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Critical-Sized Bone Defects Regeneration Using a Bone-Inspired 3D Bilayer Collagen Membrane in Combination with Leukocyte and Platelet-Rich Fibrin Membrane (L-PRF): An *In Vivo* Study

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Abstract

Objectives

We aim to develop a 3D-bilayer collagen (COL) membrane reinforced with nano beta-tricalcium-phosphate (nβ-TCP) particles and to evaluate its bone regeneration in combination with leukocyte-platelet-rich fibrin (L-PRF) *in vivo*.

Background data

L-PRF has exhibited promising results as a cell carrier in bone regeneration in a number of clinical studies, however there are some studies that did not confirm the positive results of L-PRF application.

Methods

Mechanical & physiochemical characteristics of the COL/nβ-TCP membrane (1/2 & 1/4) were tested. Proliferation and osteogenic differentiation of seeded cells on bilayer collagen/nβ-TCP thick membrane was examined. Then, critical-sized calvarial defects in 8 white New Zealand rabbits were filled with either Col, Col/nβ-TCP, Col/nβ-TCP combined with L-PRF membrane, or left empty. New bone formation (NBF) was measured histomorphometrically 4 & 8 weeks postoperatively.

Results

Compressive modulus increases while porosity decreases with higher β-TCP concentrations. Mechanical properties improve, with 89 % porosity (pore size ∼100 μm) in the bilayer-collagen/nβ-TCP membrane. The bilayer design also enhances the proliferation and ALP activity. *In vivo* study shows no significant difference among test groups at 4 weeks, but Col/nβ-TCP + L-PRF demonstrates more NBF compared to others ($P < 0.05$) after 8 weeks.

Conclusion

The bilayer-collagen/nβ-TCP thick membrane shows promising physiochemical *in vitro* results and significant NBF, as ¾ of the defect is filled with lamellar bone when combined with L-PRF membrane.

Keywords

Bone regeneration, Leukocyte and platelet-rich fibrin, Beta-Tricalcium phosphate, Rabbit

1. Introduction

In the triad of bone tissue engineering—stem cells, growth factors and scaffold—the latter has become the interest of researchers in recent years (Bastami et al., 2017). Scaffolds harbor stem cells and promote their differentiation and proliferation, allow vascularization and *de novo* bone formation, may be used for local delivery of growth factors and, finally, endure mechanical forces until they degrade (Fahimipour et al., 2017). Various scaffold designs and chemical compositions have been introduced, and their physical and biological characteristics have been evaluated *in vitro*, *in vivo* and clinically in the maxillofacial area (Pang et al., 2013; Yu et al., 2014); (Wang et al., 2014). A three-dimensional (3D) scaffold must be biocompatible and osteo-inductive. It is also favorable that the scaffold degrades at the same pace as new bone is formed. Its mechanical properties should also render the scaffold strong enough to bear stresses, while its porosity allows vessel infiltration and decent nutrient distribution (Arahira and Todo, 2014). Many studies have shown bone regeneration in critical-sized defects using different scaffold templates, but there are still shortcomings regarding mentioned qualifications (Rai et al., 2007; Zhang et al., 2016).

in vivo tissue engineering studies in the realm of oral and maxillofacial reconstruction have illustrated successful applications of different biomaterials, like natural and synthetic polymers, ceramics or composites (Dey et al., 2018; Akhlaghi et al., 2019; Jazayeri et al., 2017; Dong et al., 2002).

Beta-tricalcium phosphate (β-TCP, β-Ca3(PO4)2) is among the calcium phosphates that have been used extensively in bone tissue engineering (Eftekhari et al., 2015; Chazono et al., 2004). β-TCP has demonstrated osteo-conductive capability, phase stability and firm attachment to existing bone tissue (La Monaca et al., 2018; Annibali et al., 2015; Lai et al., 2019).

β-TCP has a Ca/P ratio similar to that of natural bone tissue (Suzuki et al., 2008). Moreover, it has displayed progressive biodegradability accompanied simultaneously by formation of normal bone structure *in vivo* (Tanaka et al., 2017).

Collagen is the most common natural polymer used, in combination with composite scaffolds (Nikpour P et al., 2018). Many studies have shown that addition of collagen has the advantage of enhancing compressive modulus and strength of the scaffold, as well as promoting proliferation and expression of osteo-conductive factors (Hao et al., 2010; Liao et al., 2014; Zhou et al., 2007). Collagen type I is commercially available for delivery of bioactive molecules in bone tissue engineering (Friess et al., 1999). Although collagen can promote bone formation, its mechanical properties as a scaffold are less than favorable, especially for use in load-bearing areas (Bulgin et al., 2013). Thus, it has been prepared in combination with β-TCP in various ratios and through different methods (Sun et al., 2003; Xiang et al., 2003; Brkovic et al., 2008; Lee and Kim, 2011). A homogenous and well-integrated structure is desired when preparing these scaffolds, so that scaffold properties (such as the porosity and weight ratios) are reliable (Zou et al., 2005). These scaffolds are commonly fabricated as collagen coated β-TCP (Hasani-Sadrabadi et al., 2014; Deschamps et al., 2017), β-TCP coated collagen (Murakami et al., 2017; Al-Munajjed and O'Brien, 2009) or composites (Inzana et al., 2014; Du et al., 2000; Kang et al., 2015). Bilayer collagen membrane has been used in the treatment of periodontal bony defects. Collagen can readily adapt to bone surface, and new bone formation has been observed (Camelo et al., 2001; Burns et al., 2000; Sakata et al., 2006). Reinforcing the bilayer collagen design with β-TCP particles provides a construct mimicking natural bone structure.

Leukocyte platelet-rich fibrin (L-PRF) is the second generation of platelet concentrates, which were first introduced in 2001 by Choukroun et al. in the regeneration of oral and maxillofacial defects (Choukroun et al., 2001). This complex network of autologous fibrin is a favorable carrier capable of releasing growth factors within 1–4 weeks and promoting micro-vascularization, bone growth and maturation (Dohan et al., 2006). This concentrate contains cytokines that have been shown to have anti-inflammatory and osteo-inductive characteristics (Saluja et al., 2011). L-PRF has exhibited promising results as a cell carrier in bone regeneration in a number of clinical studies (Öncü and Alaaddinoglu, 2015; Pradeep et al., 2015; Kumar et al., 2015). Kim et al. demonstrated promising results in the application of L-PRF combined with TCP in rabbits (Kim et al., 2014). Similarly, in another study, L-PRF proved equally successful in regenerating rabbit calvarial defects compared to hydroxyapatite TCP scaffolds. The most desirable results, however, were obtained when the defects were filled with the combination of those (Yilmaz et al., 2014).

In this study, a 3D collagen scaffold has been fabricated and reinforced with β -TCP nano-particles. A thorough characteristic evaluation of the new design was conducted, and its osteoconductive capability was tested *in vitro*. Finally, critical-sized defects in rabbit calvarias were grafted by the designed thick membrane combined with L-PRF membrane to evaluate *in vivo* bone formation (Fig. 1).

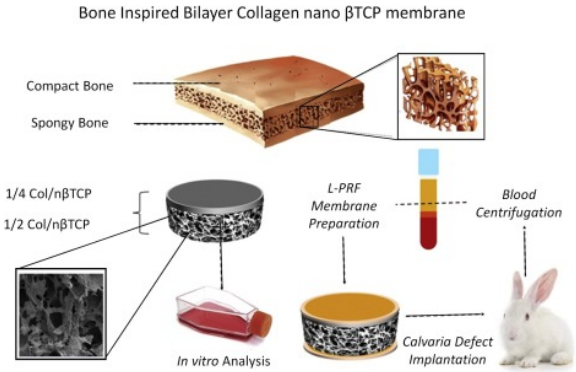


Fig. 1. Schematic design of experiment.

2. Materials and Methods

2.1. Bilayer Thick Membrane Fabrication

2.1.1. β -TCP Collagen Bilayer Membrane

Collagen type I solution (Sigma, USA), 50 mg/mL, was dispersed in 30 mL of 0.1 M acetic acid in de-ionized water (pH = 1–4) at room temperature. This concentration of collagen was the maximum attainable while still maintaining an even dispersion. Mixing was performed using a homogeniser.

Calcium phosphate powders (relative ratios of 1.5 and 1.6) were synthesized by ethanol-based sol-gel method with phosphorus pentoxide (P_2O_5, MERCK) and calcium nitrate ($Ca(NO_3)_2.4H_2O$, MERCK) as the chemical precursors, respectively (Öncü and Alaaddinoglu, 2015). Briefly, the 0.5 M phosphorus pentoxide (aqueous solution) and 1.5, 1.6 and 1.67 M of calcium nitrate in pure ethanol were obtained separately. Then phosphorus solution was slowly added into the calcium containing solution and stirred using a mechanical stirrer (1000 rpm). The obtained sol was stirred for another 30 min. The resulting transparent sol was kept in a closed container for 48 h at room temperature to obtain gel through aging process. After drying the samples in a 60 °C oven for 1 day, the powders were kept at 120 °C for 3 h to be calcined. The temperature was later increased with heating rate of 1 °C/min up to 600 °C and held at this temperature for 1 h. The final step utilized a press machine (pressure of 0.15 MPa), and the powders were stored at 1100 °C for 3 h (Pradeep et al., 2015). β-TCP reinforcements were then added at 1/2 or 1/4 (collagen/ β-TCP by weight), and then the solution was mixed for another 2 min. The collagen/β-TCP solution was degassed in a vacuum desiccator for 15 min. After a uniformly suspending mixture was formed, the mixture was poured into separate cylindrical silicone molds, with or without β-TCPs. The molds were placed in a freeze dryer (Labconco Inc., Kansas City, MO) on a specimen plate and cooled to −80 °C. Scaffolds were lyophilized for 24 h and stored at 0 °C until further use.

In order to fabricate the bone-inspired bilayer collagen/nβ-TCP membrane, a novel method with two freeze drying cycles was performed. First, the collagen/nβ-TCP membrane with the ratio of 1/4 was fabricated. The freeze-dried 1/4 collagen/nβ-TCP membrane was placed on top of the 1/2 collagen/nβ-TCP solution before freezing. After that, the bilayer membrane was frozen at -80 °C, lyophilized for 24 h and stored at 0 °C until use. All scaffolds were cross-linked using EDC/NHS in order to stiffen the collagen network. Pure collagen scaffolds were prepared as a control, and the two test groups were reinforced with β-TCP powder at 1/2 and 1/4 ratios (collagen/ β-TCP by weight), respectively. Scaffolds

at 1/2 and 1/4 ratio approximated lower and upper bounds of the mineral content present in the ECM of natural bone (Kumar et al., 2015).

2.2. Membrane characterization

2.2.1. Structure, morphology and surface topography

Evaluation of the scaffold's morphology and surface roughness were conducted by Scanning Electron Microscopy (SEM). Three of the scaffolds were randomly selected, and the dimensions of the pore size, distance between strands and the thickness of strands in scaffolds were measured using Image J software.

2.2.2. Porosity and mechanical properties

Porosity was measured using solvent displacement method, as previously reported (Kim et al., 2014). Briefly, scaffolds were weighed in dry state, then immersed in 10 ml ethanol. The specimens containing ethanol were weighed and porosity of scaffolds was calculated using the following equation:

where W_w and W_d are the weight of scaffold in wet and dry state, ρ is the solvent density, while R and T are the radius and thickness of scaffolds, respectively.

The compressive modulus and strength of the collagen-based matrices and Col/nβ-TCP constructs were measured utilizing a mechanical testing machine (Shimadzu, Japan) with cross-head speed of 1.0 $mm.min^{-1}$ and a 5 kN load cell. The cylindrical samples (n = 5) were compressed until they crushed.

3. *In vitro* analysis

3.1. Cell biocompatibility

At the first step, scaffolds were disinfected by triple immersion in 70 % ethanol, followed by rinsing with PBS for 15 min cycles and air drying. 0.5 × 106 third passaged rabbit bone marrow derived mesenchymal stem cells (MSCs) were seeded on the scaffolds and immersed in a growth medium that was refreshed every other day. [3-(4,5-dimethylthiazol-2-yl)-1,5-diphenyltetrazulium bromide] (MTT, Sigma, USA) mitochondrial reaction method was used to determine the biocompability of the scaffold. Briefly, after 24 h of cellular cultivation, the scaffolds were washed with PBS and incubated with MTT solution (5 mg/ml in PBS) for 2 h at 37 °C. The MTT solution was replaced with 0.5 ml of dimethylsulphoxide (DMSO) as an extraction solution. The absorbance of the supernatant at 540 nm was recorded by a spectrophotometric plate reader (Synergy HTX, BioTEK).

3.2. Cell proliferation and cell adhesion

PrestoBlue® (PB) assay (Invitrogen, USA) was carried out according to the manufacturer's instructions at different time intervals of 7 and 14 days after cell culture. The scaffolds were disinfected using 70 % ethanol, as described above. Afterward, 1×10^6 cells were seeded onto the scaffolds, which were placed in a non-adherent 12 wells-plate and then submerged in culture medium. The medium was changed every other day. After the incubation period, 10 % PrestoBlue was added to the scaffold-cell constructs. The fluorescence measurements (Ex: 560 nm and Em: 590 nm) were determined by a spectrophotometric plate reader (Synergy HTX, BioTEK). In order to record the difference between study groups, triplicate samples were analyzed for this experiment.

The attachment of cells on the scaffolds was examined by scanning electron microscopy (SEM). To do this, scaffolds were removed from the culture medium, washed in PBS and fixed with 4 % glutaraldehyde. The seeded constructs were then dehydrated in gradient ethanol concentrations of 50, 70, 80, 90 and 100 % to conserve their intact morphology. Samples were coated with gold by means of a GSL-1100X-SPC12 Compact Plasma Sputtering Coater instrument and scanned for their microstructure using scanning electron microscope (SEM, JEOL JSM-6510LV).

3.3. Cell differentiation

ALP activity of cells cultured on scaffolds was assessed utilizing an ALP assay kit (Abcam, USA). The samples were incubated in osteogenic medium and tested after 7 and 14 days to evaluate osteogenic differentiation. The cell-scaffold constructs were washed with PBS, and lysis buffer solution was added to lyse the attached cells. After sonication and centrifugation, 50 µl of supernatant reacted with 150 µl of p-nitrophenyl phosphate (p-NPP) for 30 min at 37 °C. The reaction was terminated with stop solution, and the absorbance of p-nitrophenol was measured at 405 nm employing a microplate reader (Synergy HTX, BioTEK) to indicate the ALP quantity. Cell lysates were analyzed for protein content using a micro-BCA Assay kit (Pierce), and ALP activity was normalized to the total protein content; alkaline phosphatase activity is expressed in arbitrary units. The results represent the mean values of three individual experiments and each in quadruplicate.

4. *In vivo* analysis

The study protocol was approved by the Ethics Committee at Tehran University of Medical Sciences. Eight New Zealand white albino rabbits were maintained and operated in accordance with the guidelines of the Institutional Animal Care and Use Committee Tehran University of Medical Sciences and the standards of the Association for Assessment and Accreditation of Laboratory Animal Care.

4.1. L-PRF preparation

The L-PRF was prepared *via* a single centrifugation of blood according to the approved protocol for a period of 12 min at 2700 rpm. The animal blood was taken in 9 mL tubes, centrifuged, and used for the experimental sites. After centrifugation, each L-PRF clot was separated from the centrifuged blood samples. Afterward, the L-PRF clot was condensed and molded on a sterile surgical plate and used as a layer of membrane.

4.2. Surgical procedure

Briefly, under general anesthesia induced by intramuscular injection of 35 mg/kg ketamine and 5 mg/kg xylazine, four defects with a diameter of 8 mm were created on the calvaria of the rabbits using a trephine bur. Defects were randomly filled with solo collagen scaffolds (Col), collagen/nano β-TCP scaffolds (Col/nβ-TCP) or Col/nβ-TCP scaffolds combined with L-PRF membrane (Col/nβ-TCP + L-PRF), while the 4th defect was left empty as a negative control. Connective tissue, at first, was sutured by vicril (4-0, SUPA, Iran) to prevent displacement of the grafted materials, and skin was closed by nylon (4-0, SUPA, Iran). The rabbits recovered with no complications and were sacrificed 4 or 8 weeks postoperatively. Harvested samples were fixed and maintained in formalin (10 % wt) for further analysis.

4.3. Histologic and histomorphometric assessments

The samples were decalcified in nitric acid for two weeks. Longitudinal slices were cut and embedded into paraffin blocks. Then, 5-micron thick slices were sectioned and stained with hematoxylin and eosin (H&E), to be evaluated under a light microscope (Nikon Eclipse E400, Nikon, Samida KU, Japan). Inflammation was evaluated in two ways: infiltration of acute and chronic inflammatory cells and the intensity of inflammation (score 0–3 for <10 %, 10–30 %, 30–50 % and >50 % inflammation, respectively). With regard to bone regeneration, type of bone (lamellar or woven) and location of new bone formation (marginal or central) were evaluated. Foreign body reaction (presence of giant cells) was also assessed. For histomorphometric analysis, the images were taken by a digital camera (Nikon Fuji HC300 Nikon) from each defect under 4X magnification of light microscope. Computer-assisted histomorphometric analysis of the new bone formation were performed using an automated image analysis software program (IHMMA, Ver. 1, SBMU, Iran). In fact, the percentage of newly formed bone and residual materials regarding the whole defect area were reported for each specimen by an experienced oral and maxillofacial pathologist blinded to the study design.

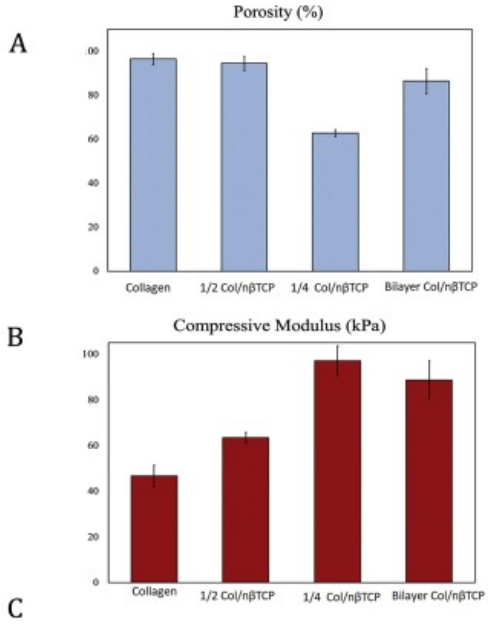
5. Statistical analysis

SPSS version 21 (Microsoft, IL, USA) was used for data analysis. Data is expressed as mean ± standard deviation. One-way and two-way analysis of variance (ANOVA) was used to investigate the quantitative data. Post-hoc comparisons were performed using Turkey's HSD test. The level of significance for all tests is set at $p < 0.05$.

6. Results

6.1. Physicomechanical characterization

The porosity of collagen and 1/2 Col/nβ-TCP scaffolds are 96.41 ± 2.6 and 94.45 ± 1.7, respectively. The mean scaffolds porosity decreases to 62.65 ± 3.1 with increased β-TCP fraction to 4 ($p < 0.05$). The mean pore size diameter is also affected by the composition of samples. The compressive modulus enhances with increased β-TCP weight fraction ($p < 0.05$). Scaffolds reinforced with β-TCP at 1/2 and 1/4 ratios (collagen/ β-TCP) exhibit an increased compressive modulus compared to the bare collagen ($p < 0.05$). An improved mechanical property with appropriate porosity of 89 % and a pore size of ∼100 μm is achieved in the bilayer collagen/ nβ-TCP membrane design. (Fig. 2)



Composision	Collagen	1:2 Collagen: nβTCP	1:4 Collagen: nβTCP
Pore Size (Mean ± SD)	108.6 ± 8.39	96.5 ± 14.67	66.8 ± 10.73

Fig. 2. (A) Porosity, (B) compressive modulus and (C) pore size of Col, Col/nβ-TCP (1/2), Col/nβ-TCP (1/4) scaffolds.

6.2. Biological results

Cell biocompatibility of the scaffolds is confirmed by MTT [3-(4,5-dimethylthiazol-2-yl)-1,5-diphenyltetrazulium bromide] assay (Fig. 3). The cellular proliferation is shown in Fig. 4A. The cells show progressive proliferation in all groups. As evident, the cells proliferate more onto the 1/2 Col/nβ-TCP, bilayer Col/nβ-TCP than COL and 1/4 Col/nβ-TCP construct. The number of cells is significantly greater after 14 days compared to 7 days ($p < 0.05$). The 3D culture of the MCT3T cells on the scaffolds shows better proliferation compared to those cultured on the tissue culture plate ($p < 0.05$) (Fig. 4). Scaffold composition demonstrates a significant effect on ALP activity by MCT3T ($p < 0.05$). By day 14, β-TCP-containing scaffold has significantly higher ALP activity than the collagen scaffolds in osteogenic media. Cells seeded on the 1/4 and bilayer thick membranes exhibit significantly higher ALP activity at both day 7 and day 14 compared to other groups ($p < 0.05$). By day 14, ALP activity is also significantly higher in the 1/2 group than the control ($p < 0.05$) (Fig. 4B). By using the bilayer design to achieve the optimal pore size and mechanical properties, the cellular proliferation and ALP activity are also optimized.

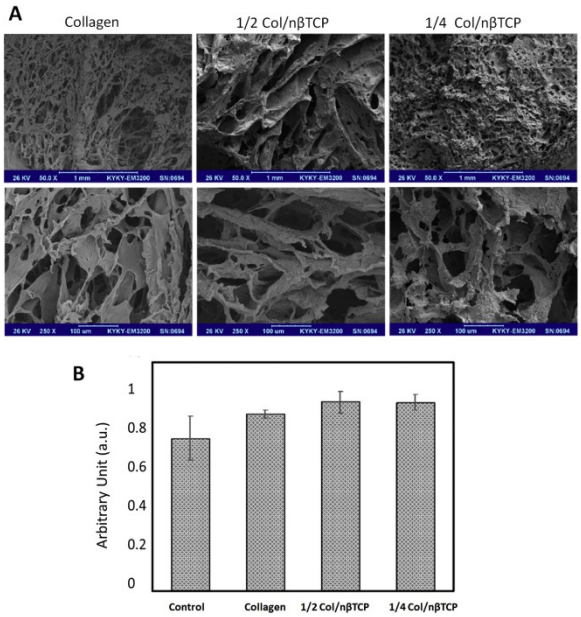


Fig. 3. (A) Scanning electron micrographs (SEM) of scaffolds with different compositions and (B) MTT assay result.

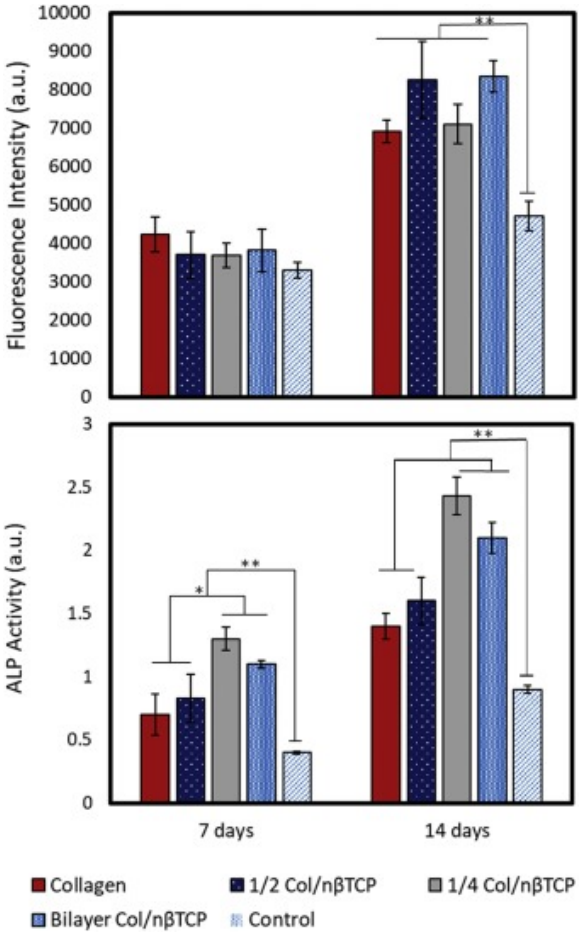


Fig. 4. (A) Cellular proliferation and (B) ALP activity on the scaffolds and tissue culture dish (control).

6.3. *In vivo* results

Minimal inflammation is observed in all study groups (below 10 %). No foreign body reaction is encountered in any of Col/nβ-TCP scaffold groups. The new bone formation after 4 and 8 weeks among different groups is demonstrated in Fig. 5.

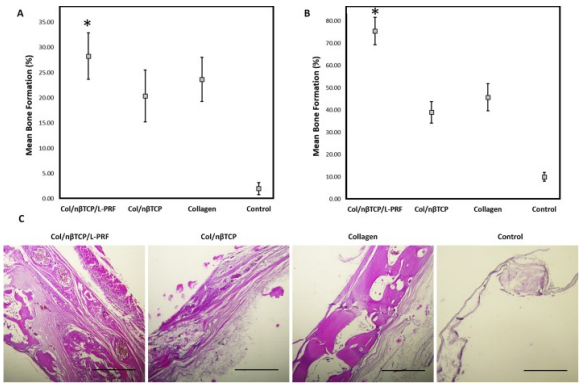


Fig. 5. New bone formation of Col, Col/nβ-TCP, Col/nβ-TCP + L-PRF and control groups after (A) 4 weeks and (B) 8 weeks, and H&E staining after 8 weeks (C).

In the Col/nβ-TCP + L-PRF group, new bone formation is 28.86 ± 2.40 % and 73.36 ± 3.74 % at 4 and 8 weeks, respectively. At week 8, newly formed bone has a fully mature lamellar structure. In the Col/nβ-TCP group, new bone is measured to be 23.6 ± 2.83 % and 41.05 ± 2.46 % after 4 and 8 weeks, respectively. In Col group, 22.88 ± 2.41 % new bone is formed after 4 weeks, which is increased to 45.18 ± 2.93 % at week 8. In the control group after 4 weeks, the defect core is empty—although a woven bony structure is developed in the peripheries, but this is statistically insignificant. At the 8th week, the percentage of newly formed bone reaches 8.7 ± 1.29 % in the peripheries, but this was of woven architecture.

7. Discussion

Endogenous mechanisms are found to be insufficient in healing critical-sized bony defects (Fahimipour et al., 2018, 2019), thus, various methods have been utilized to help accelerate the process. Autogenous bone grafts are currently assumed to be the gold standard.[20] However, it requires a two-step surgical procedure and has a risk of donor side morbidity and bone resorption (Shibuya and Jupiter, 2015). Given these downsides, other methods—such as xenografts and allografts—have been proposed. But slow bone formation and often unpredictable results, even rejection, is possible (Akhlaghi et al., 2019; Bauer and Muschler, 2000; Liu et al., 1997; Zioupos et al., 2008). Filling the defect with proper prefabricated scaffolds combined with the delivery of growth factors have also been proposed (Khojasteh et al., 2016). Ideally, a scaffold with suitable mechanical properties, as well as physical and biological characteristics, is desired for bone tissue engineering (Arahira and Todo, 2014).

In this study, a freeze-dried collagen scaffold seeded with β-TCP nanoparticles was fabricated. The combination of the ideal ratios of 1/2 and 1/4 is applied, considering the *in vitro* results. Later, 8 mm defects were created on the calvaria of New Zealand white rabbits and filled with either β-TCP/Collagen bilayer and β-TCP/Collagen + L-PRF membrane. Two remaining defects were either filled

with solo collagen scaffold or left empty. Histomorphometric analysis was run 4 and 8 weeks postoperatively. The best results are obtained by the COL/ β-TCP + L-PRF group. This design of the scaffold not only favors attachment and proliferation of MCT3T cells, but also shows significantly higher osteogenic ability compared to the control. Cell growth is observed after 7 days and significantly increases by day 14. MCT3T cells proliferate significantly better on the designed scaffolds as compared to culture plates.

Studies have investigated β-TCP alone or in combination with other material in shapes of granules, plates and scaffolds for bone tissue engineering (Ogose et al., 2002; Tanaka et al., 2012; Bulgin and Hodzic, 2012; Rajan et al., 2006; Tawil and Mawla, 2001). Collagen type I is a key component of natural bone extracellular matrix. Tawil et al. demonstrated that bilayer collagen membrane can help increase implant survival rate and quality of graft healing when used in sinus floor elevation (Baheiraei et al., 2018). In another study by Schwarz et al., peri-implantitis defects were treated with either nano hydroxyapatite or bovine-bone xenograft combined with bilayer collagen membrane. It was concluded that bilayer collagen membrane resulted in probing depth reduction and clinical attachment level gain after 6 months (Schwarz et al., 2006). Although collagen can promote bone formation, its low stiffness renders it mechanically weak, especially for load-bearing areas (Bulgin et al., 2013). Thus merging β-TCP nanoparticles with collagen results in a feasible scaffold with materials similar to those of natural bone and with enough strength to support different stages of bone regeneration. This bone-inspired design is our strategy in this study. A clinician must opt for a scaffold with suitable pore size to allow for vascular infiltration and bone ingrowth. In the current study, a pore size of about 60 μm is observed in the specimens with 96.41 (SD = 2.6) porosity, and this decreases with higher ratio of β-TCP nanoparticles.

Two different ratios of collagen to β-TCP (1/2 and 1/4) was tested i*n vitro* to determine the most convenient. While the compressive modulus increases with higher concentrations of β-TC, density of puros decreases. The proliferation rate of cells is similar between different ratios of β -TCP scaffold, while ALP activity is significantly higher in 1/4 ratio compared to other groups. Baheiraei et al. also prepared a scaffold with 1/4 ratio (collagen to β-TCP particles) and concluded that addition of β-TCP to the scaffolds significantly enhanced mechanical and biological properties (Baheiraei et al., 2018; Mojahediana et al., 2016). In our study, the compressive modulus is significantly higher in the scaffolds containing β-TCP with a mean of 83.21 (SD = 6.37) in 1/4 composition compared to the collagen alone, indicating inferior mechanical characteristics of the collagen scaffolds.

Various compositions of Col/nβ-TCP scaffold in combination with growth factors or stem cells have been documented for *in vivo* studies, although their fabrication process has been varied (Xiang et al., 2003; Brkovic et al., 2008; Lee and Kim, 2011; Zou et al., 2005; Fahimipour et al., 2018; Fahimipour et al., 2019; Khojasteh et al., 2017). In a study, Col/nβ-TCP composite scaffolds resulted in a complete bone tissue regeneration after 12 weeks *in vivo* (Zou et al., 2005). In another study, rabbit tibial defects were filled with Col/nβ-TCP composite scaffolds and fibroblast growth factor-2 (FGF-2). The authors observed more bone formation with addition of FGF-2 (Komaki et al., 2006). Inzana et al. used a 3D printed Col/nβ-TCP scaffold and showed that the addition collagen improved flexural strength and cell viability, as well as osteo-conductivity of the scaffolds (Inzana et al., 2014). Alternatively, β –TCP particles can be coated on collagen scaffolds. Murakami et al. demonstrated that compressive

strength, calcium ion release and enzyme resistance of β –TCP coated collagen scaffolds increased with β-TCP ratio (Murakami et al., 2017).

Another challenge that researchers encounter in regenerating bony defects is development of biological additives, such as platelet concentrate to reduce inflammation and promote healing (Miron et al., 2017). L-PRF is a fibrin matrix that acts as a reservoir of cytokines, growth factors and cells to be released in a specific period of time. This natural and economic material needs no further biomechanical modification and can be utilized as a resorbable membrane (Gassling et al., 2013). L-PRF has been used extensively in ridge augmentation procedures, sinus augmentation surgeries, guided bone regeneration (GBR) and treatment of periodontal and endodontic defects (Bastami and Khojasteh, 2016; Xuan et al., 2014). In 2012, Kim et al. used commercial TCP and L-PRF in the regeneration of bone in the calvarium defects of rabbits (Kim et al., 2012). They observed greatest new bone formation when TCP was combined with L-PRF 2, 4, 6 and 8 weeks postoperatively. Also, in our study, significantly more new bone formation is observed in Col/nβ-TCP + L-PRF groups compared to others. This newly formed bone has a lamellar structure, as opposed to the woven architecture of bone formed in the control group.

Conversely, in another study, Comert et al. found that adding PRF to β–TCP scaffolds did not significantly improve the new bone formation in maxillary sinus-floor elevation procedure (Comert Kilic et al., 2017). In another study, rabbit calvarial defects were filled with either PRF membrane or PRF and β–TCP. A third group of defects were left empty as controls. Authors argued that significant bone formation was observed in both groups compared to the control (Abdullah, 2016). In our study, minimal new bone (8.7 %) is formed at week 8, which is a woven structure that is limited to peripheries.

8. Conclusion

Briefly, in this study, a β-TCP-reinforced collagen scaffold with desirable physical features has been fabricated. This scaffold shows promising results in cell adhesion and proliferation, as well as osteogenic differentiation. In the conducted preclinical study, up to three-fourths of the defect is regenerated with lamellar bone when the scaffold is used in combination with L-PRF membrane. The designed scaffold can be further investigated for the delivery of cells and various growth factors to initiate and advance bone tissue regeneration.

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