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Isfahan University of Medical Sciences

Original Article

The role of gelatin and collagen in cell viability and osteogenic potential of an injectable chitosan-based scaffold containing LL37 peptide

Mahboubeh Rezazadeh[1,*], Vajihe Akbari[2], Azam Sadeghi[1], and Naser Tavakoli[1]

[1]*Department of Pharmaceutics and Novel Drug Delivery System Research Center, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, I.R. Iran.*
[2]*Department of Pharmaceutical Biotechnology and Pharmaceutical Sciences Research Center, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, I.R. Iran.*

Abstract

Background and purpose: LL37 peptide is a human antimicrobial peptide with potential application in bone tissue engineering through the stimulation of cell proliferation and osteogenesis. The current study aimed to fabricate chitosan/gelatin/glycerophosphate (CTS/G/GP) and chitosan/collagen/glycerophosphate (CTS/C/GP) thermosensitive hydrogels loaded with LL37 and compared their ability to support cell growth, proliferation, and osteogenesis.
Experimental approach: The hydrogel systems were prepared by the physical mixture of chitosan, gelatin, collagen, and GP at the concentrations of 2.5, 1, 1, and 10% w/v, respectively. LL37 was added at a fixed concentration of 1 µg/mL of the hydrogels. The viscosity, friability, release properties, and biological experiments were evaluated based on standard procedures.
Findings/Results: The viscosity of CTS/C/GP increased to 7000 cP at 35 °C in 100-120 s, while for CTS/G/GP, the viscosity and gelation time were recorded as 14000 cP and 30 s, respectively. The friability percent for CTS/G/GP after 72 h was reported as 28%, which was significantly lower than that of 38% for CTS/C/GP. LL37 was released during 8 h from both scaffold systems, and it did not demonstrate any significant differences between the hydrogel systems. Cell viability and alkaline phosphatase activity revealed that the incorporation of LL37 in the hydrogels could accelerate cell proliferation compared to empty scaffolds, and it was higher in gelatin-containing scaffolds.
Conclusion and implications: LL37 was successfully loaded into both hydrogel systems and demonstrated the ability to accelerate cell proliferation and differentiation compared to the empty scaffold.

Keywords: Thermosensitive hydrogel; LL37 peptide; Bone tissue repair.

INTRODUCTION

Bone tissue engineering is becoming a promising strategy for bone repair and growth factor-containing scaffolds have been widely evaluated for this application. However, several drawbacks, including their costs, difficult production procedure, immunogenicity, and short half-life, have limited their clinical application (1). Antimicrobial peptides with osteogenic properties are considered interesting alternatives and provide new opportunities to obtain cheaper and more effective agents for bone tissue engineering applications (1,2). Among the antimicrobial peptides, LL37, a human cathelicidin antimicrobial peptide, is considered a promising candidate for bone regeneration, because it shows neoangiogenic properties, broad-spectrum antimicrobial activity, and is commercially available (2,3).

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*Corresponding author: M. Rezazadeh
Tel: +98-3137927123, Fax: +98-3116680011
Email: rezazade@pharm.mui.ac.ir

This peptide plays a key role in cell proliferation and vascularization in the region of damaged tissue. Based on molecular studies, it was demonstrated that LL37 induced bone marrow mesenchymal stem cells (BMSCs) osteogenesis through the stimulation of the P2X7 purinergic receptor and activation of the mitogen-activated protein kinase signaling pathway to trigger an intracellular cascade resulting in the proliferation, differentiation, and migration of BMSCs (3). In recent years, the fabrication of different scaffolds for local delivery of LL37 has grown. He *et al*. developed a titanium implant loaded with LL37 and evaluated its effect in bone tissue repair (4). In another similar study, a collagen scaffold containing LL37 was implanted in the rabbit femur for bone tissue repair (5). However, *in vivo* results revealed that the effectiveness of the formulations, pathogens can accumulate on the surface of the implant material after the invasive implantation procedure, forming a biofilm that protects them from the host's defenses, which can consequently lead to surgical failure, disability, and even death (6). Thermosensitive hydrogels are extensively evaluated for biomedical applications. Therapeutic proteins, such as different growth factors and cells, are easily incorporated into the liquid form of hydrogels in a minimally invasive manner, injected into the desired tissue, and then form a gel structure *in situ* (7). The three-dimensional structure of hydrogels closely mimics the extracellular matrix tissue supporting cell attachment and proliferation (8). Jorge *et al.* developed an extracellular matrix hydrogel functionalized with LL37 and subcutaneously injected into rats to evaluate the biocompatibility and safety of the formulation. However, the formulation could be easily applied in damaged areas, the peptide was chemically attached to the hydrogel backbone, which was a complicated and extensive procedure (9). Here, we aimed to prepare thermosensitive hydrogels from the mixture of chitosan, glycerophosphate (GP), and collagen/gelatin for the local delivery of LL37 and their application for bone tissue repair. Chitosan is physically mixed with GP to prepare a thermoresponsive sol-gel system, which is liquid in room temperature and converts to gel upon exposure to the body cavities. In bone tissue engineering, chitosan has demonstrated excellent osteoconductivity and could accelerate osteoblast growth in the scaffold (10,11). Bharathi's study revealed that rat BMSCs were successfully incorporated, and growth on the surface of chitosan/GP (CTS/GP) scaffold and calcium deposition were observed in animal experiments (12). Literature review exhibited that the incorporation of collagen into the solution of CTS/GP improved its biological properties without affecting gelation time and temperature (13,14). An *in vitro* study performed on the expression of genes and osteogenic markers demonstrated that human BMSCs cultured in a collagen-chitosan scaffold are better differentiated compared to those incorporated in pure chitosan gel (15). In another study, a thermosensitive injectable CTS/collagen/GP (CTS/C/GP) was fabricated, and BMSCs were successfully harvested in an animal experiment (14). Gelatin is another biocompatible and biodegradable polymer, which is also widely used in bone tissue engineering (16,17). Arginine, glycine, and aspartate sequence in gelatin might promote cell adhesion, proliferation, and differentiation (17,18). Because of the unique advantages of LL37, the local application of the agent has been considered recently (19,20). Considering the advantages of chitosan, gelatin, collagen, and LL37 peptide, we aimed to compare the effect of collagen and gelatin on thermoresponsive and osteogenic properties of CTS/GP containing LL37 and introducing a novel scaffold containing LL37 peptide for bone tissue engineering application.

MATERIALS AND METHODS

Agents

Chitosan (deacetylation degree: 85%, molecular weight (MW): 150-300 kDa), glycerophosphate (GP), gelatin, and collagen (MW: 100-150 kDa) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). LL37 peptide was supplied by Biomatik (Kitchener, Canada), ABC kit was purchased from DNA biotech (Iran). Acetic acid, ascorbic acid, and sodium hydroxide were supplied by Merck (Darmstadt, Germany). Dulbecco's

modified Eagle medium (DMEM), fetal bovine serum (FBS), and antibiotics (penicillin and streptomycin) for cell culture were provided by Bioidea (Tehran, Iran). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), alizarin, calcein-AM, and ethidium bromide (live/dead viability kit) were obtained from Thermo Fisher Scientific (Waltham, MA, USA).

Preparation and characterization of chitosan/gelatin/glycerophosphate and chitosan/collagen/glycerophosphate

To prepare the hydrogel solutions, chitosan, along with collagen or gelatin, was dissolved in 0.1 M acetic acid at pH 3. Then, the aqueous solution of GP was added to the polymer solutions in a drop-wise manner at 4 °C and constant stirring to obtain the concentrations of chitosan, collagen, gelatin, and GP at 2.5, 1, 1, and 10% w/v, respectively. The final pH of the solutions was adjusted to 7.4 (21). To prepare LL37-loaded hydrogels, the peptide was added to the chitosan solution at a fixed concentration of 1 µg/mL in the hydrogels. The viscosities of systems were traced versus time and temperature using a digital rotary viscometer (RVDV-III U, Daiki Sciences Co., USA). The *in vitro* friability of the hydrogels was measured with respect to the hydrogel weight loss ratio as a function of incubation time in phosphate buffer solution (PBS) at 37 °C as described previously (21). Briefly, 1 mL of the hydrogel solution was prepared, freeze-dried, and weighed (W_0). Then, the hydrogel samples were incubated in BPS at 37 ºC for different times. At specific time intervals till 72 h, hydrogels were removed from PBS, freeze-dried, and then weighed out (W_t). The weight loss ratio was defined using equation (1):

$$Weight\ loss\ ratio = \frac{W_0 - W_t}{W_0} \times 100 \qquad (1)$$

In vitro release study

The amount of LL37 released from the hydrogel was measured using the ABC kit and ELISA technique as the following procedure. One mL of the hydrogel samples was placed in 25-mL flat-bottomed glass beakers and allowed to gel in an incubator at 37 °C for 5 min. Then, 10 mL of PBS (0.1 M, pH 7.4) was poured on the surface of the gels, and the vessels were shaken in a water bath shaker at 40 rpm and 37 °C. At specific time points, a 2 mL sample of the supernatant solution was withdrawn and replaced with 2 mL fresh buffer. The samples were then analyzed for LL37 using the ABC kit according to the manufacturer's protocol. Briefly, 25 µL of release medium containing LL37 peptide was added to 75 µL of the mixture of detergents A and B and incubated at 60 ºC for 60 min. The absorbance of samples was determined at 562 nm using an ELISA reader (Stat Fax-2100; Awareness Technology Inc., Palm City, FL, USA).

Cell isolation, cultivation, and osteogenic induction

Three Wistar rats (5-6 weeks old, 200-250 g body weight) were used to harvest BMSCs from the iliac of the animals. The animals were purchased from the laboratory animal center of the School of Pharmacy and Pharmaceutical Sciences (Isfahan, Iran). All animal experiments were done in agreement with the Guide for the Care and Use of Laboratory Animals provided by the National Institute of Health and obtained the approval of the Research Ethics Committee of Isfahan University of Medical Sciences (ethical code 399317).

In detail, 5 mL of bone marrow was aspirated into a 10 mL syringe containing 5000 U of heparin, and then centrifuged to remove fat and heparin. The precipitated cells were cultured in a complete medium containing L-DMEM with 10% FBS, 100 units/mL penicillin and 100 µg/mL streptomycin. Cells were incubated at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2. Once 80-90% cell confluency was reached, the cells were detached by treatment with 0.25% trypsin containing 0.01% EDTA, then subcultured and routinely passaged. For cell osteogenic differentiation, the second passage of the cells was cultured in medium containing L-DMEM with 10% FBS, 100 nM dexamethasone, 5.0 mM β-GP, and 50 mg/mL L-ascorbic acid (22). The medium was replaced every 3 days. The level of alkaline phosphatase (ALP) activity was examined on day 7 using an ALP kit (Pars Azmoon, Iran) according to the manufacturer's protocol. Mineralization was also determined by staining with alizarin red S on day 10.

Cell attachment and morphology in the hydrogel systems

BMSCs cultured in osteogenic medium for 7 days were seeded at a density of 5×10^4 cells/well in both chitosan/ collagen/glycerophosphate (CTS/C/GP) and chitosan/gelatin/ glycerophosphate (CTS/G/GP) gels for scanning electron microscopy (SEM) observation. Before seeding the cells, the scaffolds were sterilized using UV radiation for 10-20 min. Microstructure of the gels and cell growth in the scaffold were observed after glutaraldehyde fixation at day 3. On day 3, all gels were washed 3 times with PBS, and then 2.5% glutaraldehyde was added to each well for 20 min at room temperature. Then, the samples were washed again with PBS, vacuum dried, gold sputtered, and observed by using SEM (23).

Cell viability in the hydrogels

The solutions of CTS/G/GP and CTS/C/GP with and without LL37 peptide were prepared and loaded with osteoblast cells to obtain 2×10^5 cells/mL. A 0.5 mL volume of the mixed solutions was then poured into the wells of a 24-well plate and allowed to gel at 37 ºC in an incubator with a 100% humidified chamber and 5% CO_2. Then, 1 mL complete medium was added to each well, and the medium was changed every 2 days. Cell viability was assessed on days 1, 3, and 7 (n = 3) by calcein-AM and ethidium bromide. After each incubation time, 200 µL of PBS solution containing 2 µL/mL ethidium bromide homodimer and 0.5 µL/mL calcein AM was added to each well and incubated for 30 min at 37 ºC, and then the wells directly were observed under the fluorescence inverted microscope (Ceti, 3100.5000 Triton II, UK) (24).

MTT assay and the measurement of ALP activity

The number of viable cells on the hydrogel systems was also determined on days 3 and 7 after incubation using the MTT assay. Briefly, cells alone (2×10^4 cells/mL) and cell-seeded hydrogels (2×10^4 cells/mL) were placed in a 24-well plate and immediately incubated for gelation at 37 ºC. One mL of completed culture medium was added to each well, and the samples were cultured for 24, 48, and 72 h. After incubation, 100 mL of the MTT solution (5 mg/mL in 0.02 M phosphate buffer) was added to each well, and the plate was incubated for another 3 h at 37 ºC. Viable cells were able to reduce MTT into formazan crystals. Subsequently, unreacted MTT and medium were removed, and the formazan crystals in cells were dissolved in 800 mL of dimethyl sulfoxide (25). Absorbance was recorded at 570 nm using an ELISA reader (Stat Fax-2100; Awareness Technology Inc., Palm City, FL, USA). The wells containing cells alone were considered as a negative control, and the well with a blank cell culture was used as a blank control. Cell viability for each sample was calculated using equation (2):

$$\text{Cell viabillity (\%)} = \frac{\text{Ab}_{\text{sample}} - \text{Ab}_{\text{blank}}}{\text{Ab}_{\text{negative control}} - \text{Ab}_{\text{blank}}} \times 100 \qquad (2)$$

where, Ab was put instead of absorbance.

The ALP activity was also determined in different groups on days 3 and 7 according to the kit protocol (21).

Statistical analysis

Data were expressed as mean ± SD (n = 3) and analyzed by SPSS 19. An independent sample t-test was used to compare two groups. One-way ANOVA followed by an LSD post-hoc test for multiple groups. A P-value ≤ 0.05 was considered statistically significant.

RESULTS

Preparation and characterization of CTS/G/GP and CTS/C/GP hydrogel systems

The morphology and temperature-dependent gelation of both hydrogel systems containing LL 37 were shown in Fig. 1A-D. Both systems existed in a sol state at room temperature, converted to a semisolid state when the temperature was increased to 35 °C. As shown in Fig. 1C, the viscosity of CTS/C/GP increased to 7000 cP at 35 °C during 100-120 s, while the viscosity and gelation time for CTS/G/GP were recorded 14000 cP and 30 s, respectively (Fig. 1D). As shown in Fig. 1E, the friability percent of CTS/G/GP was reported 28% after 72 h which was significantly lower than that of CTS/C/GP (38%).

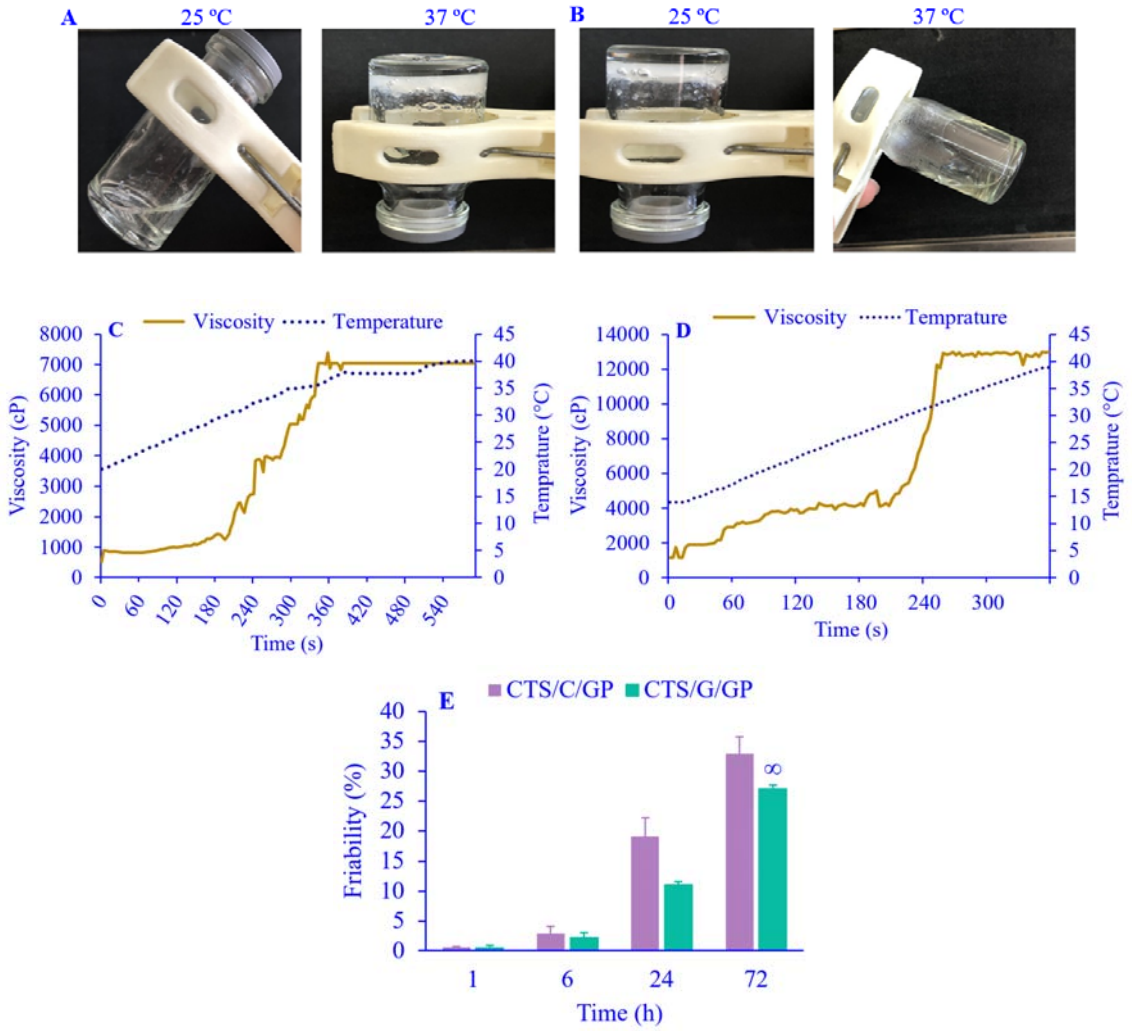

Fig. 1. Visual observation of the hydrogel systems. (A) CTS/G/GP and (B) CTS/C/GP morphology; effect of temperature on the viscosity of (C) CTS/C/GP and (D) CTS/G/GP; (E) friability of the hydrogel systems. $^{\infty}P \leq 0.05$ demonstrates a significant difference compared to the 24-h time point in the same formulation. CTS, chitosan; C, collagen; G, gelatin; GP, glycerophosphate.

In vitro release study

As shown in Fig. 2, LL37 was released during 8 h from the hydrogel scaffolds, and no significant differences were observed between the 2 hydrogel systems.

Cultivation of BMSCs and evaluation of osteogenic differentiation ability

At the 3th passage, BMSCs showed fibroblast-like adherent growth with regular morphology, and the cell counts reached 5×10^5 cells/mL (Fig. 3A). After 7 days, the cells in osteogenic medium showed higher positive ALP activity significantly than that of cells cultured in completed DMEM medium (0.04 versus 0.01 IU/ng DNA). After 10 days, alizarin red S staining also revealed the formation of mineralization nodules in osteogenic medium, while the cells in DMEM medium resulted in negative alizarin red S staining after 10 days (Fig. 3 B).

Cell attachment and morphology in the hydrogel systems

As shown in Fig. 4, both hydrogel systems showed porous heterogeneous microstructures, and osteoblast cells were easily distinguished in both systems. Cells were extended well and connected with each other in the scaffolds.

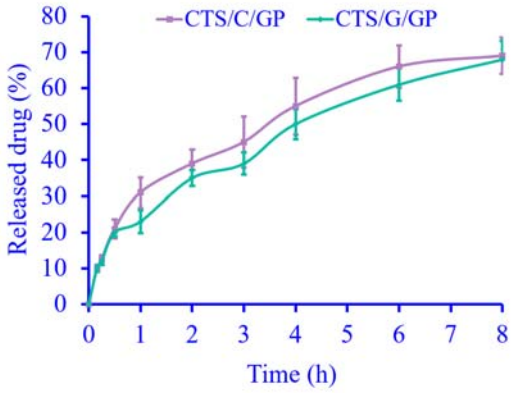

Fig. 2. *In vitro* release profiles of LL37 from the hydrogel systems. Data were expressed as mean ± SD, n = 3. CTS, chitosan; C, collagen; G, gelatin; GP, glycerophosphate.

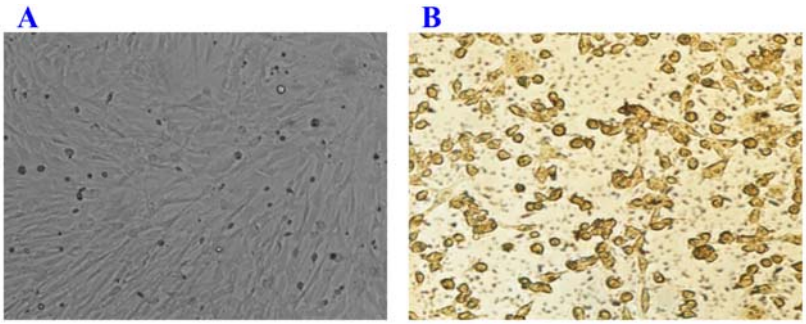

Fig. 3. (A) Microscopic observation of BMSCs after a 3-day incubation in osteogenic medium and (B) microscopic observation of BMSCs staining with alizarin red after 10 days (magnification ×200). BMSCs, Bone marrow mesenchymal stem cells.

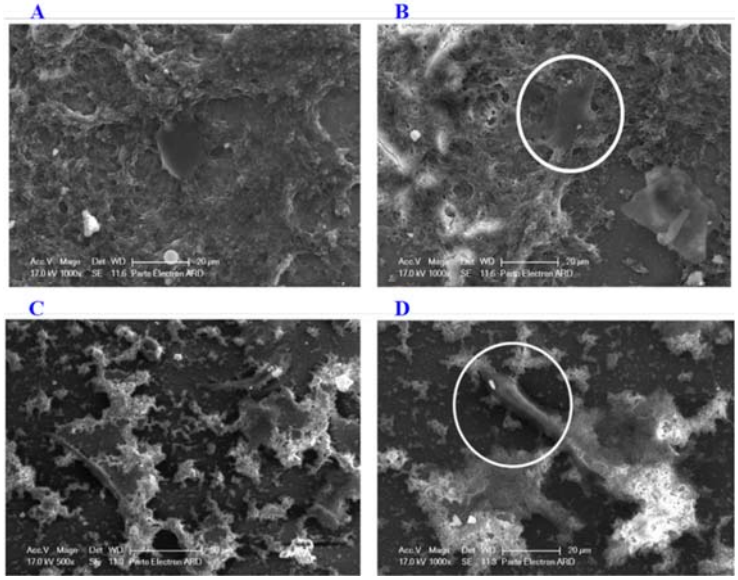

Fig. 4. Scanning electron microscopy of CTS/C/GP and CTS/G/GP hydrogels treated by glutaraldehyde at day 3. (A) Cell-free CTS/C/GP, (B) CTS/C/GP hydrogel containing osteoblast, (C) cell-free CTS/G/GP, (D) CTS/G/GP hydrogel containing osteoblast. CTS, chitosan; C, collagen; G, gelatin; GP, glycerophosphate.

Viability and ALP activity of cells loaded in the hydrogels

Calcein-AM and ethidium staining for live and dead cells showed that most cells were stained with green fluorescence, with the highest density indicating successful supported cell attachment and proliferation (Fig. 5). The cell viability was also qualitatively evaluated using the MTT assay on days 3 and 7. The results showed that the cell viability in all groups was statistically higher on day 7 compared to day 3, and the number of viable cells in LL37-loaded hydrogel was significantly higher than the scaffold without LL37 (Fig. 6A). As shown in Fig. 6B, the ALP activity was significantly higher in all of the groups compared to untreated cells.

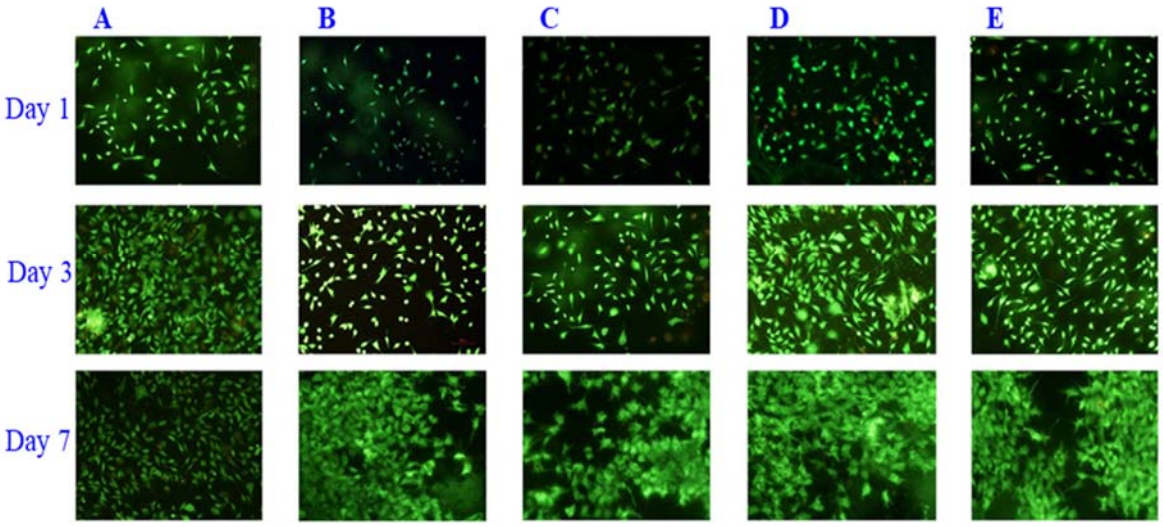

Fig. 5. Viability of osteoblast cells cultured in the hydrogel scaffolds by live/dead stain at days 1, 3, and 7. (A) Control, (B) CTS/G/GP, (C) CTS/C/GP, (D) LL37 loaded CTS/G/GP, (E) LL37 loaded CTS/C/GP. Live cells were green, and dead cells were red. CTS, Chitosan; C, collagen; G, gelatin; GP, glycerophosphate.

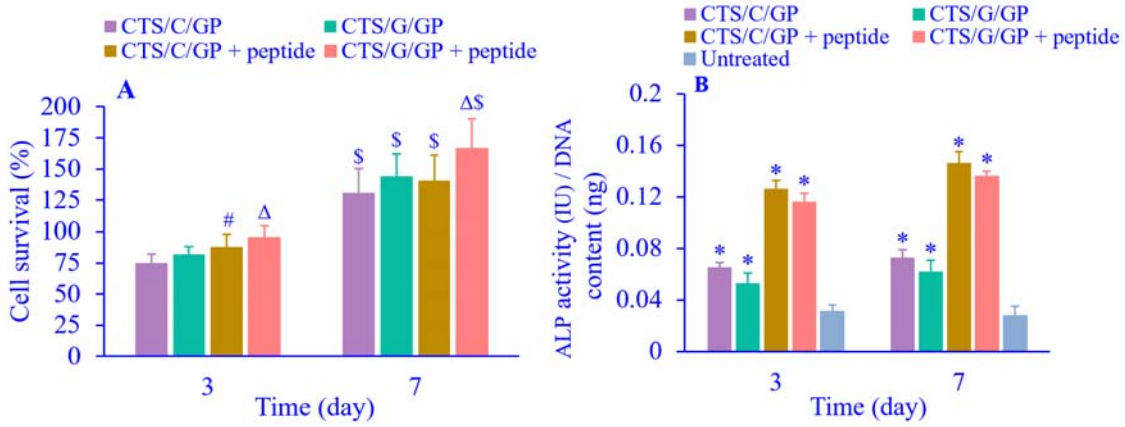

Fig. 6. (A) Cell viability and (B) ALP activity in different formulations after the 3rd and 7th day exposures. Data were expressed as mean ± SD. $^{\$}P \leq 0.05$ indicates significant differences compared to the same formulation on the 3th day; $^{\#}P \leq 0.05$ versus CTS/C/GP at the same time point; $^{\Delta}P \leq 0.05$ versus CTS/G/GP at the same time point; $^{*}P \leq 0.05$ versus untreated. CTS, Chitosan; C, collagen; G, gelatin; GP, glycerophosphate; ALP, alkaline phosphate.

DISCUSSION

Collagen is one of the major components of the extracellular matrix and is found in all of the connective tissues of the body. Gelatin is a derivative of collagen with higher solubility and lower price. The presence of amino acid consequences such as arginine, glycine, and aspartate improved cell attachment and proliferation (26). Chitosan is a naturally derived polymer with visco-elastic properties and biological function, which is widely used in the preparation of hydrogels. LL37 exerts different biological effects such as wound healing, immune response, and angiogenesis in injured tissues. Recently, it has been demonstrated that LL37 could facilitate bone repair by promoting osteogenesis (27). The current study compared the physical properties of chitosan, collagen, or gelatin hydrogel systems and evaluated the effect of adding LL37 on the proliferation and differentiation of mesenchymal stem cells to osteoblasts. CTS/G/GP showed higher viscosity reflected lower friability. While CTS/G/GP demonstrated higher viscosity, there was no significant difference between the release properties of LL37 attributed to the low molecular weight and high-water solubility of the peptide, which could easily pass through the porous structure of the hydrogel systems. The present results showed that the incorporation of LL37 in both of the hydrogel systems significantly increased cell viability and ALP activity. Anders *et al*. indicated that the high concentration of LL37 reduced human osteoblast MG63 cell viability, but the low concentration of LL37 accelerated the viability of the MG63 cell line (28). In another similar study, the osteogenic ability of LL37 on mesenchyme stem cells was shown to be concentration dependent and reached a peak at 4 µg/mL (27). In another similar study, LL37 peptide was loaded into the scaffold of nanoparticles, and the amount of peptide was determined to be 10 µg in each scaffold, whereas one scaffold was implanted in an animal study (29). In the current study, LL37 was loaded at 1 µg/mL of the hydrogels and could support and increase cell proliferation. The incorporation of LL37 peptide significantly increased cell viability and ALP activity, reflecting the prominent effect of LL37 in the induction of osteogenic differentiation compared to groups receiving scaffolds alone. ALP is a widely expressed enzyme in the bone and is responsible for the mineralization of new bone (21). The higher expression of ALP in LL37-treated groups indicated higher mineralization than the untreated and scaffold-treated groups without LL37. The higher viscosity of the CTS/G/GP system compared to the CTS/C/GP contributed to the lower friability of the gelatin-containing system; however, it did not show any significant differences in biological experiments.

CONCLUSION

This study aimed to introduce a scaffold containing LL37 peptide for bone tissue engineering. In order to achieve the goal, collagen or gelatin was added to CTS/GP hydrogel, and its effect on cell proliferation and differentiation was examined. The present results revealed that both hydrogel systems could support cell attachment, proliferation, and differentiation; however, it was higher in the gelatin-containing scaffold. LL37 was successfully loaded in both hydrogel systems and could accelerate cell proliferation and differentiation compared to empty scaffolds.

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Conflict of interest statement

All authors declared no conflict of interest in this study.

Authors' contributions

M. Rezazadeh and V. Akbari contributed to the conception and design of the study, data collection, analysis, interpretation, and writing of the manuscript; A. Sadeghi and N. Tavakoli contributed to the data collection, interpretation, and drafting of the manuscript. All authors read and approved the final version of the manuscript.

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