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A new approach in bone tissue regeneration: in vivo study of the impact of calcium aluminate cement scaffolds incorporated with mesenchymal cells

Uma nova abordagem na regeneração do tecido ósseo: estudo in vivo do impacto de *scaffolds* de cimento de aluminato de cálcio incorporado com células mesenquimais

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ABSTRACT

Objective: The objective of this study was to evaluate the bone regeneration potential of CAC-based scaffolds, with or without mesenchymal stem cells (MSC), in bone defects created in rat femurs. **Material and Methods:** Forty-eight CAC scaffolds and their blends of tricalcium phosphate (TCP), zinc oxide (ZNO), and zirconia (ZIRC) were produced, with half of these incorporated with MSC. Twenty-three Wistar rats were used, with 3 for MSC isolation and 20 for creating bone defects in both femurs. Five animals were assigned to each group, and during the defect surgery and material insertion, the animals received MSC-incorporated scaffolds on the left side and non-incorporated scaffolds on the right side, with the same type of material used in each animal to avoid different systemic effects (n=5); they were euthanized 21 days after the surgical procedure. **Results:** In the scanning electron microscopy analysis of the scaffolds, structures with open and interconnected pores, as well as cell adhesion, were observed in all groups. In the histological analysis, all groups showed newly formed bone trabeculae interspersed with bone marrow cells and connective tissue. **Conclusion:** In the histomorphometry, for the scaffolds not incorporated with MSC, the ZIRC group showed greater bone formation, and in the MSC-incorporated scaffolds, the TCP group demonstrated better results, both exhibiting a statistically significant difference from the other groups ($p<0.05$).

KEYWORDS

Biocompatible materials; Bone cements; Bone regeneration; Bone tissue; Mesenchymal stem cells.

RESUMO

Objetivo: O objetivo neste trabalho foi avaliar o potencial de regeneração óssea de *scaffolds* à base de CAC, incorporados ou não com células mesenquimais (MSC) em defeitos ósseos realizados em fêmures de ratos. **Material e Métodos:** Foram produzidos 48 *scaffolds* de CAC e suas blendas fosfato tricálcico (FOSF), óxido de zinco (ZNO) e zircônia (ZIRC), sendo que metade destes foram incorporados com MSC. Vinte e três ratos *Wistar* foram utilizados, sendo 03 para isolamento das MSC e 20 para confecção de defeitos ósseos em ambos os fêmures. Foram separados 5 animais para cada grupo, sendo que durante a cirurgia de defeito e inserção do marterial os animais receberam *scaffolds* incorporados com MSC do lado esquerdo e não incorporados do lado direito, em cada animais foi utilizado material de mesmo tipo para que não houvessem diferentes efeitos sitêmicos

(n=5); e foram eutanasiados 21 dias após o procedimento cirúrgico. **Resultados:** Na análise dos *scaffolds* por microscopia eletrônica de varredura foram verificadas estruturas com poros abertos e interconectados, além de adesão celular em todos os grupos. Na análise histológica, foi observado que todos os grupos apresentaram trabéculas ósseas neoformadas, entremeadas por células da medula óssea e tecido conjuntivo. **Conclusão:** Na histomorfometria, para os *scaffolds* não incorporados com MSC, observou-se que o grupo ZIRC apresentou maior neoformação óssea e nos *scaffolds* incorporados com MSC, o grupo FOSF demonstrou melhores resultados em comparação com os demais grupos incorporados com células mesenquimais, ambos exibindo diferença estatística para os demais grupos ($p<0,05$).

PALAVRAS-CHAVE

Materiais biocompatíveis; Cimentos ósseos; Regeneração óssea; Tecido ósseo; Células mesenquimais.

INTRODUCTION

Regenerative medicine aims to restore organs, tissues, or cells to recover compromised mechanical and biological functions due to trauma, tumors, infections, degenerative diseases, and aging [1,2]. Tissue bioengineering accelerates tissue regeneration and repair by developing new biomaterials to restore, enhance, or prevent tissue function deterioration [3,4]. Biomaterials, interacting with biological systems, can treat, enhance, or replace tissues, organs, or body functions [5].

With increased life expectancy, degenerative diseases and pathological conditions causing tissue loss, such as neoplasms and tumors, are growing, along with a higher probability of trauma and bone fractures. A major challenge of bioengineering is developing biomaterials to assist in bone tissue recovery [6]. In recent decades, new synthetic biomaterials have been developed to promote and accelerate bone regeneration without damaging healthy tissues or increasing contamination risks [7-11]. Biomaterials used as bone substitutes must be biocompatible and osteoconductive, allowing the migration of osteoprogenitor cells to the injured site and providing support for bone neoformation [5,12]. They can be presented in various forms such as powders, solid blocks, membranes, hydrogels, sponges, and scaffolds, with different origins and chemical compositions [13,14]. Three-dimensional porous scaffolds mimic the extracellular matrix environment, guiding cell migration, differentiation, and proliferation to form new tissue [15-18]. The size and quantity of pores of the scaffolds have an important influence on the progression of osteogenesis, since the greater quantity and size of pores result in greater bone growth [19-21]. The interconnection between these pores promote a key role in the migration and proliferation of blood vessels - a primary condition for tissue growth. In addition to supplying nutrients, vascularization will coordinate the activity of bone cells and their migration to the implantation site [22].

Calcium aluminate cement is an excellent material for filling bone defects, acting as a barrier against bacteria [23]. Scaffolds based on calcium aluminate cement (CAC) release calcium ions, favoring osteogenic differentiation and mineralization during bone regeneration [9,24]. Additionally, they form a layer similar to apatite or carbonated hydroxyapatite on their surface, improving osteointegration [25,26]. Other materials such as tricalcium phosphate (TCP), zinc oxide, and zirconia, when associated with CAC, show positive results in osteoblastic cell viability and the ability to induce mineralization and bioactivity [27-29]. Tricalcium phosphate presents itself as a biocompatible and bioactive bioceramic; the zinc oxide has bactericidal properties and the zirconia, besides biocompatibility, presents good resistance to corrosion, wear and compression [29,30].

Regenerative medicine and tissue engineering with stem cell therapy hold promise for bone regeneration [31,32]. They have great potential for bone regeneration because they exhibit a high capacity for regeneration, proliferation and cellular differentiation, playing na important role in the fields of medicine and dentistry [33,34]. Adult bone marrow-derived stem cells are multipotent and have the potential to repair damaged tissues [35-37].

The increased interest in cell therapy is due to the potential of mesenchymal cells to multiply, self-renew and differentiate, both in vitro and in

vivo, into cells of different lineages, presenting potential to improve repair or regeneration of damaged tissues [32,38]. Studies combine synthetic biomaterials with mesenchymal cells to enhance differentiation and bone growth in bone defects [7,8].

Preclinical trials have also demonstrated that the association of mesenchymal cells with biomaterials increases osteogenic capacity [7,39,40]. Within this context, the aim of this study was to verify whether the use of scaffolds based on calcium aluminate cement (CAC) and its blends (tricalcium phosphate, zirc oxide and zirconia), incorporated or not with mesenchymal cells can influence, favor or stimulate bone tissue regeneration, producing important information regarding the potential use of these biomaterials in cell therapy in tissue regeneration.

MATERIAL AND METHODS

Scaffolds samples

Forty-eight scaffolds were produced by foam replica method technique developed by Schwartzwalder and Somers (1963) [41], that consists in the impregnation of polyurethane foam with ceramic solution followed by thermal treatment to burn the organic part of the foam [28]. The scaffolds were produced from 3M Scotch Brite polymeric foams containing 49 pores per linear inch. These were impregnated with aqueous ceramic suspensions containing 60%-p solid content of calcium aluminate cement (CAC), followed by heat treatment at 1300°C. To form the blends, 4% by weight additives (tricalcium phosphate, zinc oxide or zirconia) were added to the calcium aluminate cement. All scaffolds were 4.0 mm in diameter and 4.0 mm in length. The pore size distribution and total porosity of the scaffolds were evaluated previously to this study, by mercury intrusion porosimetry - all of them showed porosity between 50-60% and pore distribution with peaks in diameters: 0.015; 30 (micropores) and mainly, 200 μm (macropores) [28]. These scaffolds were also previously analyzed for bioactivity and behavior in cell culture (cell adhesion, cell viability, protein production, differentiation into bone cells and mineralization nodule formation) presenting positive results [28]. To analyze the surface topography of the samples, a scanning electron microscope (SEM) (EVO/MA10) from the Central Multiuser Analytical Laboratory of the Research and Development Institute of Universidade do Vale do Paraíba (UNIVAP) was used. The samples were positioned on an aluminum platform (stub), aided by a double-sided carbon tape (3M, Sumaré SP, Brazil) and metallized with a thin gold layer by sputtering in the metallizing machine (EMITECH K550X, Sputter Coater, Qriorum Technologies), for 130s. This study was developed by our research group [28].

Ethics committee

This study was approved by the Research Ethics Committee, Brazil (CEUA, protocol 012/2019), of São José dos Campos Institute of Science and Technology - UNESP, and was conducted according to the ethical principles for animal experimentation, adopted by the Brazilian College of Animal Experimentation (CONCEA). This work also followed the guidelines recommended by ARRIVE (Animal Research Reporting of In Vivo Experiments) [38].

Isolation of mesenchymal cells

Mesenchymal cells were obtained from the bone marrow of the femurs of 3 Wistar male rats, at 3 months old, weighing about 350 g [42]. Initially, the animals were euthanized with an overdose of anesthetic using a combination of the drugs Xylazine hydrochloride (Anasedan® - Vetbrands, Jacareí - Brazil) and Ketamine hydrochloride (Dopalen® - Vetbrands, Jacarei - Brazil). Three times the recommended dose for the animal's weight was applied intramuscularly, and after confirmation of anesthesia, decapitation was performed. Subsequently, 6 femurs were removed and placed in a 50 mL falcon tube containing the transport solution composed of 95% filtered MEM alpha (minimum essential medium) and 5% gentamicin. After transport to laminar flow cabinet and cleaning of the femurs with 0.12% chlorhexidine, bone marrow cells were isolated and inserted into 250 mL and 75 cm2 cell culture flasks with alpha MEM culture medium (Gibco) supplemented with 10% Bovine Fetal Serum (SBF) and gentamicin (500 μg/mL) (Gibco). Next, the flasks were incubated in an incubator at 37°C temperature with atmospheric humidity containing 5% CO2. The culture medium was changed every three days and the progression of the culture was evaluated by inverted phase microscopy (Carl

Zeiss Microscope - Axiovert 40C, Germany). After confluence of the cells (seven days after isolation) they were enzymatically released and plated at a density of 2x10^4 cells in each well of the 96-well microplate (Kasvi) containing the scaffolds, which were previously sterilized under ultraviolet (UV) light for 15 minutes.

Surgical procedure

In the in vivo assays of this study, bone defects were made in the right and left femurs of 20 adult male rats (Rattus norvegicus, albinus, Wistar), with approximately 90 days old, weighing about 350g. Initially, the animals were weighed and anesthetized according to their weight by intramuscular injection of Xylazine hydrochloride (Anasedan® - Vetbrands, Jacareí - Brazil) and Ketamine hydrochloride (Dopalen® - Vetbrands, Jacarei - Brazil). Then in the medial region of the femurs trichotomy and antisepsis with iodized alcohol solution were performed. The incision was made with a no. 15 scalpel blade and the flap was detached to access the bone tissue. A bone defect was made with a 4.0 mm diameter spherical drill bit in both femurs, under abundant irrigation with 0.9% sodium chloride, in order to avoid heating due to the friction of the bur with the bone. Upon arrival at the local animal facility, the animals were randomly separated by the technician without any specific criteria, ensuring randomization. On the day of surgery, one animal from each cage was selected, completing the randomization process. The four groups were defined according to the bone defect filling material: CAC for the control group, CAC with a tricalcium phosphate blend (FOSF), CAC with a zirconia blend (ZIRC), and CAC with a zinc oxide blend (ZNO). The bone defect site of the right femur was filled with the scaffold without embedded cells, while in the left femur, the filling was with the scaffold embedded with mesenchymal cells. The scaffolds inserted in the right and left femurs of the rats were made of the same material, in order to avoid any systemic effect that the material might present. In all animals, the flap was repositioned and sutured with silk thread nº. 4 (Ethicon/Johnson & Johnson). It is important to emphasize that the surgeries, the intervention process, and the future histomorphometric evaluations were carried out by the same investigators.

For 5 days, analgesia was provided by Tramadol, which doesn't exhibit anti-inflammatory effect [43], at a dose of 8mg/kg, administered every 12 hours. The animals were put back in cages containing 05 animals, and have been monitored for 21 days. Then, were euthanized with an overdose of the combined solution of the drugs Xylazine hydrochloride (Anasedan® Vetbrands, Jacarei - Brazil) and Ketamine hydrochloride (Dopalen® - Vetbrands, Jacareí - Brazil) and decapitated. The femurs were removed and placed in 10% formalin for at least 48 hours, and later submitted to the histological processing for further histological and histomorphometric analyses.

Incorporarion of mesenchymal cells to scaffolds

After obtaining the mesenchymal cells and scaffolds, two scaffolds from each group were cultured with the cells for 5 days. After this period, the cells were fixed and the scaffolds were evaluated by micrographs obtained by SEM. For fixation, a dehydration protocol with increasing concentrations of alcohol was used: 10%, 25%, 50%, 75%, and 100%. The scaffolds with cells remained in each stage for 20 minutes. After these stages, the material was dried in an oven at 37°C for 16 hours.

HISTOLOGICAL AND HISTOMORPHOMETRIC ANALYSIS

After fixation with formaldehyde, the femurs with bone defects were CUT into smaller fragments and submitted to decalcificarion using the demineralization technique at the Bone Tissue Laboratory of ICT/Unesp, using 20% formic acid for approximately 90 days. Subsequently, the pices were trasversely sectioned in the center of the scaffold insertion region and the fragments were included in paraffin blocks using tissue processor (LEICA TO 1020, USA). The pieces were embedded in paraffin and submitted to the routine laboratory technique for the preparation of histological slides. Five slices were prepared for each bone fragment and stained with hematoxylin and eosin. In the histological analysis, aspects of the development of bone repair, formation of granulation tissue, new bone formation, the arrangement of bone trabeculae and bone maturation until final remodeling were observed. For the histomorphometric analysis, the histological sections were photographed with a Zeiss Axioskop 40 light microscope (Carl Zeiss Brasil), with a digital câmera coupled to Canon,

model Power Shot A640. Digital images (JPEG format) were obtained with 2x magnification in the region of the defect. These images were analyzed usign the Image J software (National Institutes of Health, Bethesda, MD), which makes it possible to quantify the newly formed bone in the scaffold insertion region. The average area of the regions corresponding to the newly formed bone repair tissue was calculated for each group.

Statistical analysis

As per previous study [44], the number of animals was estimated by a statistical calculation from simple group analysis considering the reliability estimate (Log ß), sample error estimate (Log p) and the margin of loss of animals during the experiment.

The formula used was:

$$\begin{aligned} &n = \log\beta \log p \times 1.2 \therefore = \log 0.05 \\ &\log 0.5 \times 1.2 = 5{,}18 \approx 5 \text{ animals} \end{aligned} \quad (1)$$

After the calculation, we obtained an estimated number of 5 animals per group. The data collected were initially submitted to the Shapiro-Wilk normality test. Once the normal distribution of data was confirmed, they were submitted to analysis of variance (ANOVA) for intergroup comparison and complemented by the Tukey test, when necessary, to verify the statistical differences between the means of the groups. For intragroup analysis (scaffold incorporated with cells and not incorporated with cells), the t-test was used to verify the differences. GraphPad Prism 9 statistical software (GraphPad Software, San Diego, CA, USA) was indispensable to perform the tests. For all statistical tests a 5% significance level was adopted.

RESULTS

Scaffolds characterization

To evaluate the surface topography of the CAC scaffold samples and their blends, a scanning electron microscope (SEM EVO/MA10) was used. The three-dimensional (3D) appearance characteristic of the scaffolds was observed and it was verified that all of them presented highly porous structures with open and defined pores.

These pores were interconnected with different sizes in the magnifications, as shown in Figure 1; showing that the scaffolds design is suitable for its use as a biomaterial substitute, for bone tissue - since it mimicked an extracellular matrix in 3D, enabling cell migration and multiplication inside this network of interconnected pores.

Evaluation of incorporarion of mesenchymal cells to scaffolds

Two scaffolds from each group were plated together with the cells for 5 days. After this period, the cells were fixed and the scaffolds were evaluated by micrographs obtained by SEM (Figure 2). It was noted cell adhesion in all scaffolds used in this study, irrespective of their composition.

Histological analysis

In all groups, the histological sections revealed neoformed bone tissue formed by trabeculae covered by osteoblasts, containing numerous osteocytes inside. These trabeculae were thin and widely spaced and sometimes thicker and more continuous, interspersed with bone marrow cells and sometimes fibrous connective tissue. The scaffolds were dissolved in the process of decalcification with formic acid and therefore, only residues of these could be visualized as areas of brownish pigmentation in the histological sections of all groups. There were no inflammatory processes or foreign body reaction in any group. Sometimes a bone bridge was observed between the ends of the pré-existing cortical bone, in the region of insertion of the scaffolds. It was found that this neoformation invaginated into the medullary region of the femur, occupying part of this region.

In the CAC group incorporated with mesenchymal cells, the newly formed bone tissue developed mainly in the lower region of the scaffold and proliferated towards the bone marrow of the femur (Figure 3). The bone trabeculae in this group were thicker compared to the CAC group without cells (Figure 4).

In the histological sections of the FOSF group without incorporation with mesenchymal cells, it was possible to observe that the newly formed bone tissue occurred mainly in the region underlying the anterior region occupied by the scaffolds (Figure 5). Large amounts of bone marrow cells

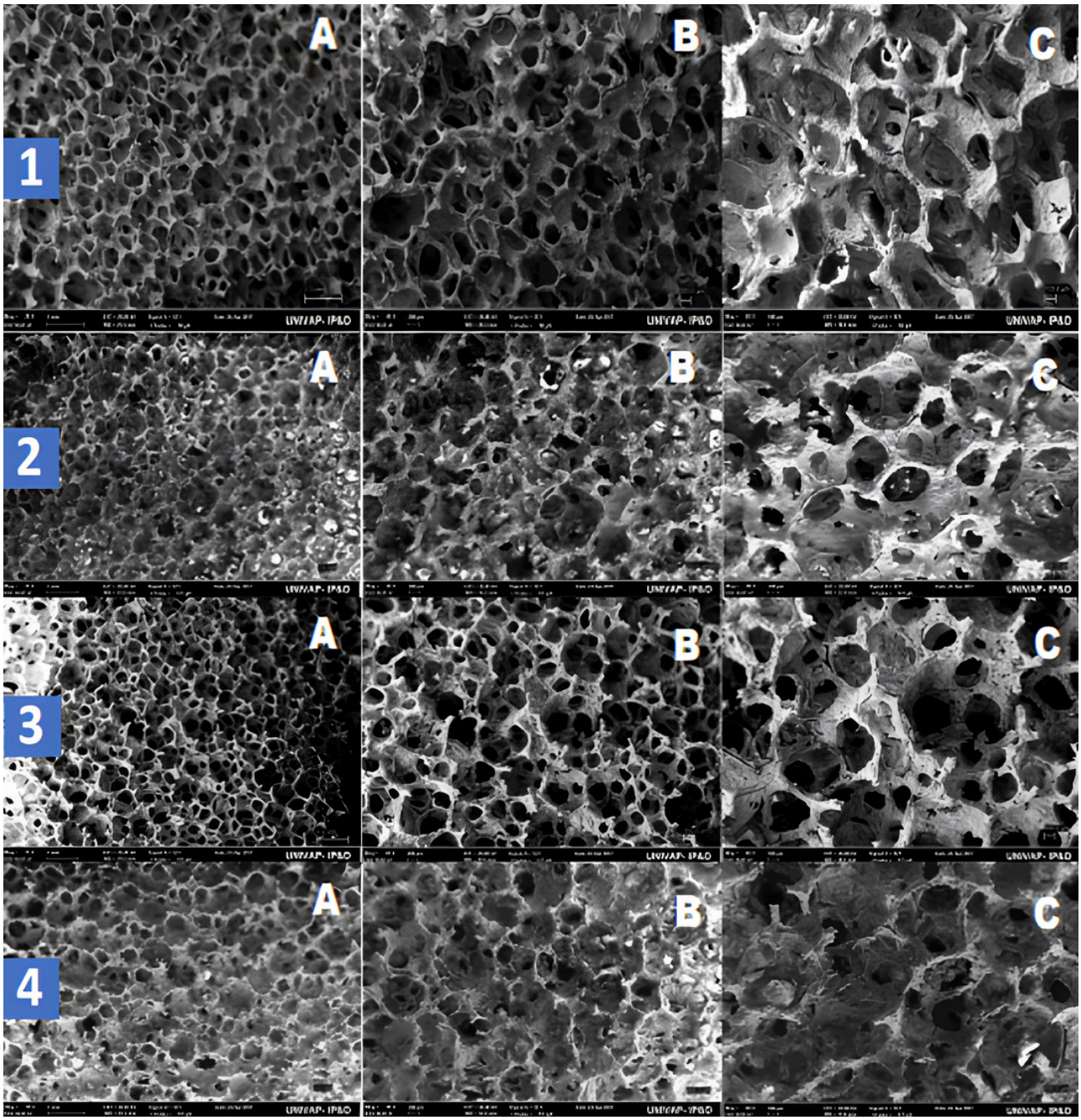

Figure 1 - Scanning electron micrographs of scaffolds prepared from sponge impregnation in aqueous suspensions of calcium aluminate cement and its blends. Legends: 1) Calcium aluminate cement and its blends containing 4%-w of: 2) FOSF group, 3) ZIRC group, 4) ZNO group. Magnificarions: A: 25x; B: 40x; C: 80x.

were visualized interspersing the bone trabeculae. When incorporated with mesenchymal cells, however, it was possible to observe new bone formation starting from the sides of the pré-existing cortical bone towards the Center of the scaffold insertion region (Figure 5). A bone bridge with tinner áreas between the córtices can be seen.

In the group with ZNO scaffolds incorporated and not incorporated with mesenchymal cells, the presence of thick bone trabecular was observed in the region of the scaffolds, without bone bridge formation (Figure 6). Figure 7 shows the ZNO group incorporated with mesenchymal cells.

In the ZIRC group without incorporation with the mesenchymal cells, it was observed that the newly formed bone tissue occurred from the sides of the pré-existing bone córtices towards the center of the scaffold insertion region, with formation of a bone brisge between the pré-existing bone cortices. In the ZIRC group

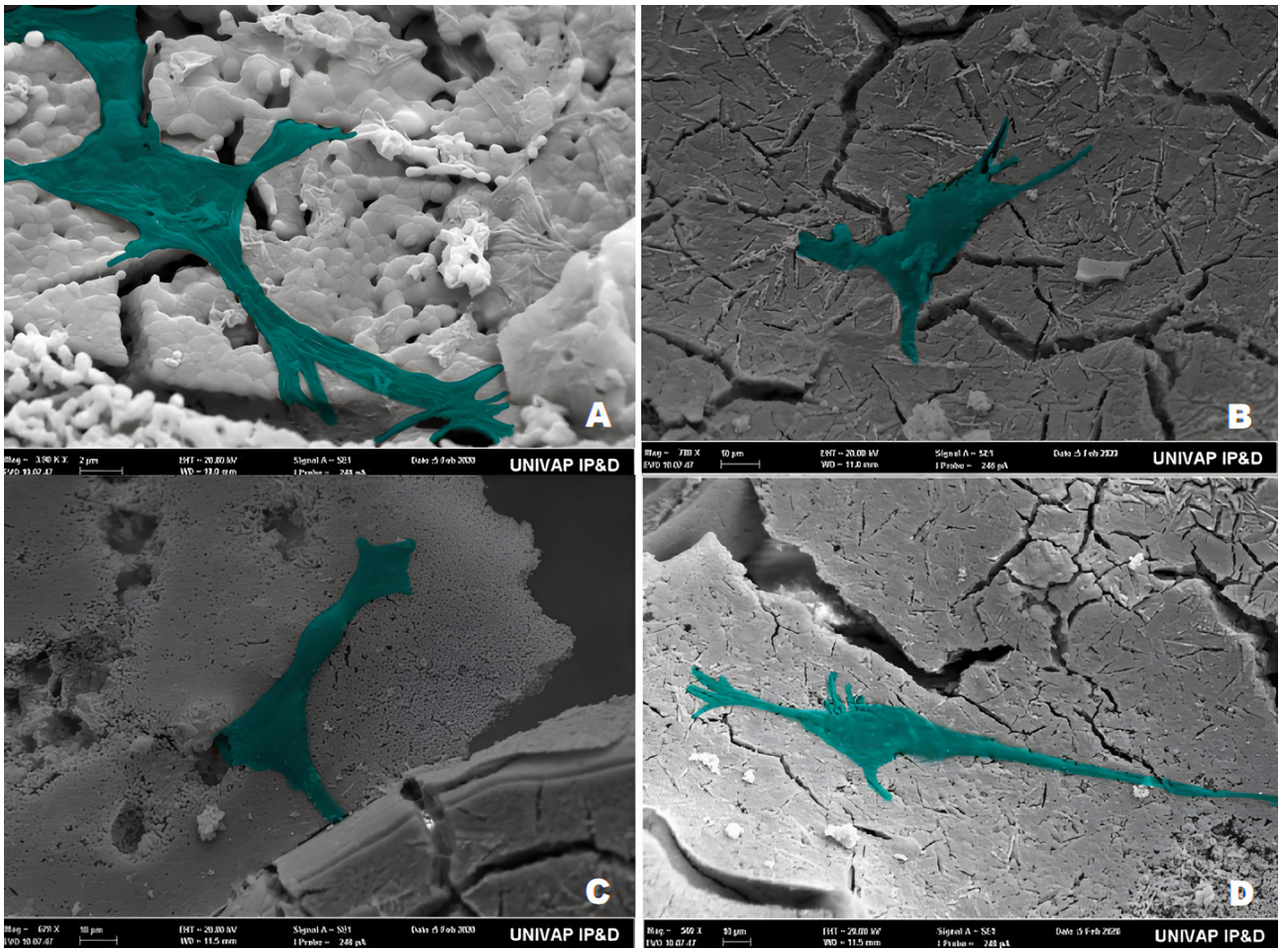

Figure 2 - Cell adhesion after 5 days on scaffolds, seen by scanning electron microscopy (SEM). Legends: In green, cells are highlighted. A) CAC - Magnification: 3.900x; B) FOSF - Magnification: 708x; C) ZNO - Magnification: 679x; D) ZIRC - Magnification: 508x.

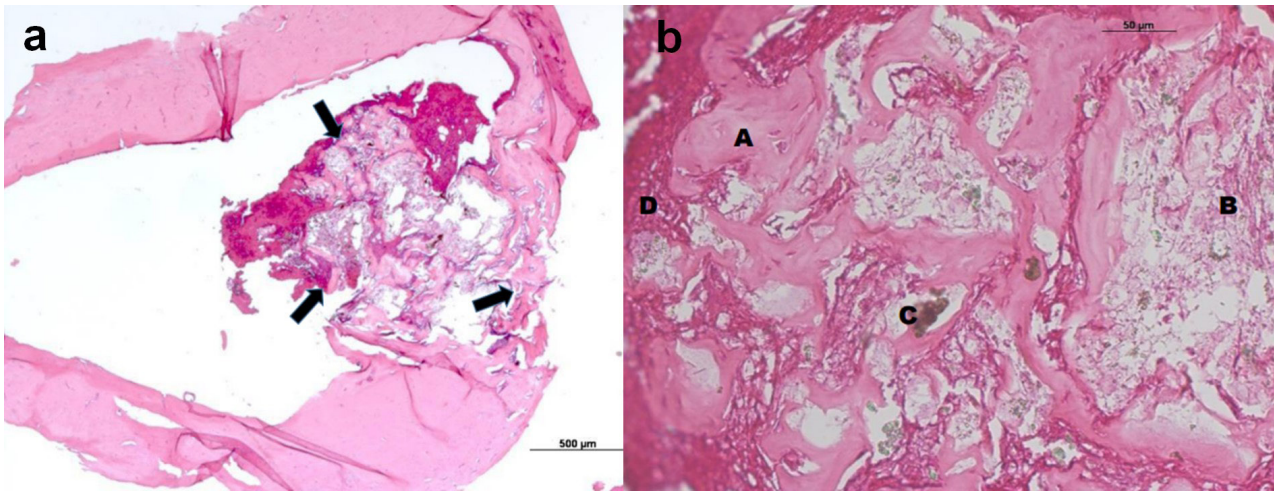

Figure 3 - Histological section observed in the: a) CAC/MSC group original magnification 20x; b) CAC/MSC group original magnification 100x. A) Newly formed bone trabeculae; B) Connective tissue between bone trabeculae; C) Residue of the scaffold; D) Bone marrow cells. The arrows indicate newly formed bone trabecular at the inferior interface of the scaffold.

incorporated with mesenchymal cells, there was formation of bone tissue at the interface with the region previously filled by the scaffold (Figures 8).

Histomorphometric analysis

The histomorphometric analysis was performed using images of histological sections

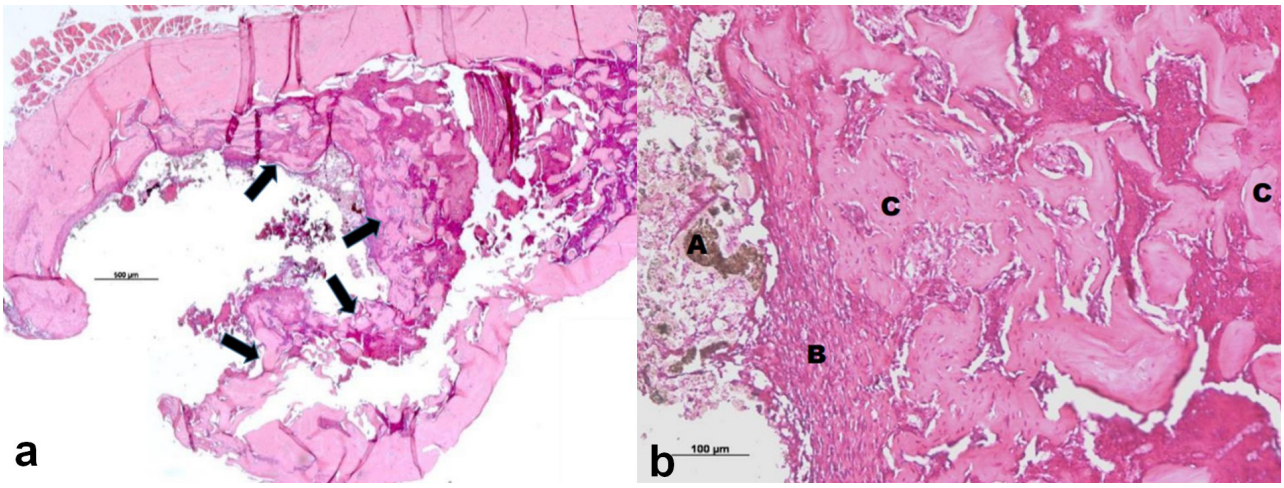

Figure 4 - Histological section observed in the: a) CAC/MSC group original magnification 20x; b) CAC/MSC group original magnification 100x. Legends: A) Residue of the scaffold; B) Connective tissue; C) Newly formed bone trabeculae. The arrows indicate newly formed bone trabecular at the inferior interface of the scaffold.

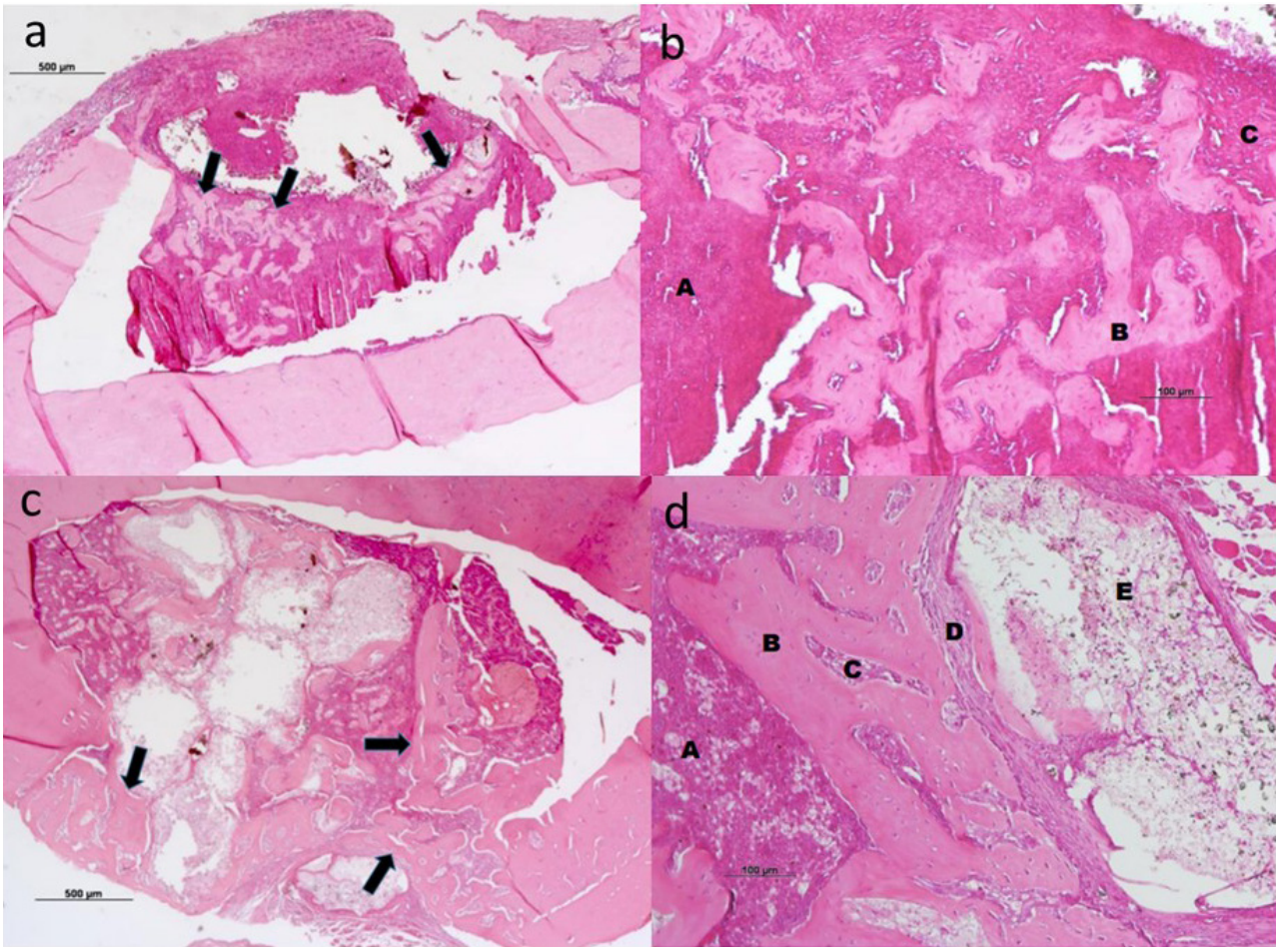

Figure 5 - Histological section observed in the FOSF group: a) FOSF group original magnification 20x; b) FOSF group original magnification 100x; c) FOSF/MSC group original magnification 20x; d) FOSF/MSC group original magnification 100x. Legends: A) Bone marrow cells; B) Neoformed bone trabécula; C) Connective tissue; D) Medullary tissue interspersed with bone trabecular; E) Connective tissue containing scaffold residue. The arrows indicate newly formed bone trabecular at the inferior interface of the scaffold.

performed in the scaffol insertion region. Image J software (National Institutes of Health, Bethesda, MD) was used to quantify bone tissue proliferation at the interface with the scaffold. Regarding the scaffolds that were not incorporated with mesenchymal cells, it was observed that the zirconia blend showed greater bone formation, showing a statistical difference when compared

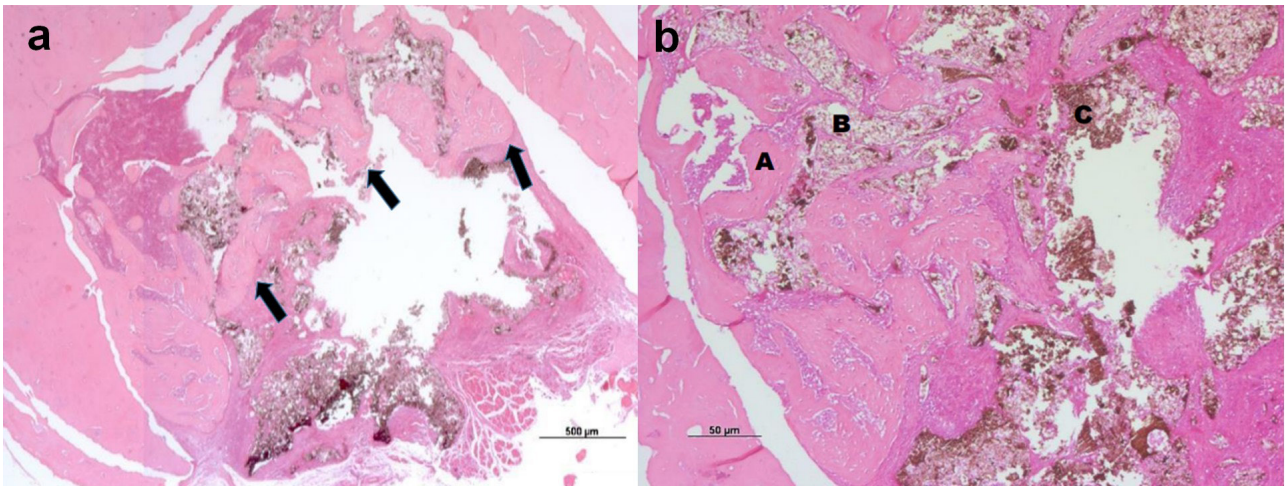

Figure 6 - Histological section observed in the ZNO Group: a) ZNO group original magnification 20x; b) ZNO group original magnification 100x; Legends: A) Represent newly formed bone trabeculae; B)Represent connective tissue; C) Represent residue from the scaffold. The arrows indicate newly formed bone trabecular at the inferior interface of the scaffold.

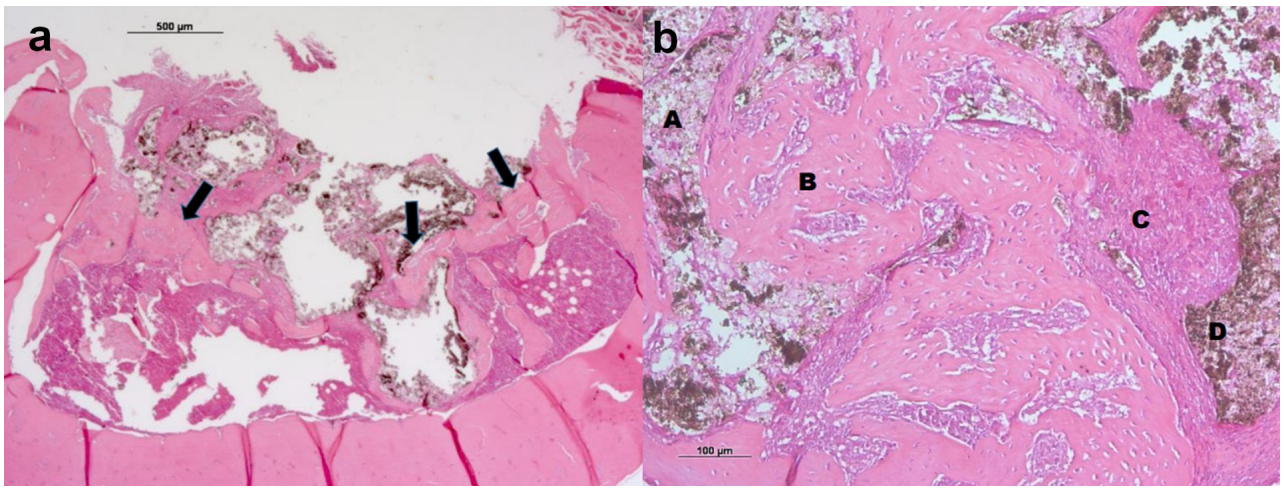

Figure 7 - a) ZNO/MSC group original magnification 20x; b) ZNO/MSC group original magnification 100x. Legends: A) Connective tissue; B) Newly formed bone trabecula; C) Fibrous connective tissue; D) Scaffold remnants. The arrows indicate newly formed bone trabecular at the inferior interface of the scaffold.

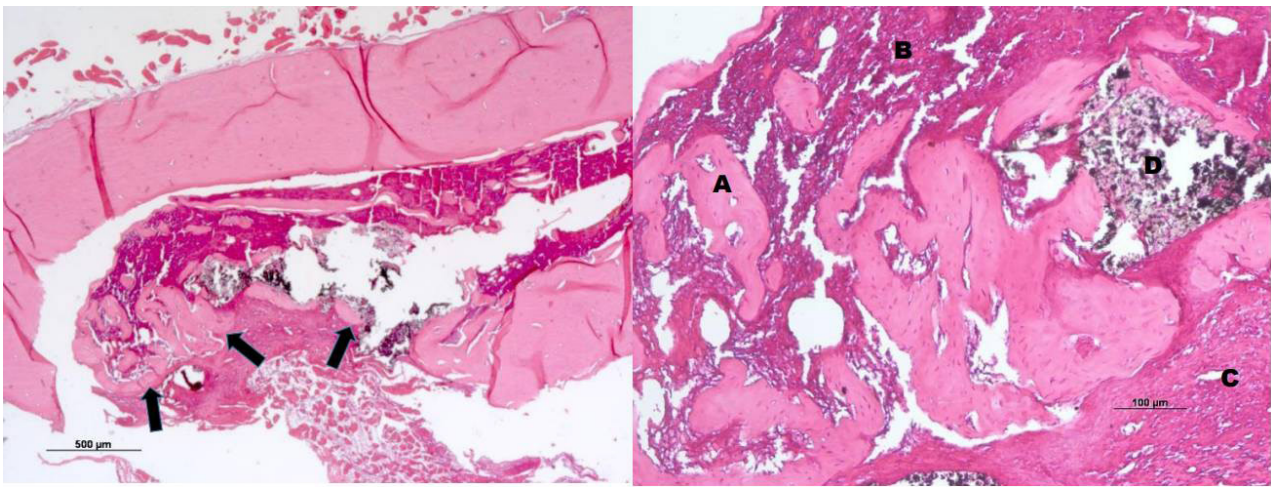

Figure 8 - Histological section observed in the ZIRC groups: a) ZIRC group original magnification 20x; b) ZIRC group original magnification 100x. Legends: A) Connective tissue; B) Newly formed bone trabecula; C) Fibrous connective tissue containing blood vessels (granulation tissue). The arrows indicate newly formed bone trabecular at the inferior interface of the scaffold.

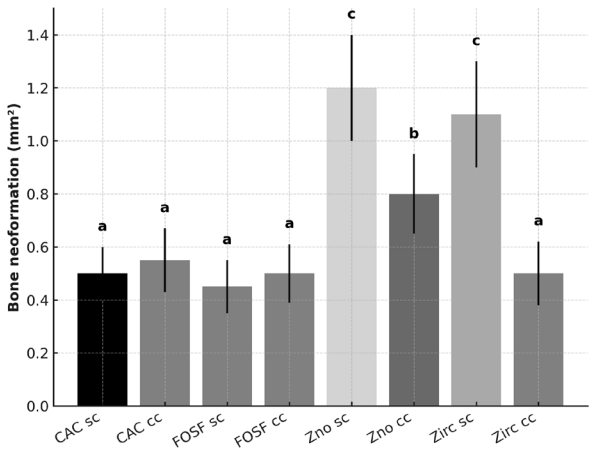

Figure 9 - Comparison between all evaluated groups. Legends: Graph of new bone formation at the interface of the insertion region of the CAC scaffolds and their blends: tricalcium phosphate(FOSF), zinc oxide (Zno) and zircônia (Zirc), whitout cells (sc) and with incorporation of cells (cc). Different letters indicate statistical difference.

to all other groups ($p<0.05$). The ZNO group was the second group with the highest bone formation, followed by the CAC and FOSF group. However, there was no statistical significance for new bone formation in these groups (ZNO, CAC and FOSF) ($p>0.05$). Regarding the scaffolds that were incorporated with mesenchymal cells, greater bone neoformation was observed in the FOSF/MSC group, which showed a statistical difference when compared to all other groups ($p<0.05$). The ZNO group was the second group with the highest new bone formation and also showed statistical difference when compared to the other groups ($p>0.05$). For the CAC group, the results obtained revealed that the bone neoformation values were similiar when these materials were incorporated with mesenchymal cells, with no statistical difference ($p>0.05$) being observed between them. The results obtained for the FOSF group revealed that new bone formation was greater when this material was incorporated with mesenchymal cells, with a statistical difference being observed ($p<0.05$). For the ZNO group, the results revealed that new bone formation was greater when this material was incorporated with mesenchymal cells, with a statistical difference ($p<0.05$) when compared to the scaffolds without cells. For the ZIRC group, the results revealed that new bone formation was greater when this material was not incorporated with mesenchymal cells, with a statistical difference being observed ($p<0.05$).

The results shown in Figure 9 refer to the comparison of new bone formation obtained at the scaffolds, in which all materials were compared, when they were incorporated and not incoporated with mesenchymal cells.

In the comparison between all groups of scaffolds of different material, incorporated or not with mesenchymal cells, it was verified that the FOSF groups incorporated with cells and the ZIRC not incorporated with mesenchymal cells presented the best results, with greater bone formation at the interface with the scaffolds, with no statistical difference between them ($p>0.05$).

DISCUSSION

In the present study, bone formation was evaluated in defects in rat femurs, which were filled with biomaterials based on calcium aluminate cement (CAC) and its blends of tricalcium phosphate (FOSF), zinc oxide (ZNO) and zirconia (ZIRC). In the configuration of scaffolds, associated or not associated with mesenchymal cells. Biomaterials are used to restore or replace some tissue, organ or function of the body [12]. In this study, biomaterials (scaffolds based on calcium aluminate cement) were used with the functions of filling and restoring the volume of lost bone tissue, providing a local mechanical function besides serving as a support for cell proliferation and stimulating bone neoformation. The aim was to contribute to the innovation of new biomaterials for the medical and dental area, through the analysis of the potential in vivo use of these scaffolds, in which important information will be obtained regarding their use in tissue regeneration associated with cell therapy. The products based on SCC come from a new generation of biomaterials that have been developed in order to perform their functions in bone tissue regeneration following the increase of life expectancy of the population [45]. The CAC possesses relevant properties for its use as biomaterial, especially in the repair of bone defects, due to its advantages of biocompatibility and high mechanical resistance when subjected to compression [45]. CAC has relevant properties for its use as a biomaterial, especially in the repair of bone defects, due to its advantages of biocompatibility and high mechanical strength when submitted to compression [45]. Furthermore, there is the advantage with regard

to its price and of its derivatives, beyond national production and less dependence on imports [45].

In vitro studies [28], CAC scaffolds with 60% solids content added with 4% additive weight of FOSF, ZNO and ZIRC were not cytotoxic, showing adequate cell viability, since all compositions showed values higher than 70% in the MTT assays. However, the ZNO and ZIRC blends showed the highest number of viable cells and were the groups that presented the highest values of alkaline phosphatase activity, indicating their ability to induce mineralization [28].

The results of this study indicated, through quantification and analysis of the newly formed bone tissue at the interface of the biomaterials insertion region, that the use of CAC scaffolds and their blends (FOSF, ZNO and ZIRC) allowed bone neoformation in this region, incorporated or not with mesenchymal cells. The incorporation of mesenchymal cells in the scaffolds was beneficial and had influenced positively in bone neoformation in all groups, except in the ZIRC blends group. However, this group of scaffolds produced with ZIRC blends not incorporated with mesenchymal cells was the one that showed the greatest bone neoformation at the interface with the insertion region of the scaffold, suggesting a better potential for bone regeneration when not incorporated with mesenchymal cells. When incorporated with mesenchymal cells, the group of the blend FOSF stood out positively, forming a greater amount of bone tissue, indicating that it is a promising material to be used in cell therapy. Comparing all groups, incorporated or not with mesenchymal cells, the groups that showed the best results were the TCP with cells and ZIRC without cell groups.

Furthermore, regarding the pore distribution of these scaffolds, verified by Mercury porosimetry [28] demonstrated that these same zirconia and zinc oxide blends had larger pores compared to the other groups, which seems to favor bone formation [19,21]. Corroborating this information, in the results of the present in vivo study, for the scaffolds not incorporated with mesenchymal calls, it was verifield that the zirconia blend was the one that presented the best results, showing greater bone neoformation compared to the other groups, presenting a significant statistical difference ($p<0.05$) with the other groups, followed by the ZNO blend, shower no statistical difference for CAC and FOSF. Therefore, these in vivo results are consistent with the in vitro results. Zirconia-based biomaterials have gained attention as a biomaterial for hard tissue reconstruction due to theis good machanical, chemical and biological properties. They have a low corrosion rate, low toxicity and low bacterial adherence [46-48], proving to be relevant for tissue engineering applications [49]. The combinarion of CAC and ZIRC in the stabilization of vertebral compression fractures resulted in high compressive strength values, similar to PMMA [50]. In the present study, the zirconia blend scaffolds presented the best results and showed a higher rato of bone neoformation when not incorporated with mesenchymal cells. The association of mesenchymal cells with scaffolds and other biomaterials offers a strategy to improve bone differentiation and growth compared to scaffolds whithout these cells [51]. This strategy has shown to be promising, showing positive results in terms of stimulating the bone regenerative process [8,39]. Preclinical tests by [7,40] Also showed that the association of mesenchymal cells with scaffolds increases the osteogenic capacity. In the present study, this premise was true and corroborates these results for all groups, except for the zirconia blend group, since in the intragroup results, comparing scaffolds incorporated or not with mesenchymal cells, it was verified that the incorporated scaffolds presented better results, due to greater bone formation. Only the ZIRC blend group presented results with lower values when the scaffolds were associated with mesenchymal cells ($p<0.05$). Thisnsatisfactory result may have occurred due to the zirconia íons had been solubilized during the 5 days of impregnation with the cells in the culture medium. Thus, the hypothesis would be that CAC scaffold would remain without the zirconia ions incorporated with the cells, ans the performance wouldbe similar to the CAC scaffolds without additives, which was exactly the result found. The CAC and zirconia blend scaffolds showed similar results when both were incorporated with mesenchymal cells ($p>0.05$). Authors evaluated blends of CAC with calcium chloride ($CaCl_2$) addociated with bismuth oxide (Bi_2O_3) and zinc oxide (ZNO) as radiopacifiers in osteogenic cell cultures and concluded that CAC with $CaCl_2$ associated with ZNO promoted a better survival rate and differentiation of osteoblastic cells, suggesting greater potential for bone repair of this blend in the contexto of endodontic therapies [52]. Zinc oxide acts

indirectly in bone repair by acting on enzymes and hormones that are related to bone growth, in addition to inhibiting osteoclasts, which are responsible for bone resorption [33]. In this present study, the ZNO blend scaffolds stood out in the intergroup comparison, ranking 2nd in bone neoformation, with statistical difference fot the other groups ($p<0.05$). In the intragroup comparison, when these were incorporated with mesenchymal cells, they showed more promising results compared to the scaffolds not incorporated with these cells ($p<0.05$). The potential of tricalcium phosphate in compositions for use asa bone substitute has been reported in the literature [29,53]. In a later study, results were obtained that showed that β-tricalcium phosphate presented a greater volume of new bone formation [53]. In others, it showed greater mineralizes matriz (mineralization nodules) in the group of scaffolds of the tricalcium phosphate blend, indicating this composition as promising for tissue engineering [28]. In this present study, the scaffolds of the tricalcium phosphate blend, when associated with mesenchymal cells, was the group with the greatest bone neoformation, proving to be a potential material for bone regeneration in the presence of cells.

CONCLUSION

When incorporated with mesenchymal cells, the blend group containing tricalcium phosphate stood out positively, forming greater amount of bone tissue, indicating that it is a promising material to be used in cell therapy. Comparing all groups, embedded or not with cells mesenchymal, the groups that demonstrated the best results were the FOSF with cells and ZIRC without cells.

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Author's Contributions

CSOB: Investigation, Resources, Formal Analysis, Data Curation, Writing – Original Draft Preparation. LADG: Validation, Formal Analysis, Writing – Original Draft Preparation. JCRA: Writing – Review & Editing, Visualization. RSG: Data Curation. ILNGV: Conceptualization, Formal Analysis, Investigation, Methodology, Validation, Visualization. IRO: Conceptualization, Methodology, Funding Acquisition. LMRV: Conceptualization, Methodology, Supervision, Project Administration.

Conflict of Interest

The authors have no proprietary, financial, or other personal interest of any nature or kind in any product, service, and/or company that is presented in this article.

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Regulatory Statement

This study was submitted and approved by the Ethics Committee of Research in Animals of the Institute of Science and Technology of Sao Jose dos Campos/UNESP (012/2019-CEUA/ICT-UNESP).

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