Does the Size of Nanohydroxyapatite Associated With Anionic Collagen Scaffolds Interferes With Osteoblasts Bioactivity?

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Research Article

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Abstract

Objectives

We aimed to evaluate the effect of nanohydroxyapatite morphology and its interaction with anionic collagen on osteoblast activity.

Materials and Methods

Murine osteoblasts were incubated with a commercial collagen scaffold (as a control) or collagen-nanohydroxyapatite scaffolds (Col-HANP) for 24 and 48 hours for viability and proliferation assessments by MTT and Ki67 immunofluorescence, respectively. The hydroxyapatite nanoparticles were synthesized in three different morphologies/sizes (labeled as Col-HANP 0h, as Col-HANP 2h, and as Col-HANP 5h) as a function of the hydrothermal synthetic approach. Osteoblast's activity was investigated by bone alkaline phosphatase activity (ALP) and Von Kossa mineralization assays. For biocompatibility evaluation, the scaffolds were implanted subcutaneously in the dorsum of male Wistar rats for 7 and 15 days.

Results

The incubation of cells with Col-HANP 5h for 48h resulted in a significant increase in their proliferation and activity. The implantation of Col-HANP 5h in the subcutaneous tissue presented decreased recruitment of inflammatory cells and IL-1β levels on day 7, as well as an increase in collagen synthesis on day 15 compared to collagen and control groups.

Conclusions

The significant effects on osteoblasts proliferation and activity illustrate the potential application of Col-HANP 5h scaffold as a promising strategy for bone tissue engineering.

Introduction

Several diseases can cause bone defects, such as traumas, tumors, avascular necrosis, and/or infections. Most bone defects undergo spontaneous repair. Bone defects more significant than 2 cm, however, cannot heal spontaneously [1] and require further surgical intervention, such as the use of a biocompatible scaffold that allows and stimulates the attachment and proliferation of osteoinductive cells on its surfaces [2, 3, 4]. The principles for guided bone regeneration are based on creating and maintaining a protected and cell-friendly environment, allowing viable cells to proliferate [2].
Autologous bone graft is the golden standard for treating bone defects [5]. However, complications of bone grafting are significant, mainly at the donor site. Therefore, the replacement for autogenous bone is the main direction of bone tissue engineering.

Bioabsorbable scaffolds are engineered to present ideal characteristics such as biocompatibility, nontoxicity properties, osteoconductivity, and adequate porosity to allow fluid perfusion and cell colonization [6]. In addition, biodegradability is desirable for programmed safe substitution of the scaffold material with osteoid deposition [7]. The reabsorption of these scaffolds occurs by cellular mechanisms, through enzymatic reactions, or by phagocytosis [8].

Studies have shown that hydroxyapatite bone graft substitutes function as well as bone allografts when considering the long-term clinical outcome, confirming their value as a biomaterial in bone replacement [9]. It has been reported that the association of hydroxyapatite nanoparticles with collagen-based biomaterials enhances their biomechanics and bioactivity [10], providing adequate support for osteogenic cell adhesion and proliferation [11]. Nano-sized hydroxyapatite has been widely used for bone regeneration due to its biocompatibility and osteoinductive properties [12, 13]. These properties are probably due to characteristics likely associated with significant physicochemical similarity with the mineral constituents of human bones [12, 13]. It has been shown that the size and shape of hydroxyapatite nanoparticles can affect the bioactive capacity of hydroxyapatite, where the nanoparticles with nanometric sizes have better biological activity [14]. The nanometric hydroxyapatite can bind along the collagen molecules forming fibers and fibrils [15]. Collagen membranes associated with nanohydroxyapatite accelerated bone healing in critical defects created in rat calvaria and did not induce inflammation [16]. In the last decade, several studies have been dedicated to incorporating nanoparticles in different biomaterials to improve their physical-chemical properties [17–19]. In fact, when HANP is associated with nanoparticles, it presents greater bio-reactivity due to its similarity with the natural hydroxyapatite found in hard human tissues. In addition, nanoparticles have greater sintering, densification, and tensile strength [20]. These characteristics are determined by their crystallite morphology and size. The synthesis of hydroxyapatite through hydrothermal treatment is a controlled method to achieve these desirable characteristics [21].

Anionic Collagen scaffolds are negatively charged at physiological pH due to the selective hydrolysis of amino acid groups residues asparagine and glutamine present in tropocollagen chains, generating new calcium-binding sites [22]. The mineralized collagen shows better biocompatibility with animal and human tissues when compared with collagen sponges without HANP treatment [16, 23]. Depending on the size, hydroxyapatite may have different morphology and bioactivity. HANP 0h is amorphous and showed the poorest bioactivity results. However, HANP 2h and HANP 5h showed better bioactivity, as they have a nanorod morphology that is similar to that of natural human bone. Furthermore, it is known that one of the cell adhesion mechanisms on hydroxyapatite surfaces is due to its size and porosity. Therefore, this study aimed to investigate the bioactivity and biocompatibility of nano-hydroxyapatite anionic collagen sponges through in vitro and in vivo studies, respectively.
Materials And Methods

2.1 Preparation of HANP

The synthesis of hydroxyapatite nanoparticles was carried out according to our previously reported procedure [20, 21] (Fig. 1). Briefly, 0.3 mol L$^{-1}$ H$_3$PO$_4$ was stirred at room temperature with 0.5 mol L$^{-1}$ of CaCl$_2$·H$_2$O (99.67% purity - Quimex, Dinamica, São Paulo, Brazil) with a Ca/P molar ratio of 1.67. A white precipitate was obtained by the addition of 30% NH$_4$OH solution (99.5% purity - Vetec, São Paulo, Brazil) until reaching pH 9. The white precipitate (labeled as HANP-0h) was washed with distilled water and vacuum filtered. This powder was dispersed in NH$_4$Cl solution (0.1 mol L$^{-1}$ with 99.5% purity - Vetec). The weight ratio of the reaction mixture was 1:10. This suspension was heated hydrothermally in a Teflon autoclave reactor at 150°C for: 2 hours (HANP 2h) and 5 hours (HANP 5h). The sample was finally vacuum filtered, washed and dried at 80°C for 4 hours and stored in a desiccator. The synthesis followed the equation:

$$10\text{CaCl}_2 + 6\text{H}_3\text{PO}_4 + 2\text{NH}_4\text{OH} \rightarrow \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 + 18\text{HCl} + 2\text{NH}_4\text{Cl}$$ (1)

Figure 1: Scheme for the synthesis of the HANP, sponge collagen preparation and the final material (composite).

2.2 Anionic Collagen and Sponge Preparation

The collagen was prepared by solubilization of collagen from bovine serosa after 72 h of treatment under alkaline conditions in presence of salts, followed by homogenization in acetic acid solution, at pH 3.5. The samples were dialyzed against acetic acid solution, at pH 3.5, and brought to a final concentration of 1%, determined by hydroxyproline. The collagen sponge was prepared by equilibrating the soluble collagen in phosphate buffer, 0.13 mol L$^{-1}$, pH 7.4, for 120 h, followed by centrifugation (10 000 rpm, 1h). The resulting gel was poured on the acrylic mold and then was freeze-dried (Fig. 1).

2.3-The composite preparation

The composite sponge (or scaffold) was cast in acrylic molds from a collagen sponge, which is soaked in a saturated solution of hydroxyapatite, and dried by lyophilization (Fig. 1). Prepared anionic collagen-based sponges were soaked in hydroxyapatite suspensions to obtain a concentration of 30% (w/w). After cooling for 24 h at approximately −4 °C, the samples were freeze-dried at −45°C. Samples receive nomenclature according to the hydrothermal treatment time that hydroxyapatite nanoparticles were subjected to a temperature of 150°C: for 0 hours (Col-HANP 0h), 2 hours (Col-HANP 2h) or 5 hours (Col-HANP 5h).

2.4. In vitro Studies

2.4.1. Culture of Osteoblasts (OFCOL II cells)
Murine osteoblast OFCOL II cells were obtained from Rio de Janeiro Cell Bank, which is managed by Paul Ehrlich Technical and Scientific Association, and cultured in DMEM-high glucose (Gibco) supplemented with 10% fetal bovine serum and 1% antibiotics (penicillin/Streptomycin) (Gibco) at 37°C with 5% CO₂ in an incubator. The culture medium was replaced every 2 days. Cells were seeded into collagen nanohydroxyapatite scaffolds: Col-HANP 0h, Col-HANP 2h and Col-HANP 5h. The control groups were: 1. DMEM: cells seeded directly in the well plates (without any scaffold) and 2. Col: cells seeded into non-mineralized polyanionic collagen scaffolds. All samples were analyzed in triplicates.

2.4.2. Cell viability assay (MTT)

The MTT assay was used to assess cell viability by evaluating the ability of live osteoblastic cells to reduce 3-(4,5-dimethyl-2-thiazole)-2,5-diphenyl-2-yl-tetrazolium bromide (Sigma-Aldrich) and form insoluble violet formazan crystals. The osteoblasts (3x10⁵ cells/well) were seeded into the scaffolds in 96-well plates and incubated for 24 and 48h. After 24 and 48h, a 10% MTT solution (5 mg/mL) diluted in PBS was added to each well. The cultures were incubated for 3 hours at 37°C and protected from light until the presence of the violet formazan crystals was observed. For formazan crystal solubilization, 100 µL of DMSO was added to each well. The absorbance at a wavelength of 570 nm was then measured as previously described [24]. The results were expressed as the percentage of cell viability relative to the control, which viability is 100%.

2.4.3. Immunofluorescence protocol

The osteoblasts (3x10⁵ cells/well) were seeded directly into the scaffold and placed in a 24-well cell culture plate. After 24 and 48 h, the cells were fixed with 500µL of 4% paraformaldehyde for 30 minutes, permeabilized, and blocked with a blocking solution (0.25% Triton X-100 and 1% BSA in PBS) for 1 h at room temperature. Then, the cells were incubated with the Ki67 antibody (1:200 Abcan) overnight at 4 ºC and the secondary antibody for 1h at room temperature. The cell nuclei were stained with DAPI (1: 2000, diluted in 1 x PBS (Invitrogen, California, USA) for 5 min. The images were acquired using a confocal microscope (LM710-Confocal-Zeiss) under selective filters for each fluorophore (200X magnification). Ten fields were acquired per slide, with 4 slides per group. The quantification was performed using Adobe Photoshop software, as previously described [25].

2.4.4. Alkaline phosphatase activity assay

Alkaline phosphatase (ALP) assay was performed to evaluate the activity of osteoblasts. The osteoblasts (3x10⁵ cells/well) were seeded directly into the scaffold and placed in a 24-well cell culture plate. After 1, 2, 5, and 7 days of incubation, the supernatant of the cells was assayed for ALP activity according to the manufacturer's laboratory (LABTEST®, Minas Gerais, Brazil). One hundred µL of the supernatant were heated at 56°C for 10 min and immediately cooled in ice. The non-bone alkaline phosphatase activity (thermostable) was determined directly on the spectrophotometer at a temperature of 30°C with absorbance readings at 405 nm, with p-nitrophenyl phosphate as a substrate. Bone fraction, in turn, was indirectly determined by subtracting the activity obtained from thermostable alkaline phosphatase from
total alkaline phosphatase [26]. The results are expressed as mean ± SEM of the ALP activity per group (U/L).

### 2.4.5. Von Kossa staining

Osteoblasts (1x10³ cells/well) were seeded directly into a scaffold placed in a 12-well cell culture plate. After 21 days of incubation, the cells were fixed with 100% ethanol (1 mL) for 30 min, rehydrated in decreasing concentrations of ethanol (100–50%, for 5 min each), and washed with distilled water twice. Then 1 mL of 5% silver nitrate solution was added to each well, and the cells were incubated in the UV light for approximately 1 h. After this period, the silver nitrate solution was removed and washed with distilled water. Each well was stained with 1 mL of thiosulfuric acid solution and warfain for 5 min. The quantitative estimation of the area of von Kossa-stained nodules was determined from digital images, acquired through the inverted microscope, of at least four different spots of each well (from three wells per group), on 40X magnification using ImageJ software (version 1.32j) as previously described [27]. The data are presented as a percentage of the area of von Kossa-stained nodules in relation to the total area of each group [27].

### 2.4.6 SEM Analysis

Osteoblasts (3x10⁴ cells/well) were seeded directly into a scaffold placed in a 12-well cell culture plate. After 1 and 7 days exposition to the scaffolds, the culture medium was removed and the cells were washed with PBS to remove unbounded cells. Subsequently, cells were fixed with 2.5% glutaraldehyde solution buffered with 0.2 M cacodylate buffer (pH 7.4) overnight at room temperature and gradually dehydrated in the ethanol/distilled water mixture graded from 50–100% ethanol for 10 min each. Then, the samples were dried, placed on carbon strips and metallized with 30 nm thick gold. The surface analysis was performed in Quanta 450 FEG - FEI Electronic Scanning Microscope with a voltage of 20 kV. The adhesion of the osteoblasts to the scaffolds was evaluated based on the cell morphology.

### 2.5. In vivo studies

#### 2.5.1. Animals

All experimental protocols were approved by the Federal University of Ceará Committee on the Ethical Treatment of Research Animals (Protocol nº 25/2017) and performed in accordance with the ARRIVE ethical guidelines. Thirty Wistar rats (weighing 180–200 g), were housed in temperature-controlled rooms and received water and food *ad libitum*. The animals were divided in three experimental groups of 6 animals per group, according to the observation time (1, 7, or 15 days). Horizontal incisions of approximately 1 cm were made at 3 locations on the dorsal surface in the lateral-lateral direction under anesthesia with ketamine (60 mg/kg; i.p) and xylazine (5 mg/kg; i.p). With blunt surgical scissors penetrating through the incision in a caudal direction, the subcutaneous tissue was divulsed to allow the placement of scaffolds (0.5 x 0.5 cm) soaked in isotonic 0.9% sodium chloride solution. The following scaffolds were implanted in rat subcutaneous tissue: a commercial collagen membrane (Col), Col-HANP 0h and Col-HANP 5h. the point where the scaffolds (5x5 mm) were positioned. An interrupted suture was
performed (silk suture 4–0), followed by asepsis with iodate alcohol. The animals were euthanized 1, 7, and 15 days after surgery.

### 2.5.2. Histopathological analysis

The scaffolds and the surrounding skin tissues were removed for histopathological analysis. The specimens were fixed in 10% buffered formalin for 24 h, dehydrated in ethanol, cleared with xylene, and embedded in paraffin. Sections of 4 µm thickness were obtained for hematoxylin and eosin (H&E) staining to evaluate, by light microscopy, inflammatory parameters, such as inflammatory cells infiltration and Masson's trichrome staining (ready-to-use kit HistokitTM-EasyPath) to evaluate the effect of scaffolds on collagen deposition, following the manufacturer’s instructions and as previously described [28]. The quantification of the collagen deposition was performed as previously reported [29].

### 2.5.3. Myeloperoxidase (MPO) activity

Myeloperoxidase (MPO) is an enzyme present in azurophilic granules of neutrophils. In this study, MPO concentrations were used to evaluate neutrophil recruitment into the skin tissues 1 and 7 days after the scaffolds (Col-HANP, collagen, and control scaffolds) implantation. Briefly, the samples were collected and placed in potassium phosphate buffer with 0.5% hexadecyltrimethylammonium bromide (pH 6.0, 50 mg tissue per ml), homogenized and centrifuged (5000 rpm, 4°C, 2 min). The supernatants were transferred to 1.5 mL and centrifuged (10 min) to remove contaminants. Then, 10 µL of the supernatant and 200 µL of the O-dianisidine solution (5 mg of O-dianisidine, 15 µL of 1% H₂O₂, 3 mL of NaPO₄ buffer, 27 mL of distilled H₂O₂) was added in 96-well plates. The absorbance was measured at 460 nm at two different times (t₀ = 0 min and t₁ = 1 min). The change in absorbance was obtained, plotted on a standard curve of neutrophils, and the values obtained were expressed as U MPO/mg tissue (MPO activity) [30].

### 2.5.4. Cytokine dosage

The scaffolds and the surrounding skin tissues were removed 1 and 7 days after the scaffold’s implantation for quantification of cytokine levels. Samples were collected and homogenized in RIPA lysis buffer (25 mmol / L Tris-HCl, pH 7.6; 150 mmol / L NaCl; 5 mmol / L EDTA; 1% NP40; 1% Triton X-100; 1% sodium deoxycholate; 0.1% SDS) to evaluate tumor necrosis factor-alpha (TNF-α) and interleukin 1 beta (IL-1β) concentrations using a commercial ELISA kit (R&D Systems) according to manufacturer’s instructions. The total protein quantification in each sample was obtained by bicinchoninic acid protein assay (Thermo Fisher), following the manufacturer’s instructions. The results are expressed as picograms per mg of protein, as previously described [31].

### 2.5.5. Immunohistochemistry for IL-1β

Immunohistochemistry for IL-1β was performed using the streptavidin-biotin-peroxidase method [32] in the surrounding skin tissues, removed 7 days after the scaffold implantation. Samples were fixed in 10% neutral buffered formalin for 24 hours, dehydrated, and embedded in paraffin. Sections (4 µm thick) were deparaffinized, and antigens were recovered by incubating the slides in citrate buffer (pH 6.0) for 20 min
at 95°C. Endogenous peroxidase was blocked with 3% H$_2$O$_2$ for 10 min to reduce nonspecific binding. Sections were then incubated overnight at 4°C with a goat polyclonal antibody against IL-1β (1:200, Santa Cruz, Texas, USA) and a biotinylated secondary antibody (1:400, Santa Cruz, Texas, USA) for 1h at room temperature. Afterward, sections were washed and incubated for 30 min with streptavidin-conjugated peroxidase complex (ABC Santa Cruz complex). Antibody binding sites were visualized by incubating the samples with diaminobenzidine–H$_2$O$_2$ (DAB, DAKO, California, USA) solution. Sections incubated with antibody diluent, without the primary antibody were used as negative controls. Four slides for each group were processed. For each slide, IL-1β positive cells were counted in five different fields, around the scaffolds, at 400X magnification. The results were expressed as the average of cells per group.

2.6 Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM). Student’s t-test, one or two-way Analysis of Variance (ANOVA) followed by the Bonferroni test, was used to compare means. $P < 0.05$ was considered significant.

Results

3.1 Collagen-hydroxyapatite nanoparticle scaffolds submitted to hydrothermal treatment (Col-HANP) increase viability and proliferation in osteoblasts in vitro

To investigate whether the size/morphology of the hydroxyapatite nanoparticles in the Collagen-hydroxyapatite scaffold could affect osteoblasts viability, we performed MTT assay. All the evaluated scaffolds increased osteoblasts viability compared to cells incubated only with supplemented DMEM medium at 24h incubation (Fig. 2A). Whereas Col-HANP 5h increased ($p < 0.05$) the viability of the osteoblasts compared to DMEM and Collagen scaffold group at 24h incubation, persisting at 48h (Fig. 2A).

Given that increased cell viability can be related to cell proliferation, we evaluated whether the HANP could stimulate osteoblasts proliferation by immunocytochemistry for Ki67, a proliferation marker. We found that the collagen-hydroxyapatite nanoparticle scaffold in a manner Hydrothermal treatment independently increased ($p < 0.05$) the number of i67 positive osteoblasts compared to DMEM and Collagen scaffold group at 24h incubation (Fig. 2B and 2C). However, at 48h incubation, only Col-HANP5h increased ($p < 0.05$) the osteoblasts proliferation compared to DMEM and Collagen scaffold group (Fig. 2B and 2C).

3.2 Collagen-hydroxyapatite nanoparticle scaffold submitted to hydrothermal treatment (Col-HANP) increases osteoblasts activity in vitro
The metalloenzyme known as bone alkaline phosphatase (BAP) is a good indicator of bone synthesis and is high in areas of extracellular matrix mineralization. Given that the levels of BAP from osteoblasts supernatant are frequently measured as an osteoblast activity marker, we evaluated whether Col-HANPs could stimulate osteoblasts to produce and release BAP by using a colorimetric assay after 1, 2, 5, and 7 days of exposition to the scaffolds. We found that only Col-HANP 5h could increase BAP levels persistently compared to DMEM and Collagen scaffold group at 2, 5, and 7 days incubation (Fig. 3A).

Next, we assessed whether the Collagen-hydroxyapatite nanoparticle scaffold submitted to hydrothermal (Col-HANP) treatment could promote mineralization staining osteoblasts by using von Kossa stain after 21 days of exposition to the scaffolds. According to Fig. 3B, Col-HANP2h (Fig. 3F) and Col-HANP5h (Fig. 3G) increased (p < 0.05) von Kossa staining in osteoblasts in vitro compared to DMEM (Fig. 3C) and Collagen scaffold (Fig. 3D) group.

### 3.3 Ultrastructural analysis of Col-HANP and increased extracellular matrix synthesis by osteoblasts exposed to this scaffold

We assessed the scaffold ultrastructure and composition by scanning electron microscope (SEM) and energy-dispersive X-ray spectroscopy. We found more significant calcium and phosphorus levels in Collagen-hydroxyapatite nanoparticle scaffolds by energy-dispersive X-ray spectroscopy (data not shown).

As shown in Fig. 4, the collagen scaffold exhibited interconnected pores and no crystals and calcium were found along the collagen fibers. Photomicrographs of Col-HANP0h, Col-HANP2h, and Col-HANP5h show a notable crystals amount adhered to the surface of the scaffold (Fig. 4). Col-HANP5h exhibited hexagonal crystals along the collagen fibers (Fig. 4).

**Figure 4-** Scanning electron microscope (SEM) micrographs and mineral composition of Collagen-hydroxyapatite nanoparticle scaffold submitted to hydrothermal treatment and collagen scaffolds. Ultrastructure images of (A) Collagen scaffold (Col), Collagen-hydroxyapatite nanoparticle scaffold submitted to hydrothermal treatment for 0 (B), 2 (C), and 5h (D). (E) Composition of hydroxyapatite crystals by energy-dispersive X-ray spectroscopy in Col-HANP.

After 7 days of incubation, we observed osteoblasts involved by crystals of calcium phosphate (Fig. 5D, 5F, and 5H). In addition, we found an intense synthesis of extracellular matrix in osteoblasts exposed to Col-HANP 5h (Fig. 5H). Taken together, Col-HANP5h demonstrated better results than Col-HANP0h and Col-HANP2h in stimulating osteoblasts proliferation and activity, as well as in promoting mineralization.

### 3.4 Col-HANP 5h causes less inflammation in rats' skin than commercials scaffolds
Next, the biocompatibility of Col-HANP 5h scaffolds in dermis tissue was assessed after 1, 7, and 15 days of implantation in rats.

After 24h (Day 1), we observed an intense inflammatory cell infiltration surrounding the control (Fig. 6B), collagen (Fig. 6C), and Col-HANP 5h (Fig. 6D) scaffolds. Notably, a decrease in inflammatory cell infiltration was observed around collagen (Figs. 6F and 6I) and Col-HANP 5h (Figs. 6G and 6J) compared to the control group (Figs. 6E and 6H) after 7 and 15 days of implantation.

To quantify the neutrophil infiltration, we measured the MPO levels in surrounding tissues after 24h (Day 1) and 7 days. A marked decrease in MPO concentrations was observed 1 and 7 days after surgery in the Collagen and Col-HANP 5h groups compared to the control group (p < 0.05, Fig. 6K).

To confirm inflammatory response after exposition to the scaffolds, we evaluated proinflammatory cytokines levels (TNF-α and IL-1β) by ELISA and analyzed IL-1β expression by immunohistochemistry. No significant differences among the groups were found regarding TNF-α concentration in surrounding skin tissues after 24h or 7 days of scaffold implantation (Fig. 7A). However, a reduction in IL-1β levels was observed in the tissues surrounding Col-HANP5h scaffolds compared to the control group (p < 0.05, Fig. 7B). In accordance, we found a notable decrease in the number of IL-1β positive cells around Col-HANP 5h (Fig. 7F) compared to control (Fig. 7D) and collagen groups (Fig. 7E). Quantitative analyses confirm the decreased (p < 0.05) number of IL-1β positive cells surrounding the Col-HANP5h scaffold compared to the control group (Fig. 7C).

### 3.5 Col-HANP5h stimulates collagen synthesis in rats' skin

As shown in our current study, Col-HANP 5h induced matrix extracellular synthesis in osteoblasts in vitro. Given that collagen is one of the main components of the dermis, we evaluated the collagen content by Masson’s trichrome stain after 15 days of implantation. We found an increased Masson’s trichrome staining in Col-HANP 5h group compared to the control group (p < 0.05, Fig. 8A-D).

**Discussion**

In this study, we proposed to evaluate the effect of the addition of hydroxyapatite nanoparticles with different sizes and morphology in osteoblast viability, proliferation, and activation (*in vitro*), and we also evaluated the biocompatibility (*in vivo*) of the scaffold, which showed the best results *in vitro* studies on rat skin tissues compared to a commercial anionic collagen scaffold.

Initially, we assessed the effect of the Collagen-hydroxyapatite nanoparticle scaffold submitted to Hydrothermal treatment (Col-HANPs) on osteoblasts. Col-HANP5h increased the number of viable cells and proliferation since day 1 exposition, whereas Collagen alone could not promote cell proliferation. Our findings can be explained by the fact that, such as bone matrix, the biomaterial used in this study has the same components, collagen and hydroxyapatite, which stimulate the adhesion and proliferation of osteoblasts, as already reported in other studies [33–36]. In addition, the incorporation of
nanohydroxyapatite to the Collagen scaffolds accelerates osteogenic differentiation when compared to the incorporation of macro-size hydroxyapatite to Collagen scaffolds or Collagen scaffolds only [33]. Therefore, the size of the hydroxyapatite particle influences the effect.

Interestingly, the scaffolds with collagen or nanomaterials that received hydrothermal treatment for a shorter time, zero and two hours, did not have the same effect as the one treated for a long time despite all having the same composition and amount of collagen and/or hydroxyapatite. A preliminary study of our group revealed that a higher time of hydrothermal treatment rebounds in the size and width of the nanoparticle, and consequently, the sticks obtained with nanohydroxyapatite have structures of crystalline hexagons closer to that of hydroxyapatite present in the bone. Thus, it seems that the time of hydrothermal treatment plays a crucial role in the proliferative effect of the collagen membranes incorporated with nanohydroxyapatite.

To investigate the influence of the scaffolds on osteoblasts activity, we evaluated ALP levels and the mineralized nodule formation in living osteoblastic cultures using the Von Kossa stain. ALP is an osteoblast-synthesized enzyme that is extremely important in the process of extracellular mineralization, releasing inorganic phosphate that enters into the cell and is stocked into vesicles when it is released, contributing to hydroxyapatite crystals formation [37–39]. Our findings showed that despite increased ALP levels found in all groups after 24h exposition, only the Col-HANP5h group maintained the high levels in a long-term exposition (Day 7). Therefore, we suggest that the morphology of hydroxyapatite interferes with the mineralization process. The ability of nanohydroxypeptides incorporated into collagen scaffolds to induce phosphatase activity increased the expression of alkaline phosphatase-related genes for up to 4 weeks [33]. Here, analysis of Von Kossa staining, a marker of calcium salt deposition, confirmed these findings. In this study, we observed that after 21 days, Col-HANP5h promoted an intense mineralization compared to the other scaffolds, as previously shown [40].

When we investigated the interaction of collagen fibers with hydroxyapatite nanoparticles by using the SEM technique, we found that the collagen sample exhibited interconnected pores of various sizes in a structure consisting of a randomly arranged array of fibers. However, no evidence of crystals along the fibers, as demonstrated by chemical analysis using EDS, was found in this sample, indicating that this specific scaffold is calcium-free and confirming, thus, the absence of hydroxyapatite. On the other hand, the sample had a phosphorus content of 8.84% P, indicating the presence of phosphate salts.

Micrographs of scaffolds Col-HANP5h and Col-HANP2h showed a remarkable distribution of embedded crystals on the surface of the samples. Values obtained by EDS for these specimens showed a Ca/P ratio close to that of hydroxyapatite (Ca / P = 1.67), indicating that the crystals in these samples are agglomerates of the hydroxyapatite nanoparticles. For Col-HANP5h scaffold, hexagonal crystals appear along the collagen fibers. Our data showed that these crystals are closely connected to the array lattice.

Considering the best results exhibited by Col-HANP5h in our in vitro studies, we performed the in vivo study to test its biocompatibility. A reduction in the inflammatory process was observed in the tissues surrounding Col-HANP5h on day 7 implantation compared to the control scaffold, showing even better
results than collagen scaffolds. The inflammatory response to collagen-based biomaterials has been demonstrated in other studies over decades [41–42]. The inflammatory reaction is essential in the repair process. Already studies concerning the toxicity of hydroxyapatite nanoparticles in vivo are scarce except for one study using fish and another work that evaluated the administration of this biomaterial orally for one year [43–44]. All other studies reveal the excellent biocompatibility of hydroxyapatite with soft tissues, such as skin, muscle, and gums, but also in the repair of hard tissues, for example, bone repair, as well as implant coating or filling as bone or teeth [45]. However, these data do not invalidate the relevance of our study when using collagen for the incorporation of nanohydroxyapatite because our scaffolds with and without nanoparticles did not generate superior or equal commercial influx but reduced the infiltration of neutrophils, as shown by a decrease in MPO activity, at sites adjacent to the membrane mainly on day 7 implantation. Of note, our data do not suggest an anti-inflammatory activity of scaffolds with and without nanoparticles, but that Col-HANP5h promoted a lower inflammatory potential, generating a lower tissue reaction when implanted subcutaneously compared to the commercial scaffold.

The data revealed scaffold insertion can trigger acute (Day 1) and chronic (Day 7) inflammation. IL-1β and TNF-α are cytokines that play an essential role in the development of tissue inflammation [46]. As noted here, TNF-α has been involved in acute and chronic inflammation triggered by the administration of scaffolds [47–49]. Collagen-derived proteins activate tissue-resident macrophages, which release TNF-α [50]. In addition, this cytokine when released, can induce the chemotaxis of inflammatory cells that are essential sources of cytokines [51]. In contrast to TNF-α, IL-1β is one of the leading promoters of pro-inflammatory response in an acute phase. Col-HANP5h group exhibited a significant reduction in IL-1β protein levels compared to the control scaffold in an acute and later exposition phase. Thus, the addition of hydroxyapatite nanoparticles did not increase cell migration or cytokines, in fact, it leads to a reduction in cell migration and IL-1β immunoexpression, decreasing both acute and chronic inflammatory response.

**Conclusion**

Based on our findings, we concluded that hydroxyapatite nanoparticles, besides being biocompatible, are potent activators of osteoblasts due to their morphology being similar to natural bone. Col-HANP may represent a promising strategy for bone tissue regeneration. However, further studies are needed to verify its activity in a model of critical bone defects in animals.

**Declarations**

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Figures

Figure 1

Scheme for the synthesis of the HANP, sponge collagen preparation and the final material (composite).
Figure 2

The effect of collagen-hydroxyapatite nanoparticle scaffolds submitted to hydrothermal treatment on osteoblasts viability and proliferation. Effect of Collagen-hydroxyapatite nanoparticle scaffold submitted to hydrothermal treatment (Col-HANP 0, 2 and 5h), collagen (Col) and control scaffold on (A) cell viability in osteoblasts and (B) percentage of Ki67 positive cells. (C) Representative photomicrographs of Ki67 (green) immunostaining and DAPI, a nuclear marker (blue) in osteoblasts after day 1 and day 2 exposed to control, Col and Col-HANP5h. Data are presented as the mean ± SEM (n = 6). * p < 0.05 versus DMEM group; # p < 0.05 versus Col group (Two-way ANOVA; Bonferroni test).
Figure 3

The effect of collagen-hydroxyapatite nanoparticle scaffold submitted to hydrothermal treatment on mineralization and alkaline phosphatase (ALP) concentrations. (A) Supernatant ALP activity was evaluated through a colorimetric assay after incubation of osteoblasts with DMEM, Col, and Col-HANP 0, 0.2, and 5h for 1, 2, 5, and 7 days; (B) Graph represents the mean ± SEM of the percentage area of von Kossa positive nodules after 21 days of incubation. (C) Representative images of nodular von Kossa
staining after 21 days of incubation of osteoblasts with DMEM, Col, and Col-HANP 0, 2, and 5h. * p < 0.05 versus DMEM group; # p < 0.05 versus Col group (ANOVA; Bonferroni test).

Figure 4

Scanning electron microscope (SEM) micrographs and mineral composition of Collagen-hydroxyapatite nanoparticle scaffold submitted to hydrothermal treatment and collagen scaffolds. Ultrastructure images of (A) Collagen scaffold (Col), Collagen-hydroxyapatite nanoparticle scaffold submitted to hydrothermal treatment for 0 (B), 2 (C), and 5h (D). (E) Composition of hydroxyapatite crystals by energy-dispersive X-ray spectroscopy in Col-HANP
Figure 5

Scanning electron microscope (SEM) micrographs of osteoblasts attached to Collagen-hydroxyapatite nanoparticle scaffold submitted to hydrothermal treatment and collagen scaffolds. Osteoblasts on collagen scaffolds (Col, A-B), Col-HANP0h (C-D), Col-HANP2h (E-F), and Col-HANP5h (G-H) after day 1-7 incubation.
Figure 6

Col-HANP5h promotes less neutrophil infiltration in rats’ skin than commercial scaffolds. (A) Schematic diagram of biocompatibility model. (B-J) H&E stain images showing the control and collagen scaffolds (Col), as Col-HANP 5h inserted in the skin after 1-15 days implantation. (K) The levels of MPO were evaluated through an o-dianosidine assay in skin tissues containing control and Col, as well as Col-HANP.
5h after 1-7 days incubation. * p < 0.05 versus CONTROL group and # p < 0.05 versus Col group; two-way ANOVA followed by Bonferroni test was used.

Figure 7

Col-HANP5h reduces levels of IL-1β, but no TNF-α, in rats’ skin. (A) Levels of TNF-α and (B) IL-1β performed by ELISA in skin tissues containing control and Col, as well as Col-HANP 5h after 1-7 days.
implantation.* \( p < 0.05 \) versus Control group and \# \( p < 0.05 \) versus Col group; two-way ANOVA followed by Bonferroni test was used. (C). The graph represents the percentage mean ± SEM of IL-1β positive cells number per field after 7 days of implantation of Control, Cols, and Col-HANP 5h. * \( p < 0.05 \) versus Control group and \# \( p < 0.05 \) versus Col group. One-way ANOVA followed by Bonferroni test. (C) Representative photomicrographs of IL-1β immunostaining after 7 days exposition to Control, Cols and Col-HANP 5h.

![Figure 8](https://example.com/graphicalabstract.tif)

Figure 8

**Col-HANP5h induces collagen synthesis in rats' skin.** (A) Representative photomicrographs of Masson's trichrome staining, showing collagen formation (blue) after 15 days exposition to control, Col and Col-HANP 5h. (B) The graph represents the percentage mean ± SEM collagen per field after 15 days of implantation of Control, Col, and Col-HANP 5h. * \( p < 0.05 \) versus Control group and \# \( p < 0.05 \) versus Col group. One-way ANOVA followed by Bonferroni.

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