and osteoconductive, leading to formation of bone tissue around and also inside pores (Al-Sanabani et al., 2013; Hirota et al., 2009). It has been used in a variety of surgical procedures with satisfactory clinical and histological findings in animals and humans (Al-Sanabani et al., 2013; Brkovic et al., 2008).

Some studies have shown that small changes in morphology, crystallinity, porosity, temperature, and pH of biomaterials during their manufacture can lead to different biological and mechanical behaviors of the bone substitutes (Aarvold et al., 2013; Cox, Jamshidi, Grover, & Mallick, 2014; Oliveira, Motisuke, Leal, & Beppu, 2008). Thus, the development of new bone substitutes with better physical, chemical and biological characteristics is an important requirement for evolution of medical and dental research on biomaterials. According to this purpose, the present study compared an experimental β-TCP with two other bone substitutes through the indicators of osteogenesis, cytotoxicity and genotoxicity of these materials in indirect contact with osteoblast-like cells.

Material and methods

This study was performed after approval by the Research Ethics Committee in humans of the Institute of Sciences and Technology, São Paulo State University (UNESP). Part of this work was associated with the Federal University of São Paulo (UNIFESP), where the experimental β-TCP was prepared and tested mechanically with satisfactory results (Oliveira et al. 2008).

The reaction for obtaining the β-TCP powder consisted of a mixture of calcium carbonate ($CaCO_3$ – Synth, Brazil) and monetite ($CaHPO_4$ – Synth, Brazil) at a molar ratio of 1:2 (Equation 1). The obtained powder was calcined at 1100°C for 6 hours in a muffle furnace (EDG 10P INOX Model 3000) and then milled in a ball mill (Model MA-500) for 48 hours, resulting in particle sizes ranging from 1 to 1.36 micrometers.

$$CaCO_3 + 2CaHPO_4 \rightarrow \beta\text{-}Ca_3(PO_4)_2 + CO_2 + H_2O \quad (1)$$
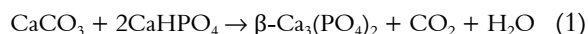
Commercially available biomaterials were used in this research. Bio-Oss® was obtained from Geistlich, with particle sizes ranging from 0.25 to 1 mm, while the β-TCP was obtained from Bionnovation, with particle sizes ranging from 0.1 to 0.5 mm. The β-TCP (Bionnovation) was mentioned in this article as β-TCP and the experimental material was called experimental β-TCP.

Procedures of cell culture

In this study, an established lineage of osteoblast-like cells (MG 63), obtained from Rio de Janeiro Cell Bank (Rio de Janeiro, RJ, Brazil), was used. The cells were cultured in DMEM (Cultilab Curitiba, Brazil) supplemented with 10% of fetal bovine serum (FBS), penicillin (100 U mL^{-1}), and streptomycin (100 mg mL^{-1}) and maintained at 37°C and 5% of CO_2. Cell culture flasks of 75 mL and 250 cm^2 were used. The culture medium was changed every two days and the cell growth was assessed by using a reverse phase microscope (Carl Zeiss Microscope Microlimaging GmbH - Axiovert 40C, Germany).

Preparation of conditioned medium

A pilot test was carried out to select which method of cell contact (direct or indirect) would be used, since both are found in the literature (Bernhardt, Lode, Peters, & Gelinsky, 2011; Doostmohammadi et al., 2011; Tavares, Castro, Soares, Alves, & Granjeiro, 2013; Vaziri, Vahabi, Torshabi, & Hematzadeh, 2012). The direct method provided higher rates of cell death compared to the indirect method, thus the latter one was chosen. In order to prepare the conditioned medium, the bone substitutes were put in contact with the medium. The proportion of the material/medium performed

in this study was 16 mg mL^{-1}, which was used in a previous study by Vaziri et al. (2012).

Determination of proliferation and cell viability

For the cytotoxicity test, 8×10^3 cells were plated in 96-well plates. It was used eight wells for each material and positive control (n=8). The cells were maintained in DMEM supplemented with 10% FBS, penicillin (100 U mL^{-1}) and streptomycin (100 mg mL^{-1}) at 37°C for 24 hours in a humidified atmosphere with 5 % CO$_2$. After this period, the old medium was removed and the cell cultures were exposed to the conditioned medium by bone substitutes and maintained in the incubator for 24 hours. Next, cell survival was determined by MTT assay (3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma, St Louis, Missouri, USA). The activity was quantified by dissolution of MTT in 0.1N NaOH (6.25 v v^{-1}%) in DMSO (dimethyl sulfoxide) and then the reading of optical density of the resulting solution was measured by spectrophotometer (Biotek - EL808IU) at 570 nm. The cytotoxicity was expressed as percentage relative to the control group (100%).

Genotoxicity test (MNT assay)

For this test, 3×10^5 cells were plated on glass slides arranged into 4-well plates. It was used four glass slides for each material and control groups (n=4). After 24 hours, the old medium was removed and the cells were exposed to the conditioned medium by bone substitutes and maintained in the incubator for 24 hours. To obtain a positive control, some glass slides were also exposed to ethyl methanesulfonate (EMS) at a concentration of 5 mM for 24 hours. After this period, the glass slides were washed, fixed, stained and micronuclei were observed and counted in an inverted light microscope (Carl Zeiss Microscope Micro lmaging, GmbH - Axiovert 40C, Germany) with immersion oil (100X). The counting was performed considering the amount of micronuclei *per* 1000 cells.

The micronuclei were identified as DNA structures clearly separated from the main nucleus, surrounded by nuclear membrane, and presenting less than 1/3 of the main nucleus. Only mononuclear cells containing less than 5 micronuclei were counted; mitotic cells and cells exhibiting nuclear fragmentation by apoptosis were not considered.

Indicators of osteogenesis

Total protein content

To assess the total protein content, the cells were plated in 24-well plates at a density of 20,000 cells well. Ten wells were used for each material and control group (n=10). After 24 hours, the old medium was removed and the cell cultures were exposed to conditioned medium by bone substitutes and kept in an incubator for periods of 7, 10 and 14 days. After these periods, the protein was extracted from each well according to the modified method of Lowry, Rosebrough, Farr and Randall (1951). Then, absorbance was measured by spectrophotometer (Shimadzu Europa GmbH UV 1203) at 680 nm and the total protein content was calculated from a standard curve determined by bovine albumin and expressed in µg mL^{-1}.

Alkaline phosphatase activity

The alkaline phosphatase activity was determined in the same lysates used to assess the total protein content, thereby, the periods of 7, 10 and 14 days were also evaluated in this test. The alkaline phosphatase activity was measured based on the release of thymolphthalein from the hydrolysis of thymolphthalein monophosphate substrate. A commercial kit was used (alkaline phosphatase Labtest 50-100), and the test was performed according to the manufacturer's instructions. The absorbance was measured by spectrophotometer (Shimadzu Europa GmbH UV 1203) at 590 nm and the obtained data were expressed as alkaline phosphatase activity normalized by total protein content. The values were presented in µmol of thymolphthalein h^{-1} mg^{-1} protein^{-1} mL^{-1}.

Qualitative and quantitative analysis of calcium deposition

For qualitative and quantitative analysis of calcium deposition the cells were placed in 24-well plates at a density of 20,000 cells/well. Ten wells were used for each material and control group (n = 10). After 24 hours, the old medium was removed and the cell cultures were exposed to the conditioned medium by bone substitutes and kept in an incubator for periods of 14 and 21 days. After these periods, the test was carried out according to Gregory, Gunn, Peister and Prockop (2004) and Rosa et al. (2009). For qualitative analysis, the calcium deposition was photographed with digital camera (Sony F828 Digital, Cyber Shot, 8.0 megapixels) coupled to an inverted light microscope (Carl Zeiss Microscope Micro lmaging GmbH - Axiovert 40C, Germany). For quantitative analysis, upon extraction of the dye, the absorbance was

measured by spectrophotometer (Biotek - EL808IU) at 405 nm.
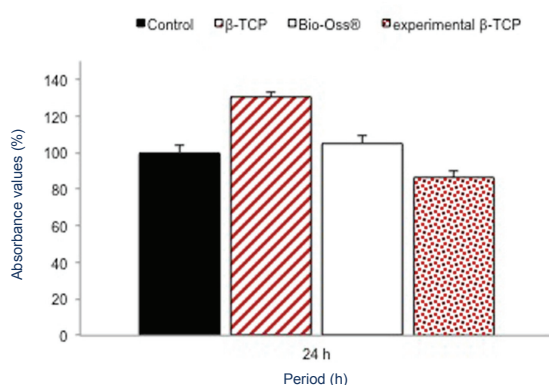
Statistical analysis

The data obtained were submitted to one-way analysis of variance (ANOVA), Tukey's multiple comparison (post hoc) and Z Test. The level of statistical significance was set at $p < 0.05$.

Results

Determination of proliferation and cell viability

Statistical analysis of the data obtained in the MTT test showed that none of the tested materials was cytotoxic. The bone substitutes tested in this study showed values of absorbance statistically different from the control group, also being statistically different between them. Both Bio-Oss® and β-TCP showed absorbance values higher than 100%, whereas experimental β-TCP had lower values (Figure 1).

Figure 1. Graph showing the mean absorbance values of the bone substitutes and control group (100 %) after 24 hours.

Genotoxicity test

Statistical analysis of the micronuclei numbers indicated that the bone substitutes tested in this study presented no genotoxicity. Only EMS (positive control) showed statistically significant differences from the negative control. The average number of micronuclei presented by the three bone substitutes in contact with osteoblast-like cells was similar to that found in the negative control and it was lower than the average of micronuclei presented by EMS (Figure 2).

Indicators of osteogenesis

Total protein content

Statistical analysis of the total protein content indicated that there were significant differences between the groups on the 7th and 14th day. In these periods, only β-TCP showed statistically significant

differences in relation to the other groups, with this material presenting the highest mean of total protein content in all periods (Figure 3 A). Concerning the period of culture, it was observed that only control and experimental β-TCP groups showed statistically significant differences between the periods, although both presented the highest values on the 10th day (Figure 3 B). In the experimental β-TCP group, differences were found between the periods of 10 and 14 days, whereas in the control group statistically significant differences were found between the periods of 7 and 10.
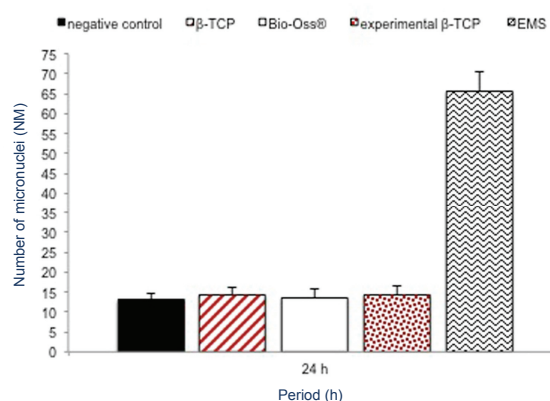
Figure 2. Graph showing the mean micronuclei number of the bone substitutes, negative control, and positive control (EMS) after 24 hours.

Alkaline phosphatase activity

Statistical analysis of the alkaline phosphatase activity showed that there were statistically significant differences between the groups on the 10th day. In this period, β-TCP showed statistically significant differences in relation to experimental β-TCP and control groups. It was observed higher alkaline phosphatase activity by the β-TCP in all periods (Figure 4 A). Assessment of the periods indicated that the three bone substitutes presented statistically significant differences between the periods, with the highest values of alkaline phosphatase activity on the 14th day (Figure 4B). The period of 14 days exhibited statistically significant differences in relation to the periods of 7 and 10 days in all bone substitutes.

Qualitative and quantitative analysis of calcium deposition

It was observed in the qualitative analyses of calcium deposition that experimental β-TCP, Bio-Oss® and control groups exhibited a large amount of calcium deposition on the 14th day (Figure 5A, B and D). On the 21st day, fewer amount of calcium deposition was visualized in these groups. The wells

containing β-TCP were excessively stained, which prevented an accurate visualization of calcium deposition in this group on the 14th (Figure 5C) and 21th days.
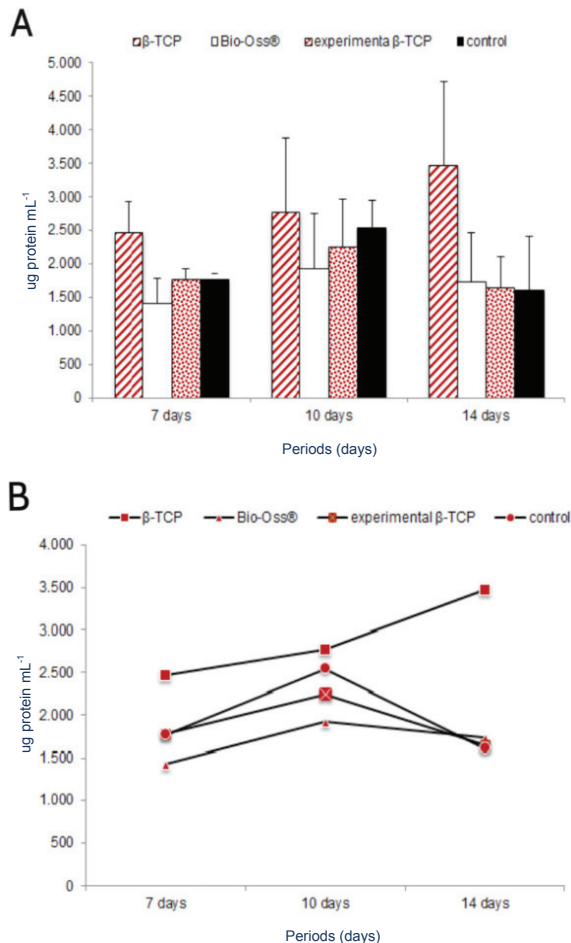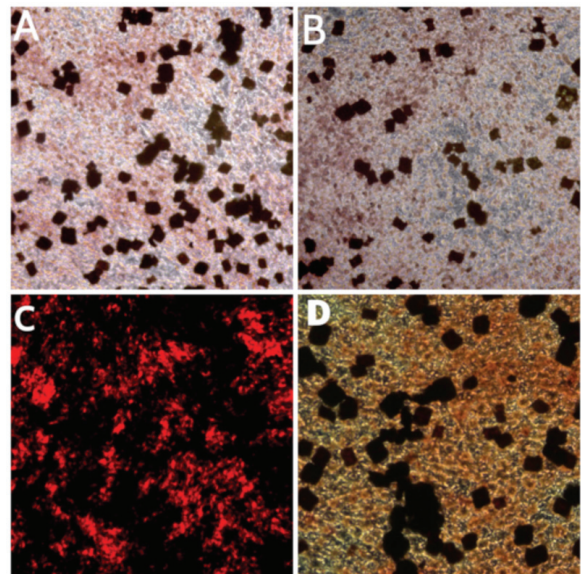

Figure 3. Figure exhibiting the content of total protein. (A) Graph showing the groups within each period. (B) Graph showing the periods within each group.

Quantitative analysis indicated that there were statistically significant differences between bone substitutes in both periods, with β-TCP differing from the other materials and presenting the highest average percentage (Figure 6A).

Both Bio-Oss® and β-TCP also presented statistically significant differences in relation to the control group (100%). Concerning the evaluation of the periods, it was observed that β-TCP and Bio-Oss® exhibited statistically significant differences between the periods of 14 and 21 days, whereas the highest average was observed on the 14th day in both materials (Figure 6B).
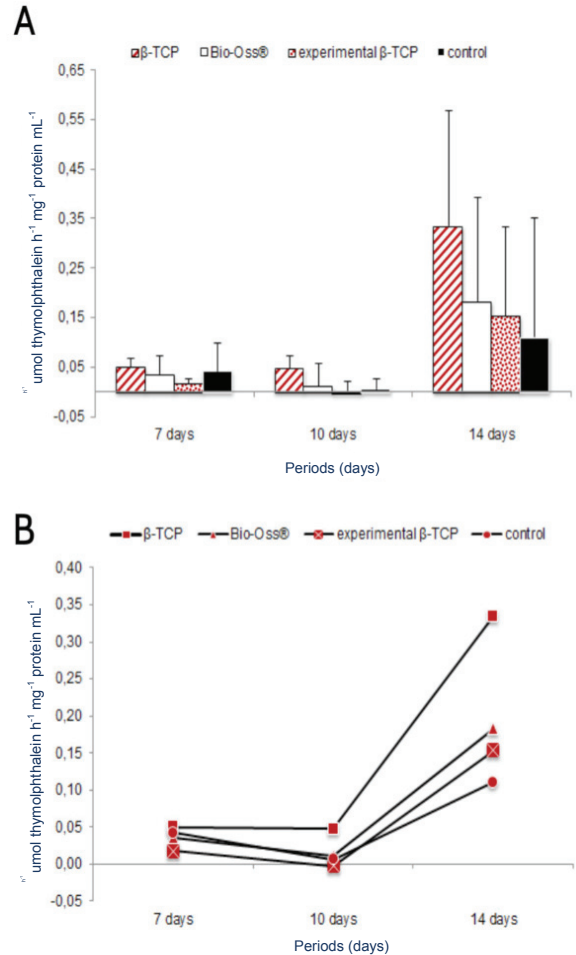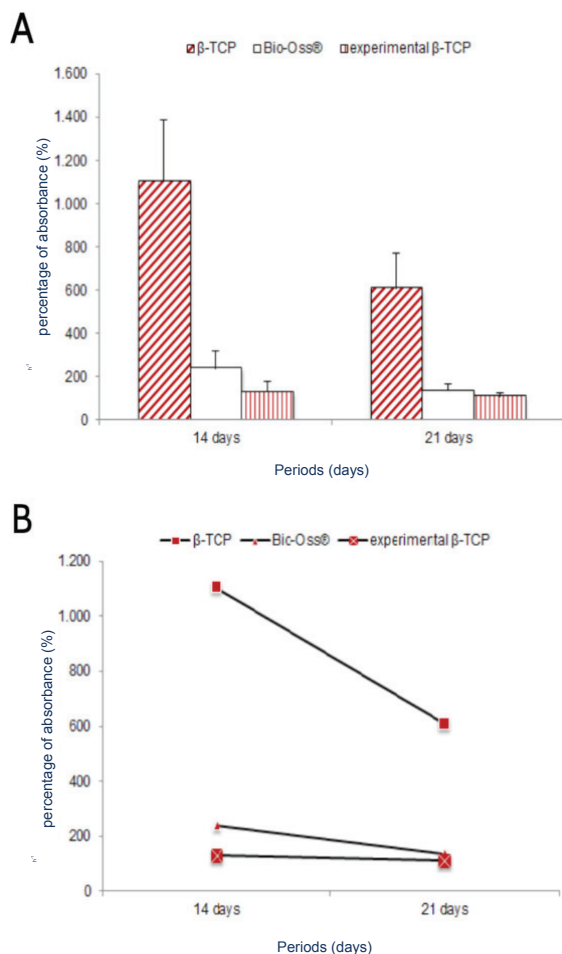

Figure 4. Figure exhibiting the activity of alkaline phosphatase. (A) Graph showing the groups within each period. (B) Graph showing the periods within each group.

Figure 5. Figure showing the calcium deposition after 14 days. Original magnification x25 (A) experimental β -TCP, (B) Bio-Oss®, (C) β -TCP, (D) control.

A

B

Figure 6. Figure exhibiting the percentage of absorbance obtained with the quantitative analysis of calcium deposition. (A) Graph showing the groups within each period. (B) Graph showing the periods within each group.

Discussion

This study has evaluated and compared *in vitro* an experimental β-TCP with two commercially available bone substitutes through indicators of osteogenesis, cytotoxicity and genotoxicity of osteoblast-like cells in indirect contact with these materials. The methodology used was based on previous studies that also examined the behavior of bone cells *in vitro* (Beloti, Martins, Xavier, & Rosa, 2008; Beloti & Rosa, 2005; Oliva et al., 2009; Quan et al., 2013; Rosa & Beloti, 2003, 2005; Simão et al., 2007).

The experimental material tested in this study corresponds to a β-TCP produced by solid-state reaction route, which showed positive mechanical results (Oliveira et al., 2008). This material has not been tested for its biocompatibility and indicators of bone formation yet. The other bone substitutes used in this study correspond to the Bio-Oss® and β-

TCP (Bionnovation). Many studies have evaluated the behavior of Bio-Oss® in which negative (Beloti et al., 2008; Bernhardt et al., 2011) and positive results were obtained *in vivo* and *in vitro* (Chaves et al., 2012; Kim et al., 2010; Liu et al., 2011; Sollazzo et al., 2010). In relation to the β-TCP (Bionnovation), no report in the literature was found. Nevertheless, in general, β-TCP has been widely studied and it has been considered a biocompatible, osteoconductive, and resorbable bone substitute (Al-Sanabani et al., 2013; Hirota et al., 2009; Shiratori et al., 2005). Tests of biocompatibility indicated that the experimental β-TCP, as well as both β-TCP and Bio-Oss®, exhibited neither cytotoxicity nor genotoxicity. Thus, these materials may be considered biocompatible in accordance with the conditions of this study.

The MTT test showed that β-TCP and Bio-Oss® induced cell proliferation by 130 and 105%, respectively, values that were higher than the control group (100%). The experimental β-TCP caused a decrease of viable cells (86%), but was not cytotoxic. Some authors found that Bio-Oss® was unable to induce proliferation of osteoblasts (Beloti et al., 2008; Bernhardt et al., 2011). One possible explanation for these results may be the direct contact between material and cells, which was the approach used by these authors. Tavares et al. (2013) used a concentration of 100 mg mL^{-1} of bone substitute/medium in the assay of proliferation and cell viability. These authors found that β-TCP did not differ from control group after 24 hours. In the present study, the concentration of 16 mg/mL was very positive for β-TCP, which showed the highest rates of proliferation and cell viability.

In relation to genotoxicity, only the EMS used as a positive control in this study was genotoxic. This result was already expected once EMS is a known genotoxic substance that causes genetic mutation and induces formation of micronuclei (Schweikl & Schmalz, 2000). As occurred in this study, Quan et al. (2013) also reported that pure hydroxyapatite does not induce genotoxicity in osteoblasts.

The indicators of osteogenesis were measured in this study by analyzing the total protein content, alkaline phosphatase activity, and qualitative and quantitative analysis of calcium deposition.

It is known that total protein content is associated with the capacity of synthesis of cells, being considered an important parameter to evaluate the osteogenesis *in vitro* (Beloti & Rosa, 2005; Rosa & Beloti, 2005). In this study, β-TCP was the material that induced the highest synthesis of total protein. Beloti et al. (2008) found an increase in the

total protein content in Bio-Oss® and control groups on the 14th. On the other hand, in this study the experimental β-TCP and control groups were the only ones influenced by the period, whereas the highest production of these materials occurred on the 10th day.

Another important indicator of osteogenesis is the production of enzyme alkaline phosphatase (Mödder & Khosla, 2008) which is associated to bone mineralization and is responsible for capturing phosphate ions during the process of osteogenesis (Delgado-Calle et al., 2011). Bernhardt et al. (2011) observed that β-TCP (Cerasorb M), in general, showed higher alkaline phosphatase activity than the synthetic hydroxyapatite. In this work, β-TCP also showed the highest alkaline phosphatase activity. Some authors observed an increase in the alkaline phosphatase activity over days (Bernhardt et al., 2011; Kübler, Neugebauer, Oh, Scheer, & Zöller, 2004), a finding similar to ours as the highest alkaline phosphatase activity was observed on the 14th day.

The process of mineralization of the matrix corresponds to the last event in the formation of bone tissue (Rosa et al., 2009) and studies using osteogenic cultures consider this process an important *in vitro* parameter that indicates the presence of cell differentiation (Hoemann, El-Gabalawy, & McKee, 2009). The qualitative analysis of calcium deposition revealed calcium deposits in Bio-Oss®, experimental β-TCP, and control groups. This deposition was higher in the period of 14 days and maintained a homogeneous pattern between the groups, including the control group. The excessively stained wells containing the β-TCP hampered the visualization of calcium in this group. Possible hypotheses may be the physicochemical characteristics of this bone substitute, which may have induced a large release of its components into the medium and favored an excessive calcium deposition as a result.

Quantitative analysis indicated that β-TCP induced the calcium deposition and that this deposition increased on the 14th day, mainly in the β-TCP and Bio-Oss® groups. The correlation between alkaline phosphatase activity and mineralized matrix production by osteoblasts has been observed (Beloti & Rosa, 2005; Rosa & Beloti, 2005; Sugawara, Suzuki, Koshikawa, Ando, & Iida, 2002). Thus, the high levels of alkaline phosphatase produced by materials on the 14th day may be related to the extensive calcium deposition in this same period.

Vaziri et al. (2012) observed that the formation of mineralized matrix by SaOS-2 cells in indirect contact with allogenic bone substitutes has increased at a concentration of 16 mg mL^{-1}. (bone substitute/medium), compared to 8 mg mL^{-1}. Therefore, the first concentration was positive for the cells, finding that is consistent with the one of this study, once we have used the same concentration with positive results.

The biocompatibility tests indicated that the experimental β-TCP as well as β-TCP and Bio-Oss® had neither cytotoxicity nor genotoxicity, thus being considered biocompatible *in vitro*. Moreover, all the materials induced osteogenesis *in vitro*, although β-TCP was found to be superior to Bio-Oss® and experimental β-TCP, according to the conditions of this study. The experimental β-TCP still needs to be tested *in vivo* regarding its biological properties before being used in clinical applications, since it is an experimental material.

Conclusion

The experimental β-TCP appears to be a promising material as a bone substitute once it was biocompatible and it has induced osteogenesis *in vitro*. Also, this experimental material may have lower cost compared to β-TCP (Bionnovation) and Bio-Oss®.

Acknowledgements

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