

ORIGINAL ARTICLE

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

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ABSTRACT

Introduction: Beta-tricalcium phosphate (β TCP) 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 β TCP. β TCP 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 analyze the cytotoxicity of a β -tricalcium phosphate–chitosan–gelatin composite scaffold as a bone substitute. **Methods:** Type of research was experimental laboratory. Freeze-drying method was used to produce a composite scaffold which was divided into two groups: chitosan-gelatin scaffold as control group and β TCP-chitosan-gelatin scaffold (each group consisted of three samples) To evaluate cytotoxicity, composite scaffolds were tested on osteoblast cells and the MTT assay was measured and assessed based on time evaluation at 24 hours and 72 hours. Cytotoxicity was determined based on the percentage of viable cells obtained from the MTT assay. **Results:** Viable cells percentage on the chitosan-gelatin scaffold was 70.32% at 24 h and increased to 99.52% at 72 h. While on the chitosan-gelatin- β TCP scaffold there were 85.11% viable cells 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 β TCP-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 as the biopolymer has the properties of biocompatibility, biodegradability, and anti-bacterial which has been long introduced to the scaffold fabrication for bone tissue engineering. While gelatin with its biocompatibility and no antigenicity could form a network with chitosan increasing its biological activity.¹⁻³ Combination of both chitosan and gelatin demonstrated a well-developed scaffold which is biocompatible, biodegradable, and could increase proliferation and differentiation of osteogenic cells.²⁻⁵ However, due to the limited mechanical properties, materials such calcium phosphate ceramics were used to improve the mechanical strength of chitosan gelatin scaffold.⁵⁻⁷

Besides hydroxyapatite, the application of beta-tricalcium phosphate (β TCP) is quite popular in dental and orthopaedic treatment.^{8–12} It degrades quicker than hydroxyapatite due to its high solubility, facilitating the new bone to be formed replacing the bone loss.^{13–16} Maji et al. has introduced β TCP into chitosan gelatin scaffold and the result exhibited a better cell proliferation compared to chitosan gelatin scaffold without the β TCP addition.⁶ On the other hand, another study conducted by Serra et al. also investigated β TCP incorporation into chitosan gelatin scaffold.⁷ The obtained scaffold reinforced by β TCP showed higher viable cells at 24 h compared to chitosan gelatin scaffold but cell proliferation after 48 h and 72 h in chitosan gelatin scaffold was higher than scaffold with β TCP.⁷

Beta-tricalcium phosphate used in numerous studies were obtained through chemical process or purchased readily.^{8,15,17} Producing β TCP could be an expensive procedure especially in some countries where the raw materials need to be imported. Limestone, that is quite an abundant natural resource, could be utilized considering that its major composition of calcium carbonate could be the source of calcium for the synthesis of β TCP.^{18–21}

Previously, Putri et al. had successfully fabricated chitosan gelatin β TCP composite scaffold where the β TCP was extracted from limestone.^{19,22,23} The obtained scaffold possessed a suitable porosity and mechanical properties which make it a potential candidate for bone substitute. However, the novelty of this research is that the biological activity of the scaffold has not been investigated yet. Therefore, the objective of this study is to analyze the cytotoxicity of β -tricalcium phosphate chitosan gelatin composite scaffold as a bone substitute

METHODS

The β TCP powder was obtained from Center for Ceramics, Indonesia, where the manufacturing process was mentioned 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 $\text{Ca}(\text{OH})_2$. This was reacted with H_3PO_4 (Ca/P = 1.5) for 6 h, left 24 h for precipitation, then dried at 100 °C for 24 h and sintered (1000 °C, 6 h) to obtain β TCP.^{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. Gelatin-in-water solution was poured into the chitosan solution and stirred for another 10 min. Then, the β TCP powder was added and manually mixed followed by the addition of 0.25% glutaraldehyde and mixed 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). Following this, the resulting scaffolds were washed with solutions of sodium borohydride and sodium hydroxide. The compositional ratio of chitosan gelatin (CG) was 50:50% and β TCP 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 (MEM α) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μ g/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 MEM α at an extraction ratio of 0.2 g/mL and kept for 24 h at 4 °C, followed by shaking and filtering. The undiluted extracts (100%) were used for cytotoxicity evaluation. For 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 μ L 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 ELISA reader. Each experimental group consisted of three samples ($n=3$), with four groups in total where the total samples were twelve. The sample size of three per group followed common practice in *in vitro* cytotoxicity assays, where experiments are performed in triplicate to ensure measurement reliability and reduce experimental variability, consistent with ISO 10993-5 recommendations.^{16,24}

Mouse osteoblast 7F2 cell lines (CRL-12557, ATCC, USA) were used for *in vitro* evaluation. Cells were grown with minimum essential medium α (MEM α) containing 10% fetal bone serum, 100 U/mL penicillin and 100 μ g/mL streptomycin which were all purchased from Gibco, USA. Cells were then detached with trypsin–EDTA (Gibco). After sterilization in ultraviolet light, both scaffold (CG and BCG) specimens were immersed in MEM α and kept for 24 h at 4°, followed by shaking and filtering. The immersion solutions were used for evaluation.

Cultured cells were added into a 96-well plate and incubated for 24 h. Mediums were replaced until the cells were confluent. The immersion solutions were then added into the well plate at 50 μ l per well and incubated for 24 h and 72 h. The viable cells number was measured using MTT Assay and read by ELISA reader operated at a wavelength of 540 nm.^{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. Both samples were visibly different, where the CG scaffold has a white with yellowish appearance (Figure. 1a) while the BCG scaffold was white (Figure. 1b).

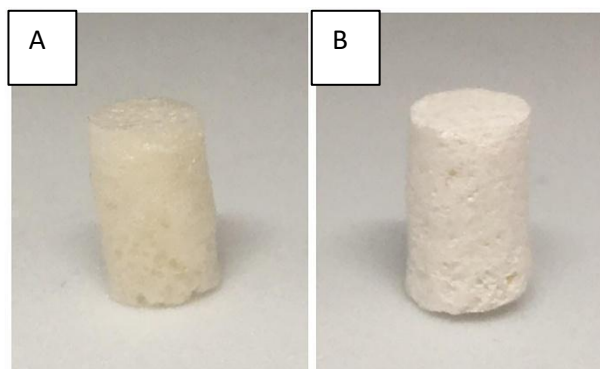


Figure 1. The images of A. chitosan gelatin and B. β TCP chitosan gelatin scaffolds.

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

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

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

Figure 2. shows the cell viability and proliferation of the scaffold containing chitosan and gelatin and scaffold containing β TCP, chitosan, and gelatin. The significant difference could be found between CG scaffold at 24 h and 72 h, indicating a significant cell proliferation. There were no significant differences between the scaffold with and without the β TCP addition, whether it was at 24 h or 72 h. Scaffold containing β TCP have no significant increase of viable cells from 24 h to 72 h.

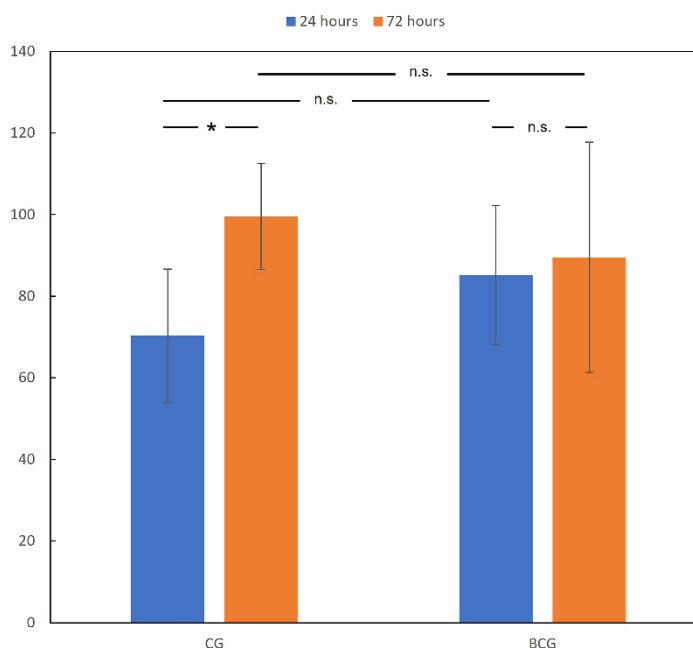


Figure 2. Viable cells at 24 h and 72 h of two scaffolds: chitosan gelatin (CG) and β TCP chitosan gelatin (BCG) (n=3). Statistical significance of differences between groups indicated as follows: n.s.: not significant; *: significant ($p < 0.05$)

Two-way ANOVA was selected because the study design included two independent variables: material type (Group CG vs Group 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 1) showed no significant effect of material type ($p = 0.895$), incubation time ($p = 0.358$), or their interaction ($p = 0.498$) on cell viability. Because no significant main effects or interaction were observed, post hoc comparisons were not performed. These findings indicate that cell viability remained comparable between the CG and β TCP/CG scaffolds at both 24 h and 72 h, and that the incorporation of β TCP did not significantly influence early cell proliferation within the tested period.

Table 2. Two-way ANOVA of cell viability for CG and β TCP–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 has successfully prepared the scaffold containing β TCP made from limestone, chitosan, and gelatin. The findings of this study are consistent with previous research reporting good biocompatibility of chitosan–gelatin– β TCP composite scaffolds. Previous studies have shown that β TCP-containing scaffolds support osteoblast viability and proliferation due to their bioactivity and gradual ion release. Compared with commonly used calcium phosphate bone substitutes, β TCP-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 β TCP does not compromise the biocompatibility of the scaffold. The appearance of the β TCP-contained scaffold is opaquer and whiter compared to the scaffold containing only chitosan and gelatin (Figure 1) because the β TCP powder itself is a typical white powder, reducing the translucency of the scaffold when mixed with chitosan and gelatin.¹⁹ The obtained scaffolds were then evaluated for cytotoxic analysis.

Cell viability of both scaffolds, which were above 70%, indicated that the materials is not toxic because according to the article written by Sirait et al., the materials is not toxic if the viable cells percentage is above 60%.²⁵ Three materials used in this experiment have bioactive property. Due to its relatively high solubility, β TCP could easily release calcium and phosphate ions. The calcium and phosphate ions play important role in bone remodelling process. The higher release of these ions would lead to higher ions concentration around the defect site, promoting higher calcium-phosphate precipitation, resulting in better osteogenesis. Additionally, higher calcium ions can stimulate osteoblast activity near the resorption site.^{26–29} Lou et al. studied the cells activity on chitosan and gelatin scaffold and exhibited that the cell viability was above 80% which indicated that both chitosan and gelatin are biocompatible.² Another research carried by Pezeshki-Modaress et al. showed that the chitosan gelatin electrospun scaffold could promote cell proliferation which was shown by the significant increase in number of cells after 14 days.³⁰

Viable cells against chitosan gelatin scaffold in current study significantly increased from 24 h to 72 h, while the ones against β TCP chitosan gelatin scaffold had no significant increase from 24 h to 72 h (Figure 2). The higher cell viability observed in the chitosan–gelatin scaffold compared to the β TCP-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 cell proliferation. In contrast, the incorporation of β TCP increases scaffold stability and results in slower dissolution, leading to a more gradual ion release and slightly lower short-term proliferation despite remaining within the non-cytotoxic range. Compared to previous study by Maji et al., it has been proven that the chitosan gelatin scaffold has higher degradability compared to chitosan gelatin which is reinforced with β TCP.⁶ This proved that higher degradability causes more component of the scaffold to be dissolved and promotes the bone cells to be more active. While in the reinforced scaffold, the degradability is lower where the solubility of the scaffold is also lower making it intact and less component would be dissolved.^{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 cell proliferation over time. This behavior may be related to the biodegradable and biocompatible nature of chitosan and gelatin, which can support cell attachment and proliferation. In contrast, the β TCP–chitosan–gelatin (BCG) scaffold showed relatively stable cell viability between 24 h and 72 h. The incorporation of β TCP may increase scaffold structural stability and reduce the degradation rate compared to CG alone, resulting in a more gradual interaction

between the scaffold and surrounding cells. Nevertheless, the high viability values observed in the BCG group confirm that the addition of β TCP does not induce cytotoxic effects and maintains the biocompatibility of the composite scaffold.

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 shown in Table 2 indicate that the incorporation of β TCP into the chitosan–gelatin scaffold did not significantly influence osteoblast viability within the 24–72 h 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 that potential biological effects of β TCP, such as calcium and phosphate ion-mediated stimulation of osteoblast activity, may require longer incubation periods to become detectable.

Based on the result of this study, the β TCP chitosan gelatin scaffold shows promising potential as a future bone graft material due to its biocompatibility β TCP. 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 β TCP, 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 the long-term degradation behavior or *in vivo* bone-forming ability. These limitations restrict the ability to predict its performance under physiological conditions. Future studies should therefore include investigation of degradation behavior and ion release kinetics, and *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 scaffolds containing β TCP chitosan gelatin and chitosan gelatin 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 β TCP 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.

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