Bone Regeneration in Rat Using a PCL/gelatin/Nanoclay Nanocomposite Scaffold Containing Silybin

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Research Article

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Abstract

Bone cells need solid structures like the extracellular matrix (ECM) for healing injured areas. Finding appropriate materials and fabrication processes for the scaffold is a challenge in tissue engineering. In this study, 3-D porous scaffold was made of Polycaprolactone/Gelatin/Nanoclay (PCL/GNF/NC) and different dosages of silybin (Sil) were loaded by a combination of electrospinning and thermal-induced phase separation (TIPS) techniques. Different experiments like assessing surface morphology, porosity, compressive strength, water contact angle, degradation rate, releasing profile, hemolysis, and cell proliferation were done to assess attributes of fabricated scaffolds. For in vivo evaluation, the calvaria defect model in rats was used and the result was evaluated by histological analysis. Based on the results, the porosity of scaffolds was in the range of 70-90%, and samples containing silybin had lower compress strength and contact angle and higher degradation rate in comparison with samples without silybin. The results showed that PCL/GNF/NC/Sil1% had better cell proliferation bone healing than other studied groups. The results of this study can be considered for further researches to assess the effect of silybin in bone defect treatment.

1. Introduction

When the size of the damaged tissue is greater than a critical size, cellular signals are incapable to be transmitted, and consequently, the body is unable to repair damaged organs. Bone healing for fractures less than 2 cm is a natural process while for a larger one, other solutions are necessary [1–3]. To healing large damages, external factors are needed, and tissue engineering scaffolds can be a good option to enhance the repair process [4, 5]. The determination of appropriate materials to support properties that need for bone tissue engineering is a big challenge [6, 7]. Gelatin (Gel) is a biocompatible polymer extracted from collagen by hydrolysis [8–10]. In addition, it has a positive effect on bone cell adhesion and migration due to Arg-Gly-Asp (RGD)-like sequences [11]. Electrospun gelatin is widely used for bone healing in pure form or combination with different materials [12, 13].

Poly (ϵ -caprolactone) (PCL) is another biomaterial that is used in different bone tissue engineering studies [14]. PCL is a hydrophobic linear aliphatic polyester and polymer, which is more flexible, higher degradation rate, and has a higher compression modulus in comparison with Poly (L-lactic) acid (PLA) [15]. A previous study informed that a blend of gelatin and PCL had higher biocompatibility and mechanical strength than PCL that is suitable for bone scaffold [16].

Thermally-induced phase separation (TIPS) is a popular technique for bone scaffold fabrication that can produce a 3D scaffold with interconnected pores [17]. In this process, first of all, a homogenous solution is prepared and heated and then it is divided into polymer and solvent-rich phases. In the end, porous construction is taken out after solvent extraction [14]. The mixture of electrospun nanofibers with a 3D scaffold structure can develop in vitro and in vivo functions of the final scaffold [18]. A previous study showed that the combination of the nano-scale mat with a solution can change water permeability, improve resistance, and increase drug release rates [19].

To improve the mechanical and biological properties of bone scaffolds, Nanoclay (NC) can be used [20, 21]. A previous study showed the effectiveness of NC in combination with PCL for bone tissue engineering [22]. Nanocomposites are used because a small volume of reinforcement is required to deliver the requisite growth in properties. NC is a toxic material but a previous study showed that low concentrations of it are not toxic for biological application [23, 24].

The efficiency of the scaffold can be improved by loading different drugs and materials into the scaffolds. Silymarin is a mixture of silybin (Sil), silychristin and silydianin is a flavonolignan complex [25]. Among its components, silybin is the most biologically active component that has antioxidant and free radical scavenging potential [26]. Also, silybin is considered an old drug for liver cancer treatment [27].

Accordingly, we fabricated 3D scaffold for bone tissue engineering by using PCL containing Gel nanofibers (GNFs) and NC containing different amounts of Silybin. The hypothesis of this study is that PCL served as the matrix, GNFs as the simulation of the bone extracellular matrix (ECM), NC for improving mechanical properties and stimulate bone regeneration, and Silybin as the healing agent. The combination of PCL/GNF/NC/Sil has not been used for bone tissue engineering in the form of a 3D scaffold.

2. Material And Methods

2.1. Materials

Silybin (C₂₅H₂₂O₁₀, Molecular Weight:482.44), Gelatin powder (bovine skin, type B), Nanoclay (Al₂Si₂O₅(OH)₄ · 2 H₂O, Molecular Weight: 294.19), Poly (ε-caprolactone) [PCL; Mw = 48–90 kDa], Glutaraldehyde, 1,4-Dioxane, neutral buffered formalin (NBF) were purchased from Sigma-Aldrich (St. Louis, MO). Acetic acid, phosphate-buffered saline (PBS), normal saline, Dimethyl sulfoxide (DMSO), Ethylenediaminetetraacetic acid (EDTA), purchased from Merck Chemicals (Darmstadt, Germany). Fetal Bovine Serum (FBS), Penicillin–Streptomycin (Pen-Strep), MTT ((3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide), Dulbecco's Modified Eagle Medium(DMEM): Nutrient Mixture F-12 (DMEM/F-12), and Trypsin–EDTA were purchased from Gibco (Germany). Ketamine and Xylazine were taken from Alfasan (Woerden, Netherlands). Male adult Wistar rats were kindly provided by Shahroud University of Medical Sciences, Shahroud, Iran.

2.2. Gelatin nanofibers fabrication

The electrospinning technique was used to prepare gelatin nanofibers. Acetic acid aqueous solution [75% (v/v)] was used to dissolve gelatin powder to prepare a 40% (w/v) solution. The solution was loaded into the disposable syringe (10-mL) with a blunted 20-gauge stainless needle, and then it was placed in the holder of the electrospinning machine (Fanavaran Nano-Meghyas, Tehran, Iran). The operating parameters were: applied voltage: 20kV, flow rate: 0.40 mL/h and distance between nozzle and collector

was 15 cm. The product was crosslinked with the vapor of glutaraldehyde (20% (v/v)) at 37°C for 6 h and then they were washed and kept in a nitrogen tank for one night and finally cut into small pieces.

2.3. Preparation of scaffolds

PCL solution (5% (w/v)) was prepared by dissolving it into 1,4-dioxane and then GNFs were added to prepare a solution with the ratio of 10% (w/w). In the end, NC was loaded at the weight ratio of 1:10 (NC: PCL), and it was mixed (24 h) and sonicated (20 min). The fabricated solution was split into 4 groups and the silybin powder (0%, 0.1%, 0.5%, and 1%) was added. The solutions were frozen at -80°C and then freeze-dried for 72 h.

2.4. Scaffold Characterization

2.4.1. Surface evaluation

To evaluate morphology and microstructure of GNFs, PCL/GNF/NC, and PCL/GNF/NC/Sil1%, they were coated by gold for 250 s by using a sputter coater (SCD 004, Balzers, Germany), and then their pictures were taken by a scanning electron microscope (SEM; KYKY Technology Development, Beijing, China) at 15 kV. To assess the effect of Sil on diameters of pores, Image J (National Institutes of Health, Bethesda, USA) and Origin Pro 2015 software (Origin Lab, Northampton, USA) were used to analyze a total of 20 random points in PCL/GNF/NC, and PCL/GNF/NC/Sil1% pictures.

2.4.2. Porosity measurement

The porosity of different scaffolds was measured by the fluid displacement method and the following equation [28].

$$\text{Porosity \%} = \frac{V_1 - V_3}{V_2 - V_3} \times 100 \text{ (Eq. 1)}$$

V_1 , V_2 and V_3 are the volume of the primary liquid (ethanol) in a graduated cylinder, the secondary volume of the liquid after immersion of the scaffold, and the volume after removing the scaffold from the liquid, respectively.

2.4.3. Compressive strength evaluation

A mechanical testing machine (Santam, IRI) with a crosshead speed of 0.5mm/min was used for strength evaluation. Scaffolds were cut into a cylindrical shape with a height of 16 mm and a diameter of 8 mm and compressed vertically until the height dropped to half [29, 30].

2.4.4. Wettability evaluation

The wettability of scaffolds was determined with a sessile drop method by using static contact angle measurements (G10, KRUSS, Germany) [31].

2.4.5. In-vitro degradation

The scaffolds were formed into a cylindrical shape (1 cm^2) and they were weighted (W_i). Next, scaffolds were kept in PBS at 37°C for 14 and 28 days. In specific time points, scaffolds were dried and weighted (W_f). Equation 2 was used to calculate the degradation rate [32].

$$\text{Weight loss \%} = \frac{W_f - W_i}{W_i} \times 100 \text{ (Eq. 2)}$$

2.4.6. The release profile of silybin

Silybin releasing from PCL/GNF/NC scaffold was evaluated by putting PCL/GNF/NC/Sil 1% into the PBS (10 mL) at 37°C . In specific time points, 1 ml of the supernatant was evaluated by UV-VIS spectrophotometer (Cole-Parmer UV/Visible Spectrophotometers, USA) at 288nm [33].

2.4.7. Blood compatibility

For blood compatibility assessment, scaffolds were immersed into the 0.2ml of a combination of human blood (2ml) and normal saline (2.5 ml) at 37°C for 60 min. Blood samples were withdrawn, centrifuged (10 minutes, 1500 rpm) and their absorbances at 545nm were evaluated by the microplate reader (D_t). Negative control (D_{nc}) was mixture of 0.2 ml of diluted blood and 10 ml of normal saline while a combination of 0.2 ml diluted blood and 10 ml of deionized water was positive control (D_{pc}). Equation 3 was used to calculate hemolysis [34].

$$\text{Hemolysis \%} = \frac{D_t - D_{nc}}{D_{pc} - D_{nc}} \times 100 \text{ (Eq. 3)}$$

2.4.8. Cell proliferation

The cell viability of prepared scaffolds was evaluated by an indirect MTT test. Scaffolds were sterilized by UV and then they were put in DMEM medium for 24h and 72h. After the mentioned time, the supernatants were extracted and filtered. On the other hand, bone marrow mesenchymal stem cells (BMSCs) which were isolated from rats based on the previous study was used for this test [35]. 1×10^4 cells were cultured in a 96-well plate by using extracted supernatants cell culture medium. At each time point, the culture medium was replaced with PBS three times and then replaced with 200 μL MTT solution (0.5 mg/ml) and it was kept in an incubator for 4 hours. Then, MTT solution was replaced with 100 μL of DMSO, and absorption was read at 570 nm using a microplate reader after 10 minutes.

2.4.9. In vivo studies

Based on the previous study [28], thirty healthy Wistar rats (250g) rats were anesthesia by injecting Ketamine 5% / Xylazine 2% (70 mg ketamine and 6 mg Xylazine /1 kg body weight). A trephine (Meisinger) was used to create a 7 mm spherical incision in the calvaria (skull) of rats and then they were separated into 6 groups, randomly and treated with the following scaffolds with a diameter of 7mm and thickness of 1mm: PCL/GNF, PCL/GNF/NC/Sil 0%, PCL/GNF/NC/Sil 0.1%, PCL/GNF/NC/Sil 0.5%, PCL/GNF/NC/Sil 1% and negative control (Fig. 1). In the end, periosteum and skin were closed with No.6.0 nylon suture and No. 3.0 nylon suture, respectively.

2.4.10. Histological analysis

After three months, animals were euthanized and their collected tissue (implanted site) was fixed in 10% NBF (PH. 7.26) for 48 h, then decalcified in 19% EDTA, processed, and embedded in paraffin. The sections with 5µm thickness were stained with hematoxylin and eosin (H&E) and Masson's trichrome (MT) and then an independent reviewer assessed slides by using light microscopy (Olympus BX51; Olympus, Tokyo, Japan). The reviewer evaluated the newly formed bone and leftover scaffold and inflammation and biocompatibility of scaffolds. Histomorphometric analysis was done by using computer software Image-Pro Plus® V.6 (Media Cybernetics, Inc., Silver Spring, USA) to calculate and analyze the number of different cells such as fibroblast, fibrocyte, osteoblast, osteocyte, osteoclast, osteon, and other constituents such as blood vessels, new bone tissue formation.

2.5. Statistical Analysis

OriginPro software (2015) (Statistics on Rows) was used and outcomes were reported as a mean $\pm$ standard deviation. In this study, $p < 0.05$ was considered statistically significant.

3. Results

3.1. Morphology

The SEM image of the GNFs, PCL/GNF/NC, and PCL/GNF/NC/Sil1% scaffolds, are shown in figure 2. GNFs were distributed randomly, and they had a consistent shape with smooth morphology. Regarding scaffolds, PCL/GNF/NC and PCL/GNF/NC/Sil1% had connected pores and their average pore size is $36 \pm 11.6 \mu\text{m}$ and $57 \pm 9.3 \mu\text{m}$, respectively. It shows that adding Sil increased pore size.

3.2. Porosity

The scaffolds had a porosity of 70 to 90% which is good for cell penetration and migration (Table1) [36]. The results show that by increasing the volume of Silybin, the porosity decreased. However, the porosity of the PCL/GNF/NC/Sil1% scaffold is still proper for cell migration and proliferation.

3.3. Mechanical strength

Table1 shows that the increasing amount of silybin in scaffolds significantly reduced the mechanical strength. The mechanical strength of the PCL/GNF/NC scaffold was $9.05 \pm 4.1 \text{ MPa}$, while it was $5.3 \pm 3.2 \text{ MPa}$ for PCL/GNF/NC/Sil1%.

3.4. Wettability

wettability of scaffold due to its direct effect on biological response and cell adhesion is considered an important factor in scaffold fabrication [37]. PCL and silybin are strong hydrophobic polymers while gelatin is water-soluble material [38, 39]. Table1 shows the increase of water contact angle after adding silybin.

3.5. Evaluation of Degradation

The weight loss outcomes are shown in Fig. 3A. Based on it, silybin due to its hydrophobicity had a direct effect on the degradation rate and by increasing the percentage of Silybin, the degradation decreased.

3.6. Release

Releasing silybin from PCL/GNF/NC/Sil1% scaffold was evaluated in 9 different time points and results are indicated in Fig. 3B. According to the results, 61.09% of silybin was released after 28 days.

3.7. Hemolysis

Based on the results shown in Fig. 4A, the differences between the positive control group and other experimental groups were highly significant which shows the hemocompatibility of prepared scaffolds. The result also shows that by increasing the amount of silybin in the scaffold, the blood compatibility decreased but it is not significant.

3.8. Cell viability of scaffold

The effect of different scaffolds on cell proliferation was evaluated by indirect MTT assay and results are shown in Fig. 4B. The results indicated that Silybin improved cell viability and proliferation.

3.9. Histopathological analysis

The result of histological evaluation of the negative control group showed that the defect area was filled with fibrous connective tissue (FT) and it didn't have any sign of significant new bone formation (NBF) (Fig. 5). Micrographs of PCL/GNF treatment showed a close similarity to the control group at this time point with the presence of the fibrous connective tissue and the loose areolar connective tissue (LACT) in the defect site.

The histology of the PCL/GNF/NC and PCL/GNF/NC/Sil 0.1% groups indicated that the defect area was filled with FT. NBF was negligible, and remnants of the scaffold also existed in the defect area (Fig. 5).

The histopathological evaluation of PCL/GNF/NC/Sil 0.5% and PCL/GNF/NC/Sil 1% treatments showed new bone formation, however, the majority of defect sites were still filled by FT and remnant of implanted scaffolds.

Table 2 shows Histomorphometric analysis and the results indicated significant differences in NBF, number of osteoblasts-osteocytes, and osteon between PCL/GNF/NC/Sil 0.5% and PCL/GNF/NC/Sil1% and other experimental groups. The number of osteon in these groups was significantly higher than others. The histomorphometric analysis indicated a significant difference in the number of fibroblasts between Ctrl and other experimental groups (Table 3).

Table.1. Characterization of prepared scaffolds.

Samples	Porosity (%)	Compressive strength (MPa)	Contact angle (°)
PCL/Gel/Sil 0%	91.4±5.2	9.05 ± 4.1	80.1 ± 6.09
PCL/Gel/Sil 0.1%	90.1±4.7	8.6 ± 3.3	88.3±7.1
PCL/Gel/ Sil 0.5%	87.6±4.2	7.9 ± 2.6	95.2±5.2
PCL/Gel/ Sil 1%	79.9±5.8	5.3 ± 3.2	103.2 ± 5.4

Table 2

Histomorphometric findings of new bone formation (%) and fibrous tissue formation (%) in the defect area

Valuable	Ctrl	PCL-Gel	PCL-Gel-NC	PCL-Gel-NC+0.1Sil	PCL-Gel-NC+0.5Sil	PCL-Gel-NC+1Sil
NBF (%)	2.4±1.3	3.1 ± 1.1	4.6 ± 2	5.8± 1.9	15 ± 3.6	14 ± 2.6
FTF (%)	91.6±2	90.1 ± 3.4	91.3 ± 4.5	87.8± 3.2	78.7 ± 2.9	81 ± 3.6

Table 3
Histomorphometric findings of bone tissue regeneration in the defect area

Valuable	Ctrl	PCL-Gel	PCL-Gel-NC	PCL-Gel-NC+0.1Sil	PCL-Gel-NC+0.5Sil	PCL-Gel-NC+1Sil
Fibroblast+fibrocyte	126.8 ± 12.3	104.3 ± 10.9	73.3± 7.7	53.6 ± 8.6	53.7 ± 6.2	47.6± 7.3
Osteoblast+osteocyte	4.6± 1.5	9.6 ± 3	9.3± 2.5	12.8± 2.2	29.5 ± 4	30.3± 5.6
Osteon	0	0	0	0.8± 0.8	3.7 ± 0.9	5.6 ± 0.5

4. Discussion

The purpose of this study was evaluation the effect of different dosages of silybin on bone healing. To assess it, PCL/GNF/NC scaffold was used to carry different amounts of silybin to the bone defect site in the rat model.

Biomaterials that are used for drug delivery applications significantly improve bone healing in defect sites. Moreover, each biomaterial used in drug delivery and scaffold fabrication has distinct advantages. PCL is used because of its resorb ability and its capacity to merge with different polymers [40]. Its fabrication process is affordable and almost easy but the biggest problem in using this polymer is its low degradation rate, consequently, it is difficult to use it alone [41, 42]. In addition, it is a high hydrophobic material and by adding gelatin fibers, the hydrophobicity of the scaffold decreases, and cell attachment on the surface of the scaffold increases [43]. Another advantage of using gelatin is that it increases the similarity of scaffold to the trabecular bone and gelatin has the ability to increase angiogenesis and pre-osteoblast cell differentiation by stimulating host cellular response[44–46]. NC improves tensile and compress strength, bioactivity and has been investigated in the processing of bone cement [47]. So, PCL/GNF/NC scaffold was used in this study to not only improve bone regeneration but also carry silybin for more regeneration.

Silybin is the main active component of silymarin that is used for the therapy of different liver illnesses such as cirrhosis, jaundice, and hepatitis [48]. Hayder GaeedOufi showed cytogenetic effects of silybin on the somatic cells of bone marrow. He concluded that silybin has a slight clastogenic effect, and it enhances the inhibitory effect of methotrexate on the mitotic index in a mixture with methotrexate and decreases chromosomal aberrations in bone marrow cells [49]. In this study, different dosages of silybin were loaded into the PCL/GNF/NC to evaluate its effect on bone regeneration.

Freeze-dried (TIPS) scaffolds are a good choice for bone applications because of the similarity of structure to the ECM. The blend of electrospun nanofibers with freeze-dried scaffolds improves the mechanical properties of the scaffold and leads to slower drug-releasing [50]. A scaffold made by the Freeze-drying technique has a porosity of further than 95% with a micro-to-nanoscale dimension [51]. A

previous study showed that cancellous bones have 70% porosity and a scaffold with this porosity is appropriate for bone tissue engineering [52]. Here, scaffolds had an average porosity of 80% which is good for bone regeneration. The compressive strength of bone scaffolds also should be considered in bone scaffold fabrication and it should be in the range of 4-12 MPa to avoid damage and irritations [53]. The mechanical properties of scaffolds in this study was in the range of 5.5-8 MPa which is acceptable as bone scaffold. Loading hesperetin decreases compress strength because its molecules located between scaffold chains.

Some studies reported that the appropriate hemolysis range is less than 2% - 2.5% and some other sources considered below 5% as an acceptable rate [54]. The results showed that adding silybin increased the amount of erythrocyte hemolysis, which is due to the toxicity effect in high dosages but it is still good for biomedical application [48].

Bone and cartilage ECM is made of glycoproteins, hyaluronan, and proteoglycans that are the best source for bone cell stimulation. ECM has a dynamic structure to control releasing various factors and molecules under different conditions [55]. ECM simulation is always considered in scaffold design and fabrication due to its role in reconstruction [56]. The result of this study shows that PCL/GNF/NC scaffolds with and without Sil improve cell growth and they have good communication with the cells. A previous study reported that Sil has a low clastogenic effect, and it has a dose-dependent inhibitory effect on the mitotic index [49].

Histological images indicated the NBF area after three months while the rate of progression and complete bone formation are different [28, 57].

Different studies used various scaffolds for bone healing. In a study, bone regeneration was improved by using collagen–hydroxyapatite scaffolds containing doxycycline [28]. They reported that the best bone regeneration was in the rat treated with collagen/nano-hydroxyapatite/doxycycline after eight weeks. In this study, the higher bone formation happened in PCL/GNF/NC/Sil1% group after four months. Salehi et al used poly (lactic acid)/hydroxyapatite nanoparticle scaffold containing nandrolone decanoate for bone tissue engineering [58]. The prepared scaffold had a porosity of 80% and mechanical compress strength of 6.5 MPa which are the same as fabricated scaffolds in this study.

5. Conclusion

In the current study, PCL/GNF/NC scaffolds containing various concentrations of Silybin were fabricated, and prepared scaffolds were characterized with different in vitro experiments and their effect on bone healing was evaluated with in vivo test. Results confirmed that the PCL/GNF/NC scaffolds containing silybin have good properties for use as bone scaffolds. *In vivo* study outcomes showed the bone regeneration effect of the scaffolds had silybin compared with the scaffold without silybin.

6. Declarations

6.1. Ethics approval and consent to participate

The protocol of present research was reviewed and approved (No.IR.SHAHED.REC.1399.133) by the Shahed University of Medical Sciences Ethics Committee. All experiments were followed the guidelines of the Iran Animal Care Committee.

6.2. Consent for publication

All of the authors confirm the publication of this paper.

6.3. Availability of data and materials

Not available.

6.4. Competing interests

The authors have no conflicts of interest.

6.5. Funding

Not Applicable

6.6. Authors' contributions

All of the authors contributed to different parts of this paper.

6.7. Acknowledgement

Not Applicable

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Figures

Figure 1

The spherical incision in rat calvaria with the scaffold.

Figure 2

SEM image to evaluate structure of (a) Gelatin nanofiber, (b) PCL/GNF/NC, and (c) PCL/GNF/NC/Sil 1%.

Figure 3

(A) Degradation of prepared scaffolds, (B) The cumulative release of Silybine from the PCL/GNF/NC scaffold (n=3).

Figure 4

(A) Results of Hemolytic Rate Experiment, (B) The result of MTT assay to evaluate effect of different scaffolds on BMSCs proliferation and viability (n=3).

Figure 5

Histopathological images of calvarial defects in different experimental groups. Dotted line area: defect, OB: old bone, FT: fibrous connective tissue, NB: new bone formation, SC: Scaffold, LACT: loose areolar connective tissue, H&E, and MT staining. Scale bar 40x: 200µm Scale bar 200x: 100µm

Supplementary Files

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