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Preliminary study of bone repair in a non-critical defect after the implantation of wollastonite and tricalcium phosphate granules

Avaliação da fase inicial do reparo em defeito ósseo não crítico após a implantação de grânulos de wollastonita e fosfato tricálcio

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Abstract

Introduction: *tissue bioengineering is a multidisciplinary area that seeks to develop new techniques and biomaterials that replace injured bone tissue or stimulate bone regeneration. Ceramic biomaterials are a promising alternative due to their biocompatibility, osteoconductivity, bioactivity and bioresorption. Therefore, association of wollastonite and tricalcium phosphate ceramics results in a composite biomaterial with superior osteogenic properties when compared to its use alone. The aim of this study was to histomorphologically evaluate the initial phase of repair of a non-critical bone defect after the implantation of wollastonite (W) and β-tricalcium phosphate (β-TCP) granules in different proportions.* ***Metodology:*** *eighteen animals were randomly distributed into three experimental groups, with six animals in each one of them: i) W60 – group with granules of 60% of W and 40% of β-TCP on a non-critical bone defect; ii) T20 – group with 80% W and 20% β-TCP granules on a non-critical bone defect; III) CG – control group without implantation of biomaterials on a non-critical bone defect. All groups were evaluated 15 days after surgery.* ***Results:*** *histological analysis at 15 days evidenced a discrete reparative bone neoformation, restricted to the edges, moderate granulomatous chronic inflammation with few multinucleated giant cells, filling of the defect with granules throughout the entire extension and loose connective tissue formation amongst the biomaterials.* ***Conclusion:*** *W/β-TCP composites evaluated in this study, at the biological point of 15 days, were biocompatible, osteoconductive and bioactive, which indicates high potential for use as bone substitutes.*
Keywords: *Biomaterials; bone regeneration; Wollastonite; Tricalcium phosphate; mouse.*

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Resumo

Introdução: a bioengenharia tecidual é uma área multidisciplinar que busca desenvolver novas técnicas e biomateriais que substituam o tecido ósseo lesionado ou estimulem a regeneração óssea. Os biomateriais cerâmicos constituem uma alternativa promissora devido à biocompatibilidade, osteocondutividade,

bioatividade e biorreabsorção. Nesse contexto, a associação das cerâmicas wollastonita e fosfato tricálcico resulta em um biomaterial compósito com propriedades osteogênicas superiores em relação a seus constituintes, quando utilizados de forma isolada. O objetivo deste estudo foi avaliar histomorfologicamente a fase inicial do reparo de defeito ósseo não crítico após a implantação de grânulos de wollastonita (W) e β-fosfato tricálcico (β-TCP), em diferentes proporções. **Metodologia:** dezoito animais foram aleatoriamente distribuídos em três grupos experimentais, compostos por seis animais em cada: i) W60 – grupo com grânulos de 60 % de W e 40% de β-TCP, em defeito ósseo não crítico; ii) T20 – grupo com grânulos de 80% de W e 20% de β-TCP, em defeito ósseo não crítico; III) GC – grupo de controle, sem implantação de biomateriais no defeito ósseo não crítico. E foram avaliados após 15 dias de pós-operatório. **Resultados:** a análise histológica evidenciou, aos 15 dias, discreta neoformação óssea reparativa, restrita às bordas, inflamação crônica granulomatosa moderada, com escassas células gigantes multinucleadas, preenchimento do defeito com grânulos por toda a extensão e formação de tecido conjuntivo frouxo de permeio aos biomateriais. **Conclusão:** os compósitos de W/β-TCP avaliados neste estudo, no ponto biológico de 15 dias, foram biocompatíveis, osteocondutores e bioativos, o que indica elevado potencial para uso como substitutos ósseos.
Palavras-chave: Biomateriais. Regeneração óssea. Wollastonita. Tricálcico fosfato. Rato.

INTRODUCTION

The bone tissue is a highly resistant type of connective tissue, due to the association of organic phases, mainly type I collagen, and inorganic, represented substantially by hydroxyapatite (HA) crystals. This tissue is metabolically active, due to constant remodeling activity throughout life; and highly vascularized, which enables the transport of oxygen, growth factors and nutrients, migration of cells responsible for osteogenesis and bone resorption, and removal of metabolic waste[1-6].

When an injury occurs, after the rupture of blood vessels, a clot is formed with a subsequent contribution of inflammatory cells, followed by the migration and proliferation of osteogenic cells, for tissue repair. However, it is known that, when there is extensive tissue loss, repair occurs with the formation of connective tissue, which can affect the functionality and aesthetics of the affected area[7-9].

Under these conditions, it is necessary to use biomaterials that can replace the lost tissue or stimulate tissue neoformation, since the autogenous graft, considered the gold standard, has limitations, such as a reduced volume of donor tissue, the need for a second surgical intervention with increased morbidity and risk of infection[4,10-12]. At this point, ceramic materials become a promising alternative due to their biocompatibility; osteoconductivity; chemical similarity with the mineral composition of bone tissue;[12-17] feasibility of performing anionic and cationic substitutions, with the purpose of improving physicochemical and biological properties; possibility of being produced in different formats and forms of presentation[15,18-25].

Among these, wollastonite (W) stands out, a calcium silicate whose chemical formula is CaSiO3, composed by 51.7% silicon dioxide and 48.3% calcium oxide[26]. This mineral has two polymorphs, classified according to the synthesis temperature: pseudo-wollastonite (α-CaSiO3), synthesized at high temperature, between 1125 and 1436 °C; and β-wollastonite (β-CaSiO3) developed at low temperature, below 1125 ºC.[27,28] This type of biomaterial has low contraction, reduced moisture absorption, reduced thermal expansion and electrical constancy, in addition, it is biocompatible, osteoconductive and bioactive, important characteristics to improve bone regeneration[22,29-34].

Another ceramic which is widely used in medicine and dentistry is β-tricalcium phosphate (β-TCP), represented by the chemical formula β-Ca3(PO4)$_2$,[35-37] due to its chemical composition be very similar to the inorganic matrix of bone tissue and because it is more soluble compared to hydroxyapatite (HA). This ceramic is biodegradable, bioresorbable, bioactive, biocompatible, osteoconductive and osteoinductive[36-38]. Thus, the association of W and β-TCP ceramics results in a composite with osteogenic properties, capable of stimulating new bone formation[22,34,39 – 41.] Given the above, the aim of this study was to evaluate the potential for new bone formation in the repair of a non-critical bone defect after the implantation of W and β-TCP granules.

METHODOLOGY

This study was approved by the Committee on Ethics in the Use of Animals of the ICS-UFBA, under the protocol nº 5241060423, and was developed in accordance with the Brazilian Guide for Production, Maintenance or Use of Animals in Teaching Activities or Scientific Research,[42] with the Normative Resolution n° 55 – Brazilian Guideline for the Care and Use of Animals in Teaching or Scientific Research Activities of CONCEA (Conselho Nacional de Controle de Experimentação Animal)[43], and with the Normative Resolution n° 37 – Guideline for the practice of euthanasia of CONCEA[44].

The biomaterials evaluated in this study were synthesized, processed and characterized at the Instituto de Cerâmica y Vidro (CSIC), Madrid, Spain. The synthesis, processing and characterization are described in Vasconcelos et al.[34] (2023).

Sample

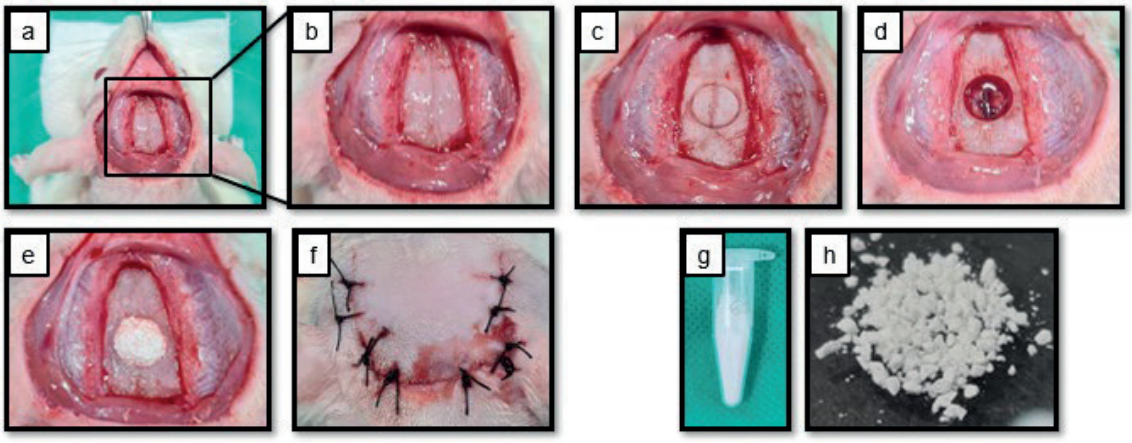
Eighteen male Wistar rats, weighing between 300 and 370 grams, aged between two and three months, were randomly distributed into three experimental groups, with six animals in each: i) W60 – non-critical bone defect filled with granules composed of 60% W and 40% β-TCP; ii) T20 – non-critical bone defect filled with granules composed of 80% W and 20% β-TCP; iii) GC – non-critical bone defect without implantation of biomaterials (control group), all being evaluated at the biological point of 15 days.

Surgical Procedure

The surgical technique used was the same as described by Miguel et al.[8] (2013) and illustrated by Santos et al.[45] (2019), however it is noteworthy that, on both cited studies, the authors used an 8.0 mm trephine bur to create a critical bone defect, while in this work, a 5.0 mm diameter trephine bur was used to make a non-critical bone defect with approximately 5.2 mm diameter[46].

First, the animals were submitted to anesthesia, analgesia, and sedation, respectively, with intraperitoneal administration of ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (10 mg/kg). Subsequently, trichotomy was performed on the region of the calvaria bone, followed by antisepsis with 0.5% alcoholic chlorhexidine and positioned in ventral decubitus. Then, a bicoronal semilunar skin incision of approximately 3.0 cm was made, followed by divulsion, elevation of the skin flap and removal of the periosteum to access the calvaria bone (Fig. 1a and 1b). Afterwards, a non-critical bone defect with approximately 5.2 mm diameter (Fig. 1c and 1d) was created using a 5 mm trephine bur. Subsequently, the biomaterial (Fig. 1g and 1h) was implanted (Fig. 1e) according to the experimental group, except for the CG. Then, the skin flap was repositioned and sutured with 4.0 nylon suture (Fig. 1f).

Figure 1 – *surgical procedure to obtain bone defect and biomaterials implantation.*



Source: Elaboration of the authors

At the biological point of 15 days postoperatively, the animals were euthanized with an intraperitoneal lethal dose of ketamine hydrochloride (300 mg/kg) and xylazine hydrochloride (30 mg/kg) association, to obtain tissue samples to subsequent histological analysis.

Laboratory stage

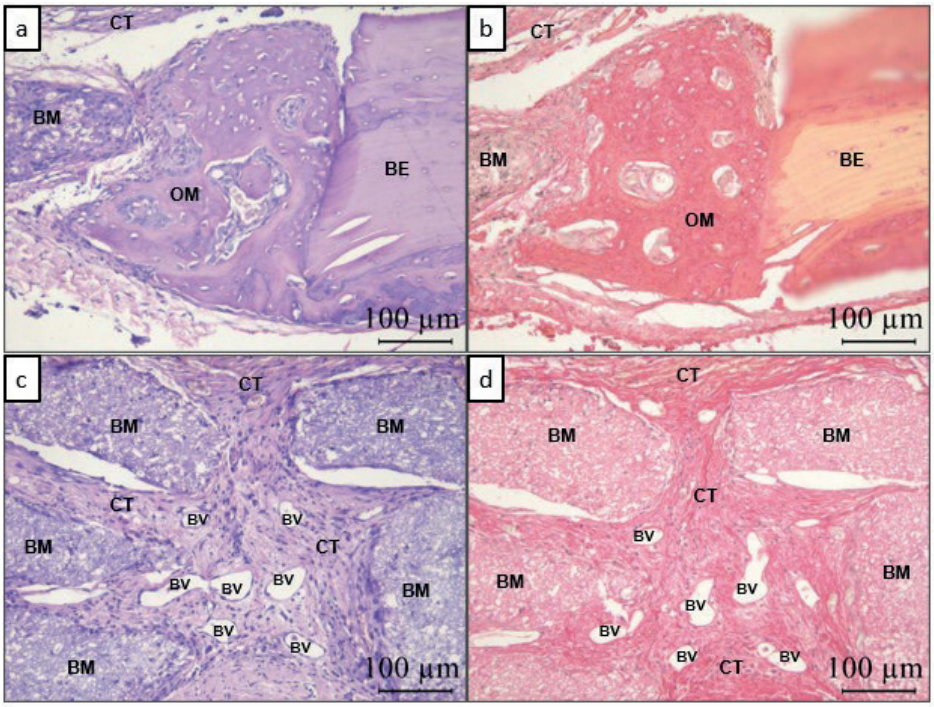
The specimens obtained were fixed in 4% buffered formalin and after 48 hours reduced and transversely sectioned on the central region of the defect obtaining two fragments, anterior and posterior. Then, the posterior portion was sent for routine histological processing. The blocks were cut with 5.0 µm thickness and stained with hematoxylin and eosin (H.E.), routine stain, and picrosirius red (PIFG), to identify collagen fibers and newly formed connective tissue. For the histomorphological analysis, a common light optical microscope (DM6B – Leica®, Wetzlar, Germany) with a coupled digital camera (DFC7000 T – Leica®, Wetzlar, Germany) and the software Leica Application Suite (LAS) v. 4.12 (Leica®, Wetzlar, Germany) for image capture.

RESULTS

The histomorphological analysis at 15 days showed osteoid matrix neoformation restricted to the bone edges for all three groups. In the W60 and T20 groups, this finding was seen towards the center of the bone defect associated with the granules (Figure 2A, 2B, 3A and 3B). In these groups the biomaterials were arranged in single or bilayers along the defect.

A mild to moderate chronic inflammatory response was noted with the presence of edema (Figure 2D, 3D, 5B) on all groups. The groups in which the biomaterials were implanted, the inflammation found was classified as a moderate granulomatous type with scarce multinucleated giant cells (Figure 4). On the residual area of the defect there was formation of connective tissue, rich in blood vessels throughout its extension, on all the three groups studied. In the CG, this tissue showed reduced thickness when compared to the bone edges (Figure 5).

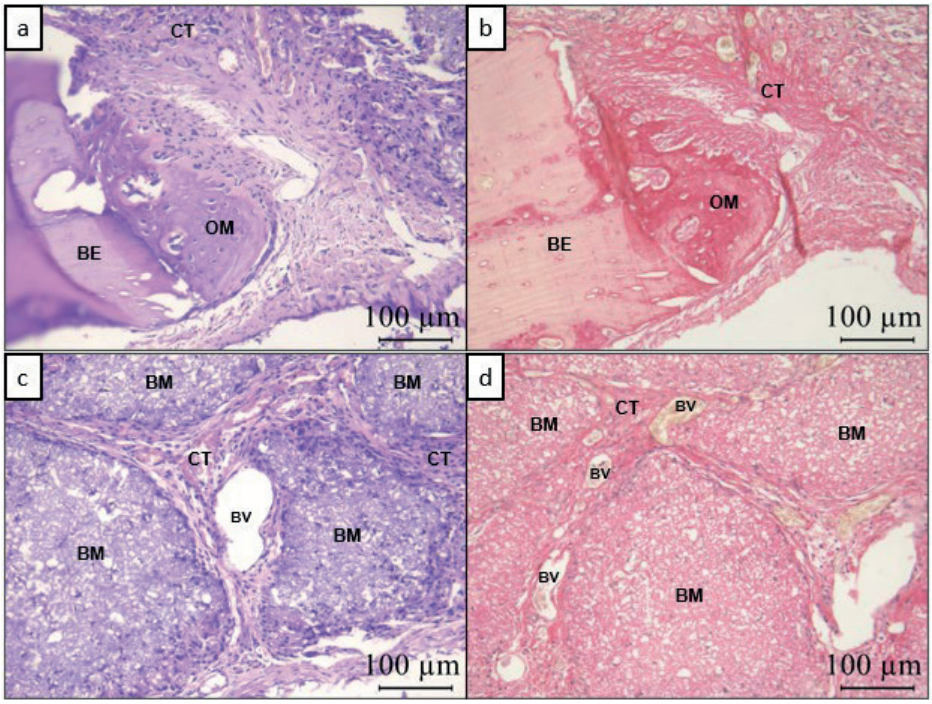
Figure 2 – *Optical micrograph of W60 after 15 days of implantation*



(a) and (b): formation of reactive osteoid matrix (OM) from the remaining bone rim (BE) in W60. Note the interaction between new bone formation and a W60 granule (BM). (c) and (d): center of the bone defect with W60 granules, on multiple layers with the presence of blood vessels (BV) and loose connective tissue (CT) on the interstitium. (a) and (c): HE staining, for visualization of inflammatory cells infiltration. (b) and (d): PIFG staining, with visualization of collagen fibers and newly formed connective tissue.

Source: Elaboration of the authors

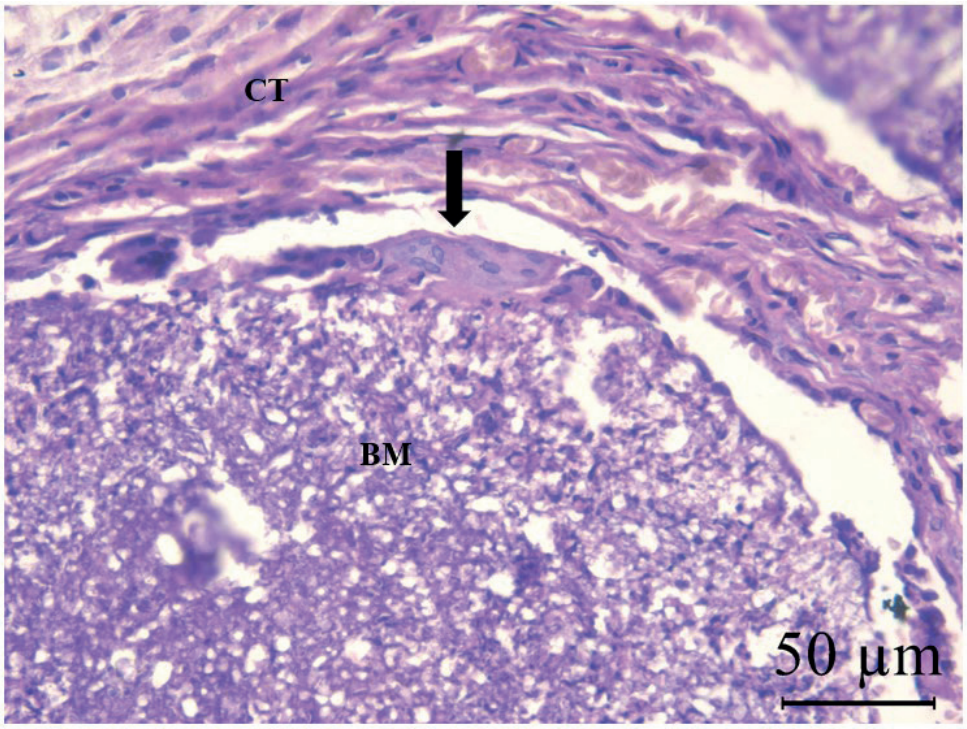
Figure 3 – *Optical micrograph of T20 after 15 days of biomaterial implantation*



(a) and (b): neoformed bone tissue (OM) adjacent to the bone edge (BE) of the defect in T20. (c) and (d): Central area of the bone defect with T20 granules (BM), on multiple layers with the presence of blood vessels (BV) and loose connective tissue (CT) intermingled with the biomaterial. (a) and (c): HE staining for visualization of inflammatory cells infiltration. (b) and (d): PIFG staining, with visualization of collagen fibers and newly formed connective tissue.

Source: Elaboration of the authors

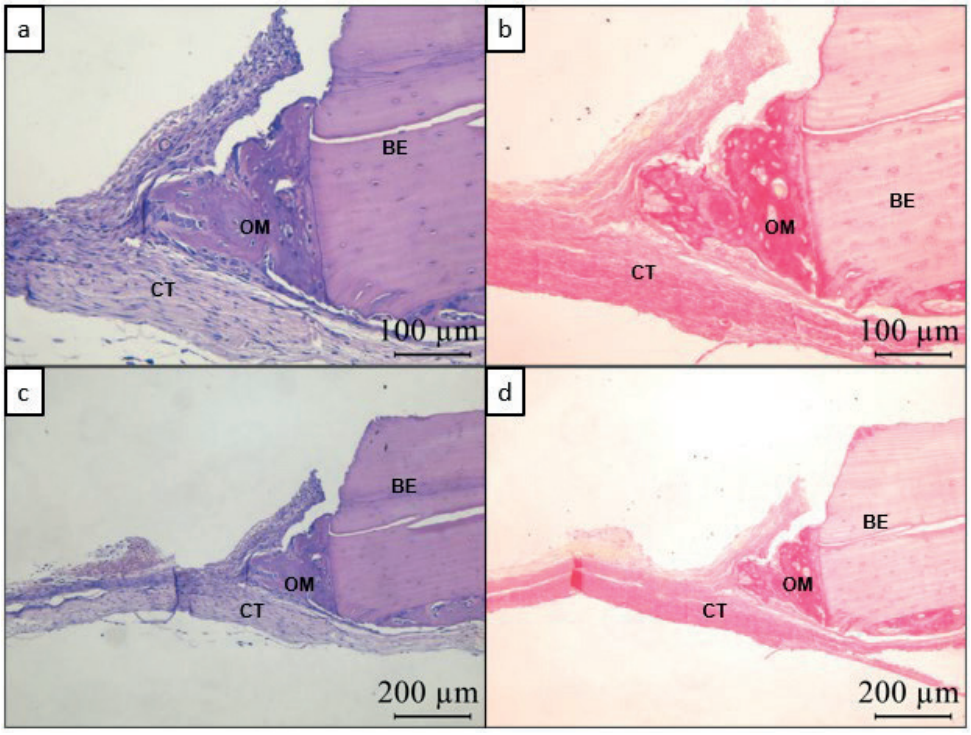
Figure 4 – *Optical micrograph of a multinucleated giant cell (MGC) adhered to a W60 biomaterial granule (arrow).*



The presence of MGC (black arrow) adhered to the W60 granule (BM) confirms the granulomatous inflammatory response. Loose connective tissue (TL) surrounding the granule.

Source: Elaboration of the authors

Figure 5 – *Optical micrograph of GC after 15 days*



(a) and (b): Bone neoformation alongside the margin of the defect. (c) and (d): Newly formed loose connective tissue along the defect, in thin thickness. (a) and (c): HE staining, for visualization of infiltration of inflammatory cells. (b) and (d): PIFG staining, with visualization of collagen fibers and newly formed connective tissue. (CT) Loose connective tissue; (OM) Osteoid matrix; (BE) Bone edge.

Source: Elaboration of the authors

DISCUSSION

The use of W and β-TCP biomaterials has gained prominence in recent years, especially when associated with other materials to produce composites, given their favorable physicochemical characteristics and promising biological properties for bone regeneration and repair. Thus, this study was conducted to evaluate the initial phase of bone repair after the implantation of W and β-TCP granules, in two different proportions.

Histomorphological analysis of the groups with implantation of biomaterials showed a chronic moderate inflammatory response of the granulomatous type. This finding corroborates with Miguel et al.[7] (2006), Santos et al.[22] (2021b), Ribeiro et al.[25] (2023), Vasconcelos et al.[34] (2023) and Almeida et al.[48] (2018) who also evaluated different materials in bone defect in rat calvaria bone. Therefore, the biomaterials used were biocompatible as well as the composites of calcium silicates and tricalcium phosphates used by Santos et al.[22] (2021b), Vasconcelos et al.[34] (2023), Barbosa et al.[40] (2019), Wang et al.[47] (2012) and Liu et al.[49] (2013). Biocompatibility is an essential property for materials to be used clinically. The bioactivity and cytocompatibility of W were tested in vitro by Palakurthy et al.[30] (2019), on our trial the two composites used for the W60 and T20 groups showed to be bioactive and biocompatible endorsing what was found in other experiments carried out in vivo[22,40,50].

The organization of the granules on the area of the bone defect at this trial formed a framework that enabled angiogenesis by supplying nutrients and conduction of cells, followed by growth factors secretion. Such findings are aligned with Miguel et al.[7] (2006), Carmo et al.[19] (2018), Santos et al.[22] (2021b), Ribeiro et al.[25] (2023), Vasconcelos et al.[34] (2023) and Ding et al.[51] (2021), who observed new vessels formation, a fundamental factor to support bone tissue repair, beyond facilitating the action of mesenchymal stem cells and osteogenic cells. At the present study, these findings were evident in the groups in which composite biomaterials were implanted for bone neoformation, with the presence of fibroblasts and mononuclear inflammatory infiltrates.

At our study the initial phase of bone repair new bone formation was limited and restricted to the edges of the defect on all three groups, which is consistent with the findings observed by Santos et al.[22] (2021b), in despite of being performed by critical defect of 8.0 mm observed the same results at the same biological point of this study. Ribeiro et al.[25] (2023) corroborates with these findings using nanostructured microspheres of hydroxyapatite (HA) replaced by strontium, however these authors used the biological starting point of 30 days.

The study carried out by Ge et al.[52] (2019), W associated with HA showed high performance in bone regeneration with 34.68% and 96.78% of mineralized osteoid matrix formation at six and twelve weeks, respectively. Nevertheless, when these materials were implanted alone, W showed a percentage of 8.79% and 16.77% of new bone formation; and for HA, 28.46% and 78.58%, at the same periods of analysis, which indicates the relevance of the association of W with other biomaterials, especially ceramics.

Tissue repair on the residual areas of the non-critical defect was completed with connective tissue formation, indicated by the interacting of the biomaterial granules for the W60 and T20 groups. The CG had no framework and there was deposition of a thin thickness fibrous connective tissue, similar to what was described by Abbassi et al.[11] (2020), Daltro, Barreto, Rosa [46] (2016), Ge et al.[52] (2019), who also used 5 mm defects. This feature presented by the GC is a critical bone defects characteristic which do not regenerate throughout the animal`s life Schmitz, Hollinger [53] (1986), as observed by Miguel et al.[7] (2006), Miguel et al.[8] (2013), Santos et al.[22] (2021b), Ribeiro et al.[25] (2023), Vasconcelos et al.[34] (2023), Daltro, Barreto, Rosa [46] (2016), Almeida et al.[48] (2018) and Barletta et al.[54] (2020), which worked with 8 mm defects.

CONCLUSION

The W and β-TCP composites proved to be biocompatible, osteoconductive and bioactive along with bone neoformation potential to repair non-critical bone defect.

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