Fabrication of Decellularized Engineered Extracellular Matrix through Bioreactor-Based Environment for Bone Tissue Engineering

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ABSTRACT: Extracellular matrix (ECM)-contained grafts can be achieved by decellularization of native bones or synthetic scaffolds. Limitations associated with harvesting the native bone has raised interest in preparing in vitro ECM bioscaffolds for bone tissue engineering. Here, we intend to develop an ECM-contained construct via decellularizing an engineered gelatin-coated β-tricalcium phosphate (gTCP) scaffold. In order to find an optimal protocol for decellularization of cell-loaded gTCP scaffolds, they were seeded with buccal fat pad-derived stem cells. Then, four decellularization protocols including sodium dodecyl sulfate, trypsin, Triton X-100, and combined solution methods were compared for the amounts of residual cells and remnant collagen and alteration of scaffold structure. Then, the efficacy of the selected protocol in removing cells from gTCP scaffolds incubated in a rotating and perfusion bioreactor for 24 days was evaluated and compared with static condition using histological analysis. Finally, decellularized scaffolds, reloaded with cells, and their cytotoxicity and osteoinductive capability were evaluated. Complete removal of cells from gTCP scaffolds was achieved from all protocols. However, treatment with Triton X-100 showed significantly higher amount of remnant ECM. Bioreactor-incubated scaffolds possessed greater magnitude of ECM proteins including collagen and glycosaminoglycans. Reseeding the decellularized scaffolds also represented higher osteoinductivity of bioreactor-based scaffolds. Application of Triton X-100 as decellularization protocol and usage of bioreactors are suggested as a suitable technique for designing ECM-contained grafts for bone tissue engineering.

1. INTRODUCTION

The new generation of bone biografts has delivered on its promise of promoting bone regeneration without cell recruitment in clinical setting. The rationale for developing this system is to eliminate the necessity of cell transmission which has the potential of graft rejection and infection transfer.\(^1\) Cell-free scaffolds are biodesigned based on their capacity to evoke endogenous preosteoblastic and mesenchymal stem cells (MSCs) to the defect site, as well as stimulation of bone regeneration.\(^2,\) Fabrication of these scaffolds is achievable either by incorporation of chemoattractive factors to an acellular osteoinductive construct or by decellularization of extracellular matrix (ECM)-contained grafts.\(^3,\) However, fabrication of a bone-mimicking synthetic structure by introducing an adjusted amount of biomolecules to the scaffolds is a laborious task or even impossible. Hence, decellularized ECM-contained scaffolds have gained more interest in recent studies.\(^4−11\) These scaffolds referred to as bioscaffolds provide suitable structural support as well as biochemical cues for direction of cellular
alkaline solutions, and ionic and nonionic agents, each possess widely used for decellularization of bone tissues, including acids, synthetic graft ultrastucture. Chemical reagents that have been removes cell materials, without detrimental in which retains as much of the ECM components and adequately native bone, the purpose is to adopt the most suitable reagent of structure disruption on synthetic grafts similar to that of engineering.

Although all decellularization methods cause various degrees of structure disruption on synthetic grafts similar to that of native bone, the purpose is to adopt the most suitable reagent which retains as much of the ECM components and adequately removes cell materials, without detrimental influence on the synthetic graft ultrastructure. Chemical reagents that have been widely used for decellularization of bone tissues, including acids, alkaline solutions, and ionic and nonionic agents, each possess specific functions and disadvantages. Considering the possible toxicity of chemical reagents, decellularization of grafts using mechanical or physical methods has been introduced as a complementary or alternative choice. Grafts that undergo only physical procedures are mostly considered as devitalized instead of decellularized tissues since cell debris still remains within the source tissue even after decellularization. Therefore, mechanical methods are often used in conjunction with chemical and enzymatic procedures to eliminate the need for higher percentage of reagents or longer exposure times. Enzymatic agents are advantageous because of their specific activity on biologic substrates and are mostly used after chemical treatment to further accelerate the removal of residual cellular materials.

Decellularized grafts have been obtained either by decellularizing native bone tissues or by synthetic fabrication of ECM-contained scaffolds. Incorporation of naturally derived bioscaffolds into bone defects has brought a desirable potential to promote tissue repairs that might not be achievable with synthetic bioscaffolds. However, native bone-derived decellularized grafts suffer from several drawbacks including limitations associated with harvesting tissue from cadavers, graft mismatch with the recipient tissue, risk of pathogen transmission, and influence of donor age which showed to cause outcome variations. Besides, younger ECM encompasses fibrillin-2 and tenascin which can modulate cellular growth positively, while adult ECM contains higher concentration of decorin, biglycan, and thrombospondin-1 that decrease cell proliferation and can induce cell apoptosis. Therefore, there has been increased interest in producing in vitro ECM for bone tissue engineering.

In this study, initial attempt was made to find an optimal protocol for decellularization of in vitro engineered gelatin-coated β-tricalcium phosphate (gTCP) scaffold. Then, the effectiveness of the best-suited decellularization protocol was evaluated and compared with gTCP scaffolds incubated under static or dynamic, that is, rotating and perfusion (R&P) bioreactor, conditions. Finally, the cytotoxicity and osteogenic capability of these scaffolds were evaluated after reseeding with MSCs.

2. RESULTS

2.1. Characterization of Human Buccal Fat Pad-Derived Stem Cells. The flow cytometry analysis showed that the isolated buccal fat pad-derived stem cells (BFPdSCs) expressed CD90 (100%), CD73 (100%), CD105 (100%), CD34 (0.024%), and CD45 (0.315%). The data affirmed positive expression of CD90, CD73, and CD105 as well as negative expression of CD34 and CD45. These results demonstrated that isolated BFPdSCs represented MSC-defined markers with high population (Figure 1a).

Imaging showed fibroblastic-shaped morphology of BFPdSCs (Figure 1b). Calcium deposition confirmed the osteogenic induction of stem cells visualized by Alizarin Red staining (Figure 1c). Toluidine blue staining revealed proteoglycan formation of cartilage matrix, that is, chondrogenic differentiation (Figure 1e). Also, observation of neutral lipid vacuoles after induction of adipogenic conditions and staining with Oil red O supported multilineage differentiation capacity of isolated cells (Figure 1d).
2.2. Evaluation of Decellularization Protocols (Phase I).

2.2.1. Macroscopic Evaluation. Following decellularization, scaffolds were observed macroscopically. The 3D structure of decellularized scaffolds was similar to that of untreated scaffolds, except for protocol no. 4 in which the scaffolds were destroyed after addition of trypsin. Upon visual inspection, other scaffolds had an almost intact structure for further assessments (Figure 2a).

2.2.2. DAPI Staining. 4′,6-Diamidino-2-phenylindole (DAPI) staining was conducted to confirm complete cell removal (scale bar = 50 μm) and the evaluation of SEM images confirmed that all protocols could completely remove cells from the constructs (unseeded scaffold = 200 μm, seeded scaffold = 200 μm, protocol no. 1 = 300 μm, protocol no. 2 = 300 μm, protocol no. 3 = 300 μm, and protocol no. 4 = 200 μm). A little alteration of the 3D structure is detectable after decellularization in all groups.

2.2.3. Masson’s Trichrome and Sirius Red Staining. Masson’s trichrome (collagen fibers in blue-red arrow) and Sirius Red (collagen network in red, black arrow) staining demonstrated the presence of collagen fibers in slides. ImageJ analysis of Sirius Red revealed that the residual collagen fibers after decellularization with Triton X-100 (Protocol no.1) was almost similar to the untreated group (seeded scaffold group) and significantly greater than protocol nos. 2 and 3 (P < 0.05). Error bars are standard deviations (N = 3).

Figure 2. (a) Macroscopic view of decellularized scaffolds. Application of trypsin (protocol no. 4) damaged the scaffold structure (scale bar = 2 mm). (b) DAPI staining approved complete cell removal (scale bar = 50 μm) and (c) the evaluation of SEM images confirmed that all protocols could completely remove cells from the constructs (unseeded scaffold = 200 μm, seeded scaffold = 200 μm, protocol no. 1 = 300 μm, protocol no. 2 = 300 μm, protocol no. 3 = 300 μm, and protocol no. 4 = 200 μm). A little alteration of the 3D structure is detectable after decellularization in all groups.

Figure 3. (a) Masson’s trichrome (collagen fibers in blue-red arrow) and Sirius Red (collagen network in red, black arrow) staining demonstrated the presence of collagen fibers in slides. (b) ImageJ analysis of Sirius Red revealed that the residual collagen fibers after decellularization with Triton X-100 (Protocol no.1) was almost similar to the untreated group (seeded scaffold group) and significantly greater than protocol nos. 2 and 3 (P < 0.05). Error bars are standard deviations (N = 3).
removal after all decellularization protocols. Images of fluorescent microscopy illustrated that all decellularization methods left no visible cells in the scaffold sections (Figure 2b).

2.2.3. Microscopic Analysis. Decellularization process, amount of ECM production on gTCP scaffolds, and microscopic structure of treated scaffolds were evaluated by scanning electron microscopy (Figure 4).

Figure 4. (a,b) DAPI staining (scale bar = 50 μm) and SEM evaluation illustrated that decellularization of Control Neg, Control Pos, and Test groups were almost complete. (c) Photographic view of the R&P bioreactor. (d) Quantification of DNA showed that Control Pos and Test groups had higher amount of remaining DNA. However, all groups had less DNA than standard level for decellularization, that is, 50 ng/mL. *, #P < 0.05: comparison between groups. Error bars are standard deviations (N = 3).

Figure 5. (a) H&E staining was carried out to confirm complete cell removal and measure the thickness and density of semitabecular structures (red line). (b) ImageJ analysis showed that the Test group had significantly greater semitabecular density. (c,d) Masson’s trichrome staining illustrated higher amount of collagen fibers (red arrow) in the Test group, followed by Control Pos and Control Neg, respectively. (e,f) Results of staining GAG proteins (black arrow) by Alcian blue staining was similar to collagen in which the Test group had the most GAG concentration. *, #P < 0.05: comparison between groups. Error bars are standard deviations (N = 3) (Scale bar = 0.09 μm).
electron microscopy (SEM) micrographs. Microscopic architecture of scaffolds remained intact after decellularization process, which is the subsequent effect of chemical reagents and also deposition of ECM through the pores of seeded scaffolds. Microscopic evaluation confirmed that all protocols could completely remove cells from the constructs. Sheets of ECM with shade of cell nucleus were observed in seeded scaffolds which filled the pores of engineered grafts in all groups (Figure 2c).

2.2.4. Histological Evaluation. Assessment of collagen organization by Sirius Red and Masson’s trichrome staining sections revealed that collagen fibers were detected in all decellularized groups. Quantification of collagen amounts within Sirius Red images showed that, among all decellularized samples, bioscaffolds treated with protocol no. 1 had closer collagen content to the seeded scaolds (Figure 3). The results indicated that decellularizing gTCP scaolds using Triton X-100 could result in retaining of most ECM and least reduction in collagen levels compared to sodium dodecyl sulfate (SDS) and trypsin reagents. Therefore, protocol no. 1 was used for further experiments of phase II.

2.3. Evaluating the Efficacy of Decellularized Protocol in Static and Dynamic Conditions (Phase II). 2.3.1. DAPI Staining and SEM Evaluation. Successful removal of cells from scaffolds was confirmed by the absence of detectable cells in DAPI images and SEM micrographs in all groups (Figure 4a,b).

2.3.2. DNA Counting. DNA quantification showed that treatment of all groups significantly reduced DNA content which comply with the safe magnitude for preventing the immune response to bioscaffolds, that is, 50 ng/mL. Mean DNA content was $14.37 \pm 0.174$, $25.24 \pm 0.491$, and $25.44 \pm 0.412$ ng/mL for negative control (Control Neg), positive control (Control Pos), and Test groups, respectively. Data represented that the DNA amount was significantly lower in Control Neg compared with those in Control Pos and Test groups ($P < 0.05$), suggesting that higher ECM content in Control Pos and Test groups resulted in more entrapment of cells and consequently more residual DNA after decellularization (Figure 4d).

2.3.3. Histological Evaluation. 2.3.3.1. H&E Staining. General histomorphologic evaluation by H&E staining demonstrated successful decellularization of all groups with no detectable cells within the stained sections (Figure 5a). The density of semitrabecular structures, as an indicator of ECM deposition, was measured by ImageJ. Quantification of semitrabeculae indicated that the Test group had significantly higher overall density of these structures ($P < 0.05$). Mean thickness of semitrabecular constructs was measured as $117.08 \pm 41.14$, $120.03 \pm 103.99$, $98.88 \pm 41.14$, and $139.25 \pm 120.03\%$ for Control Neg, Control Pos, and Test groups, respectively (Figure 5b). Furthermore, thicker semitrabeculae in the Test group was almost distributed in the center and margin of the scaolds. This indicated the role of bioreactor culturing in the uniform diffusion of medium to seeded cells through the entire scaffold, and the cells followed the scaffold 3D pattern for deposition of ECM, and propagation of trabecular structures was initiated from the primary framework of the scaffold.

2.3.3.2. Masson’s Trichrome Staining. Masson’s trichrome staining was performed to discriminate collagen fibers from...
ECM-contained scaffolds on histological slides. ImageJ analysis of samples indicated that the Test group had significantly higher levels of collagen followed by Control Pos and Control Neg, respectively (Control Neg: 1.08 ± 0.55%, Control Pos: 4.46 ± 0.10%, and Test: 6.91 ± 0.04%) (Figure 5c,d). Five regions were selected randomly in each slide for this analysis.

2.3.3. Alcian Blue Staining. Alcian blue staining was intended to demonstrate the amount of secreted glycosaminoglycans (GAGs) as a part of the newly developed ECM. ImageJ analysis carried out on five randomized sites from each group confirmed the presence of GAGs in all slides. One-way analysis of variance (ANOVA) test and Tukey HSD revealed that increase in GAG levels was considerably more in the Test group (8.68 ± 0.24%). Also, Control Pos had greater amount of stained GAG compared with Control Neg (4.70 ± 0.175% vs 3.75 ± 0.13%) (Figure 5e,f).

2.3.4. Levels of Calcium and Phosphate. The amount of calcium and phosphate in scaffolds could represent the amount of mineralization through the constructs by osteoblastic differentiated cells. Since the gTCP scaffolds encompass calcium and phosphate within its composition, a control unseeded scaffold incubated in standard medium for a similar period was proposed as a zero indicator to eliminate the influence of scaffold degradation in the medium. Calcium and phosphate levels (mg/g) were measured: 8.66 ± 2.08, 48.28 ± 1.32 for Control Neg, 40.66 ± 1.52, 96.62 ± 1.31 for Control Pos, and 37.33 ± 2.51, 101.33 ± 0.52 for Test groups. The amounts of measured calcium and phosphate in the Control Pos and Test groups were significantly greater compared to those in Control Neg. However, the difference between the Control Pos and Test groups was not statistically significant for both calcium and phosphate amounts (Figure 6a,b).

2.3.5. Mechanical Evaluation. The amount of stress required to make a distinct strain in the scaffolds was measured by the UTM machine and expressed in strains of 10−50% (Figure 6c,d). One-way ANOVA and Tukey HSD analysis showed that the Test group in all five strains was significantly higher than the other groups. Also, the Control Neg group was significantly higher in the 10, 20, and 40% strains than the Control Pos group, except for the 30% strain, where there was no significant difference. Also, the Control Pos group in 50% strain was significantly higher than the Control Neg (P value ≤ 0.05).

2.4. Evaluating the Cytotoxicity and Osteogenic Capability of Decellularized Scaffolds (Phase III).

2.4.1. Alamar Blue Staining. Alamar Blue staining demonstrated that decellularization does not have any toxic effects on BFPSCs. Analysis of one-way ANOVA and Tukey HSD showed that the Test group was significantly higher than the other groups. Error bars are standard deviations (N = 3).
2.4.2. Alkaline phosphatase Activity Assessment. After 14 days, the activity of alkaline phosphatase (ALP) enzyme in Control Neg, Control Pos, and Test groups was reported with an absorption rate of 2.97 ± 0.00, 3.15 ± 0.94, and 3.51 ± 0.010, respectively. This result indicates an increase in the induction of bone differentiation on cells seeded within scaffolds containing more produced ECM in the bioreactor. Data showed that the Test group had significantly higher differentiated cells compared with Control Neg, but the difference in enzyme activity between Control Neg and Control Pos was not significant (Figure 7b).

3. DISCUSSION
The main purpose of the current study was to produce an in vitro synthetic construct close to the bone structure, mimicking the microarchitecture and bioactivity of the native bone. Generation of decellularized bone matrix requires ridding the ECM of the laid cells. However, decellularization of natural bone/cartilage has a longer history of inception for bone regeneration.27,28 Naturally derived ECM can be utilized in the form of decellularized intact bony blocks or can be processed to produce components incorporating in a supportive synthetic scaffold.29–31 Several studies have fabricated scaffolds by integrating powdered decellularized ECM (dECM), dECM particles, or solubilized ECM into polymers, ceramics, and hydrogels.30–33 Considering the limitations associated with native dECM grafts which include donor-to-donor and tissue-type variations, development of in vitro engineered dECM has been recently concentrated by researchers.34

Decellularization methods have been reported to cause various degrees of structure disruption on synthetic grafts similar to the native bone. Hence, the purpose is to adopt the most suitable reagent which retains as much of the ECM components within the grafts. Developing an appropriate decellularization technique for our scaffold requires comprehensive information around various methods and agents applied for native tissue decellularization. Acids have been shown to function by solubilizing cytoplasmic ingredients and disrupting nucleic materials resulting in detachment of DNA from the ECM. Similarly, alkaline treatment causes cell degradation and removal of the cellular components, especially nucleic acids. Treating with bases is considered as a harsh technique which terminates in excessive loss of GAGs13,15 since the presence of GAGs within the ECM benefits tissue engineering greatly.13,15 Non-ionic detergents such as Triton X-100 have been shown to destroy the cell membrane by disrupting the lipid–lipid interactions of the lipid bilayer while leaving the protein–protein interactions intact. Maintaining the native structure of proteins has rendered nonionic detergents to be considered as a gentle agent, and hence it is suggested for decellularization of synthetic scaffolds composed of protein components. Ionic detergents such as SDS are strong agents that disrupt cell membranes and effectively denature proteins. SDS can unravel noncovalent bonds between proteins and alter their native conformation.15,34 Reduction of GAGs by 50%35 and diminishing the tissue-embedded growth factors have also been reported by exertion of SDS for decellularization objectives.36 Administration of enzymatic agents is advantageous because of their specific activity on biologic substrates and are mostly used after chemical treatment to further accelerate the removal of residual cellular materials. Nuclease, including DNase and RNase, function by hydrolyzing phosphodiester bonds of DNA and RNA chains, respectively. Trypsin is among commonly used proteases that selectively cleaves peptide chains and disrupts cell adhesion to the tissue surface.13,15 Targeting peptides by trypsin can intensively damage ECM proteins such as collagen and subsequently decrease the mechanical strength of decellularized synthetic or native grafts.27 Considering the possible toxicity of chemical reagents, decellularization of grafts using mechanical or physical methods have been introduced as complementary or alternative choices. These techniques include application of freeze-thaw cycles and hydrostatic pressure and agitation and immersion of tissue that work to lyse cells and damage cell adherent proteins concomitantly.25

Scientific investigations concerning the efficacy of engineered bioscaffolds have been conducted by several research groups.19,20 Pati et al. developed 3D printed polycaprolactone (PCL)/poly(lactic-co-glycolic acid) (PLGA) and PCL/PLGA/TCP scaffolds and seeded them with MSCs. After 14 days of incubating in spinner flask bioreactors, the specimens were decellularized by freeze and thaw cycles to obtain ECM-contained structures. DNA counting verified that the decellularization process was nearly complete, and the measured residual DNA content was lower than 3% in vitro. In addition, after seeding the mineralized ECM by MSCs, the expression of osteogenic-related markers was significantly above the reported amount for ECM-free scaffolds. Implantation of scaffolds in rat calvarial defects showed that not only bioscaffolds did not elicit inflammatory response in the site but also ECM-contained scaffolds represented higher amount of new bone formation and bone ingrowth.20 In contrast to Pati report, numerous studies have asserted that the freeze-thaw technique is one of the primary steps for decellularization, during which, merely cell lysis occurs and the remaining genetic materials possibly evoke immunorejection.13,22,25

In a study, Wei et al. engineered ECM from 2D culturing of human adipose-derived stem cells, which underwent decellularization using Triton X-100, and then coated the decellularized ECM on a poly sebacoyl diglyceride mesh. After loading the biocomposites with bone marrow mesenchymal stem cells (BMSCs) and implanting in rat calvarial defects, the coated scaffolds illustrated a considerably greater new bone area (78.49%) compared to uncoated (48.29%) groups.19 Nevertheless, decellularization of engineered bioscaffolds is more challenging. Accordingly, in the first phase of our study, we attempted to select an appropriate protocol for decellularization of gTCP scaffolds which can retain as much of the secreted ECM within the scaffolds, along with separating DNA and RNA effectively. Concentration of tissue is the main determining factor that should be considered for defining the most suitable protocol and reagents for effective decellularization. Having known that the concentration of synthetic ECM and collagen amount is orders of magnitude lower than the native ECM of bone, removing entrapped cells from the former is easier and can be performed with less destructive methods. Hence, decellularization techniques preferred for engineered bony ECM can be acquired from the procedures performed for looser tissues such as urinary bladder, trachea, or intestine. In the present study, decellularization techniques for gTCP scaffolds were taken from methods utilized for annulus fibrosus and bone tissues.34,37 Engineered gTCP scaffold treatment with all four methods implied successful decellularization and maintenance of the porous structure, except for protocol no. 4 with trypsin which spoiled the whole construct. Other three treatments maintained the mechanical features of ECM to justify further examinations. This result could be an approval for the detrimental effect of trypsin on structural fibers and proteins explained by Grauss et
They found that trypsin 0.5% treatment of porcine aortic valves resulted in fragmentation of elastic fibers and collagen depletion and subsequently modified the configuration of normal tissue substantially. Similarly, Meyer et al. represented that enzymatic decellularization of rat aortic valve by means of trypsin 0.5% causes massive degradation of matrix composition. Although majority of researchers believe in the damaging effects of trypsin solutions on ECM integrity, Cui et al. claimed subtle trace of trypsin in comparison to an equivalent concentration of Triton X-100. They revealed that Triton X-100 left more structural modifications and disordered collagen fibers compared to trypsin. In contrary to the scrutiny study by Cui et al., our data implied that applying 3% Triton X-100 on engineered gTCP scaffolds could retain further amount of collagen than other treatment agents. Implantation of 3% Triton X-100-treated tracheal grafts in mice and canine models demonstrated that decellularized transplants supported site-specific re-epithelialization with adequate mechanical properties. For investigating the efficacy of Triton X-100 in the cell removal of porcine soft tissue, Faulk et al. subjected the basement membrane complex of urinary bladder to either 3% Triton X-100 or 1% SDS and evaluated the content of DNA, GAG, and collagen. They reported that exposing the bladder tissue to SDS resulted in less residual DNA, but for all that, it rendered noticeable alterations of collagen fibers, while Triton X-100 was well tolerated and the treated specimen architecture resembled more to the untreated group. Comparably, full decellularization of anterior cruciate ligaments with 1% SDS led to remarkable reduction of GAG levels and alteration of collagen sensitivity to gelatinase enzyme trypsin, which subsequently accelerated collagen degradation. Considering that the degenerative effects of SDS solutions correlated with the concentration of the agent, we reduced the percentage of SDS to 0.5%, according to the study by Xu et al. However, the findings of the present study are in line with the outcome of utilizing 1% SDS, and decrease in collagen amounts was approved by ImageJ analysis. Also, subjecting the engineered gTCP scaffolds to protocol no. 4 with more limited exposure time to 0.5% SDS exhibited collagen depletion similar to protocol no. 2. This strongly indicates the innate potency of SDS in the alteration of matrix. Lower percentage of 0.1% was also suggested to hamper the protein loss of grafts. In this regard, Mirdasraee et al. achieved whole cell removal of pericardial matrix with no significant changes in ultimate strength, collagen, and GAG content after decellularization with 0.1% SDS. Although performing 0.1% SDS on human pericardia could preserve the mechanical features of the graft, it failed to maintain compressive stiffness when applying for porcine cartilage decellularization. Driven by our data on SDS implementation besides the reported influence of SDS on the biomechanical properties of tissues, it seems that SDS is not a favored choice for decellularization of synthetic-based ECM scaffolds. In the present study, decellularization of gTCP using Triton X-100 solution produced a well-preserved ECM compared to other treatment groups.

The controversies associated with the practicability of decellularization solutions for withdrawing cell components could be explained by variations in decellularization protocols such as presence of accompanied agents, agitation of samples during treatment or exposure time, and temperature of each agent, together with differences in species and tissue types. Therefore, making the final judgment about the optimum concentration of these solutions for decellularization of various tissues is not feasible yet and requires further experiments which eliminate the confounding factors to precisely determine the desirable conditions.

After selecting the best-suited protocol for decellularization of engineered gTCP scaffolds, the second phase was designed to evaluate the effectiveness of chosen treatment on scaffolds with different ECM contents. BFPSCs were chosen for producing ECM within the scaffolds. Based on the study conducted by Wei et al., these cells demonstrated higher potency in depositing ECM in comparison with BMSCs. They showed that adipsic-derived matrices contained higher amount of collagen and fibronectin proteins with well homogenous aligned fibers. Perceived from our previous study, exertion of dynamic condition on scaffolds via R&P bioreactor generated higher amount of ECM significantly, supported by increased ALP activity. Therefore, gTCP scaffolds incubated under the static condition in growth or osteogenic medium was compared with the ECM of the bioreactor-based group.

The term of effective decellularization has not been strictly defined by quantitative matrices, and various publications introduced distinct methods for this aim. However, the extent of decellularization plays a critical role in clinical outcomes. Three criteria have been suggested as parameters of adequate cell clearance which have shown definitive differences in host responses: (1) absence of visible nuclei in the histological images of tissue sections via H&E and DAPI stainings, (2) less than 50 ng of measured double-strand DNA per mg of dry material weight, and (3) less than 200 base pairs in length of the remaining double-strand DNA. In the present study, complete decellularization was approved by the absence of cell and its nucleus in DAPI, histological assessments, and SEM imaging. Measurement of DNA content was also consistent with previous observations, and the trace amount of residual DNA was less than 26 ng for all groups, which is approximately half the permitted level. The herein shown disparity in the minimum range of remnant DNA might account for the difference of initial cells within the native tissue and the seeded scaffolds before decellularization. Therefore, it seems that the permitted range should be up to date to be tailored for in vitro engineered constructs. The measured DNA in the Control Neg group incubated in standard medium exhibited significantly lower amount rather than Control Pos and Test groups with osteogenic medium. This could be attributed to the fact that secretion of ECM throughout the scaffolds results in embedding the cells within the ECM proteins and fibers, which makes cell removal more difficult.

Collagen and GAG are the main components of bone ECM. Histological assessment of engineered gTCP bioscaffold sections using H&E, Masson’s trichrome, and Alcian blue were conducted to measure the produced amounts of ECM proteins, and ImageJ analysis was performed on the histograms. Our observation demonstrated that the Control Neg group was considerably inferior on the remaining level of GAG and collagen. Higher content of collagen and embedded GAGs within them not only provide physical support for cell infiltration and proliferation but also negatively charged GAGs have shown to carry certain bonded growth factors. Thus, attenuation of GAGs could result in loss of various growth factors. Following this hypothesis, Wei et al. quantitatively assessed the release amount of bone morphogenetic protein-2 and insulin-like growth factor-1 after decellularization for 4
weeks. Found in his study, the adipose-derived matrix constitutively released growth factors in a time-dependent way with burst release within the first 3 days.\(^\text{19}\) Similarly, we tested the osteoinductivity of decellularized engineered gTCP and confirmed via measuring the ALP activity of decellularized bioscaffolds after reseeding with MSCs for 14 days where the bioreactor group with higher GAG amount represented better osteogenic capacity. Besides the amounts of GAGs and collagen, the efficacy of fiber distribution within the construct is a matter for debate. Upon visual inspection, a homogenous distribution of fibers through the center and margin of gross sections was observed in the bioreactor group, while the static condition induced more fiber condensation throughout the borders of the scaffold, farther from the centric hole. The rotating motion of the bioreactor axis besides the homogenous diffusion of the medium to the resident cells has been considered as the vantage point of bioreactors in histological examinations and all other conducted experiments.

Although sufficient papers are not available similar to our study design which fabricate engineered bioscaffolds for bone engineering, the studies of Pati et al.\(^\text{20}\) and Wei et al.\(^\text{19}\) partly resemble our plan. Briefly, Pati et al. developed 3D printed PCL/PLGA and PCL/PLGA/TCP scaffolds. After seeding with MSCs and incubating in a spinner flask bioreactor, the 3D scaffolds were decellularized. Implantation of scaffolds in rat calvarial defects showed that the ECM-contained scaffolds represented higher amount of new bone area compared to the controls. Their results, their outcome illustrated a considerably greater new bone area with the bioreactor group with higher GAG amount represented better osteoinductivity of decellularized engineered gTCP and freeze-dried in −80 °C for 6 h. Then, gelatin-coated scaffolds (gTCP) were cross-linked with glutaraldehyde (1 wt%) for 24 h. Residual amount of glutaraldehyde was washed out by rinsing the samples with distilled water for three times and soaking in distilled water for 24 h, followed by 24 h of freeze-drying cycle.

5.2.2. Cell Isolation. Buccal fat pad tissue (BFP) was extracted surgically from one healthy patient that underwent oral and maxillofacial surgery in the Department of Oral and Maxillofacial Surgery of Shahid Beheshti university of Medical Sciences, Tehran, Iran. BFP was maintained in a sterile PBS solution and supplemented with 5% penicillin/streptomycin and 1% amphotericin B. Then, the tissue was cut into small pieces and immersed in a digestive solution consisting 3 mg/mL collagenase type 1 and 1% penicillin/streptomycin for 1 h at 37 °C. Cells were washed prior to centrifugation. Isolated cells were seeded in a 25 cm\(^2\) culture flask in growth medium containing DMEM-high glucose supplemented with 15% FBS and 100 U/mL penicillin/streptomycin in a humidified atmosphere of 5% CO\(_2\) at 37 °C. After reaching the confluency of 90%, the cells were detached and passaged using 0.25% trypsin/1 mM EDTA. Cells at passages 3 and 4 were used for all experiments.

5.2.2.1. Characterization of BFDPdSCs. Expression of mesenchymal cell surface markers (flow cytometry): Passage 3 undifferentiated BFDPdSCs were detached using 0.25% trypsin/EDTA. To characterize the MSC-specific marker, 10\(^6\) cells per sample were stained with fluorescein isothiocyanate-conjugated antibodies (anti-CD90, anti-CD73, and anti-CD45) and phycoerythrin-conjugated antibodies (anti-CD105, and anti-CD34) for 30 min in a dark room. After incubation period, the cells were washed twice with PBS prior to performing the flow cytometry test by an Attune Acoustic Focusing Cytometer (Applied Biosystems, Foster City, CA, USA). Data was analyzed using FLOWJO 7.6.1 software (TreeStar, San Carlos, CA, USA).

Multilineage differentiation potential: The multilineage differentiation capability of BFDPdSCs toward osteoblasts, chondroblasts, and adipocytes was also evaluated. For this aim, P3 cells were cultured in 24-well plates at a concentration of 10\(^4\) cells/well and incubated in either osteogenic medium, chondrogenic, or adipogenic medium for 14 days. Osteogenic medium was prepared by DMEM-low glucose, 10% FBS, 50 μg/mL ascorbate-2-phosphate, 10\(^{-8}\) M dexamethasone, and 10 mM b-glycerophosphate. Chondrogenic medium contained DMEM-

4. CONCLUSIONS

Application of Triton X-100 as a decellularization protocol and using bioreactors are suggested as a suitable technique for designing ECM-contained engineered grafts for bone tissue engineering.

5. EXPERIMENTAL SECTION

5.1. Materials. Dulbecco’s modified Eagle’s medium (DMEM)-high glucose, DMEM-low glucose, fetal bovine serum (FBS), trypsin/ethylenediaminetetraacetic acid (trypsin/EDTA), and ascorbate-2 phosphate were purchased from Thermo Fisher Scientific Waltham, MA, USA. Penicillin/streptomycin, TRIzol solution, and amphotericin B were purchased from Life Technologies, CA, USA. Glutaraldehyde, ethanol, and sodium tripolyphosphate pentabasic (TPP) were purchased from Merck, Kenilworth, NJ, USA. DAPI, EDTA, Triton X-100, ribonuclease A (RNase A), deoxyribonuclease I (DNase I), SDS, Tris, collagenase type 1, phosphate-buffered saline (PBS), gelatin, dexamethasone, b-glycerophosphate, transforming growth factor (TGF)-β1, sodium pyruvate, Alizarin Red, Toluidine blue, indomethacin, Oil Red O, calcium phosphate powder, and colloidal silica were purchased from Sigma-Aldrich, St. Louis, MO, USA. Anti-CD105 and anti-CD34 were purchased from EXBio, Vestec, Czech Republic. Anti-CD45 and anti-CD73 were purchased from BD Bio-
| groups                        | descriptions                                                                 |
|------------------------------|-----------------------------------------------------------------------------|
| **phase I**                  |                                                                             |
| unseeded scaffold            | gTCP scaffold without BFSCs.                                                |
| seeded scaffold              | gTCP scaffold seeded with BFSCs incubated in standard and osteogenic mediums for 3 and 14 days, respectively. |
| protocol no. 1               | the seeded scaffolds were immersed in hypotonic Tris–HCl buffer (10 mM, pH 8.0) with 0.1% EDTA (Sigma-Aldrich, St. Louis, MO, USA) at 4 °C for 48 h. Then, the specimens were agitated in Tris–HCl buffer with 3% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) and 0.1% EDTA at 4 °C for 72 h, and the solution was changed every 24 h. Then, the constructs were incubated with 0.2 mg/mL RNase A (Sigma-Aldrich, St. Louis, MO, USA) and 0.2 mg/mL DNase I (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 24 h. Finally, decellularized gTCP scaffolds were soaked in PBS for 24 h to remove residual reagents. All steps were conducted under continuous shaking.34 |
| protocol no. 2               | the seeded scaffolds were frozen at −80 °C for 3 h and thawed at RT for 4 h three times. Constructs were decellularized with 10 mM Tris–HCl buffer containing 0.5% SDS (Sigma-Aldrich, St. Louis, MO, USA) and 0.1% EDTA at RT for 72 h, and the solution was exchanged every 24 h. Then, decellularized scaffolds were incubated with 0.2 mg/mL RNase A and 0.2 mg/mL DNase I at 37 °C for 24 h and washed with PBS for 24 h to remove residual reagents. All steps were conducted under continuous shaking.34 |
| protocol no. 3               | the seeded scaffolds were placed in the solution consisting of 0.1% EDTA (wt/vol) in 450 mL of deionized water and 50 mL of 10× PBS for 1 h at RT under a shaker. The constructs were transferred to solutions 0.1% EDTA (wt/vol) and 10 mM Tris (Sigma-Aldrich, St. Louis, MO, USA) for 12 h at 4 °C. The scaffolds were then placed in 10 mM Tris and 0.5% SDS (wt/vol) at RT for 24 h. Finally, the constructs were incubated with 50 units/mL DNase I, 1 unit/mL RNase A, and 10 mM Tris at 4 °C for 5 h at RT and rinsed extensively with PBS and deionized water.36 |
| protocol no. 4               | the seeded scaffolds were incubated under continuous shaking in hypotonic Tris–HCl supplemented with 0.5% trypsin, 0.2% EDTA, RNase A (20 mg/mL), and DNase I (0.2 mg/mL) at 37 °C for 72 h. The trypsin/EDTA solution was refreshed every 24 h. Decellularized constructs were washed with PBS for 24 h to remove residual substances.37 |
| **phase II**                 |                                                                             |
| negative control (control neg) | gTCP scaffolds seeded with BFSCs and incubated in standard medium at static condition for 24 days. Then, decellularization was performed according the most efficient protocol. |
| positive control (control pos) | gTCP scaffolds seeded with BFSCs and incubated in standard and the osteogenic mediums at the static condition for 3 and 21 days, respectively. Then, decellularization was performed according to the most efficient protocol. |
| bioreactor R&P (test)        | gTCP scaffolds seeded with BFSCs and incubated in standard and osteogenic mediums at the static condition for 3 and 5 days, respectively. After this period, the scaffolds were transferred to an R&P bioreactor containing osteogenic medium for 16 days. Then, decellularization was performed according to the most efficient protocol. |
low glucose, 10% FBS, 1 μM ascorbate-2 phosphate, 10⁻⁷ M dexamethasone, 10 ng/mL TGF-β1, and 1% sodium pyruvate. Adipogenic medium was made using DMEM-low glucose, 10% FBS, 50 μg/mL ascorbate-2 phosphate, 10⁻⁷ M dexamethasone, and 50 μg/mL indomethacin. After incubation period, the cells were washed and fixed by 4% paraformaldehyde for 20 min. Staining was conducted with Alizarin Red (A3757), Toluidine blue, and Oil Red O to assess osteogenesis, chondrogenesis, and adipogenesis, respectively. The stained samples were imaged by inverted light microscopy.

5.2.3. Study Design. Scaffolds were sterilized using ethanol 75% followed by a five-time wash with PBS (at least 5 min each). Scaffolds with cylindrical shape, that is, 12 ± 0.5 mm diameter and 5 ± 0.5 mm thickness, porosity 70%, and pore size 72.8–334.8 μm, were incubated in the growth medium overnight. Then, the scaffolds were loaded by cells at a density of 1 × 10⁶ viable cells/scaffold.

The study has three main phases described in the following. The first phase aimed to develop and introduce the desired decellularization protocol of cell-loaded gTCP scaffolds in which the scaffold morphology and the secreted ECM structure are retained the most, and complete cell removal is achieved. For this purpose, cell-loaded gTCP scaffolds were incubated in standard medium and osteogenic medium for 3 and 14 days, respectively. After this period, the specimens were subjected to various decellularization techniques as described in Table 1.

The second phase was designed to evaluate the efficacy of the desired protocol in removing cells from gTCP scaffolds containing various amounts of ECM. For this aim, gTCP scaffolds were seeded with BF-PsdSCs and incubated in standard medium (3 days) and osteogenic medium for 5 days. Samples were then incubated in either static or dynamic condition for 16 days. Different concentrations of ECM were attained by incubating the scaffolds in dynamic and static conditions. Finally, in the third phase, these scaffolds were decellularized with the desired protocol, derived from phase I assessment, and evaluated in terms of cytotoxicity and osteoinductive potentials after reloading with BF-PSCs for 14 days (Figure 9).

5.2.3.1. Evaluation of Various Decellularization Protocols (Phase I). Four decellularization protocols were evaluated with modification as described in previous studies. Different protocol steps are explained in detail in Table 1.

5.2.3.2. Evaluating the Efficacy of Selected Decellularized Protocol in Static and Dynamic Conditions (Phase II). In this phase, three groups were designed: (1) Test group: gTCP scaffolds seeded with BF-PSCs and incubated in standard and osteogenic mediums at the static condition for 3 and 5 days, respectively. After this period, the scaffolds were transferred to an R&P bioreactor, that is, with a rotation rate of 1 rpm and a perfusion rate of 1–2 mL/min, containing osteogenic medium for 16 days. Then, decellularization was performed according to the most efficient protocol from phase I. (2) Control Neg: gTCP scaffolds seeded with BF-PSCs and incubated in standard medium at static condition for 24 days. Then, the decellularization was performed according to the most efficient protocol from phase I. (3) Control Pos: gTCP scaffolds seeded with BF-PSCs and incubated in standard medium at static condition for 3 and 21 days, respectively. Then, the decellularization was performed according to the most efficient protocol from phase I (Table 1).

5.2.3.3. Evaluating the Cytotoxicity and Osteogenic Capability of Decellularized Scaffolds (Phase III). In this phase, previous groups described in phase II were examined for possible toxicity and osteoinductive capacity after being recellularized by BF-PSCs for 7 and 14 days, respectively.
5.2.4. Biological, Biochemical, and Mechanical Assessments. 5.2.4.1. DAPI Staining (Phase I and II). The scaffolds were washed and fixed with 4% paraformaldehyde for 20 min. Then, they were exposed to DAPI (1 μg/mL) for 1 min at RT in dark condition. Then, they were imaged by fluorescence microscopy (N = 3).

5.2.4.2. SEM (Phase I and II). Decellularized scaffolds were imaged by SEM to visualize the effect of treatments on the porous structure, pore size, and also trace of remnant cells. Scaffolds were fixed using 2.5% glutaraldehyde for 45 min at 4 °C and dehydrated in a graded series of ethanol. Fixed samples were sputter-coated with gold. Images were captured using SEM (Akishima Tokyo, Japan) (N = 3).

5.2.4.3. Histological Analysis (Phase I and II). Initially, specimens were fixed using 10% solution of buffered formalin for at least 48 h. Afterward, the samples were decalcified using 10% solution of nitric acid for 48 or 96 h, 10% solution of formic acid for 48 h, and 5% solution of formic acid for 48 h separately (N = 3), followed by embedding in paraffin as described by Carriel et al.

After decalcification, the samples were cut into 4 μm thickness and were stained using H&E for histological assessment and general morphology (phase II), Masson trichrome staining (phase I and II), Sirius Red staining (phase I) for collagen network organization, and finally Alcian blue staining (phase II) for proteoglycan content evaluation. All microscopic slides were evaluated by using a light microscope (Olympus, BX40, Japan). Quantification of collagen content was conducted using ImageJ software on five randomly selected slides of Sirius Red and Masson’s trichrome staining. Similarly, proteoglycan content, that is, GAG, was measured by analysis of ImageJ on Alcian blue staining.

5.2.4.4. DNA Counting (Phase II). DNA quantification was carried out using the salting-out method, as described previously. Briefly, decellularized scaffolds were lysed using TRizol solution for 10 min. Next, phase separation was conducted by addition of chloroform and centrifugation of mixture at 12,000 rpm for 10 min at RT. The supernatant solution was collected and dissolved in NaOH (8 mM) solution. The DNA concentration was quantified by a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA) at 260 nm (N = 3).

5.2.4.5. Calcium and Phosphorus Contents (Phase II). Phosphorus content was determined using an ICP-OES Varian 73S ES configuration torch redial instrument. Determination of calcium was performed by a GBC flame atomic absorption spectrometer model 932 (Victoria, Australia) which was equipped with a Ca hollow cathode lamp and an air-acetylene burner. A deuterium background correction was also used for Ca determination. Instrumental parameters of both techniques were adjusted based on the manufacturer’s recommendation (N = 3).

5.2.4.6. Mechanical Characterization (Phase II). Decellularized scaffolds were immersed in PBS at RT for 2 h. Analysis was performed using a uniaxial test system (SANTAM, STM-20, Iran) with a compression rate of 0.5 mm/min. As a flexible structure, these scaffolds are not expected to have a net point of break down. So, the compressive strength of these scaffolds is generally expressed as the stress in a distinct strain. In our study, the sample strength was expressed in the strain level of 10−50% (N = 3).

5.2.4.7. Alamar Blue Assay (Phase III). To evaluate the viability/proliferation of the cells on decellularized scaffolds, 1 × 10⁶ cells were seeded per bioscaffold. Cell viability/proliferation was assessed using Alamar Blue after 1, 5, and 7 days. To do this, growth medium was exchanged with medium containing 10% Alamar Blue and incubated for 4 h. Alamar Blue fluorescence was measured at 590 nm using an ELIZA reader (BioTek, Winooski, VT, USA) (N = 3). 5.2.4.8. ALP Activity Assay (Phase III). The osteoinductive effect of decellularized scaffolds was assessed through culturing of BFPSCs on scaffolds in osteogenic medium. BFPSCs at a density of 1 × 10⁶ cells were seeded on each bioscaffold and cultured in osteogenic medium for 14 days in static condition. After the culturing period, ALP activity test was conducted to assess the osteoinductive capacity of decellularized scaffolds (N = 3). On the test day, the scaffolds were removed from the medium and were washed twice with PBS. Each scaffold was soaked in 400 μL of cell lysis buffer and placed over a shaker for 30 min. Next, the supernatant solution was collected and centrifuged at 20,000g for 15 min at 4 °C. For each specimen, the cell lysate was incubated with p-nitrophenyl phosphate substrate in a dark place. After 30 min, 3 N NaOH was used to quench the reaction. The absorbance of ALP was measured by an ELISA reader (BioTek, Winooski, VT, USA) at 405 nm (N = 3).

5.2.5. Statistical Analyses. The results are expressed as mean ± standard deviation. One-way ANOVA and independent sample T-test with Tukey HSD as a supplementation test were performed by SPSS software for Windows (version 15.0; SPSS, Chicago, IL). Differences were considered with a confidence interval 95% (P value ≤0.05) for all analyses.

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