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Morphological analysis of the initial regenerative potential of collagen scaffold in critical bone defects

Análise morfológica do potencial regenerativo inicial de um andaime de colágeno em defeitos ósseos críticos.

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Abstract

Introduction: *under physiological conditions, bone tissue undergoes constant remodelling and regeneration. In situations that lead to a breakdown in homeostasis and extensive tissue damage, the regenerative capacity of this tissue is limited, and, as a result, much of the bone lesion is filled with fibrous connective tissue, which characterises a critical bone lesion. In these cases, it is essential to use bone grafts, biomaterials, or technologies that can aid bone regeneration. Among the materials available for this purpose, collagen has been widely used in bone tissue bioengineering because of its properties, mainly biocompatibility, osteoconduction, and biointegration.* ***Objective:*** *to investigate, through an initial phase study, the regenerative potential of collagen scaffolds in repairing critical bone defects in rat calvaria.* ***Methodology:*** *six adult male albino Wistar rats were used to create an 8.0 mm critical bone defect for the implantation of a porcine collagen scaffold. Histomorphological analysis was performed using a standard light microscope at the biological point 15 days postoperatively.* ***Results:*** *it was possible to observe the formation of mineralisation nuclei, the presence of osteoblasts, and neoformation of bone tissue from the edges of the defect. Osteocytes were identified trapped in bone lacunae with their extensions, indicating bone matrix vitality. Furthermore, integration of the collagen scaffold with the neoformed bone matrix was noted. Mild chronic inflammation was identified, observed by the presence of giant cells in small quantities, with no indication of oedema. In addition, neoangiogenesis was observed through the formation of new blood vessels.* ***Conclusion:*** *the collagen scaffold has been shown to be biocompatible, osteoconductive, and to have regenerative potential and biomimicry with bone tissue.*

Keywords: *Bone Regeneration; biocompatible materials; collagen; osteogenesis; Tissue Scaffold.*

Resumo

Introdução: *em condições fisiológicas, o tecido ósseo apresenta constante remodelação e regeneração. Em situações que levem à quebra de sua homeostase, com vasto comprometimento tecidual, a capacidade regenerativa deste tecido torna-se limitada e, assim, grande parte da lesão óssea é preenchida por tecido conjuntivo fibroso, o que caracteriza uma lesão óssea crítica. Nestes casos, torna-se imprescindível a utilização de enxertos ósseos, biomateriais ou tecnologias que possam auxiliar a regeneração óssea. Dentre os materiais disponíveis para este fim, o colágeno tem sido amplamente empregado na bioengenharia tecidual óssea por apresentar propriedades, principalmente de biocompatibilidade, osteocondução e biointegração.* **Objetivo***: investigar, por meio de estudo de fase inicial, o potencial regenerativo do scaffold de colágeno no reparo de defeito ósseo crítico em calvária de rato.* **Metodologia:** *seis Rattus norvegicus da linhagem wistar, albinos, machos e adultos foram utilizados para a confecção de defeito ósseo crítico de 8,0 mm para implantação de scaffold colagênico de origem porcina. A análise histomorfológica realizada por microscopia de luz comum, procedeu-se no ponto biológico de 15 dias de pós-operatório.* **Resultados:** *foi possível observar formação de núcleos de mineralização, a presença de osteoblastos e neoformação de tecido ósseo a partir das bordas do defeito. Identificou-se osteócitos aprisionados em lacunas ósseas com seus prolongamentos, o que indica vitalidade matriz óssea. Ainda, notou-se a integração do scaffold colagênico com a matriz óssea neoformada. Foi identificada inflamação crônica discreta, observada pela presença de células gigantes em pequenas quantidades, não houve indicação de edema. Além disso, observou-se a presença de neoangiogênese pela formação de novos vasos sanguíneos.* **Conclusão***: o scaffold de colágeno demonstrou ser biocompatível, osteocondutor, apresentou potencial regenerativo e biomimetismo com o tecido ósseo.*

Palavras-chave: *Colágeno; osteogênese; material biocompatível; regeneração óssea.*

INTRODUCTION

Bone tissue is vascularized, mineralised, and characterised by its hardness, resistance, and regenerative and remodelling capacities. The ex-

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tracellular matrix of this tissue is composed of both an organic fraction, consisting mainly of type I collagen protein, as well as non-collagenous proteins, peptides, lipids, glycoproteins, polysaccharides, and citrates; and by the inorganic fraction, whose components are calcium phosphates, carbonates, sodium salts, magnesium, and fluoride[1]. This tissue comprises the musculoskeletal system, provides structural support and protection to vital organs, and is also involved in mineral storage and hematopoietic tissue housing[1-4].

The spontaneous regenerative capacity of bone tissue has long been recognised in the literature, provided that the bone injury does not involve extensive loss[5]. When this is observed, due to trauma, fractures, congenital diseases, or tumors that can cause bone injuries that are difficult to treat[6,7], spontaneous regeneration of bone tissue is not expected to occur, making it necessary to stimulate its formation in order to restore the form and function of the injured tissue[3], considering that inadequate repair can cause deformity or more serious consequences, such as loss of function[5]. Given this situation, it is essential to develop new biomaterials and technologies that can accelerate and optimise regeneration in order to promote more efficient and complete recovery[1,2].

In this regard, in recent decades, biomaterials have represented a significant fraction of the products used in healthcare, due to the various types of biomedical devices and materials used. Defined as any substance or combination of substances, of synthetic or natural origin, that can be used, for a period of time, totally or partially, to treat, augment, or replace any tissue, organ, or function of the body, these materials can have diagnostic or therapeutic applications[8]. The development of biomaterials involves several stages, from identifying the need for clinical application to their development, manufacture, and sterilisation, preclinical and clinical evaluation, and decellularisation, when cells are present. The evaluation of the biological properties of biomaterials, such as hemocompatibility, cytotoxicity, biofunctionality, and biocompatibility, is fundamental to their clinical efficacy[9,10].

Among the many types of raw materials available for the synthesis of biomaterials, collagen is a protein easily found in natural tissues, compatible with the human body, widely used as a haemostatic agent and as a dressing[5].The widespread use of this material in the field of tissue bioengineering is associated with its natural properties, which include low immune response; low toxicity; ability to promote cell growth, adhesion, proliferation, migration, and differentiation; homeostasis; and, as a biomaterial, it aids in bone regeneration[11-13].

Collagen also has excellent biocompatibility and is absorbed by the body over time[14]. Due to its interaction with cells, molecules, and the extracellular matrix, this raw material enables cell adhesion, insertion, migration, and proliferation, thanks to its physicochemical properties, which include haemostatic capabilities, low antigenicity, interconnected pore architecture, easy processing, and hydrophilicity[13,15,16]. Furthermore, it is non-toxic and aids in the deposition of hydroxyapatite crystals within its structure, which contributes to the mineralisation of the bone matrix. In addition to its low preparation cost, it is commercially available, which facilitates its acquisition[16-18].

Type I collagen is formed by triple helices of polypeptides that bind together to form collagen fibrils, which are important for providing mechanical strength to bone tissue, as they guide the deposition of hydroxyapatite crystals and contribute to the organisation of bone mineralisation. In addition, its three-dimensional architecture acts as a structural support for the migration and adhesion of osteoprogenitor cells and, thus, promotes the initial events in bone tissue formation[6,10,16].

The Hemospon® collagen scaffold is commercially available and is indicated during or after surgical procedures, mainly dental, as it promotes local hemostasis and tissue repair. In addition, it also acts to stabilise clots and fill spaces resulting from tooth extractions, cyst removal, tumours, biopsies, among other procedures[16].

Although there are several studies in the literature on resorbable and non-crosslinked collagen scaffolds for bone regeneration, the material evaluated in this study is widely used in different biomedical applications. However, as it has no primary clinical indication for bone tissue regeneration purposes, studies validating its application in bone regeneration deserve further investigation, which reinforces the need for experimental studies to validate its clinical application in different contexts[12,16]. Thus, through an initial phase study, the regenerative potential of the collagen scaffold, Hemospon®, was analysed in the repair of critical bone defects in rat calvaria.

METHODOLOGY

Ethical considerations

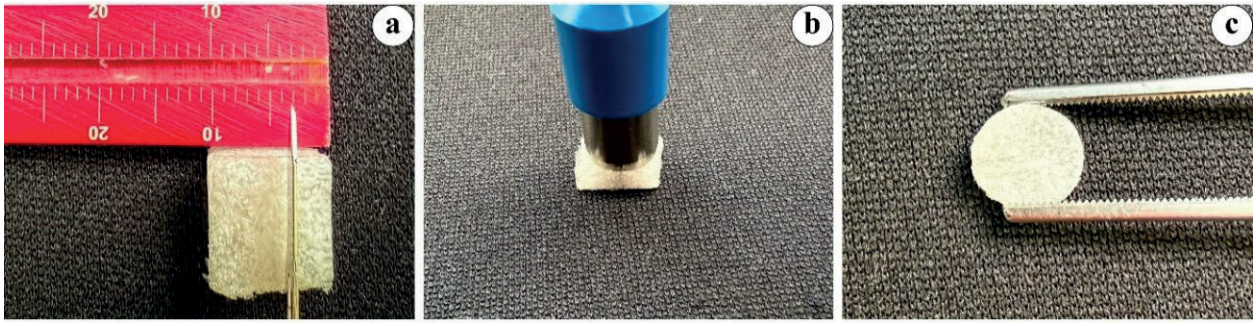
This study was approved by the Animal Use Ethics Committee (CEUA) of the Institute of Health Sciences of the Federal University of Bahia (ICS-UFBA), under protocol CEUA No. 8036250325. After approval, the study was conducted in accordance with the Brazilian Guide for the Production, Maintenance, or Use of Animals in Teaching or Scientific Research Activities, Resolution 55 – Brazilian Guideline for the Care and Use of Animals in Teaching or Scientific Research Activities of CONCEA (National Council for the Control of Animal Experimentation), and Normative Resolution No. 371, to ensure compliance with all relevant ethical and regulatory standards.

Biomaterials

The biomaterial used in this study was the collagen scaffold Hemospon®, a freeze-dried hydrolysed collagen sponge of porcine origin. To adapt the collagen matrix

to the dimensions of the bone defect, this material was initially cut into 3.0 mm thick blocks using a sterile millimetre ruler and a 15C scalpel blade, and then cut with an 8.0 mm sterile surgical punch (Figures 1a, 1b, and 1c). Before insertion into the bone defect, the biomaterial was soaked in sterile saline solution.

Figure 1 — *Collagen scaffold cutout for insertion into a critical bone defect.*



Caption: *Collagen scaffold block to be cut to a thickness of 3.0 mm (Figure 1a); 8.0 mm surgical punch positioned to cut the scaffold (Figure 1b); scaffold ready to be implanted in the bone defect (Figure 1c).*

Source: own authorship (2025)

Sample and experimental procedures

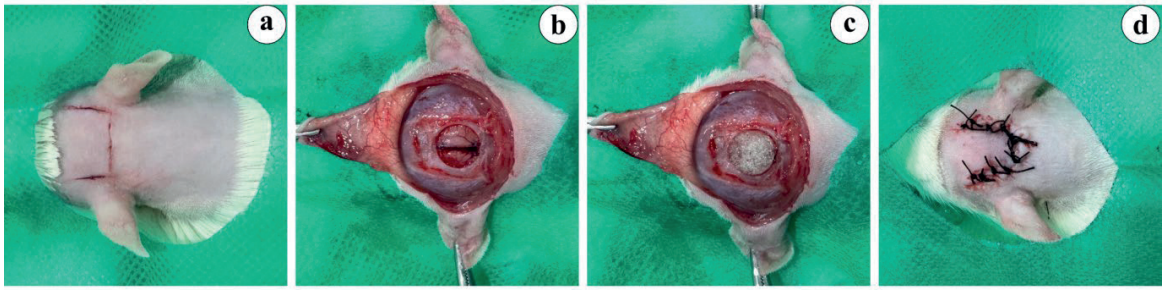
Six adult male albino Rattus norvegicus Wistar rats weighing approximately 350 to 400 g were used, supplied, bred, and maintained by the ICS-UFBA animal facility. During the experiment, the animals were kept in an air-conditioned environment, received water and feed ad libitum, and had a 12-hour light:12-hour dark photoperiod. The animals were included in a single experimental group and evaluated at the 15-day biological point, the GMC – collagen scaffold group, creation of a critical bone defect with subsequent implantation of the collagen matrix.

In consideration of the 3Rs principles (reduction, refinement, replacement),[19,20] his study did not include a control group with critical bone defects without biomaterial implantation, since this result has already been reported in publications by our research group[10,17,21-29].

For the surgical procedure, the animals were anaesthetised with 10% ketamine hydrochloride (100 mg/kg) and analgesia and sedation with xylazine hydrochloride (10 mg/kg) administered intraperitoneally. After trichotomy and asepsis with 2% chlorhexidine of the region to be operated, a bicoronal skin flap incision was made with a number 15C scalpel blade (Bencton Dickinson®), approximately 3.0 cm in length, to expose the calvarium. Next, the periosteum of this region was removed with a Molt periotome (SS White®) to expose the cortical bone and create the critical defect, posterior portion of the calvarium, with an 8.0 mm diameter bone trephine drill (Dentoflex® – Brazil) coupled to a contra-angle (Dabi Atlante® – Brazil) with a 16:1 reduction and a surgical motor (Driller® BLM 600 Plus – Brazil) at 1500 revolutions per minute (rpm), under abundant and constant irrigation with saline solution. Subsequently, a circular transfixed bone defect approximately 8.5 mm in diameter was created in the middle portion of the calvarium, between the vertices of the anterior and posterior sutures, with removal of the portion of the calvarium[24]. Next, the collagen scaffold was implanted in the defect under irrigation with saline solution, and subsequently, the flap was repositioned and sutured with simple stitches using 4 – 0 silk thread (Ethicon – Johnson & Johnson® – United States) (Figures 2a-d). For postoperative analgesia, ketoprofen was administered subcutaneously at a dose of 5.0 mg/kg once daily for three days. Fifteen days after surgery, the animals were euthanised with a lethal injection of ketamine hydrochloride, 300 mg/kg, and 30 mg/kg of xylazine via the peritoneal route.

The sample processing stage was carried out at the Tissue Bioengineering and Biomaterials Laboratory (LBTB) of the (ICS – UFBA). The upper portion of the calvarium was removed, fixed in 4% formalin for 72 hours, and decalcified in 7% nitric acid for approximately 2 hours. The specimens underwent histological processing in an automatic tissue processor (LEICA® – Germany). The slides were stained with hematoxylin and eosin (HE), a routine stain, and Picrosirius Red (PIFG), a technique used to highlight collagen. The sections were examined by light microscopy (DM6B Microscope, Leica® – Germany) for histomorphological evaluation.

Figure 2 — *Stages of the surgical procedure and collagen scaffold implantation.*



Caption: *The bicoronal skin incision (Figure 2a); observe the bone defect (Figure 2b); collagen scaffold implantation (Figure 2c), and suture (Figure 2d).*

Source: own authorship (2025)

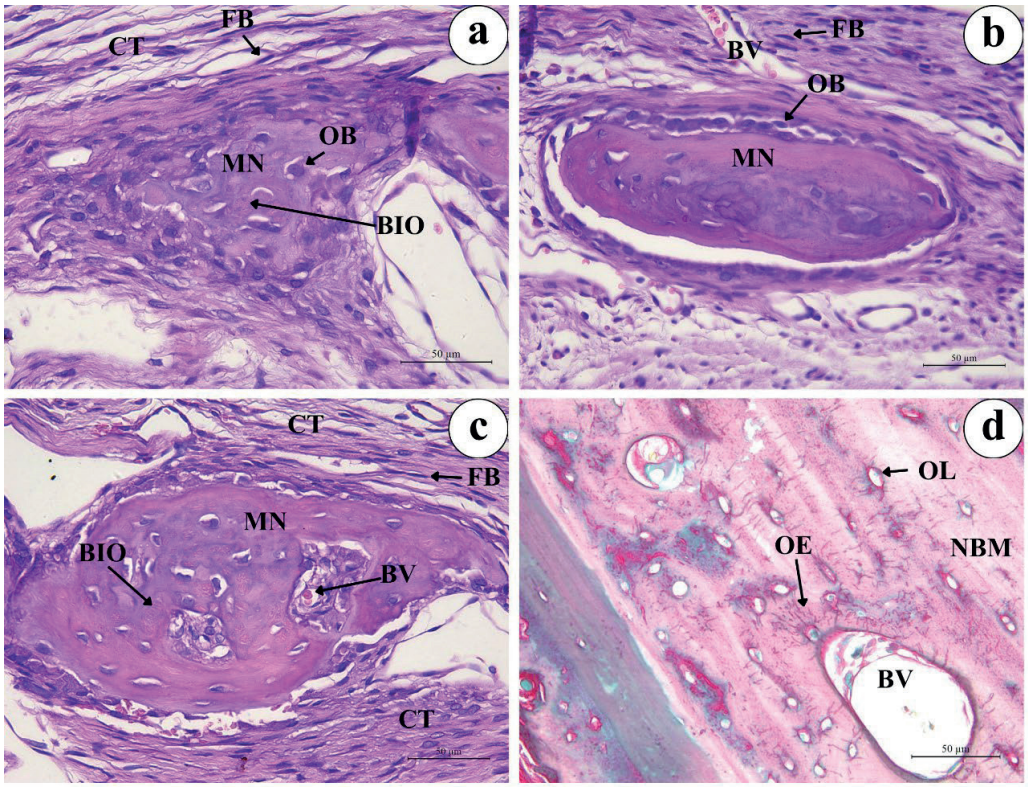
RESULTS

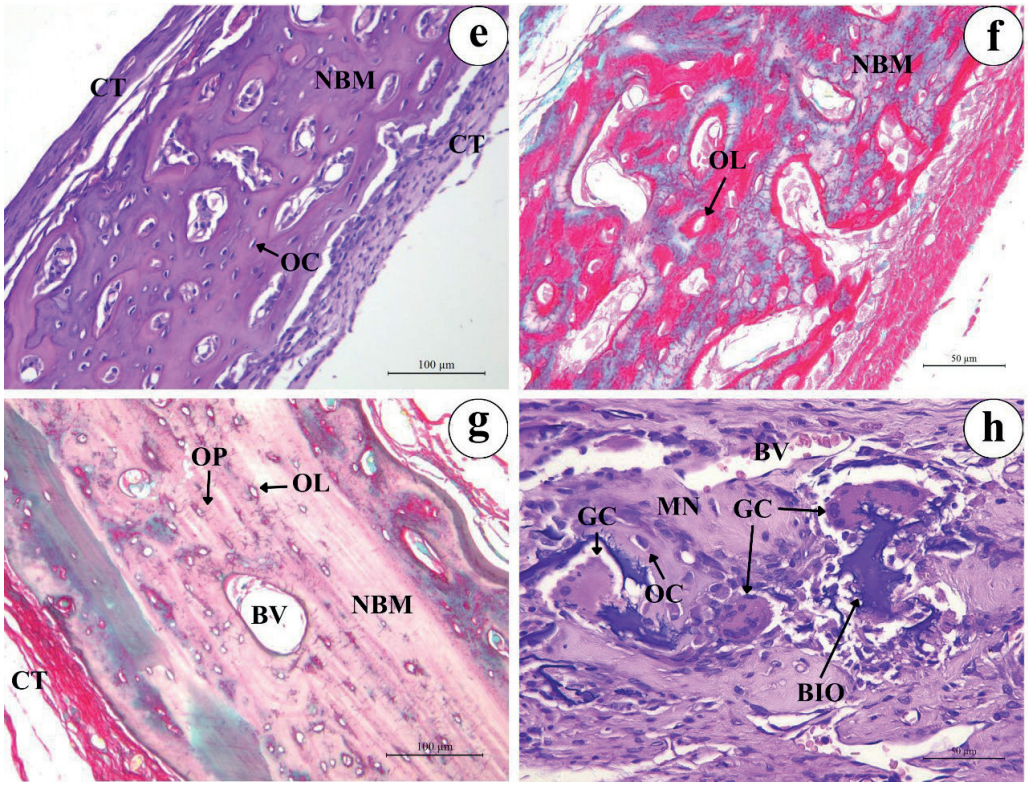
At 15 days, bone neoformation was evident in continuity with the margins of the defect, indicating progress in bone regeneration toward the centre of the lesion and nuclei of bone neoformation in the central region of the defect (Figures 3a, 3b, 3c, 3h). The bone matrix was present in a disorganised manner, pointing to recent bone tissue formation (Figures 3d, 3g). In addition, osteoblasts were noted surrounding the mineralisation nuclei (Figures 3a, 3b), and osteocytes were also observed in lacunae with their extensions.

The implanted collagen scaffold showed signs of biodegradation and integration with the newly formed bone tissue. The biomaterial is very similar to the host's connective tissue, suggesting that the scaffold integrated with the mineralisation nuclei, which formed along the bone defect (Figures 3a, 3c).

Connective tissue was observed in the non-mineralised regions (Figures 3a, 3c, 3e, 3g), with an abundance of fibroblasts in the extracellular matrix (Figures 3a, 3b, 3c). In the analysis of the inflammatory profile, repair was noted after 15 days, with the onset of mild chronic inflammation and no indication of oedema, and the identification of small numbers of multinucleated giant cells, indicating degradation of the collagen scaffold (Figure 3h). In addition, a few thin blood vessels were observed (Figures 3b, 3c, 3d, 3h, 3g).

Figure 3 — *Photomicrographs of GMC.*





Caption: *Figure 3a: Mineralisation nuclei (MN) in the centre of the defect, connective tissue (CT) along the bone defect and around the MN, presence of fibroblasts (FB), osteoblasts (OB), biomaterial (BIO) integrated into the MN. Figure 3b: MN in the centre of the defect, OB surrounding the MN, presence of FB, blood vessels (BV) inside the defect. Figure 3c: MN in the centre of the defect, CT along the bone defect, presence of FB, VS in the centre, and BIO. Figure 3d: Osteocyte lacunae (OL) with osteocyte and osteocyte processes (OP), osteocyte extensions (OE), presence of BV and newly formed bone matrix (NBM). Figure 3e: Presence of osteocyte (OC), CT, and NBM. Figure 3f: OL with osteocyte and NBM. Figure 3g: Presence of OP, OL, and BV in the centre of NBM, presence of CT. Figure 3h: BV inside the defect, MN, giant cells (GC) in contact with BIO, OC along the defect.*

Source: own authorship (2025)

DISCUSSION

Several factors are essential for effective bone regeneration. Among these is angiogenesis, an essential mechanism that enables the supply of oxygen, nutrients, growth factors, minerals, and osteoprogenitor cells through the formation of new blood vessels[12,13,18]. Angiogenesis is dependent on the activity of macrophages, which act as key cells in modulating the regenerative microenvironment, as these phagocytes not only participate in the degradation of the scaffold, but also stimulate the formation of new blood vessels through the release of proangiogenic mediators, such as VEGF[12,13,18], although this molecule was not evaluated in the present study. Thus, the formation of new blood vessels begins with the inflammatory response caused by surgical trauma and the implantation of the biomaterial, which causes additional inflammation and may affect the dynamics of repair[30]. In this study, small blood vessels were observed in proximity to the biomaterial. The presence of giant cells, also close to blood vessels, may be related to the onset of mild and controlled chronic inflammation, which justifies initial angiogenesis.

Another essential factor in bone regeneration is the biomaterial's biocompatibilit[14,31]. The collagen scaffold showed biocompatibility, evidenced by the integration of the biomaterial into the bone tissue and the slight presence of foreign body reactions[31], as it did not induce side effects or adverse effects[32,33], nor did it stimulate an exaggerated inflammatory response[12,15], thus also favouring bone regeneration.

Similarly, the structure of the biomaterial is also important for regenerative success, since some collagen scaffolds may have ideal porosity for the onset of bone regeneration, as the pores allow the diffusion of fluids, oxygen, nutrients, and bioactive substances, which are important for cell growth and proliferation[34,35]. In this study, in areas adjacent to the biomaterial, the presence of immature connective tissue, associated with cellular and vascular components, indicated the establishment of an environment favourable to bone tissue regeneration.

One of the main challenges associated with the use of collagen scaffolds is matching the rate of collagen matrix resorption to the time required for bone regeneration, so that collagen is gradually degraded and

biointegrated as new bone tissue forms[14,31,34]. Although non-crosslinked collagen scaffolds of porcine origin have lower mechanical strength[35], they have a greater capacity to stimulate the formation of new blood vessels due to the triple helix organisation of their collagen fibres, which can consequently promote bone regeneration[12]. This study demonstrated that the collagen scaffold acted as an effective temporary framework, with biomimicry by integrating with bone tissue throughout bone regeneration[12,30.] Thus, it was possible to observe the formation of mineralisation nuclei, possibly due to the proliferation of blood vessels and the permeation of cells in the interconnected pores of the biomaterial, independent of bone regeneration from the edges of the defect[10,17,36].

The inflammatory response is also crucial for effective bone regeneration. Macrophages and neutrophils interact with proteins present on the surface of the biomaterial and direct an inflammatory response pattern through the release of cytokines. Macrophages are cells that play an important regulatory role and deserve special mention, as they can present as the pro-inflammatory M1 phenotype and the anti-inflammatory M2 phenotype[13,31].

Giant cells (GC) are formed by the fusion of several macrophages and participate in the degradation of implanted biomaterials that are more resistant or larger. In this study, they demonstrated a foreign body cell pattern and observed it adjacent to the biomaterial, but in small quantities[13,31]. After implantation of the collagen-based biomaterial, M1 cells change to M2 cells within 7 to 14 days[13]. Like macrophages, these cells also have pro-inflammatory and anti-inflammatory phenotypes, respectively, and thus influence the regenerative response of bone tissue[13,31].

The degradation period of collagen scaffolds can vary from 1 to 4 weeks[37]. This mechanism occurs due to the action of several factors, among which the action of macrophages and multinucleated giant cells stands out, which are responsible for completely degrading the collagen scaffold within a period of 30 to 60 days[30]. However, another study identifies the presence of collagen scaffold structures in the defect region where the biomaterial was implanted over a period of 120 days[10]. Such differences, and therefore the behaviour of collagen scaffolds, such as degradation time, may be related to the origin of the collagen and its degree of cross-linking, since the use of a membrane derived from bovine cortical bone was observed in the first study, and fresh bovine pericardium subjected to selective hydrolysis, cross-linked and non-cross-linked, in the second study.

The results observed in the present study reinforce the role of collagen scaffolds as versatile matrices for bone regeneration. Their ability to integrate with bone tissue, as well as the controlled inflammatory response after implantation[13,30,31], the presence of bone tissue with osteoblasts in proximity to the biomaterial, the arrangement of osteocytes in the newly formed bone matrix, vascular neoformation, and the findings of mineralization nuclei along the defect, as reported by other authors[10,12,17,30,36], validate that this biomaterial has the potential to aid in the regeneration of this tissue.

This research is an early-stage study, but promising results have been observed. However, the study must be continued to further highlight the potential of this work and yield even more favourable results when observed at later biological stages.

CONCLUSION

The collagen scaffold used in this study proved to be biocompatible and osteoconductive, and demonstrated osteogenic potential through the formation of mineralisation nuclei and biomimicry of bone tissue. However, further investigations, with analyses of later biological stages, are necessary to confirm the role of the collagen scaffold as a strategy for bone regeneration. Based on the results presented, the use of the collagen scaffold and its relevance for future clinical applications for regenerative purposes and treatment of critical-sized bone defects was demonstrated.

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