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Research Article

Efficacy of Luteolin-Loaded Polycaprolactone-Chitosan Polymer Scaffold for Regeneration of Calvarial Bone Defects in Rats

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Abstract

Introduction: There are several occasions that a surgeon needs to perform bone grafting in the Maxillofacial region, such as when there are developmental defects and acquired defects due to trauma. Biological signals, degradable scaffolds, and host cells are used to induce the body's natural regenerative response and reinstate tissue function. Combining natural and synthetic polymers as scaffolds can increase cell interactions and provide mechanical stability. The effect of luteolin on extracellular matrix (ECM) in combination with a synthetic polymer has only been evaluated in skin tissue engineering. This study aimed to synthesize and assess the efficacy of a polycaprolactone-chitosan (PCL/CHT) polymer scaffold containing nanohydroxyapatite (nHA) loaded with luteolin for the regeneration of calvarial bone defects.

Method: In this histological study, 30 rats were randomly assigned to 6 groups (n=5); 8 mm defects were created in each rat calvaria. The defects remained empty in group 1 (negative control), filled with scaffold in group 2 (positive control), and loaded with 0.01, 0.1, 1, and 10% luteolin in groups 3 to 6, respectively. A combination of PCL with fish ECM and CHT was used to improve the polymeric properties of the scaffold. The rate of new bone formation (NBF) was assessed histomorphometrically at 8 weeks after surgery. After an in vitro assessment of the physical and mechanical properties of the scaffold and their agreement with native ECM requirements, an in vivo assessment of the PCL/CHT scaffold was conducted. Data were analyzed using ANOVA and the Tukey test.

Results: The rate of osteogenesis in the 0.01% luteolin-loaded scaffold group was significantly higher than that in the other concentrations (P=0.00). The lowest mean number of inflammatory cells was recorded in the 0.1% group; 0.01% luteolin was the most effective for calvarial bone regeneration in rats.

Conclusion: Proper luteolin concentration on the PCL/CHT/nHA scaffold can enhance osteogenesis in bone tissue regeneration.

Keywords: Polymers, Regeneration, Bone, Allograft.

Introduction

Maxillofacial Trauma could lead to loss of hard and soft tissue with subsequent need for bone grafting. Using bone grafts could be of great assistance in the restoration of bone volume and correction of deformity. Bone grafting induces new bone formation (NBF) in fractures or bone defects and enhances bone regeneration in dental implantation [1]. The osteogenic property of bone grafts is the most critical parameter in bone regeneration [2]. Considering the limitations of autografts and allografts, researchers have focused on

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applying bone graft substitutes according to tissue engineering principles to improve osteogenic properties by using bone progenitor cells and growth factors to enhance cell proliferation in a scaffold [3].

Currently, biological signals, biodegradable scaffolds, and host cells are used to elicit natural regenerative responses of the body to reinstate tissue function [4]. An ideal scaffold possesses the biochemical properties of the target tissue. It serves as a host for both endogenous host cells and grafted cells by enhancing cell adhesion, proliferation, migration, and differentiation and ensuring phenotypic stability [5]. Natural scaffolds are polymers extracted from relatively biocompatible tissues; however, they may occasionally have immunogenicity. However, synthetic scaffolds are biocompatible and resorbable polymers easily converted to three-dimensional matrices with various structures [6].

Polycaprolactone (PCL) is a synthetic hydrophobic FDA-approved polymer with optimal flexibility and biocompatibility, insignificant antigenicity, and non-toxicity, which has biomedical applications [6-8]. It has the potential for use in bone and cartilage regeneration. However, its application in complex tissue engineering is questionable due to its slow degradation speed and low mechanical strength. Its combination with other polymers may be an excellent strategy to accelerate its biodegradation and improve its mechanical properties [9].

Chitosan (CHT) is a natural amino-polysaccharide with a unique structure and several optimal properties (biodegradability, biocompatibility, non-toxicity, antibacterial properties, and no antigenicity). Due to its positive charge, it reinforces cell adhesion and cellular attachment [10]. A study on a PCL-CHT composite scaffold showed that CHT improved the wettability, permeability, and hydrolytic degradation of PCL [11].

The extracellular matrix (ECM) is extensively used in medicine due to its biological source, biodegradability, availability, and low cost [12]. Moreover, due to bioactive elements, ECM plays a pivotal role in the cellular environment and the regulation of signaling activity, and it affects cell migration, proliferation, and differentiation [13]. Enhancement of cell adhesion and function, guiding cell proliferation, and enhancing the function of ECM are among the advantages of using polymers as a scaffold. Thus, synthesizing composite polymers is an effective method to synthesize a suitable biocomposite [14,15]. Synthetic polymers can mimic the physical and dimensional properties of collagen in native ECM; however, due to poor hydrophilicity and the absence of cell recognition sites, they decrease cell-scaffold interactions [16,17]. Thus, ECM as a biopolymer can be mixed with PCL to create a bioactive electrospun scaffold, similar to native ECM in structure and mechanical and biochemical stability [12].

Bone tissue mainly comprises hydroxyapatite (HA), collagen, and non-collagenous proteins. HA is used in bone regeneration due to its structural and compositional similarity to bone [18]. However, since it is fragile and has low resistance, it was combined with synthetic biomaterials in previous studies [19].

Currently, there is a growing interest in the use of herbal products in tissue engineering and the assessment of their efficacy for this purpose. Luteolin is a flavonoid found in different plant extracts with a broad biological activity [20-22]. Animal studies that used luteolin in resorptive bone defects reported a significant increase in bone mineral density [23]. The efficacy of flavonoids in bone regeneration has been previously confirmed. Also, PCL/CHT has been previously used in tissue engineering [24]. Thus, attempts have been made to improve cell-scaffold interactions through improvement of the hydrophilicity of the scaffold and cell recognition sites in nano-fibers [25]. However, the effects of luteolin on ECM in combination with synthetic polymers have only been assessed in skin tissue engineering [26]. This study was the first to assess the efficacy of PCL/CHT and PCL/ECM containing HA nanoparticles (nHA) loaded with luteolin to regenerate calvarial bone defects in rats and possible application in bone regeneration treatments.

Methods

In this study (ethical code: IR.SHAHED.REC.1400.161), 30 rats were randomly assigned to 6 groups (n=5). An 8 mm defect was created in the calvaria of each rat by a trephine bur. The defects remained empty (filled with a blood clot) in group 1 (negative control), were filled with scaffold in group 2 (positive control), and scaffold plus different concentrations of luteolin in groups 3 to 6. The rate of osteogenesis [new bone formation (NBF)] was assessed histologically and histomorphometrically at 8 weeks after surgery. Considering the absence of cells and immunogenicity in tissue engineering using two

different species, the number of viable cells was analyzed using a DNA content assay. For this purpose, the samples containing fish ECM were assessed after centrifugation. However, since the DNA content of fish, ECM was estimated to be over 50%, it could not be used as a scaffold, and the in vivo phase of the study was carried out using a PCL/CHT scaffold.

For the fabrication of PCL/ECM and PCL/CHT polymer scaffolds containing luteolin-loaded nHA, initially, the PCL, ECM, and CHT polymers with 5%(w/v) concentration were dissolved in acetic acid. Next, nHA with a 1wt% concentration of PCL and CHT polymers was added to the solution, and the mixture was stirred for 24 hours. It was then ultrasonicated for 20 minutes at 40 KHz. Finally, the mixture was equally transferred into five bakers. Luteolin was added to each baker in 0.01, 0.1, 1, and 10% concentrations. After one hour of stirring at 50°C, the mixture was freeze-dried at-80°C. The scaffolds were synthesized as such. For the in vitro and in vivo tests, the scaffolds loaded with different concentrations of luteolin were sectioned into pieces with 7 mm diameter and 2 mm thickness 27.

(A) In vitro tests were conducted in the following eight steps at Shahid University to confirm the physical, mechanical, and physiological properties of the scaffold and ensure characteristics comparable to those of native ECM:

1. Seron Technology, South Korea, performed a scanning electron microscopy (SEM) assessment (AIS2100) to assess the scaffolds' surface morphology. For this purpose, 10 mm specimens were prepared from PCL/CHT/nHA scaffolds with and without luteolin. Each piece was then gold sputter-coated to receive a 20 nm coating of gold nanoparticles for conductivity by the chemical vapor deposition technique.
2. The water contact angle was measured using the sessile drop method (Kruss, Germany) to assess the scaffolds' wettability.
3. The degradation speed of scaffolds was calculated by measuring the weight change of scaffolds within one month.
4. The scaffolds' compressive strength was measured by a compression testing machine (Zwick/Roell, Germany).
5. The porosity percentage was calculated by the liquid displacement method.
6. The blood compatibility of scaffolds was assessed according to ISO 10993-4 and calculation of the percentage of hemolysis.
7. The DNA content of fish ECM scaffolds was calculated after centrifugation.
8. The methyl thiazolyl tetrazolium (MTT) assay was conducted to assess the possible cytotoxic effects of luteolin in high concentrations and scaffolds containing different concentrations of luteolin for MG-6 cells. For this purpose, the scaffolds' 24- and 72-hour extracts were added to the culture medium, and cells (7000 cells/well) were added to the wells.

The plates were then incubated at 37°C for 24 hours. After the complete differentiation of cultured cells in the plate, the cells were treated with extracts for 12 hours. The MTT powder was added to sterile phosphate-buffered saline in 0.5 mg/mL concentration, 200 µL of the MTT solution was added to each well, and the plates were incubated again. After 4 hours, the culture medium was removed, 100 µL of dimethyl sulfoxide was added to each well, and the plates were placed in the dark for 10 minutes. Three repetitions were considered for each concentration, and the optical density values were read at 24 and 72 hours by an ELISA reader at 570 nm wavelength.

(B) In vivo tests were carried out in the animal room of the School of Pharmacy of Tehran University of Medical Sciences. For scaffold implantation, 30 male Wistar rats weighing 250 g were randomly assigned to 6 groups (n=5).

First, 8 mm defects were created in each rat calvaria. The defects remained empty (filled with a blood clot) in group 1 (negative control), were filled with scaffold in group 2 (positive control), and scaffold loaded with 0.01, 0.1, 1, and 10% luteolin in groups 3 to 6, respectively. For this purpose, the rats were anesthetized by intraperitoneal injection of ketamine and xylazine. After shaving, a 2-2.5 cm vertical incision was made in the calvaria with a surgical scalpel. The soft tissue was retracted, and an 8-mm defect was created in the distance between bregma and lambda. The defect was filled with a scaffold measuring 8 x 2 mm. The surgical site was sutured in two layers of periosteum and dermis with 4/0 vinyl and nylon sutures (Figure 1).

(C) Histological assessments 2 months after surgery: The calvariae were removed and immersed in neutral 10% formalin in screw-top containers. Fixed specimens were decalcified in 10% EDTA and embedded in

paraffin blocks. They were then sectioned into 5 μm slices, and the slides were inspected under a light microscope (BX51, Olympus, Japan). The percentage of NBF in the entire defect surface area was calculated. For histomorphometric analysis, the mean number of inflammatory cells in 100 cells was calculated using Media Cybernetics, Image-Pro Plus V.6 software.
(D) SEM micrographs obtained from the scaffolds with and without luteolin revealed irregular and inter-connected porosities with internal diameters < 10 to 200 μm.
(E) the rats were sacrificed for histopathological assessment, and the defect area was removed and fixed in formalin for 48 hours. The specimens were processed, embedded in paraffin blocks, sectioned into 5 μm slices, and inspected under a light microscope. The mean percentage of NBF in the entire defect surface area was quantified. The mean number of inflammatory cells in 100 cells was calculated for histomorphometric analysis.
Data were analyzed using GraphPad Prism 8, ANOVA, and the Tukey test. The level of significance was set at 95%.

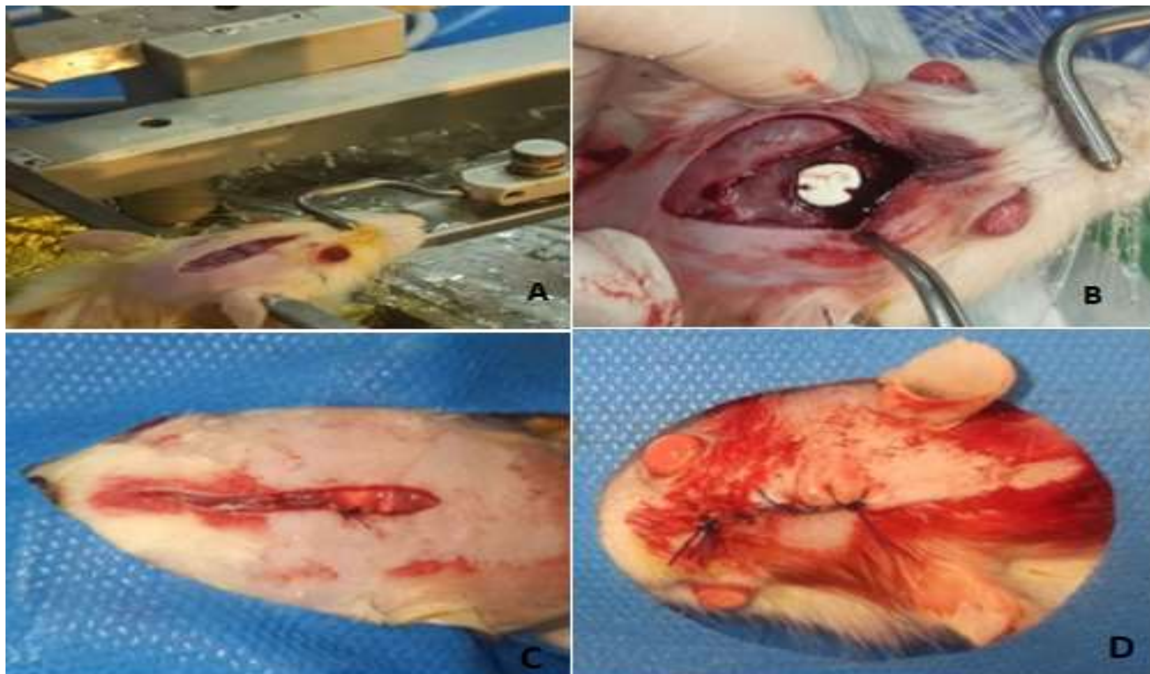

Figure 1: (A) 8-mm defect created by trephine bur, (B) application of scaffold in the defect, (C) suturing of the periosteum, (D) suturing of dermis

Results

Based on the limited sample size, the Shapiro-Wilk test was employed to assess the normality of the data within each group. This statistical test is widely recognized for its robustness in detecting normality, distinguishing it from other normality tests, such as the Kolmogorov-Smirnov test. Notably, it excels, particularly when confronted with small sample sizes, demonstrating a notably elevated level of accuracy (Table 1,2).

Table 1: Shapiro–Wilk test to check normality of Infla

Groups	Statistics	Degree of freedom	P_value
control	0.997	4	0.989
PCL/CHT without Luteolin	0.959	5	0.800
PCL/CHT with 0.01% luteolin	0.953	5	0.760
PCL/CHT with 0.1% luteolin	0.971	4	0.850
PCL/CHT with 1% luteolin	0.990	5	0.980
PCL/CHT with 10% luteolin	0.876	4	0.321

Table 2: Shapiro–Wilk test to check normality of NBF

Groups	Statistics	Degree of freedom	P_value
control	0.836	4	0.272
PCL/CHT without Luteolin	0.951	5	0.742
PCL/CHT with 0.01% luteolin	0.905	5	0.437
PCL/CHT with 0.1% luteolin	0.993	4	0.972
PCL/CHT with 1% luteolin	0.929	5	0.587
PCL/CHT with 10% luteolin	0.848	4	0.220

The results of Table 2 show that the normality assumption is accepted. Additionally, the contact angle in luteolin-free scaffolds was equal to or smaller than that in luteolin-loaded scaffolds. Approximately 9% of luteolin-free scaffolds were degraded in the first 2 weeks, and 30% of luteolin-loaded scaffolds were degraded after 4 weeks. High concentrations of luteolin increased the speed of degradation of the scaffold to some extent; however, the effect of luteolin in this respect was insignificant on scaffolds containing deficient concentrations of luteolin (Table 3).

The mean compressive strength of luteolin-free scaffolds and those containing 10% luteolin was 9.3±2.7 and 6.8±2.1 MPa, respectively. The results indicated that the presence of luteolin decreased the compressive strength of scaffolds.

The highest viability percentage was noted in a scaffold containing 0.01% luteolin, and as the concentration of luteolin increased, the rate of hemolysis increased to some extent.

Table 3: Mean degradation rate of scaffolds containing different concentrations of luteolin after 14 and 28 days

Sample	14 days	28 days
PCL/CHT with 10% luteolin	12%	32.5%
PCL/CHT with 1% luteolin	10.5%	30.5%
PCL/CHT with 0.1% luteolin	8.5%	30%
PCL/CHT with 0.01% luteolin	7%	29%
PCL/CHT without Luteolin	7%	28%

To enhance the reporting of the histopathological results, luteolin-free scaffolds (positive control) and scaffolds containing 0.01, 0.1, 1, and 10% luteolin were coded A, B, C, D, and E, respectively. In the negative control group, bone defects had been filled with loose areolar connective tissue (LACT) and fibrous connective tissue (FCT), and hemorrhage was still evident at the defect site. The micrographs of the regular group showed mature lamellar bone. The defect was filled with residual scaffold material and FCT in

group A. The percentage of NBF was insignificant, and osteogenesis was limited to areas adjacent to old bone. In group B, the NBF percentage was higher than in other groups. Osseointegration had been completed in this group; however, the scaffold material was still detectable. In group C, residual scaffold material and FCT were detected at the defect site, just as in group A. In group D, NBF was noted at the defect site, and the defect had been filled with FCT and residual scaffold material. However, osseointegration was incomplete in this group. Micrographs in group E showed higher inflammation than other treatment groups, and the defect was almost filled with FCT (Figure 2).

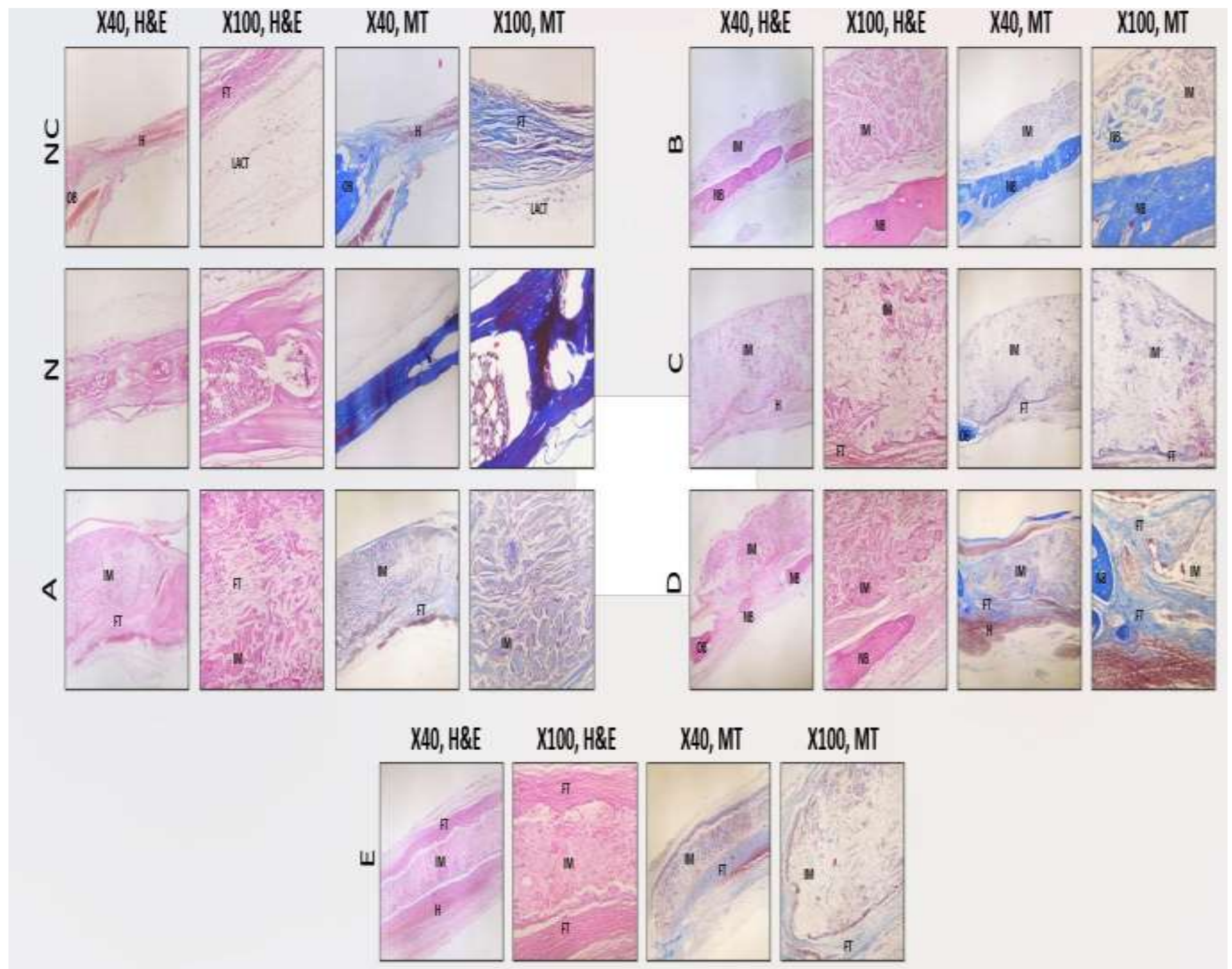

Figure 2: Histological findings in calvarial defects in different treatment groups; IM: Implanted material (scaffold), NB: New bone formation, FT: Fibrous tissue, LACT: Loose areolar connective tissue; H & E and MT staining

Histomorphometric analysis showed a higher percentage of NBF in groups B (20.8±4.3) and D (15.2±4.7). However, the number of inflammatory cells in group E (46.2±10.6) was higher than in other groups. The highest and the lowest mean percentages of inflammation were recorded in 10% and control groups, respectively (Table 4).

ANOVA showed a significant difference in the percentage of inflammation among the groups with different concentrations of luteolin (P=0.000).

According to the rate of inflammation, it may be concluded that luteolin affected bone regeneration in rats. The Tukey test showed that the mean percentage of inflammatory cells was not significantly different between the 1% scaffold and the luteolin-free scaffold (P=0.836). However, this difference was significant between the 10% and 1% groups (P=0.004). Only the difference between the 10% group and all other groups was significant (P<0.05). The lowest mean percentage of inflammatory cells was noted in the control group, followed by the 0.1% group; however, the difference between the 0.1% group and other concentrations was insignificant in this respect (P>0.05, Table 5).

Table 4: Percentage of inflammation in different groups.

Group	Sample size	Minimum	Maximum	Mean	Std. error	Std. deviation
Control	4	11.00	25.00	17.50	2.93	5.85
Luteolin-free scaffold	5	18.00	32.00	24.00	2.34	5.24
0.01%	5	15.00	27.00	21.00	2.21	4.95
0.1%	4	15.00	23.00	18.50	1.71	3.41
1%	5	22.00	35.00	28.60	2.16	4.83
10%	4	31.00	55.00	46.25	5.34	10.69

Table 5: Pairwise comparisons of the groups with different concentrations of luteolin regarding the number of inflammatory cells

Group 1	Group 2	Mean difference	Std. error	P value	95% interval	
					Lower bound	Upper bound
Luteolin-free scaffold	Control	6.25	4.09	0.652	-6.55	19.05
Luteolin-free scaffold	0.01%	3.00	3.86	0.968	-9.07	15.07
Luteolin-free scaffold	0.1%	5.50	4.09	0.758	-7.30	18.30
0.01%	Control	3.25	4.09	0.965	-9.55	16.05
1%	Luteolin-free scaffold	4.60	3.86	0.836	-7.47	16.67
0.01%	0.1%	50.2	4.09	0.989	10.30	15.30
1%	0.01%	7.60	3.86	0.391	-4.47	19.67
0.1%	Control	0.75	4.31	1.00	-12.77	14.25
1%	Control	10.85	4.09	0.128	-1.95	-23.65
1%	0.1%	10.10	4.09	0.179	-2.70	22.90
10%	Control	28.50	4.31	0.000	15.00	42.00
10%	Luteolin-free scaffold	22.25	4.09	0.000	9.44	35.05
10%	0.1%	25.25	4.09	0.000	12.44	38.05
10%	0.1%	27.75	4.31	0.000	14.25	41.25
10%	1%	17.65	4.09	0.004	4.84	30.45

The rate of inflammation only in 10% group was significantly different from that in other groups (P<0.05). Thus, injection of PCL/CHT by up to 1% concentration did not cause a significant change in inflammation, indicating no sensitivity to luteolin with up to 1% concentration (Diagram 1).

The percentage of NBF was assessed in different groups to assess the effect of polymer scaffold on bone regeneration. The highest and the lowest mean NBF were recorded in 0.01% luteolin and the control group, respectively.

ANOVA showed a significant difference in NBF among different groups with different concentrations of luteolin (P=0.000). Thus, luteolin affected the amount of NBF in rat calvarial defects. Pairwise comparisons were carried out using the Tukey test to determine which luteolin concentration had higher efficacy for NBF. The results showed no significant difference in NBF percentage

between 1% and luteolin-free scaffold (P=0.097). The difference in the mean percentage of NBF was significant between the 0.01% and 10% groups (P=0.000). Also, the 0.01% group had significant differences with all other groups except for the 1% group in this regard (Table 6).

The negative control group, the positive control group, and PCL/CHT scaffolds containing 0.01%, 0.1%, and 1% luteolin had almost similar and optimal performance concerning the amount of inflammation. However, 0.01% scaffold resulted in the highest mean percentage of NBF, with significant differences with other groups (P=0.000, Diagram 2).

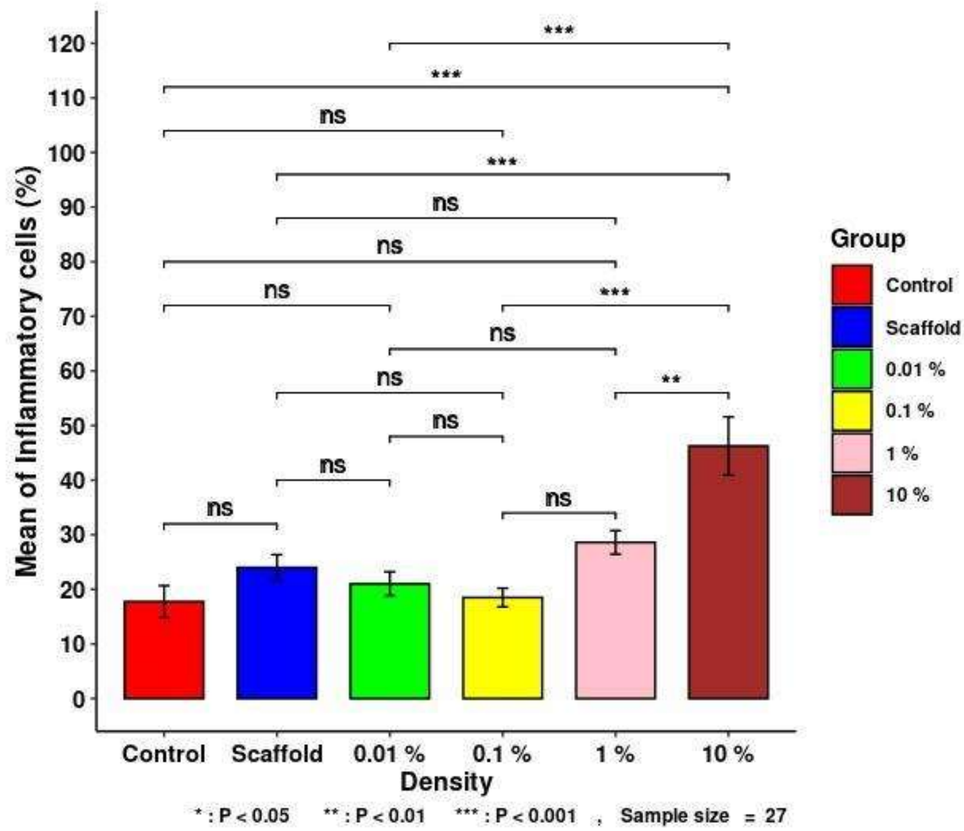

Diagram 1: Mean rate of inflammation in presence of PCL/CHT scaffold containing different concentrations of luteolin, and also in the positive and negative control groups.

Table 6: Pairwise comparisons of the groups regarding the mean percentage of NBF.

Group 1	Group 2	Mean difference	Std. error	P value	95% confidence interval	
					Lower bound	Upper bound
Luteolin-free scaffold	Control	3.15	2.20	0.707	-3.72	10.02
0.01%	Luteolin-free scaffold	11.40	2.07	0.000	4.92	17.88
0.1%	Luteolin-free scaffold	0.10	2.20	1.000	-6.77	6.97
0.01%	Control	14.55	2.20	0.000	7.67	21.42
1%	Luteolin-free scaffold	5.80	2.07	0.097	-0.68	12.28
0.01%	0.1%	11.30	2.20	0.001	4.43	18.17
0.01%	1%	5.60	2.07	0.116	-0.88	12.08
0.1%	Control	3.25	2.32	0.725	-3.99	10.49
1%	Control	8.95	2.20	0.006	2.08	15.82
1%	0.1%	5.70	2.20	0.143	-1.17	12.57
10%	Control	0.50	2.32	1.000	-6.74	7.74
Luteolin-free scaffold	10%	2.65	2.20	0.829	-4.22	9.52
0.01%	10%	14.05	2.20	0.000	7.18	20.10
0.1%	10%	2.75	2.32	0.838	4.46	10.00
1%	10%	8.45	2.20	0.011	1.58	15.32

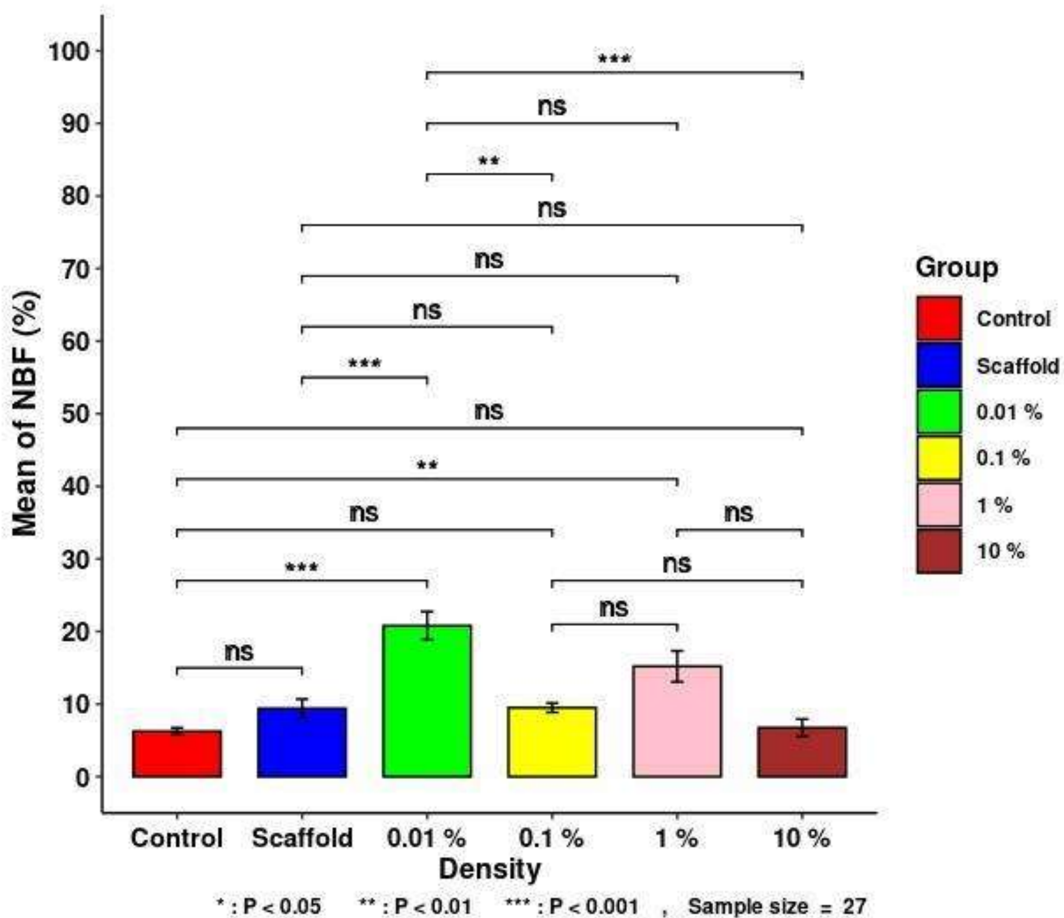

Diagram 2: Mean percentage of NBF in different groups

Discussion

The present results regarding the mean degradation rate of scaffolds containing different concentrations of luteolin after 1 and 2 weeks showed that over time, the scaffold's structure became loose, and its degradation speed increased. Also, high concentrations of luteolin had a significant accelerating effect on the degradation rate, which may be because CHT is degraded in biological systems over time, and its degradation rate can be easily adjusted by controlling the rate of deacetylation during its manufacturing process. It enables the controlled release of medications at the proper site [28]. The present study reported the lowest percentage of inflammation and the highest percentage of NBF in the presence of 0.01% luteolin. Thus, this concentration appears to be the most efficient. By increasing the concentration of luteolin, the hemolysis rate increased to some extent, possibly due to higher inflammation and a higher number of inflammatory cells. The significant difference in the mean number of inflammatory cells was only related to the difference between a 10% concentration of luteolin and other concentrations, and lower concentrations had no significant difference with each other in this regard. Therefore, lower concentrations of luteolin are more suitable for therapeutic purposes.

Zhang et al. [29] evaluated the effect of PCL scaffolds fabricated by freeze-drying. Also, Cho et al. 30 assessed rat calvarial defects and showed optimal efficacy of PCL/HA scaffolds for bone regeneration, consistent with the present findings. The reason is that PCL is a synthetic polymer with a low melting point and optimal porosity [9]. Moreover, since it is bioresorbable and has a slow degradation rate, it is ideal for bone regeneration, and its combination with other polymers is a suitable strategy to improve its mechanical properties [31,32]. In the present study, the authors used CHT as a natural polymer for the scaffold to improve the properties of PCL and create ideal conditions for cell nutrition, growth, and proliferation [32]. CHT, in combination with PCL, improves the wettability and permeability of the scaffold, accelerates the hydrolytic degradation of PCL, and enhances cell recognition sites of PCL [11]. Moreover, PCL as an additive polymer to CHT is used to optimize the morphology and uniform distribution of CHT nanofibers [6-8].

Neves et al. [33] reported the high potential of PCL/CHT scaffolds for use in terms of edema, inflammation, degradation, and mechanical properties. In the in vitro phase, they showed favorable cell adhesion, no cytotoxicity, a high cell proliferation rate, and optimal cell biocompatibility.

In vitro tests are suitable for assessing manufacturing quality and the potential harms of new materials. However, they are conducted on specific cell types and cannot clearly elucidate tissue reaction to the materials [33]. Therefore, the results of the present study's in vivo phase regarding this scaffold's properties have higher reliability.

In the present study, nHA in a 1wt% concentration was added to the PCL/CHT scaffold due to its unique properties. nHA is a bioceramic with properties similar to those of the mineral phase of bone and can be used as a synthetic bone graft material for drug delivery. The addition of nHA to the scaffolds can improve the in vitro and in vivo function of materials because bioactive compounds can directly bond to adjacent tissues [34].

The present study evaluated the possibility of applying ECM as a modifier of PCL polymer. The in vitro phase showed that this matrix had structural and mechanical properties and porosity rates similar to native ECM. Thus, the DNA content test was performed for the fish ECM after centrifugation to assess the possibility of using this acellular matrix in animal models. However, the results showed a DNA content of over 50% and very high toxicity for use as a biomaterial. The results revealed a high possibility of immunogenicity and foreign body reaction when using fish ECM in combination with PCL. Thus, fish ECM was unsuitable for tissue engineering use as a safe scaffold. However, due to their acellular nature, CHT scaffolds were safe and did not have immunogenicity so that they could be used for the in vivo phase. Thus, the scaffolds containing four different concentrations of luteolin were evaluated in the final phase. Consistent with the present results, Lee et al. [35] showed that native luteolin can inhibit osteoclast differentiation and function by inhibiting the related signaling pathway and impairment of actin ring formation [35]. Kim et al., [36], in an earlier study, evaluated the efficacy of luteolin as a phytoestrogen for the treatment of osteoporosis in rats. They reported higher serum levels of alkaline phosphatase in groups with higher concentrations of luteolin and significantly lower levels of osteocalcin and

CTX in luteolin groups compared with OVX groups. Notably, in vivo studies (including animal and human studies) can reveal the biocompatibility of materials. However, conducting a study on animal models does not necessarily allow the conduction of human studies [37,38].

Conclusion

Applying a proper concentration of luteolin for bone regeneration is a suitable strategy for enhancing osteogenesis. In the present study, loading of luteolin in 0.01% concentration on PCL/CHT/nHA scaffold increased osteogenesis compared with other groups. This method could be applied in patients with maxillofacial trauma with a considerable reduction in morbidity and complications. Considering the limitations of this study (complex and high cost of synthesis of scaffold), future studies with a larger sample size are required to assess the effect of luteolin on osteogenesis over different periods in larger animal models and compare with other bone substitutes.

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None.

Conflict of Interest Disclosures

The authors declare that they have no conflict of interests.

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None.

Authors' Contributions

All authors contributed equally to accomplishing this study.

Ethical Statement

This research was confirmed by the ethics committee of Shahed University, Tehran, Iran, with the ethics code: IR.SHAHED.REC.1400.161.

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