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# Assessment of the regenerative potential of macro-porous chitosan-calcium simvastatin scaffolds on bone cells

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**Abstract:** This study evaluated the bioactive potential of a macro-porous chitosan scaffold incorporated with calcium hydroxide (CH-Ca) and functionalized with bioactive doses of simvastatin (SV) for bone tissue regeneration. Initially, the bioactive dose of SV in osteoblastic cells (SAOS-2) was determined. For the direct contact experiment, SAOS-2 cells were plated on scaffolds to assess cell viability and osteogenic differentiation. The second assay was performed at a distance using extracts from scaffolds incubated in culture medium to assess the effect of conditioned medium on viability and osteogenic differentiation. The initial screening showed that 1 μM SV presented the best biostimulating effects, and this dose was selected for incorporation into the CH-Ca and pure chitosan (CH) scaffolds. The cells remained viable throughout the direct contact experiment, with the greatest cell density in the CH-Ca and CH-Ca-SV scaffolds because of their higher porosity. The CH-Ca-SV scaffold showed the most intense bio-stimulating effect in assays in the presence and absence of osteogenic medium, leading to an increased deposition of mineralized matrix. There was an increase in the viability of cells exposed to the extracts for CH-Ca, CH-SV, and CH-Ca-SV during the one-day period. There was an increase in ALP activity in the CH-Ca and CH-Ca-SV; however, the CH-Ca-SV scaffold resulted in an intense increase in the deposition of mineralized nodules, approximately 56.4% at 7 days and 117% at 14 days, compared with CH (control). In conclusion, functionalization of the CH-Ca scaffold with SV promoted an increase in bioactivity, presenting a promising option for bone tissue regeneration.

**Keywords:** Chitosan; Simvastatin; Tissue Engineering.

## Introduction

Bone defects frequently occur as a result of trauma, tumors, bone infections, avascular necrosis, or inherited malformations. The high frequency of bone vulnerability to trauma and fractures has attracted extensive research in the field of tissue regeneration.[1] Any part of the

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missing bone must be replaced with a suitable, functional substitute, and this has been challenging because of the hierarchical and complex structure of the bone tissue that supports several mechanical, biological, and chemical functions.[1]

The natural bone healing process begins with an inflammation stage, starting immediately after the fracture and lasting up to several days. During this stage, the blood clot present in the fracture site creates a stable structure for new bone formation. Later, this clot is replaced by fibrous tissue and collagen, which are mineralized weeks after the fracture. Thus, bone remodeling can occur over several months. The most common approaches for the reconstruction of bone defects comprise the use of autografts, allografts, xenografts, and alloplastic substitutes. In some situations, these materials are associated with possible immunogenic rejection of the recipient patient, donor site with limited availability, or disease transmission.[2] Thus, the use of these grafts in large bone wounds remains challenging.[1]

Tissue engineering has introduced new therapeutic strategies for bone regeneration, involving the combination of three-dimensional (3D) scaffolds that provide a structured and temporary extracellular matrix model, and biofactors, which guide cell activity in the target tissue and promote bone regeneration[3] effectively. Scaffolds can be designed in terms of macro-, micro-, and nano-architecture; chemical composition; and mechanical performance to promote optimal cellular activity and ideal degradability.[3] This technology is based on the implantation of scaffolds in patients to induce tissue regeneration mediated by resident cells (cell-homing), or by incubating cell-seeded scaffolds in vitro to speed up tissue deposition in vivo (cell transplantation).[4]

In the cell-homing strategy, scaffolds must have an interconnected macro-porous structure and be associated with bioactive factors that promote the chemotaxis of resident cells, their osteogenic differentiation, and new tissue deposition. In our group's prior studies, a technology was developed to modulate the porosity of chitosan scaffolds based on the incorporation of calcium hydroxide ($Ca(OH)_2$) as a bioactive mineral phase, generating a chitosan-calcium scaffold (CH-Ca) containing a highly organized, interconnected macro-porous network, which positively influenced the infiltration and osteo/odontogenic differentiation of human dental pulp cells.[5] The incorporation of simvastatin (SV) as a bioactive molecule at predetermined doses substantially increased the potential of this scaffold as a cell-homing platform to modulate dentin regeneration.[6]

Several studies have demonstrated that when scaffolds are associated with statins, their capacity to promote bone tissue regeneration increases in vitro and in vivo.[7,8] This finding occurs because statins can induce the osteoblastic phenotype in mesenchymal stem cells (MSCs) from bone marrow,[9] as modulate inflammation and angiogenesis in several tissues.[10,11] Recent studies on this topic have concluded that the local administration of statins incorporated into biomaterials seems more reliable than systemic administration for bone regeneration. However, depending on the dose released, statins can accelerate or delay the formation of new mineralized tissue.[12] Thus, this study evaluated the bioactive potential of a CH-Ca scaffold incorporated with bioactive SV doses for bone tissue regeneration. We hypothesized that the chitosan-calcium structure associated with a low SV dose has a synergistic bioactive effect, favoring osteoblastic differentiation and the deposition of mineralized matrix in vitro.

## Methodology

### Screening for bioactive doses of SV

Initially, dose- and time-response assays of bioactive SV concentrations were performed at different pre-treatment times on osteoblastic lineage cells obtained from osteosarcoma (SAOS-2) to determine the biostimulation pattern of cells promoted by the drug. The cells were seeded into 96-well plates ($2\times10^3$ cells/well) for 24 h. Next, the culture medium was replaced with complete Dulbecco's modified eagle medium (DMEM) (supplemented with 15% fetal bovine serum (FBS), L-glutamine, and 1% penicillin-streptomycin—called regular medium; GIBCO Invitrogen, Thermo Fisher Scientific, Waltham, USA), supplemented with or

without SV at concentrations of 1, 0.1, and 0.01 μM for 24 h, 72 h, or 7 days. After the pre-treatment period (PT), the cells were incubated in culture medium without SV for 14 days. Cell viability was evaluated using MTT assay at 1, 7, and 14 days after PT (cells were incubated in regular medium), and calcium-rich matrix deposition (o-cresolphthalein complexone (OCC) method) was performed at 14 day period (cells grown in osteogenic medium—complete DMEM supplemented with 50 mg/mL ascorbic acid and 5 mm b-glycerophosphate (Sigma-Aldrich, San Luis, USA).

For MTT assay, the cells were incubated for 4 h with MTT solution (5 mg/mL; Sigma-Aldrich) in regular culture medium without fetal bovine serum (FBS) (1:10). Subsequently, the supernatant was discarded, and formazan crystals were dissolved in acidified isopropanol (Sigma-Aldrich). The absorbance was measured (570 nm; Synergy H1, BioTek, Winooski, USA), and the cell viability data were converted into/percentages, considering that the negative control group (CN; cells grown in the absence of SV) had 100% cell viability at each time-point (n = 6). For the OCC assay, cells were incubated overnight at 4°C in 50 μL of 1 N HCl (Sigma-Aldrich). Next, 100 μL of the working reagent containing the OCC substrate (Calcio Liquicolor, Centerkit, Campinas, Brazil) was added, and the samples were incubated at approximately 24°C for 10 min. The absorbance was read at 570 nm (Synergy H1), and the calcium concentration in the samples was determined using a standard curve. The mean values of the CN group were used as a parameter for 100% calcium deposition (n = 6) to calculate the percentage of calcium deposition for all experimental groups.

## Fabrication of scaffolds

The scaffolds were prepared as previously described (Soares et al., 2020). In brief, a 2% chitosan (CH) solution was prepared by dissolving chitosan powder (310,000–375,000 Da; 75%–85% deacetylated, pH 3.5, Sigma-Aldrich) in 2% aqueous solution of acetic acid (Sigma-Aldrich) for 24 h at room temperature and incorporated drop by drop with an aqueous suspension of $Ca(OH)_2$ at 1% w/v (pH 12.0, Sigma-Aldrich) at a proportion of 2:1 CH:$Ca(OH)_2$ (1,000–1,500 rpm), followed by gradual freezing (−20°C/−80°C/−198°C) and lyophilization (−56°C for 24 h; Liotop L101, Liobras, São Carlos, Brazil) (Figure 1). Cross-linking was performed by positioning the prepared scaffolds on a platform in a desiccator containing 10 mL 25% glutaraldehyde solution at the bottom (Sigma-Aldrich), followed by incubation under vacuum for 6 h to create glutaraldehyde vapor. The scaffolds were immersed in 70% alcohol under vacuum and washed in phosphate-buffered saline (PBS). The scaffolds were stored in a desiccator at room temperature for up to 7 days before performing the experiments. The architecture and chemical composition of the prepared scaffolds were confirmed using scanning electron microscopy (SEM) or energy dispersive spectroscopy (EDS) (JMS-6610V Scanning Microscope; JEOL, Tokyo, Japan) at an accelerating voltage of 12–15 kV on lyophilized samples placed on metallic stubs using carbon tape and sputter coated with gold.

For the incorporation of SV, the scaffolds were aseptically immersed and incubated at 37°C for 24 h in SV solution (proportion of 1.5 μL/$mm^2$), followed by a 10 min wash in PBS to eliminate the non-adsorbed drug. The concentration of 1 μM SV was selected based on the dose- and time-response tests. SV was incorporated immediately before initiating the experiments. The following experimental groups were established: chitosan scaffolds (CH) (considered as 100% control of cellular parameters), chitosan-calcium scaffolds (CH-Ca), chitosan-simvastatin scaffolds (CH-SV), and chitosan-calcium-simvastatin scaffolds (CH-Ca-SV).

## Direct contact biological experiment

Samples of scaffolds with a 6 mm diameter and 1 mm thickness were obtained. In a laminar flow chamber, the materials were positioned on 48-well plates, and a total of $1\times10^5$ SAOS-2 cells in a 5 μL drop were seeded, followed by 30 min incubation at 37°C and 5% $CO_2$ to allow the initial adhesion of cells on the biomaterial.[5] The constructs were then cultivated in 500 μL culture medium at 37°C and 5% $CO_2$. The viability test was performed at 1, 3, 7, and 14 days of cell culture in regular medium (n = 2) with a live/dead cell viability/cytotoxicity kit (Invitrogen), and the surface of the scaffolds was analyzed using a fluorescence

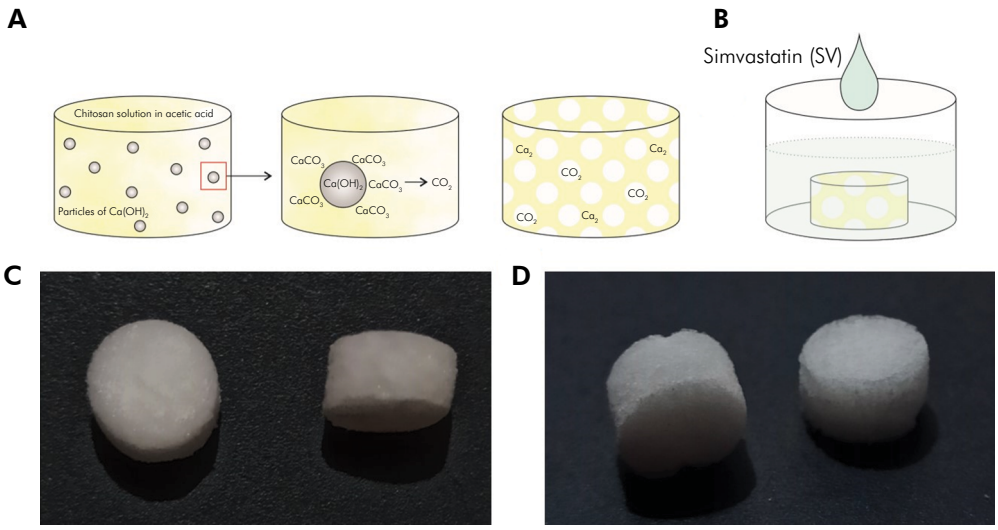



**Figure 1.** Manufacturing step of scaffolds. (a) Schematic representation of the manufacturing protocol of the CH-Ca Scaffold. $Ca(OH)_2$ particles are incorporated into the chitosan solution in acetic acid, generating their carbonation ($CaCO_3$) with a consequent release of $CO_2$, expanding the porous network and complexion of calcium to chitosan; (b) simvastatin (SV) is then incorporated by immersion into the pre-selected concentration in dose- and time-response tests; (c) and (d) are digital images of the CH and CH-Ca scaffolds, respectively, after lyophilization and cross-linking. The spongeous-like architecture obtained is displayed in the image.

microscope (Floidâ, Life Technologies, Carlsbad, USA).[7] Osteogenic differentiation was evaluated by ALP activity assay and deposition of mineralization nodules at 3, 7, and 14 days of cell culture (n = 6) in regular and osteogenic media, as described by Soares et al.[5] In brief, ALP activity was measured in constructs subjected to lysis by immersing the scaffolds in 500 µL 0.1% laurel sodium sulfate (Sigma-Aldrich) in a 1.5 mL centrifuge tube (Eppendorf, Hamburg, Germany) and subjecting them to maceration with a pistil containing a conic tip (Eppendorf). The supernatant was collected (4,000 rpm for 2 min) for reaction with the thymolphthalein monophosphate substrate (LabTest Diagnosis SA, Lagoa Santa, Brazil). Total protein in the same samples was determined using the Lowry assay for data normalization[5]. Mineralized matrix deposition (n = 6) was determined using the Alizarin red assay (Sigma-Aldrich) with cetylpyridinium chloride solution (for dissolution of nodules) followed by absorbance measurement (Synergy H1, Biotek). Scaffolds without cells were used to eliminate background (blank).[5]

## Assay with conditioned medium (extracts)

An assay was performed with extracts from biomaterials to evaluate the modulating effect of components released by the scaffolds. The SAOS-2 cells were cultured ($1\times10^4$ cells/well) in 96-well plates (Corning Inc., Corning, USA) and maintained until a confluency of 80% was attained (24 h). The scaffolds were immersed in 500 µL regular DMEM (no osteogenic supplement) and incubated at 37°C for 48 h. Subsequently, the culture medium in contact with the scaffold, called the extract, was collected and stored for application to cells. A new culture medium was added to the scaffold for the continualcollection of extracts every 48 h for 14 days. Next, 150 µL aliquots of the extracts collected every 48 h were applied to the previously seeded cells and incubated for 48 h. This process was repeated once per 48 h for 14 days. Thus, cells were incubated throughout the experimental period in culture medium containing products released from the scaffolds to evaluate the effect of sustained release on viability (MTT assay) and osteogenic differentiation (ALP activity and Alizarin Red) as described (except for the maceration/centrifugation step).

## Statistical analysis

Two independent experiments were performed, and the data were compiled for statistical analysis. For SV screening, data analysis was performed using

one-way analysis of variance (ANOVA) followed by Dunnett's test for comparisons among the experimental groups and the negative control. For experiments with scaffolds, one-way ANOVA was applied, followed by Tukey's test to conduct comparisons among all groups. In all analyses, a *p* value smaller than 0.05 was considered statistically significant.

## Results

### SV screening assay

According to the graphs shown in Figure 2a–c, the SV-tested doses had a negative effect on SAOS-2 viability in all study periods. Figure 2d shows the calcium deposition of cells at 14 days in the different PTs. All evaluated SV concentrations promoted a numerical increase in the values of calcium deposition relative to CN, in which the concentration of 1 μM showed significantly higher values for all treatment periods than others concentrations did ($p < 0.05$). Conversely, the concentration of 0.1 μM promoted a significant increase compared with the CN for PT 24 and 72 h ($p < 0.05$). The concentration of 0.01 μM showed no significant difference from CN ($p > 0.05$). Based on these results, 1 μM was selected for incorporation into the scaffolds.

### Scaffold characterization

Figure 3 presents representative SEM and EDS images of CH and CH-Ca surfaces. The CH scaffold presented a disorganized, porous structure with an irregular shape, whereas the CH-Ca scaffolds featured an organized pore network containing round pores and an interconnected network. A pore-inside-pore structure can be easily detected (white arrow in Figure 3b. EDS analysis demonstrated the presence of calcium only on the CH-Ca scaffold.

### Cell/scaffold constructs

The live or dead images demonstrated that cells remained viable throughout the experiment in all groups, with the presence of greater cell agglomerations in groups with the CH-Ca scaffold than in the group with the CH scaffold, especially at 14 days (Figure 4a). The cell biostimulation assays demonstrated that in the presence of osteogenic medium, the CH-Ca-SV group showed the highest ALP activity values at 3 and 7 days but the values displayed no significant difference with that of the CH-Ca group (Figure 4c) ($p > 0.05$). Notably, the bio-stimulating effect of the CH-Ca and CH-Ca-SV groups on ALP activity was observed even in the absence of osteogenic medium. This emphasizes that that the CH-Ca-SV group showed a significant increase in ALP activity compared with that of the CH group at 3 and 7 days , whereas for the CH-Ca group, this effect was observed at 7 days (Figure 4b). The Alizarin Red assay demonstrated that the CH-Ca, CH-SV, and CH-Ca-SV groups showed higher mineralized matrix values after 14 days than that displayed by the CH group, and the CH-Ca-SV group presented significantly higher values than the other experimental groups (Figure 4b and 4c).

### Extract assay

There was a significant increase in the viability of cells exposed to the extracts in the CH-Ca, CH-SV, and CH-Ca-SV groups after 1 days, and in subsequent periods, this effect was not observed (Figure 5a). An increase in ALP activity was observed in the CH-Ca and CH-Ca-SV groups compared with that of the CH group, with the highest values observed in the CH-Ca-SV group (Figure 5b). With respect to the deposition of mineralized nodules, this study demonstrated an improved biostimulating effect of the CH-Ca-SV group, with an approximate increase of 56.4% at 7 days and 117% at 14 days, compared to that of the CH group (Figure 5c). In the images of mineralized nodules, this group showed well-defined, large nodules after 14 days, which was not observed in the other groups (Figure 6).

## Discussion

This study proposes the development of macroporous chitosan scaffolds enriched with calcium hydroxide (CH-Ca) and SV (CH-Ca-SV) as potential biomaterials for bone regeneration. CH-Ca provides a structure containing a network of interconnected macropores alongside the complexation of calcium ions to the chemical structure of chitosan, which provides the release of calcium ions. This structure

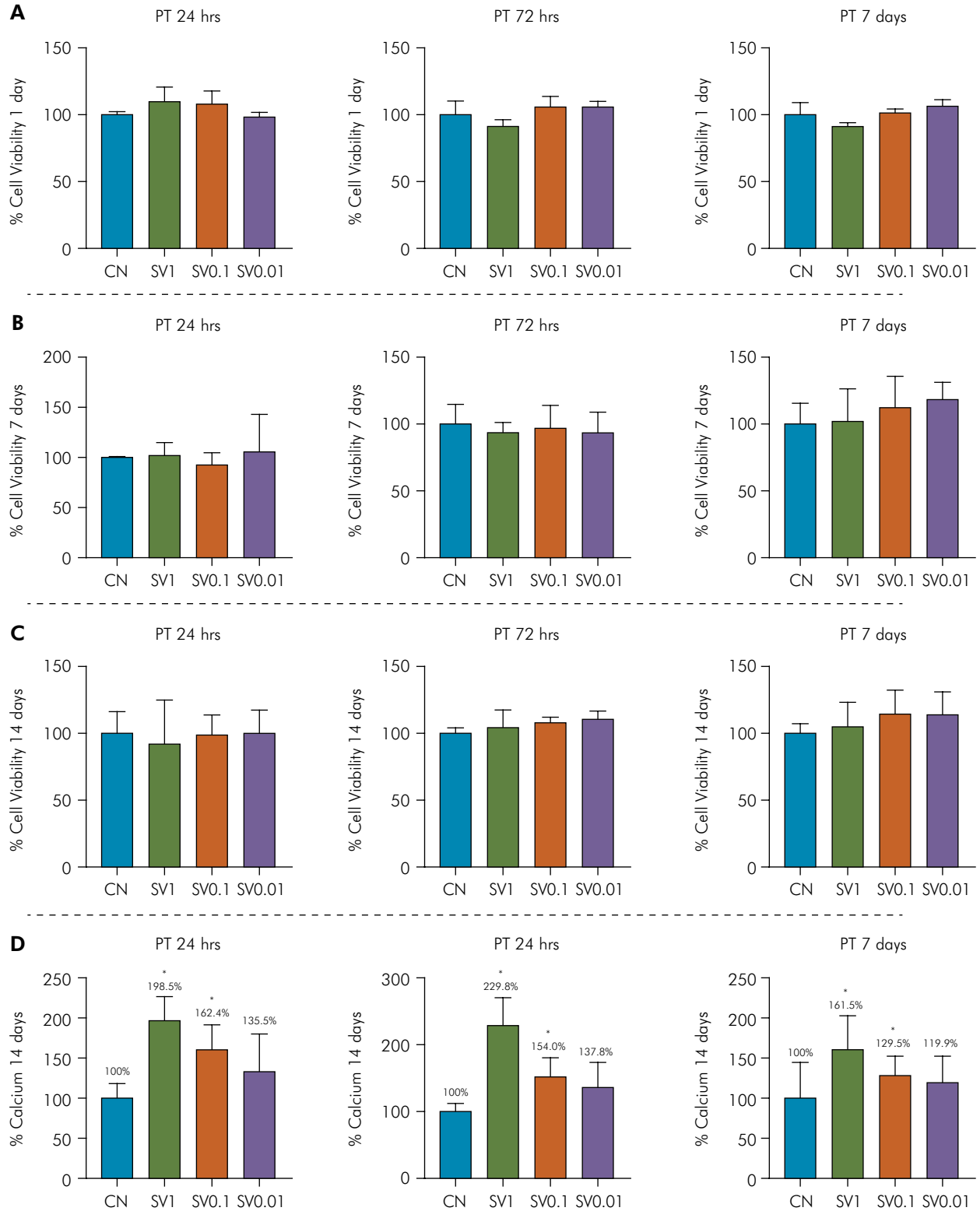



**Figure 2.** SV Screening Assay. Mean bar graph (standard deviation) of cell viability values at 1 (a), 7 (b), and 14 (c) days, and calcium deposition at 14 days (d), according to the experimental groups and pre-treatment periods (PT 24 h, 72 h, and 7 days). Values represent mean and * indicate statistically significant differences from the negative control group (CN) (one-way analysis of variance (ANOVA) /Dunnett's. n = 6, $p < 0.05$).

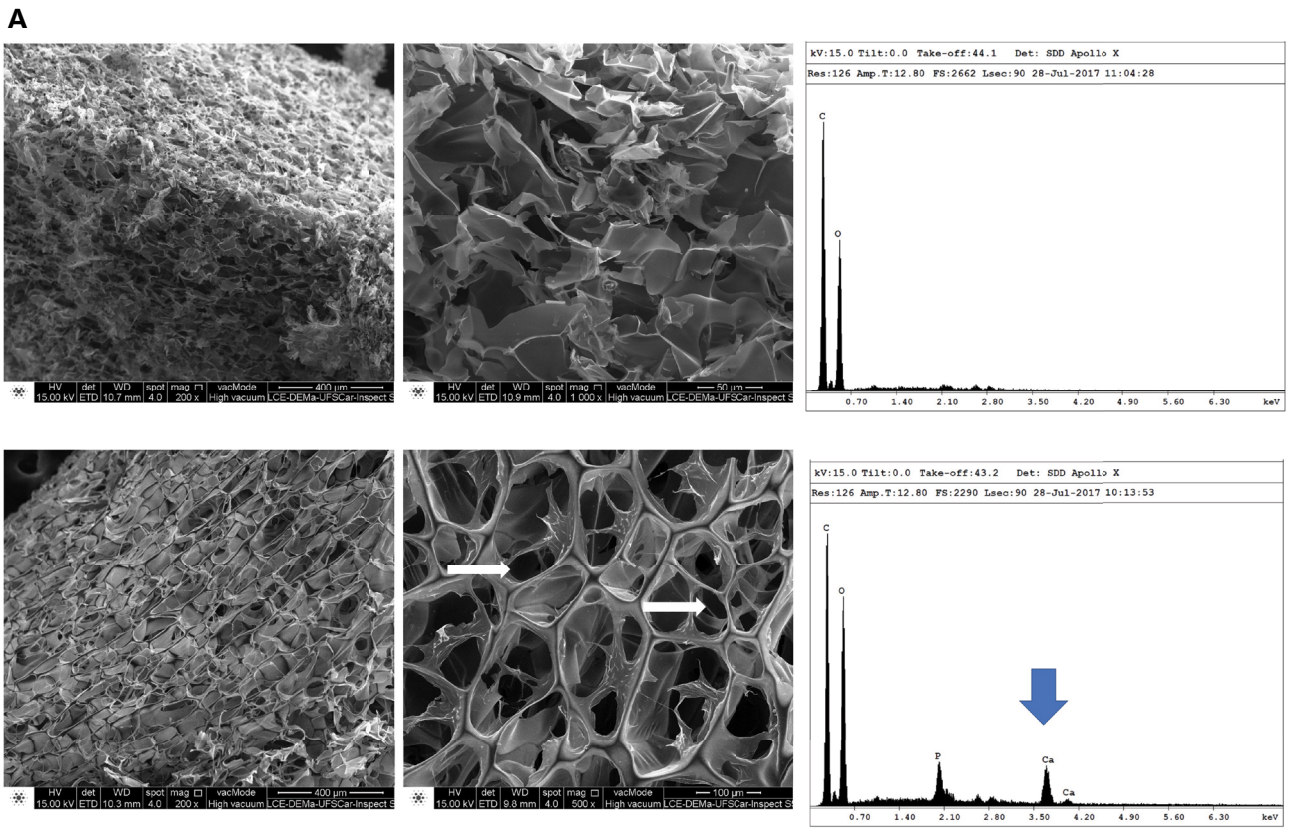



**Figure 3.** Representative images of scanning electron microscopy (SEM) or energy dispersive spectroscopy (EDS) analysis of the CH and CH-Ca scaffolds. The disorganized, porous architecture of the CH scaffold and the organized, interconnected porous network of the CH-Ca scaffold, with the presence of the Ca component identified using EDS is displayed. White arrows indicate pore-inside-pore on CH-Ca. The blue arrow indicates Ca identification on the EDS graph of CH-Ca.

has been proven to facilitate cell spread, proliferation, and mineralized matrix deposition by MSCs of dental origin.[5] In addition to evaluating the mineral phase, this study evaluated the incorporation of SV as a bioactive cue for CH and CH-Ca scaffolds to apply them as a cell-homing strategy for bone regeneration. SV is a statin that inhibits 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, an enzyme involved in cholesterol biosynthesis.[13] Thus, SV is widely used in cases of hypercholesterolemia to avoid the formation of mevalonic acid from HMG-CoA reductase.[13,14] However, researchers have reported the pleiotropic effects of this drug, such as anti-inflammatory action, angiogenesis induction, increased endothelial cell activity, and bio-stimulating effects on the collagen matrix formation and mineralization process, which are advantageous for tissue regeneration.[7,13-15] The literature has shown that SV can be easily incorporated by adsorption onto the surface of chitosan scaffolds by a simple, low-cost method, which provides a significant increase in bioactivity on cells of pulpal origin. However, the drug release pattern is fast, occurring within the first 24 h after immersion in a humid medium.[6] This finding occurs because of the lipophilicity of SV, resulting in its dissolution and progressive diffusion on scaffold surfaces immersed in a wet environment.[16]

In this study, we initially aimed to determine the ideal standard for drug release from biomaterials to optimize the process of new tissue deposition. For this reason, bioactive doses of SV (1, 0.1, and 0.01 μM) were screened to assess their effects on the viability and calcium deposition capacity in SAOS-2 cells. This cell line was chosen because our main goal was to develop a cell-free scaffold capable of stimulating resident osteoblasts and improving bone regeneration.

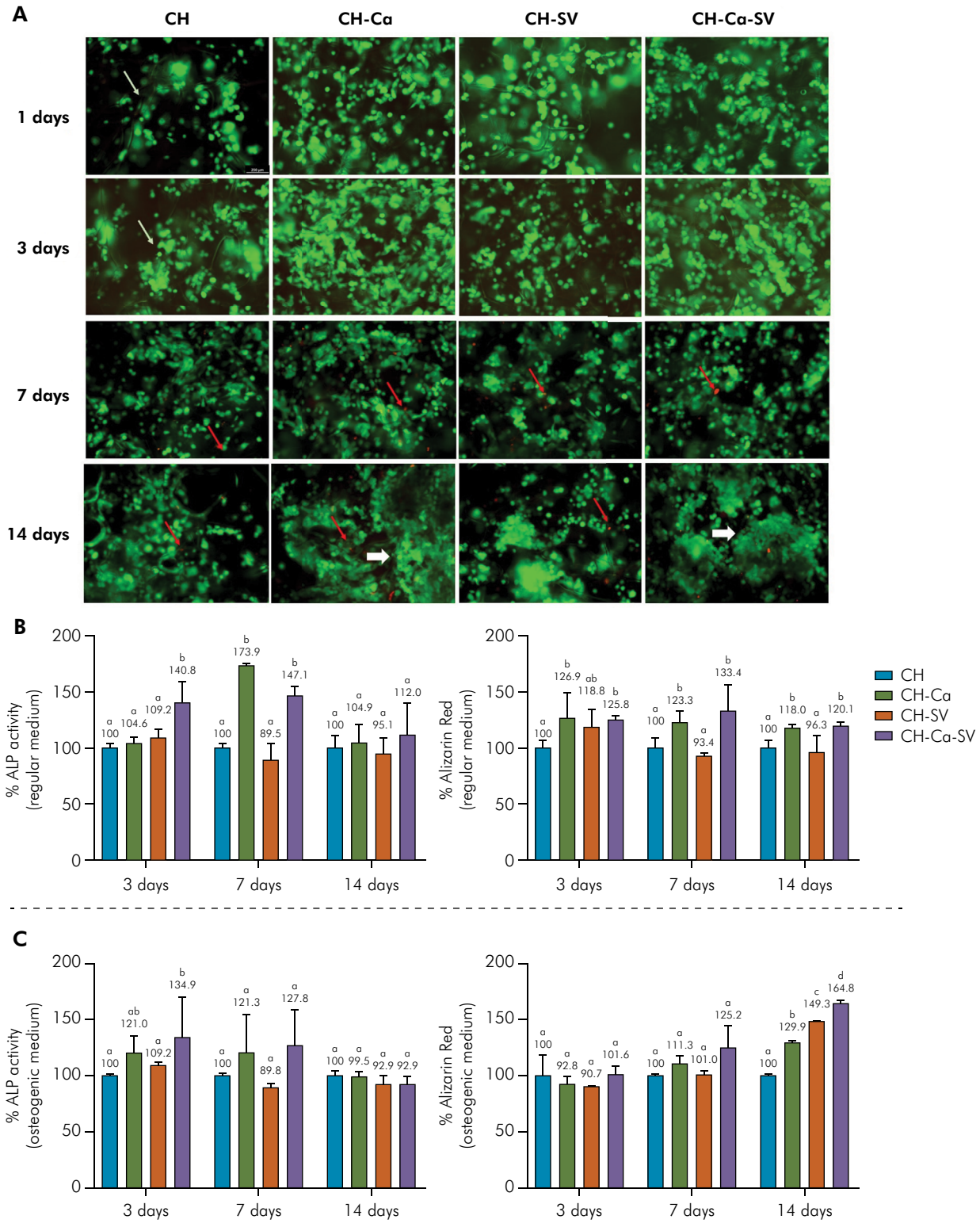



**Figure 4.** Direct contact experiment. (a) Representative images of live/dead (20x) assay. Green = live; Red = dead. Red arrows indicate the nuclei of dead cells stained with ethidium homodimer 1. Blue arrows indicate live cells stained with calcein AM. Large arrows indicate cell agglomerates at 14 days; (b) and (c) graphs representing the ALP activity and Alizarin Red tested with regular and osteogenic medium, respectively. Values represent means, and different letters indicate statistically significant differences between groups (one-way ANOVA followed by Tukey's test. n = 6, $p < 0.05$).

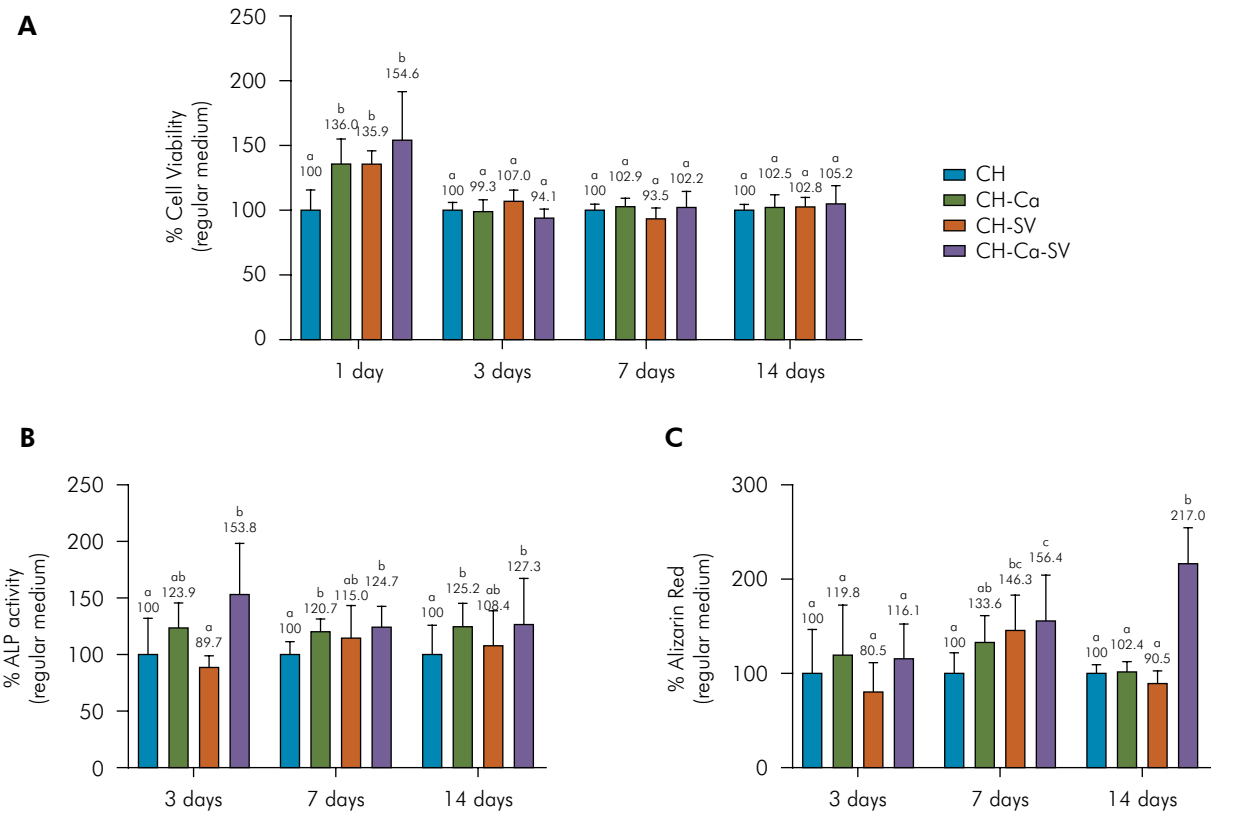
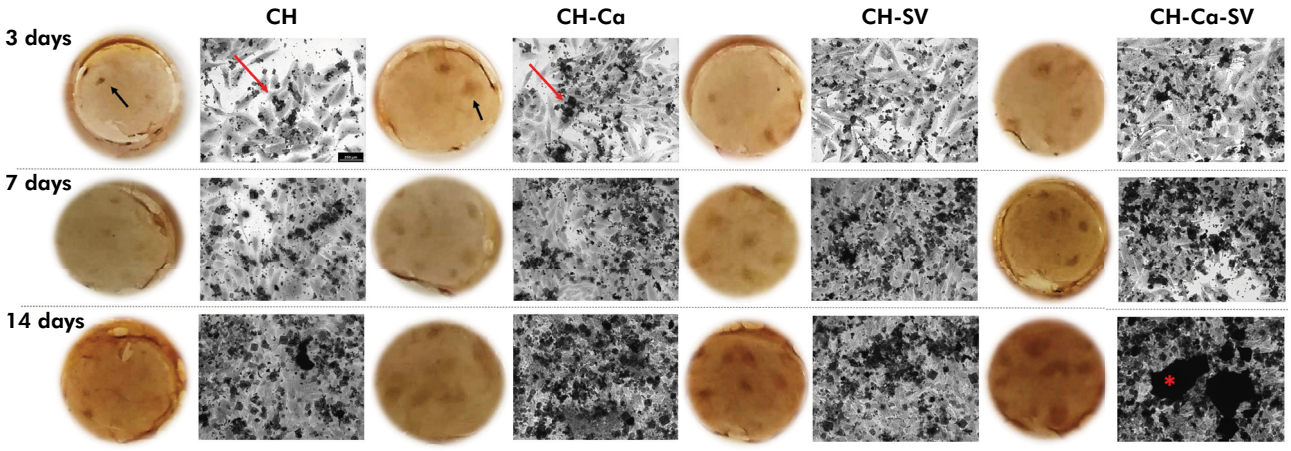



**Figure 5.** Extract assay. (a), (b), and (c) representative graphs of cell viability, ALP activity, and Alizarin Red assays, respectively. Values represent means, and different letters indicate statistically significant differences between groups (one-way ANOVA followed by Tukey's. n = 6, p <0.05).




*indicates large and well-defined mineralization nodule.

**Figure 6.** Representative images of the Alizarin Red assay. Black arrows indicate nodules observed on digital pictures of wells. Red arrows indicate small mineralization nodules on microscopic images (20x magnification).

Despite its osteosarcoma origin, SAOS-2 has been fully characterized as an osteogenic phenotype, involving the gene expression of osteoblastic markers in vitro and in vivo, mineralized matrix deposition, and osteocyte differentiation.[17,18] This cell line has been used as a model of human osteoblasts for tissue

engineering applications.[19,20] Therefore, pre-treatment with 0.1–1 μM SV for 1, 3, or 7 days was performed to simulate the effects of short-time SV release on human osteoblast cells. Similar to the literature,[9,15,21-23] this study did not observe impairment in cell viability with the use of any of the evaluated doses or pre-treatment times. However, only the 1 μM dose resulted in a significant increase in calcium deposition after 14 days of culture after pre-treatment for 1, 3, and 7 days, resulting in increases of approximately 98.5%, 129.8%, and 61.5%, respectively. These results corroborate those in the literature on the differentiation of osteoblastic cells in vitro. 1 μM SV improved the osteogenic differentiation and mineralization of bone-derived cells through the phosphorylation of protein kinases 1 and 2 regulated by extracellular signals and upregulation of BMP-2 expression via a mechanism that seems to be independent of the mevalonate, thereby enhancing bone regeneration in bone defect sites in vivo when applied locally.[9,15,21-23] Additionally, Liu et al.[21] observed that SV in concentrations ranging from 0.1 to 0.2 μM significantly increased the migration of bone marrow stem cells by 30.7% and 36% after 24 h of incubation. However, the effect of SV on osteogenic differentiation after short periods of exposure has not been well demonstrated. According to our results, 1 μM is also effective when applied for short periods (24–72 h) because it promoted a more intense biostimulation than that observed for longer periods. A similar result was found by Leite et al.[24] for MDPC-23 odontoblast-like cells, a dental pulp derived cell line with a phenotype similar to that of osteoblasts, in which pre-treatment with 0.1 and 0.01 μM of SV for 24–72 h had more intense bioactive effects than continuous treatment, with an increase of 73.2%–82.4% and 46.9%–30.4%, respectively, in the deposition of mineralized matrix.

On the basis of the results of the dose- and time-response tests, CH and CH-Ca scaffolds were incubated in a 1 μM SV solution for functionalization. This protocol was established by Soares et al.,[6] who demonstrated the formation of hydrogen bonds between the hydroxyl groups present in chitosan and SV. This interaction generated a weak bond, resulting in a burst release with a peak at 2 h, releasing approximately 25.26% for CH-SV and 30.03% for CH-Ca-SV, extending up to 21 days, when there was a tendency for it to decrease. This binding capacity of chitosan with liposoluble drugs such as SV improves the solubility of the drug and its availability for absorption by cells.[25] Notably, in the presence or absence of SV, the CH-Ca scaffolds presented calcium ion release, with burst release in the initial periods (1–3 h), that was maintained for up to 21 days. The scaffolds biomodified with SV and $Ca(OH)_2$ showed a pattern of gradual degradation over 14 days, losing approximately 9–17% of their mass, whereas the scaffold without functionalization (CH) remained stable.[6] This controlled degradation feature is highly desirable for creating a stable structure for cell interaction and stabilization, which can be gradually replaced by new tissue.[5]

The direct contact assay was the first set of experiments performed for the biological characterization of the developed scaffolds because this analysis is considered essential after biomaterial development, even when the clinical translation does not involve cell transplantation. This methodology enables the characterization of cell-scaffold interactions inside the 3D structure of the scaffold. Therefore, it is possible to observe how porosity and chemical composition affect cell infiltration, proliferation, and differentiation, leading to mineralized matrix deposition.[4] The live/dead assay of cells seeded onto the scaffolds indicated the presence of viable cells in all groups and study periods. We detected increased cell density in the CH-Ca and CH-Ca-SV groups owing to the high degree of porosity, which provides an increased surface area for cellular interaction and improved capacity for cell infiltration within the scaffold. Nevertheless, only the scaffold surface was evaluated, and this assay only indicated dead cells after membrane disruption. This finding was also detected for the CH-Ca scaffold seeded with pulp cells in vitro, in which the cells had a broad cytoplasm spreading throughout the biomaterial structure, whereas the cells seeded in the CH scaffolds were clustered with little interconnectivity.[7] The preparation method of the CH-Ca scaffolds may have influenced this macro-porous characteristic. Traditionally, scaffolds were incorporated by adding the powder of the mineral phases directly into the polymeric chitosan solution.

This method leads to the formation of agglomerates, thereby reducing the viscosity of the solution, which interferes with the formation of porosities.[26,27] The incorporation of a dispersed suspension of $Ca(OH)_2$ assists in maintaining the viscosity of the polymeric solution and aids the formation of macropores by $CO_2$ release, resulting in an organized, interconnected macro-porous network.[7] Other studies have also reported that $CO_2$ incorporation from chemical or gas-foaming reactions in the biomanufacturing process of different scaffolds obtained using the phase-separation technique enables the formation of an organized macro-porous network that directly influences the pattern of infiltration and 3D organization of the cells inside the scaffolds.[28]

The osteogenic differentiation tests of cells in direct contact were replicated with or without osteogenic medium to evaluate the effect of SV and $Ca(OH)_2$ added to the scaffolds under different experimental conditions. Ascorbic acid and beta-glycerophosphate are usually supplemented in osteogenic differentiation assays for pre-osteoblastic cells and MSCs because they promote osteoblastic maturation, improve collagen synthesis, and provide ions and inorganic phosphate for mineralization.[29] However, eliminating osteogenic supplementation when testing novel biomaterials demonstrates the potential of these biomaterials to induce osteogenesis,[30] especially when working with mature osteoblasts, such as the SAOS-2 lineage. According to our results, in the presence of osteogenic supplementation, the CH-Ca-SV scaffold featured the best biostimulation results, improving ALP activity at 3 and 7 days, and the mineralized matrix deposition by approximately 64.8% at 14 days, which were significantly higher than those of the other groups. Notably, in the absence of osteogenic medium, both CH-Ca and CH-Ca-SV improved mineralized matrix deposition, with slightly high values observed in the presence of SV. However, the difference was not significant.

A second set of experiments was performed with scaffold extracts to evaluate the bioactive effect of the release of SV and calcium ions promoted by the experimental formulations. This assay was performed to demonstrate the potential of the developed scaffolds as a cell-homing strategy for bone regeneration, modulating cell differentiation of precursor cells at the implantation site.[4] Monolayer cells were continuously cultured with the released components of scaffolds for 14 days because the previous characterization demonstrated that both SV and calcium ions were released during this period.[5,6] This assay was performed in regular medium to observe the bioactive potential of continuously released cues in the absence of a pro-osteogenic microenvironment because a direct contact assay demonstrated that SAOS-2 can deposit a mineralized matrix under these experimental conditions. The MTT assay detected increased cell viability in the CH-Ca, CH-SV, and CH-Ca-SV groups on day 1 compared with that in the CH group, which demonstrates an initial bioactive effect of the experimental formulations. ALP activity was positively modulated in the early stages of culture (3 days) in the CH-Ca and CH-Ca-SV groups, with high expression values for the functionalized formulation with SV. Consequently, the CH-Ca-SV group presented the highest values of deposition of mineralization nodules in the periods of 7 and 14 days, with a substantially increase after 14 days (117%), when it was possible to detect the presence of large, well-defined mineralization nodules.

According to the data obtained, detecting the positive biological effects of CH-Ca-SV from the synergistic effect of SV and calcium ions present in the scaffold structure was possible. Incorporating a mineral phase in scaffolds is an important strategy for bone regeneration because this stimulus contributes to the differentiation of osteoprogenitor cells into osteoblasts, increases the osteoconductive capacity, accelerates the regenerative potential, and optimizes the deposition of the mineralized matrix. The positive role of SV in bone regeneration has been observed in association with several biomaterial compositions using different pre-osteoblast and MSCs lineages, including bone marrow and periodontal ligament, because it induces osteoinduction by increasing osteoblast activity and differentiation and inhibiting their apoptosis.[31] In vivo assays demonstrated that local delivery of SV at low doses and dosages is more effective than systemic that for bone regeneration.[32] Recent studies have

also demonstrated the bursting effect of hybrid scaffolds containing SV in combination with different calcium- and phosphate-rich mineral phases, such as amorphous calcium magnesium phosphate,[33] hydroxyapatite, and beta-tricalcium-phosphate.[34] The positive effect observed on bone regeneration has been discussed as a synergistic action of ions released from the inorganic phase and SV, which creates a pro-osteogenic microenvironment prone to mineralized tissue deposition.

Montazerolghaem et al.[35] observed an effect similar to that detected in this study on SAOS-2 cells by associating a calcium phosphate cement with low-dose SV. The released components of this composite biomaterial demonstrated improved cell proliferation and ALP activity, upregulating the mineralization by approximately four-fold compared to that displayed by the released components from single-component formulations. In our prior research on human pulp cells, the synergistic effect of bioactive mineral phases with low-dosage SV has been observed for chitosan-calcium hydroxide[4,5] and chitosan-calcium aluminate formulations,[36] wherein an increase in odontoblastic markers was observed in the presence of adsorbed 1 μM SV, indicating a beneficial action of this association for the cell differentiation and deposition of the mineralized matrix. The incorporation of SV in CH-Ca scaffolds also has a chemotactic effect on human pulp cells.[6] Migration, adhesion, infiltration, and odontogenic differentiation were observed in scaffolds mediated by the chemical composition containing SV and $Ca(OH)_2$, which was superior to that displayed by biomaterials containing only one supplementation.[6] In vivo studies have demonstrated that scaffolds loaded with SV and implanted as a cell-homing strategy in different critical defect models in animals have a positive effect on the migration of resident cells to their structure and on the increase in new bone tissue deposition. The promise of this effect increases when the scaffold contains an associated mineral phase.[37]

The mechanisms controlling the bioactive potential of CH-Ca-SV may be derived from the influx of calcium ions into the intracellular medium by non-specific calcium channels; activation of the SMAD 1/5/8 and ERK1/2 signaling pathways; and increased expression of BMP-2, OCN, FGF-2, OPN, DSPP, and ALP, contributing to proliferation, differentiation, and mineral deposition in osteoprogenitor cells. Although the mechanism of action of SV on bone metabolism is not fully understood, this drug can activate the MAPK/ERK signaling pathway, presenting an action similar to that of TGF-β and BMP-22[9,15] signaling, and induce an active form of RhoA and RUNX-2/BMP-2, acting on cell proliferation and differentiation. SV also influences cytoskeleton organization, focal adhesion, and cellular tension in bone marrow stem cells.[9] However, the biostimulating effect of SV is dose-dependent, and high concentrations can lead to cell death, inhibition of angiogenesis, and cell differentiation.[38,39]

Thus, this study demonstrated the potential of highly porous matrices loaded with stable and low-cost bioactive factors as a strategy with easy translation for mineralized tissue regeneration. Although the methodology used to create CH-Ca-SV scaffolds is promising, some limitations should be mentioned. SV adsorption by immersion in a solution with drug dilution is a viable option, but its release occurs in the first hours of contact with the culture medium.[22] Considering that a slow release is favorable for the regenerative process, the use of other techniques for incorporating the drug into scaffolds, such as the use of controlled release systems, can be a more advantageous approach.[40] However, we found that even with fast release, the incorporation of $Ca(OH)_2$ and SV into chitosan scaffolds favored the expression of a regenerative phenotype in osteoblastic lineage cells in vitro, confirming this study's hypothesis. Notably, direct and indirect contact assays have substantial limitations, and the results of in vitro biological characterization should be interpreted with caution. Direct contact assays use cells seeded directly onto the scaffold structure, which can easily penetrate the porous network. In the extract assay, an expectation was that the release pattern would be different when the biomaterial is surrounded by an extracellular matrix in vivo. In addition, monolayer cells were used in the absence of a 3D network, which changes cellular phenotypic expressions.[4]

## Conclusions

This study demonstrated the regenerative potential of a chitosan scaffold containing an organized, interconnected macro-porous network developed by a simple, low-cost technology, involving the incorporation of $Ca(OH)_2$ as a modulating mineral phase associated with a bioactive dose of SV. The process generated a synergistic effect on the proliferation and differentiation of osteoblastic lineage cells seeded over the scaffolds and at a distance in the absence of osteogenic supplementation in the culture medium, demonstrating its potential as a cell-free platform for the regeneration of mineralized tissues.

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