ORIGINAL ARTICLE

Cytotoxicity of β-tricalcium phosphate chitosan gelatin composite scaffold as a bone substitute: in vitro study

Tansza Setiana Putri[1]*
Eddy Eddy[1]
Deviyanti Pratiwi[1]
Florencia Livia Kurniawan[1]
Thet Thet Swe[2]

[1]Department of Dental Materials, Faculty of Dentistry, Universitas Trisakti, Jakarta, Indonesia
[2]Department of Physics, University of Mandalay, 05032 Mandalay, Myanmar

* Correspondence:
tansza@trisakti.ac.id

Received: 11 January 2026
Revised: 13 Februari 2026
Accepted: 20 March 2026
Published: 31 March 2026
DOI: 10.24198/pjd.vol38no1.63795

p-ISSN 1979-0201
e-ISSN 2549-6212

Citation:
Putri, TS. Eddy, E, Pratiwi, D, Kurniawan, FL, Swe, TT. Cytotoxicity of β-tricalcium phosphate chitosan gelatin composite scaffold as a bone substitute: in vitro study. Padjadjaran J. Dent, March 2026; 38(1): 44-50.

ABSTRACT

Introduction: Beta-tricalcium phosphate (βTCP) has higher solubility than hydroxyapatite (HA), allowing it to be more easily resorbed and replaced by newly formed bone. This higher solubility enables the release of calcium and phosphate ions that play important roles in bone remodeling and osteoblast activity; however, excessive ion release may lead to cytotoxic effects. Limestone, mainly composed of calcium carbonate ($CaCO_3$), can serve as a calcium source for the fabrication of βTCP. βTCP scaffolds can be combined with organic components such as chitosan and gelatin to form composite scaffolds for bone tissue engineering. Therefore, this study aimed to evaluate the cytotoxicity of a β-tricalcium phosphate–chitosan–gelatin composite scaffold as a bone substitute. **Methods**: This study employed an experimental laboratory design. The freeze-drying method was used to produce composite scaffolds, which were divided into two groups: a chitosan-gelatin scaffold (control group) and βTCP-chitosan-gelatin scaffold (each group consisted of three samples). To evaluate cytotoxicity, the scaffolds were tested on osteoblast cells using the MTT assay at 24 and 72 hours. Cytotoxicity was determined based on the percentage of viable cells obtained from the MTT assay. **Results**: The percentage of viable cells on the chitosan-gelatin scaffold was 70.32% at 24 h and increased to 99.52% at 72 h. In the βTCP–chitosan–gelatin scaffold, 85.11% viable cells were observed at 24 h and increased to 89.54% at 72 h. Statistical analysis using one-way ANOVA showed no significant difference among all groups ($p>0.05$). However, Fisher's LSD test indicated a significant difference in cell viability between 24 hours and 72 hours within the chitosan gelatin group. **Conclusion**: The βTCP-chitosan-gelatin composite scaffold demonstrated no cytotoxic effect on osteoblast cells, indicating its biocompatibility and potential suitability as a bone substitute material.

KEYWORDS

Cytotoxicity, β-tricalcium phosphate, chitosan, gelatin, scaffold

INTRODUCTION

Chitosan, a biopolymer, possesses biocompatibility, biodegradability, and antibacterial properties and has been long used in scaffold fabrication for bone tissue engineering. Gelatin, which is biocompatible and non-antigenic, can form a network with chitosan, enhancing its biological activity.[1–3] The combination of both chitosan and gelatin has been shown to produce scaffolds that are biocompatible, biodegradable, and capable of enhancing the proliferation and differentiation of osteogenic cells.[2–5] However, due to their limited mechanical properties, materials such as calcium phosphate ceramics are often incorporated to improve the mechanical strength of chitosan gelatin scaffolds.[5–7]

Besides hydroxyapatite, beta-tricalcium phosphate (βTCP) is widely used in dental and orthopedic treatment.[8–12] It degrades more rapidly than hydroxyapatite due to its high solubility, facilitating new bone formation to replace lost bone.[13–16] Maji et al. introduced βTCP into chitosan gelatin scaffolds, and the results demonstrated improved cell proliferation compared to scaffolds without βTCP.[6] On the other hand, Serra et al. also investigated the incorporation of βTCP into chitosan gelatin scaffolds.[7] The βTCP-reinforced scaffolds showed higher cell viability at 24 h compared to chitosan gelatin scaffolds; however, cell proliferation at 48 h and 72 h was higher in the control group (chitosan–gelatin scaffold).[7]

Beta-tricalcium phosphate used in numerous studies is typically synthesized through chemical processes or commercially obtained.[8,15,17] However, its production can be costly, particularly in regions where raw materials must be imported. Limestone, an abundant natural resource, can be utilized as a calcium source because its primary component, calcium carbonate can serve as a calcium source for the synthesis of βTCP.[18–21]

Previous studies by Putri et al. reported the fabrication of a chitosan gelatin βTCP composite scaffold using βTCP derived from limestone.[19,22,23] The resulting scaffold exhibited suitable porosity and mechanical properties, making it a potential candidate for bone substitutes. However, the novelty of this study lies in the evaluation of the biological activity of the scaffold, particularly its cytotoxicity toward osteoblast cells, which has not been previously investigated. Therefore, the objective of this study is to evaluate the cytotoxicity of a β-tricalcium phosphate chitosan gelatin composite scaffold as a bone substitute.

METHODS

The βTCP powder was obtained from the Center for Ceramics, Indonesia, where the manufacturing process was described in previous articles.[19,22] Briefly, limestone ($CaCO_3$) was calcined at 900 °C for 6 h to produce CaO, which was crushed and wet-milled (water-to-powder = 1) for 3 h to form $Ca(OH)_2$. This was reacted with H_3PO_4 (Ca/P = 1.5) for 6 h, allowed to precipitate for 24 h, then dried at 100 °C for 24 h and sintered (1000 °C, 6 h) to obtain βTCP.[19,22] Chitosan (190,000–310,000 Da) and gelatin (bovine skin) were purchased from Sigma Aldrich.

The fabrication of the composite scaffold was previously reported.[19,22] Initially, chitosan powder was dissolved in 2% acetic acid and stirred for 10 min at 45°C. A gelatin solution was added to the chitosan solution and stirred for another 10 min. Then, the βTCP powder was added and manually mixed, followed by the addition of 0.25% glutaraldehyde and stirred for an additional 10 min.

The prepared mixture was transferred into molds measuring 6 mm in diameter and 12 mm in height, then subjected to freeze-drying (VirTis Benchtop K Freeze-Dryer; SP Industries, USA). The resulting scaffolds were subsequently washed with sodium borohydride and sodium hydroxide solutions. The compositional ratio of chitosan gelatin (CG) was 50:50% and βTCP chitosan gelatin was 70:15:15%.

Mouse osteoblast 7F2 cell lines (CRL-12557, ATCC, USA) were used for in vitro evaluation. Cells were cultured in minimum essential medium α (MEMα) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin (all purchased from Gibco, USA). Cells were detached using trypsin–EDTA (Gibco) when reaching confluence.

After sterilization under ultraviolet light, both scaffold specimens (CG and BCG) were immersed in MEMα at an extraction ratio of 0.2 g/mL and kept for 24 h at 4 °C, followed by shaking and filtering. The resulting undiluted extracts (100%) were used for cytotoxicity evaluation. For the cytotoxicity assays, 7F2 cells were seeded into 96-well plates at a density of 1×10^4 cells/well and incubated for 24 h to allow attachment. The culture medium was then replaced with 50 µL of each extract dilution, and cells were incubated for 24 h and 72 h.

Cell viability was determined by MTT assay, and absorbance was measured at 540 nm using an ELISA reader. Each experimental group consisted of three samples (n=3), with a total of four groups (n=12). This sample size is consistent with standard *in vitro* cytotoxicity assays performed in triplicate to ensure measurement reliability and reduce experimental variability, in accordance with ISO 10993-5 recommendations.[16,24]

The statistical analysis in this study was conducted using one-way analysis of variance (ANOVA), followed by Fisher's least significant difference (LSD) post hoc test. All analyses were performed with KaleidaGraph version 4.01 (Synergy Software, Reading, USA), with a significance level set at $p < 0.05$.

RESULTS

The macroscopic images of the two samples are shown in Figure 1.The two scaffolds exhibited visible differences: the CG scaffold has a whitish–yellow appearance (Figure. 1a), whereas the BCG scaffold appeared white (Figure. 1b).

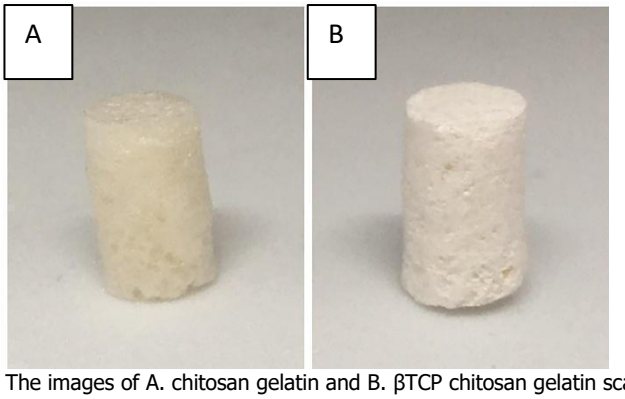

Figure 1. The images of A. chitosan gelatin and B. βTCP chitosan gelatin scaffolds.

Table 1 presents the percentage of viable cells for chitosan–gelatin (CG) and βTCP–chitosan–gelatin (BCG) scaffolds at 24 h and 72 h. The CG scaffold showed cell viability of 70.32 ± 16.33% at 24 h, which increased to 99.52 ± 12.97% at 72 h. Meanwhile, the BCG scaffold exhibited 85.11 ± 17.10% viable cells at 24 h and 89.54 ± 28.24% at 72 h. Overall, both scaffolds demonstrated cell viability values above 70% at all time points.

Table 1. Percentage of cell viability for chitosan-gelatin (CG) and βTCP–chitosan-gelatin (BCG) scaffolds

Sample	Viable cells (%)	
	24 h	72 h
CG	70.32 ± 16.33	99.52 ± 12.97
BCG	85.11 ± 17.10	89.54 ± 28.24

Figure 2 shows the cell viability and proliferation of the CG and BCG scaffolds. A significant difference was observed in the CG scaffold between 24 h and 72 h, indicating increased cell proliferation. No significant differences were observed between scaffolds with and without βTCP at either time point. The scaffold containing βTCP did not show a significant increase in viable cells between 24 h to 72 h.

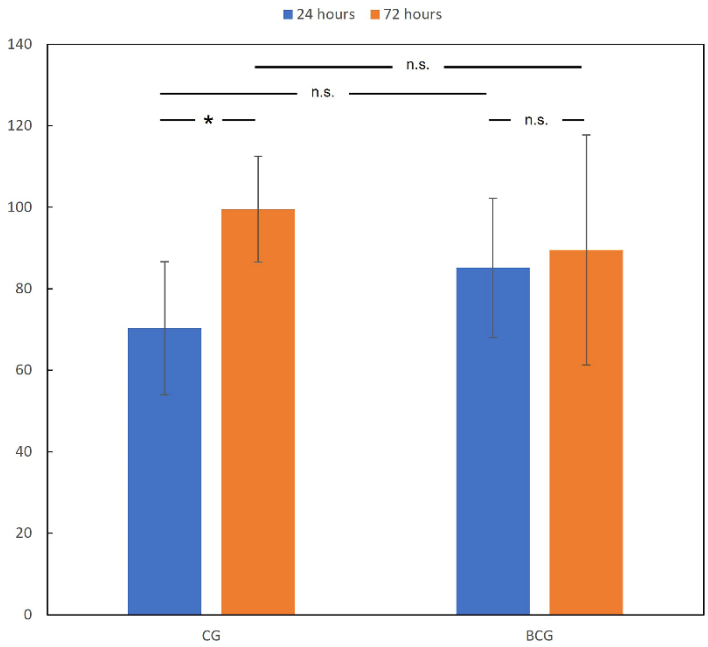

Figure 2. Cell viability at 24 h and 72 h of two scaffolds: chitosan gelatin (CG) and βTCP chitosan gelatin (BCG) (n=3). Statistical significance is differences between groups indicated as follows: n.s.: not significant; *: significant ($p < 0.05$)

Two-way ANOVA was selected because the study design included two independent variables: material type (CG vs BCG) and incubation time (24 h vs 72 h). This factorial approach allows simultaneous evaluation of the effect of material, the effect of time, and the interaction between material and time on cell viability. The results of the two-way ANOVA (Table 2) showed no significant effect of material type (p = 0.895), incubation time (p = 0.358), or their interaction (p = 0.498) on cell viability. As no significant main effects or interaction were observed, post hoc comparisons were not conducted. These findings indicate that cell viability remained comparable between the CG and βTCP/CG scaffolds at both 24 h and 72 h, and that the incorporation of βTCP did not significantly influence early cell proliferation within the tested period.

Table 2. Two-way ANOVA of cell viability for CG and βTCP–CG scaffolds.

Source	DF	F	p-value
Between Material	1	0.0178	0.895
Between Time	1	0.872	0.358
Material x Time	1	0.473	0.497

DISCUSSION

This study successfully prepared a βTCP-containing scaffold derived from limestone, chitosan, and gelatin. The findings are consistent with previous research reporting good biocompatibility of chitosan–gelatin–βTCP composite scaffolds. Previous studies have shown that βTCP-containing scaffolds support osteoblast viability and proliferation due to their bioactivity and gradual ion release. Compared with commonly used calcium phosphate bone substitutes, βTCP-based materials exhibit favorable osteoconductivity and biodegradability.[67] The comparable cell viability observed between CG and BCG scaffolds in the present study indicates that the incorporation of βTCP does not compromise the biocompatibility of the scaffold. The βTCP-containing scaffold appeared more opaque and whiter compared to the scaffold containing only chitosan and gelatin (Figure 1), likely due to the intrinsic white color of βTCP powder, which reduces scaffold translucency when mixed with chitosan and gelatin.[19] The obtained scaffolds were subsequently evaluated for cytotoxicity.

Cell viability of both scaffolds was above 70%, indicating that the materials are non-toxic, as reported by Sirait et al., who state that materials are considered non-toxic when cell viability exceeds 60%.[25] All three materials used in this study exhibit bioactive properties. Due to its relatively high solubility, βTCP can release calcium and phosphate ions, which play an important role in bone remodelling processes. Increased ion release may elevate local ion concentrations, promoting calcium-phosphate precipitation and enhancing osteogenesis. Additionally, higher calcium ion levels can stimulate osteoblast activity near the resorption site.[26–29] Lou et al. reported that cell activity on chitosan-gelatin scaffolds showed viability above 80%, indicating that both materials are biocompatible.[2] Another study conducted by Pezeshki-Modaress et al. demonstrated that the chitosan gelatin electrospun scaffolds can promote cell proliferation which, as shown by a significant increase in cell number after 14 days.[30]

Viable cells on chitosan gelatin scaffolds in the current study significantly increased from 24 h to 72 h, whereas those on βTCP–chitosan–gelatin scaffolds showed no significant increase over the same period (Figure 2). The higher cell viability observed in the chitosan–gelatin scaffold compared to the βTCP-containing scaffold at 72 h may be attributed to differences in degradation behavior. Chitosan–gelatin scaffolds tend to degrade more rapidly, which may enhance cell–material interactions and promote proliferation. In contrast, the incorporation of βTCP increases scaffold stability and results in slower dissolution, leading to more gradual ion release and slightly lower short-term proliferation, while remaining within the non-cytotoxic range. A previous study by Maji et al., has shown that chitosan gelatin scaffolds exhibit higher degradability compared to βTCP-reinforced composites.[6] This suggests that higher degradability leads to greater dissolution of scaffold components and promotes increased bone cell activity. In contrast, the reinforced scaffold exhibits lower degradability and solubility, helping it remain intact and resulting in less component dissolution.[6,26]

The results in Table 1 show that both scaffolds exhibited cell viability values above 70%, indicating that the materials are non-cytotoxic and biocompatible toward osteoblast cells. The chitosan–gelatin (CG) scaffold demonstrated a marked increase in cell viability from 70.32% at 24 h to 99.52% at 72 h, suggesting active proliferation. This behavior may be attributed to the biodegradable and biocompatible nature of chitosan and gelatin, which can support cell attachment and proliferation. In contrast, the βTCP–chitosan–gelatin (BCG) scaffold showed relatively stable cell viability between 24 h and 72 h, likely due to increased structural stability and reduced degradation rate, resulting in a more gradual interaction between the scaffold and surrounding cells. Nevertheless, the high viability values observed in the BCG group confirm that βTCP incorporation does not induce cytotoxic effects and maintains scaffold biocompatibility.

The two-way ANOVA results demonstrated that neither material type ($p = 0.895$) nor incubation time ($p = 0.358$) had a statistically significant effect on cell viability, and no significant interaction between these variables was observed ($p = 0.498$). These findings indicate that βTCP incorporation did not significantly influence osteoblast viability within the 24–72 hour observation period. The comparable viability values between groups suggest that both scaffolds exhibit similar levels of cytocompatibility under the tested conditions. Since the evaluated time points represent early cellular responses, the absence of significant differences may also reflect the possibility that potential biological effects of βTCP, such as calcium and phosphate ion-mediated stimulation of osteoblast activity, may require longer incubation periods to become detectable.

Based on these findings, the βTCP chitosan gelatin scaffold shows promising potential as a future bone graft material due to its biocompatibility. However, the current results are limited to *in vitro* cytotoxicity assessment, so further comprehensive evaluation is needed before clinical application.

Future research should focus on optimizing the ratio of βTCP, chitosan, and gelatin to achieve improved mechanical strength and controlled degradation rates, as well as conducting *in vivo* evaluations to confirm the scaffold's bone regeneration potential.

This study has several limitations. It only assessed short-term cell viability and did not evaluate long-term degradation behavior or *in vivo* bone-forming ability. These limitations restrict the ability to predict performance under physiological conditions. Future studies should therefore include investigations into degradation behavior and ion release kinetics, as well as *in vivo* experiments to evaluate bone regeneration, vascularization, and host tissue responses. Optimizing scaffold porosity and incorporating osteoinductive factors may also enhance its regenerative performance and accelerate translation toward clinical use.

CONCLUSION

Both the βTCP–chitosan–gelatin and chitosan–gelatin scaffolds exhibited no cytotoxic effects on osteoblast cells, indicating that both materials are biocompatible and suitable for bone tissue engineering applications. The implication of this study is that incorporating βTCP into a chitosan gelatin matrix can enhance the mechanical and osteoconductive properties of the scaffold without compromising its biocompatibility. These findings contribute to the development of composite scaffolds that balance biodegradability and bioactivity for potential use as bone substitute materials.

Acknowledgement
We thank the Center for Ceramics in Indonesia for providing resources for this research, and Faculty of Dentistry, Universitas Trisakti for the support in the process of publishing this article.
Author Contributions: Conceptualization, methodology, investigation, writing original draft preparation, writing and editing, T.S.P; investigation, writing and editing, E.E.; validation, resources D.P.; formal analysis, project administration F.L.K.
Funding: This research was funded by Universitas Airlangga grant number 388/UN3.14/PT/2020.
Institutional Review Board Statement: Not applicable
Informed Consent Statement: Not applicable
Data Availability Statement: The data supporting the findings of this study are available from the corresponding author upon reasonable request.
Conflicts of Interest: The authors declare no conflict of interest

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