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ENHANCED OSTEOGENIC DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS BY FLEXIBLE β-TCP/PLA BONE GRAFTS WITH SILICATE ADDITIVE

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Highlights

- β-tricalcium phosphate（β-TCP)-poly (lactic acid) (PLA) composite scaffolds containing two different concentrations (0.8% and 1%) of silicate additives were designed.
- The characterization results demonstrated that β-TCP-PLA-based scaffolds had porous and flexible structures.
- The designed synthetic flexible bone grafts with 0.8% and 1% silicate-additive significantly encouraged the proliferation and osteogenic differentiation of human bone marrow-derived mesenchymal stem cells (hMSCs). Moreover, 0.8% silicate-additive β-TCP-PLA grafts showed increased alkaline phosphatase (ALP) activity.

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ABSTRACT: In recent years, ceramics, polymers, and composites have been used to develop biologically and mechanically suitable bone scaffolds. β-tricalcium phosphate（β-TCP) is a widely used bioceramic in bone tissue engineering. It shows excellent osteoconductivity, osteoinductivity, and good biocompatibility properties, as its chemical composition is similar to the original chemical structure of bone. Herein, we designed β-TCP-PLA composite scaffolds containing two different concentrations of silicate additives. We aimed to investigate the effect of silicate-additive with varying concentrations (0.8% and 1%) on osteogenic differentiation of human bone marrow-derived mesenchymal stem cells (hMSCs) seeded flexible bone grafts. The morphological structure of β-TCP-PLA-based bone grafts was assessed by scanning electron microscopy (SEM) and the tensile strength of grafts was evaluated. The results showed that scaffolds had porous and flexible structures. hMSCs osteogenic differentiation was evaluated with the alkaline phosphatase (ALP) activity and DNA content measurements. Compared with β-TCP-PLA grafts, these designed synthetic flexible bone grafts with 0.8% and 1% silicate-additive significantly promoted hMSCs proliferation and osteogenic differentiation. Moreover, 0.8% silicate-additive β-TCP-PLA grafts showed increased ALP activity. The outcomes of the present study suggest that synthetic flexible bone grafts with silicate-additive might be useful for encouraging the regeneration of bone.

Keywords: *Bone Tissue Engineering, B-TCP/PLA Bone Grafts, Silicate, Osteogenesis*

1. INTRODUCTION

Bone diseases which correspond to half of the chronic diseases among people over 50 years old, continue to exist as a crucial problem in the clinic [1, 2]. Due to the enhanced life expectancy and aging population, there is a fast increment in musculoskeletal cases and disorders including fractures, osteoporosis, and bone metastases. Thus, bone-associated medical treatments and costs are increasing gradually [3]. Although bone has an exceptional capability for regenerating and healing itself, large bone defects and complex bone breakages lead to important difficulties in medicine [3]. To repair bone defects, the autologous bone grafting technique is accepted as the "gold standard". However, it possesses drawbacks such as secondary damages, donor site morbidity, limitation of a specific form, inadequacy of autologous bone tissue, and long surgery times [3, 4]. These shortcomings limit the use of autografts in clinical settings. Therefore, bone tissue engineering strategies as novel alternative treatment approaches aim to repair or regenerate the damaged bone tissue after a bone loss, defect, or injury by replacing three-dimensional (3D) scaffolds to build a supportive environment for the growth of bone [5]. Bone scaffolds should ideally be osteoinductive to encourage the differentiation of progenitor cells down an osteoblastic lineage, be osteoconductive to help the growth of bone tissue and promote ingrowth of surrounding bone tissue, and also have the capability for osseointegration to integrate scaffold into surrounding bone tissue [6]. Also, a bone scaffold should have proper mechanical features such as having high porosity to allow vascularization and migration of the cells and have the ability to fill irregular shapes to avoid insufficient micro-cavities around the bone defect area, and lastly have the possibility for commercialization [7, 8].

In recent years, ceramics, polymers, and composites have been used by researchers to produce biologically and mechanically suitable bone scaffolds [6]. Ceramic materials possess a huge compressive

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strength and low ductility and show good resistance to deformation [9]. β-tricalcium phosphate（β-TCP), commonly utilized ceramic in bone tissue engineering, demonstrates excellent osteoconductivity, osteoinductivity, and good biocompatibility features since the chemical composition of β-TCP ceramics is resembling the chemical composition of native bone [10-13]. A main drawback of β-TCP is that it has a sintered solid and hard form resulting in difficulties for the surgeon to ensure the needed form of the graft in the surgical field and insufficient micro-cavities in the bone defect area. Giving shape to the graft substance in the surgical field during the surgery extends the surgical period and enhances the infection risk [14, 15]. Therefore, during a surgical operation, to allow the surgeon for giving the needed form to the scaffold in the surgical field, flexibility is very important in clinical applications. Collagen fibers and polymeric biomaterials like poly (lactic acid) (PLA) have been used to provide flexibility to ceramic materials [16]. PLA-based materials are advantageous since they possess perfect mechanical features, processability, biological compatibility, and suitable degradation rates, and don't cause an important inflammatory reaction. Nevertheless, their main disadvantage of them is the absence of bioactivity for bone regeneration [16].

Silicate-based bioceramics show perfect bioactivity that could encourage the reproduction of osteoblasts, increase the creation of bone and trigger the osteogenic differentiation of stem cells [17]. Although calcium phosphate ceramics such as β-TCP and their composites with PLA have great biocompatibility, they do not have an evident stimulatory impact on osteoblasts proliferation and differentiation [17]. Since a certain concentration of silicate additive increases bone regeneration capacity and osteogenesis, silicate-substituted calcium phosphate materials have been used as novel and commercialized porous ceramic graft substitutes for bone tissue, and their crystal construction is doped by silicate ions [18-20]. Silicate contribution resulted in better *in vitro* and *in vivo* osteogenic differentiation and controllable biodegradability compared to β-TCP [21, 22]. For instance, Coathup *et al.* aimed to assess the impact of silicate substitution on calcium phosphate's osteoinductive ability without exogenously given growth factors and reported that silicate substitution remarkably enhanced the quantity of bone created and the quantity of bone tissue that is bonded to the surface of the implant [23]. Furthermore, researchers investigated whether hydroxyapatite (HA) or silicate-substituted HA can encourage mesenchymal stem cells (MSCs) osteogenic differentiation without soluble factors, and reported that silicate-substituted calcium phosphate assisted the adhesion and proliferation of MSCs and also triggered the osteogenesis to a larger degree than HA [24]. Moreover, Chan *et al.* studied the silicate substituted calcium phosphate substances having different strut porosities in *in vivo* ectopic ovine model for comparing its osteoinductivity, and reported that bone graft substitutes having higher strut porosity are more osteoinductive [25].

Importantly, a bone scaffold must have an interconnected porous structure, and for needed nutrients and oxygen diffusion, the size of pores would be at least 100 μm in diameter [26]. The pores facilitate the homogeneous distribution of cells in the scaffold, the connection of newly created tissue with neighboring tissues, and the carriage of nutrients and oxygen to cells so that new tissue formation and vascularization can occur more quickly [27]. Particulate-leaching is an easy method to produce 3D porous tissue engineering scaffolds using polymer solution mixed uniformly with salt or porogen particles of a certain diameter. Then the solvent is evaporated, and it leaves at the back a polymer matrix in which salt or porogen particles are embedded. Lastly, whenever composite material is submerged in water, embedded particles start to reach out to create a porous structure [28, 29]. By changing the size of the particle, this method can simply control the pore size and thickness of the septum of the 3D porous scaffold construct [28]. Scaffolds produced by solvent casting and particle-leaching techniques possess a porosity between 50% and 90%. It is a relatively simple and low-cost methodology, and also the developed scaffolds possess high porosity and the ability for tuning the pore size [30]. In the current study, by adding porogen particles to the bone scaffolds, it was aimed to gain the scaffolds a highly porous structure.

It is reported that the newer generation of ceramics has been formulated at 0.8 wt% Si and supported significantly more bone formation (Hing, Revell et al. 2006, Nagineni, James et al. 2012). Herein, we decided to alter the silicate amount to observe the effect of silicate amount in the scaffold. To the best of

our knowledge, the present study investigated for the first time the followings: (i) combining β-TCP-PLA with silicate-additive to fabricate a composite scaffold; (ii) characterization of the morphological structure, and mechanical features of these three different composite scaffolds; and (iii) observing the effect of β-TCP-PLA composite scaffolds with two different ratios of silicate-additive (0.8% and 1%) on osteogenic differentiation of human bone marrow-derived mesenchymal stem cells (hMSCs). In this study, we aimed to produce porous β-TCP-PLA composite scaffolds with silicate additive to increase osteogenic differentiation by mimicking the extracellular matrix (ECM) with its polymeric structure and by mimicking the bone tissue with its ceramic structure with osteoconductive and osteoinductive properties. Furthermore, flexibility and porosity were gained from the designed scaffolds to provide convenience to the surgeon during surgery to give the intended shape, and to allow vascularization and migration of the cells, respectively. First, β-TCP was mixed with PLA for imitating bone ECM and for providing flexibility to osteoconductive and osteoinductive β-TCP. Then, porogen was added and removed via the particulate leaching method to make the scaffold porous. β-TCP-PLA flexible grafts were used as the control group, while 1% and 0.8% silicate-additive flexible grafts are experimental groups to investigate the effect of silicate-additive with ranging concentrations on osteogenic differentiation by seeding hMSCs on bone grafts and incubating in an osteogenic medium for 21 days. hMSCs osteogenic differentiation on synthetic bone grafts were evaluated concerning the ALP activity and DNA content measurements. We demonstrated that designed synthetic flexible bone grafts encouraged the proliferation and osteogenic differentiation of hMSCs. The incorporation of silicate in synthetic β-TCP bone grafts significantly promotes the expression of ALP which is an early osteogenic marker by seeded hMSCs. Our results suggest that these synthetic flexible bone grafts may be useful for promoting the repair and regeneration of bone tissue.

2. MATERIALS and METHOD

2.1 Fabrication of Synthetic Flexible Bone Grafts

Bone grafts were fabricated in Bonegraft Biyolojik Malzemeler San. ve Tic. A. Ş. During the fabrication of the synthetic flexible bone graft, firstly β-TCP and silicate in determined ratios (0.8% and 1%) were mixed and added into %10 Poly (D, L-lactide-co-caprolactone) polymer solution. Then, porogen with the size of 1/1 ratio 100-250 μm and 250-500 μm were incorporated into the mixture in determined quantity for giving it a porous structure. The material was thoroughly mixed in the beaker, and after that, it was poured into Teflon-coated mold structures. The flexible bone graft was held in distilled water in a shaker water bath for removing the porogen and to acquire a porous structure of the material. For the analyzes to be performed in a sterile environment, the grafts were used after being exposed to ultraviolet (UV) for 1 hour, and stored for the following characterizations.

2.2 Characterization of Synthetic Flexible Bone Grafts

To image synthetic bone grafts, a Scanning Electron Microscope (SEM; Carl Zeiss Microscopy, Germany) at 3 kV accelerating voltage upon coating with gold (QUORUM; Q150 RES; East Sussex; United Kingdom) was used at 20 mA for 60 seconds. The scale bars found in the pictures were obtained with SEM software.

A universal testing machine with a 5 kN load cell (Shimadzu AGS-X Model, Japan) was used to perform Tensile Test. The tensile test of bone grafts was carried out according to the ASTM D638 standard, and the speed was 50 mm/min. For checking the repeatability of experiments, the tensile test was repeated at least three times.

2.3 Cell Culture and Cell Seeding

hMSCs (HMSC-AD-500, CLS cell lines Service, Lot #102, Eppelheim, Germany) were cultured within

the basal media (DMEM including 10% FBS, 250 ng/mL fungizone, 100 units/mL penicillin, 50 μg/mL gentamicin, 100 μg/mL streptomycin) in flasks. L929 cells (kindly donated from Ege University Research Group of Animal Cell Culture and Tissue Engineering Laboratory) were cultured within the basal media (DMEM including 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin) in flasks. When the cells achieve 80-90% confluency, they were passaged by utilizing a 0.25% trypsin/EDTA solution and taken to a novel flask containing fresh medium. For obtaining enough stocks in the related passage number, cells were frozen in the freezing medium at every passage and stored at -196 °C in a liquid nitrogen tank (ThermoScientific, Bio-cane 47). Before cell seeding, synthetic bone grafts were sterilized by UV radiation and washed with 70 % ethanol for 30 minutes, and then rinsed with sterile phosphate-buffered saline (PBS) three times. Following this step, synthetic flexible grafts were conditioned within the basal medium for 1 hour, and then cell suspension (10^6 cells / cm^3) was seeded on each sample in the basal medium. The cell-seeded grafts were incubated for the adhesion of cells for 24 hours. For cell viability and osteogenic differentiation experiments, L929 cells and hMSCs were seeded on scaffolds, respectively. For cell viability, the L929 cells were cultivated for 7 days in basal media. For osteogenic differentiation, the media on hMSCs seeded scaffolds was changed with osteogenic medium (basal medium supplemented with 10 mM ß-glycerophosphate, 50 μg/mL ascorbic acid, and 100 nM dexamethasone) and cells were cultivated for 21 days.

2.4 Cell Viability Analysis of L929 Cells on Synthetic Bone Grafts

3- (4, 5-dimethylthiazol-2-yl)- 2, 5-diphenyltetrazolium bromide (MTT) (Vybrant® MTT Cell Proliferation Assay Kit, Invitrogen, Waltham, MA, USA) test was used based on the manufacturer's instructions at 1., 4. and 7. days of the cell culture. For evaluating cell viability and proliferation, L929 (Mouse fibroblast cell line) cells were used for MTT assay. First, the culture medium was removed from cell-seeded grafts found in the well plate. 10% MTT dye in a culture medium was added to the cell-seeded grafts. Bone grafts were incubated in the incubator for 4 hours at 37°C and 5% CO_2. Next, the medium was removed from grafts again, and then 500 μL dimethyl sulfoxide (DMSO, Sigma-Aldrich, Steinheim, Germany) was given on cell-seeded grafts to dissolve the formed formazan crystals. After incubation for 15 minutes, a microplate reader (Biotek Synergy HTX, Winooski, VT, USA) was used to measure the absorbance values of each well at 570 nm.

2.5 Osteogenic Differentiation of hMSCs on Synthetic Bone Grafts

For stem cell differentiation studies, pure β-TCP-PLA without silicate-additive was utilized as the control group, and β-TCP-PLA with 0.8% silicate-additive and β-TCP-PLA with 1% silicate-additive were utilized as experimental groups. As additional information, the biological replicate number of the stem cell differentiation experiment was 3 meaning experiments were carried out with 3 replications from each group.

At each time point (7, 14, and 21 days), first, the cell-seeded grafts were washed with PBS, and then they were lysed with 10-mM Tris supplemented with 0.2% Triton in PBS. The lysed samples were used for the measurement of DNA content and ALP activity. To observe primary cellular concentration on bone grafts, the DNA content at day 0 was measured. Double-stranded DNA content and ALP activity of the samples were measured with DNA Quantification Kit (Sigma Aldrich, St. Louis, MO, USA) and QuantiChrom ALP assay (Bioassay Systems, Hayward, CA, USA), respectively, based on the manufacturer's directives [31]. In brief, the bisBenzimide H 33258 Solution was prepared and added to lysed samples found in 96-well plates. Then, fluorescence intensity (excited at a wavelength of 360 nm) was measured using a spectrophotometer (BioTek, Winooski, VT, USA) at an emission wavelength of 460 nm, and at ambient temperature. By using the ALP kit, ALP activity was evaluated by p-nitrophenylphosphate (*p*NPP) at 405 nm in an alkaline solution. Firstly, 50 μL of lysed sample to 200 μL total reaction volume were utilized for starting the reaction by the addition of assay buffer, 5 mM magnesium acetate, and 10 mM *p*NPP in a 96-well plate. We measured the optical density (OD) at 405 nm

for the first time (t = 0) and after 4 minutes (t = 4 min) with a multi-plate reader (BioTek, Winooski, VT, USA). Lastly, obtained ALP activities were normalized to cell numbers by dividing to DNA contents at every time point.

2.6 Statistical Analysis

All the obtained information was statistically analyzed via one-way ANOVA (SPSS 12.0, SPSS GmbH, Germany) and the Student-Newman-Keuls method as a post hoc test. Significant differences among groups were defined using p-values lower than 0.05. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3. RESULTS

3.1 Characterization of Synthetic Flexible Bone Grafts

SEM imaging was used to assess the morphology of flexible synthetic bone grafts. SEM images of three bone grafts were demonstrated in Figure 1. In SEM analysis, it was observed that these synthetic bone grafts have a porous structure as seen in Figure 1A-C. Although silicate particles were observed in 0.8% and 1% of silicate-additive β-TCP-PLA groups, the density of silicate was higher in the 1% silicate-additive β-TCP-PLA group as expected. SEM images show that the pore size and porosity of β-TCP-PLA composite scaffolds were decreased with the increased silicate ratio. The porosity of the scaffolds was measured by using Image J. The porosity of the control, 0.8% silicate-additive β-TCP-PLA, and 1% silicate-additive β-TCP-PLA scaffolds were found as 67.98%, 38.65%, and 42.14%, respectively.

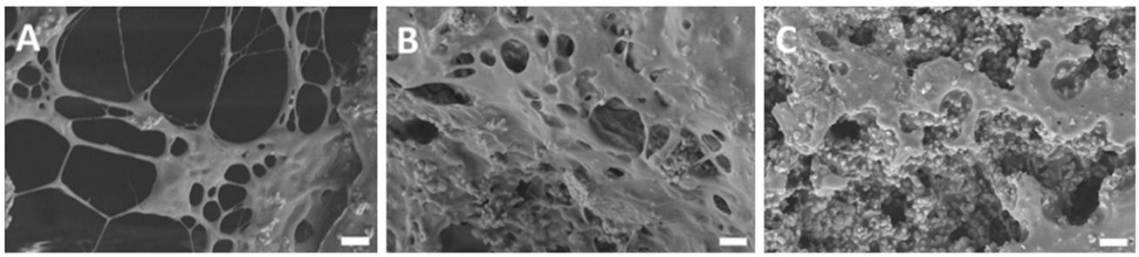

Figure 1. Scanning electron microscopy images of β-TCP-PLA (A), β-TCP-PLA with 0.8% silicate-additive (B), and β-TCP-PLA with 1% silicate-additive (C) grafts (Scale bar represents 2 μm).

Tensile strength analysis was fulfilled to observe the mechanical properties of the synthetic scaffolds (Figure 2). The tensile strength of control, 0.8% silicate, and 1% silicate groups were measured as 451,8 ± 35,06 kPa, 390,63 ± 7,21498672 kPa, and 343,02 ± 20,9923835 kPa, respectively. As demonstrated in Figure 2, tensile strength was decreased with the increase of silicate additive.

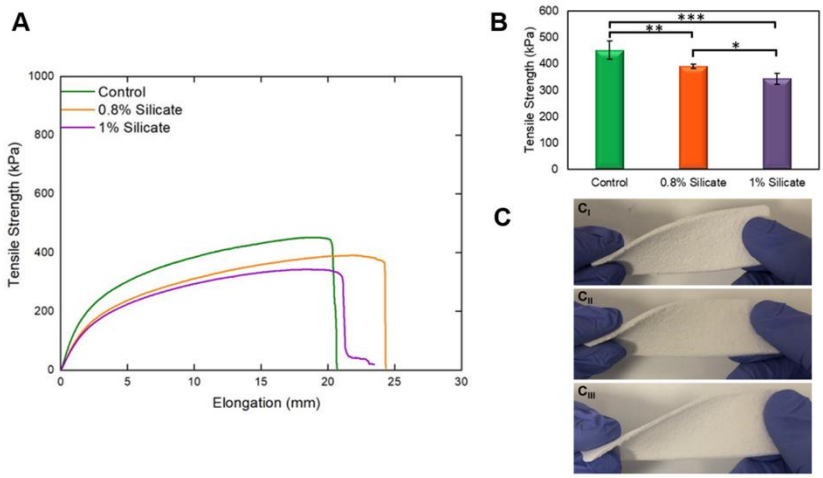

Figure 2. Tensile strength of β-TCP-PLA-based scaffolds (A-B) ((*p\0.05, **p\0.01, ***p\0.001)). Images of control (CI), 0.8% silicate (CII), and 1% silicate (CIII) group flexible grafts.

3.2 Cell Viability Analysis of Cells on Synthetic Flexible Bone Grafts

In this study, the effect of β-TCP-PLA-based bone scaffolds on cell viability and proliferation was assessed by MTT analysis. For this assay, L929 cells were seeded on bone grafts and incubated for 7 days. MTT analysis was carried out at 1., 4., and 7. days of the cell culture. As demonstrated in Fig.3, cell numbers of all three groups increased from Day 1 to Day 4, and then decreased from Day 4 to Day 7. At all time points during 7 days of cell cultivation, the cell numbers for 0.8% silicate-additive and 1% silicate-additive β-TCP-PLA bone grafts were higher than the cell numbers for the control group. This result shows the positive effect of silicate-additive bone grafts on cellular viability. Moreover, the cell numbers for 1% silicate-additive β-TCP-PLA bone grafts were the greatest at all time points.

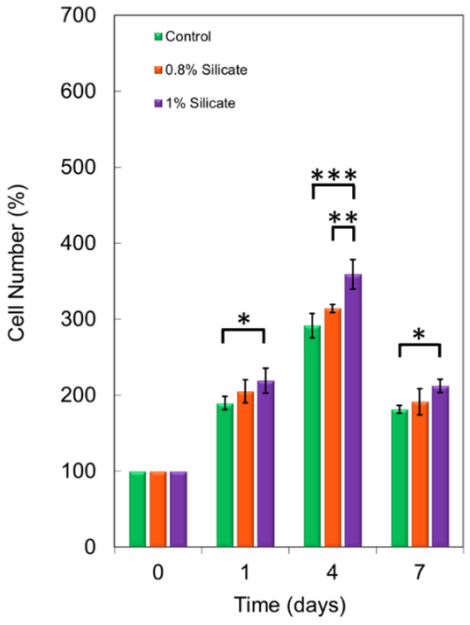

Figure 3. Cell proliferation analysis of L929 cells seeded in control, 0.8% silicate, and 1% silicate grafts. Error bars indicate mean ± SE (n = 3) [significant differences were defined by one-way ANOVA [Newman–Keuls multiple comparison tests, (*p\0.05, **p\0.01, ***p\0.001)].

3.3 Osteogenic Differentiation of hMSCs on Synthetic Flexible Bone Grafts

Osteogenic differentiation capability of hMSCs seeded on the control group, 0.8% silicate-additive and 1% silicate-additive bone grafts were evaluated for 7, 14, and 21 days. DNA content and ALP activity of the scaffolds were quantified for 21 days. The measured ALP activities were normalized to cell numbers by dividing DNA contents at each time point.

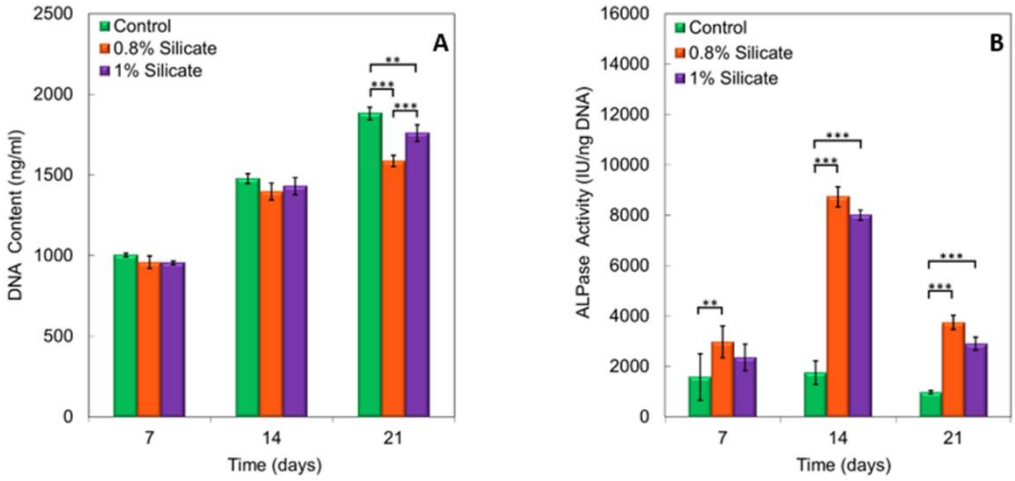

Figure 4: DNA content (A), ALPase activity (B), of hMSCs seeded in control, 0.8% and 1% silicate-additive grafts, and incubated in the osteogenic medium for 21 days. Error bars indicate mean ± SE (n = 3) [significant differences were defined by one-way ANOVA [Newman–Keuls multiple comparison tests, (*p\0.05, **p\0.01, ***p\0.001)].

hMSCs were seeded and incubated on control, 0.8% silicate, and 1% silicate graft groups. While the control group always had the highest DNA content depending on time, the DNA content of the groups containing 0.8% silicate and 1% silicate increased with time, but this increase was less than the control group. As shown in Fig.4, on Day 21, DNA contents of control, 0.8% silicate, and 1% silicate graft groups were measured as 1881,359333 ± 38,86 ng/ml, 1586,487333 ± 34,67 ng/ml, and 1759,564667 ± 50,88 ng/ml, respectively. As shown in Figure 4, the ALP activity of three graft groups peaked on Day 14 and began to decrease from Day 14 to Day 21, which was consistent with previous findings [32]. It was seen that hMSCs ALP activity increased significantly with silicate addition to grafts. For instance, peak ALP activity of the groups containing 0.8% silicate, and 1% silicate increased to 8737,517667 ± 400,8848672 IU/ng DNA, and 8005,144667 ± 201,9006029 IU/ng DNA, respectively whereas that of the control group was 1748,337333 ± 464,6124603 IU/ng DNA (***p < .001). Moreover, the ALP activity of the group containing 0.8% silicate was the highest at all times.

4. DISCUSSIONS

Herein, our objective was to develop porous β-TCP-PLA composite scaffolds with silicate additive to enhance osteogenic differentiation of hMSCs, and to assess the effect of silicate additive with ranging concentrations on osteogenic differentiation. For this purpose, we have designed three different graft groups which were β-TCP-PLA, β-TCP-PLA with 0.8% silicate-additive, and β-TCP-PLA with 1% silicate-additive. Briefly, SEM imaging and the tensile test were performed, and then hMSCs osteogenic differentiation was evaluated concerning the ALP activity and DNA content analysis. The major findings of this study include; all graft types have a porous structure confirmed by SEM imaging, the tensile strength was decreased with silicate-additive, and silicate addition resulted in enhanced ALPase activity and osteogenesis.

Vascularization of bone tissue scaffolds is of great importance for mimicking complex natural tissues, enhancing bone ingrowth and osseointegration, and especially for clinical applications. At this point, it is necessary to highlight the significance of porosity for the vascularization of bone tissue constructs. Adequate porosity with proper size and interconnections among pores ensures an environment for encouraging infiltration and migrating of cells, vascularization, transfer of nutrients and oxygen, and elimination of wastes, as well as being capable of withstanding the external stresses [33, 34]. Scaffolds porosity and pore size take a significant role in bone creation both *in vitro* and *in vivo* [35]. For bone formation, pores are needed since they let migration and reproduction of osteoblasts and mesenchymal cells, as also vascularization [35, 36]. Within the scope of this study, for fabricating synthetic bone grafts, the particulate-leaching method was used and porogen particles were added to the scaffolds. By incorporation of porogen particles, it was aimed to make the scaffold porous, and our SEM images showed that fabricated synthetic bone grafts have a highly porous structure. In the SEM images, pores can be observed, and these pores allow the synthetic bone tissue scaffolds to be vascularized better.

The production of composite scaffolds is a favorable approach since the combination of benefits of two or more materials may ensure better mechanical and physiological necessities for the host bone [21]. In addition, the formability of polymer materials in participation with a controlled-volume fraction of bioactive ceramic might result in the mechanical reinforcement of produced scaffold constructs and also increase the low bioactivity of most polymers [37]. In the current study, it was observed that the tensile strength of β-TCP grafts was decreased with increasing concentration of silicate-additive. Dalgic *et al.* used graphene oxide (GO)-incorporated silicate-doped nano HA composite materials with different ratios and examined the potential usage for bone tissue engineering via producing and reinforcing electrospun poly(ε-caprolactone) (PCL) scaffold construct. It was reported that PCL tensile feature did not enhance by the addition of HA to the fiber. Young's modulus also declined upon the addition of HA and silicate-doped hydroxyapatite (SiHA) to PCL. Similar to our results, tensile strength values have further declined via the incorporation of SiHA. It was also reported that silicate substitution may alter the surface properties of HA. When compared with HA, SiHA could have further moved off from PCL using structural resemblances and there might be a lower number of interfacial bonding ending up with decreased tensile strength [38]. To stabilize metallic implants, suitable flexible scaffolds should be placed on the surgical site just before surgical site closure. Higher elasticity is suitable for bone defects in the pelvis and lower extremities [39]. However, both flexibility and osseointegration are critical around the implant site. The composition of the scaffold used in this study allows osseointegration with the silicate additive by providing osteoinductivity. Therefore, the group with a high elastic modulus among silicate-containing groups which is 0.8% silicate might be considered optimal, although the silicate additive-free group has a higher elastic modulus than the silicate-containing groups of 0.8% and 1%, respectively.

The silicate-substitution stimulates the negative surface charge of material and importantly increases bone formation performance in comparison to present materials by the influence of it on the activity of bone-forming and resorbing cells [40-44]. This permits the vascularization of silicate-substituted calcium phosphate (Si-CaP) matrix, assisting differentiation of cells, making easy the rapid bone ingrowth, a larger volume of bone growth, and encouraging nutrition transfer to host bone tissue, with following graft remodeling to mature bone [45]. Herein, the effect of β-TCP-PLA-based scaffolds on the viability and proliferation of L929 cells was determined based on MTT analysis. Cell numbers for 0.8% silicate-additive and 1% silicate-additive bone grafts were higher than that of control grafts at all time points. This finding suggests that the functionalization of β-TCP-PLA grafts by silicate-additive induces cell proliferation, and silicate-additive bone grafts facilitated the proliferation of L929 cells compared with the control group. However, the cell numbers of the three scaffold groups increased from Day 1 to Day 4 and then decreased from Day 4 to Day 7. Figure 3 demonstrates that the cell number decreased significantly after Day 4. At the end of Day 7, it was observed that the excessive cell multiplication within the composite scaffolds led to the death of seeded cells due to the restricted area, It can be explained by the excessive cells which could not adhere to the scaffold surface. Since these excessive cells affected proliferation negatively, the cell number on Day 7 decreased in the cell number. Moreover, cell numbers on 0.8% silicate-additive and 1%

silicate-additive bone grafts suggested that silicate-additive graft groups did not have a toxic effect on cells. Furthermore, cell numbers for 1% silicate-additive grafts were the highest at all time points. Similarly, DNA content measurement results revealed that 1% silicate-additive bone grafts showed more amount of DNA content when compared to 0.8% silicate-additive bone grafts. This result suggests that the DNA content was correlated with the cell number.

hMSCs differentiation into osteoblastic lineage is a complicated procedure, containing the attachment, reproduction, maturation, differentiation, and mineralization of them [46]. The most important parameters which can be used for assessing osteogenic differentiation are known as adequate cellular growth, ALP activity, calcium deposition, and also osteogenic markers expression [31]. hMSCs osteogenic differentiation capability on bone grafts was quantified by measuring DNA content and ALP activity over 21 days. At that step, cells were seeded and then incubated on β-TCP/PLA-based grafts for 7, 14, and 21 days, and at the termination of every culture time point, we measured the hMSCs DNA content. Our results demonstrate that the control group showed greater DNA content than other groups, suggesting that hMSCs on the control group grafts proliferated more. On the other hand, 0.8% silicate and 1% silicate groups showed less increase in DNA content than the control group. However, the DNA content of silicate-additive groups enhanced with time during 21 days of incubation, indicating that cells showed enhanced proliferation at each time point. ALP activity measurement was evaluated utilizing the time-dependent *p*NPP creation in an alkaline solution. In correlation with prior findings, ALP activity of whole groups seemed to increase from Day 7 to Day 14 and began to decline with a longer period of incubation with mineralization [18]. The ALP activity of 0.8% silicate and 1% silicate groups was importantly greater than the ALP activity of the control group at all time points during 21 days of incubation. Functionalization with silicate promoted the ALP activity of hMSCs on β-TCP/PLA grafts. Similarly, in literature, silicate addition ended up with better *in vitro* and *in vivo* osteogenic differentiation of cells, and also guidable biodegradability properties when compared with β-TCP [21, 22]. Moreover, the ALP activity was the greatest in the 0.8% silicate group at all time points when compared with other groups proposing that hMSCs did not take as much osteogenic induction in control and 1% silicate grafts, and this indicates the positive effect of 0.8% silicate concentration on osteogenic differentiation. Cameron *et al.* used silicate-substituted calcium phosphate (Si-CaP) and nonmodified HA for testing if they can encourage MSCs osteogenic differentiation in the nonexistence of soluble factors or not, and reported that Si-CaP assisted the MSCs' attachment and reproduction and also triggered the osteogenesis to a larger degree than HA, as proofed by osteoblast-related genes upregulation [24]. Nagineni *et al.* aimed to evaluate the clinical and radiographical findings in spinal fusion processes by using Si-CaP material and reported that Si-CaP can be an alternative to autogenous bone material for spinal arthrodesis processes, and for a 12-month follow-up period, they observed high grades of bony fusion by utilizing Si-CaP in integration with several surgical spinal methods. In another study, researchers evaluated the osteoinductivity of silicate-substituted calcium phosphate and stoichiometric calcium phosphate using ectopic implantation. Silicate substitution importantly enhanced the quantity of bone created and the quantity of bone that is bonded to the surface of the implant, and it was reported that utilizing silicate-substituted calcium phosphate in place of stoichiometric calcium phosphate for orthopedic surgeries might significantly increase bone tissue repair and regeneration [23].

In summary, our results revealed that 0.8% silicate additive is the best concentration of silicate addition. Also, in this study, a mechanically targeted issue was to provide convenience to the surgeon during the application by adding the PLA polymer to the graft and to provide flexibility to give the desired shape. Herein, the flexible structure of these synthetic bone grafts will provide an important advantage by facilitating the surgeon for giving the intended form of the graft substance in the surgical area throughout the surgical process. Moreover, for a tissue scaffold, having a highly porous structure is very crucial, and these synthetic flexible bone grafts will have a positive effect on the pre-vascularization of scaffolds and differentiation of hMSCs due to their porous structure and interconnected pores.

5. CONCLUSIONS

In summary, β-TCP-PLA composite scaffolds were produced with two different concentrations of silicate-additive. Characterization tests showed that designed scaffolds had flexibility and porosity. We found that 1% and 0.8% silicate-additive flexible grafts improved the osteoinductive capacity of the β-TCP-PLA composite scaffolds, and encouraged the proliferation and osteogenic differentiation of hMSCs. Moreover, 0.8% silicate-additive flexible grafts showed sharply increased ALP activity when compared with 1% silicate-additive flexible grafts and β-TCP-PLA grafts. Our findings revealed that β-TCP-PLA composite scaffolds functionalized with 0.8% silicate-additive possessed more potential for inducing osteogenesis. It is believed that our results will assist scientists in enhancing and further optimization of the activity of composite scaffolds for more effective and better bone tissue repair and regeneration.

Declaration of Ethical Standards

The authors declare that all ethical guidelines including authorship, citation, data reporting, and publishing original research are followed.

Credit Authorship Contribution Statement

Günnur Onak Pulat: Performing experiments, Writing-reviewing-editing the manuscript. **Gülşah Sunal:** Performing experiments, Writing-reviewing-editing the manuscript. **Ozan Karaman:** Designing experiments, Writing-reviewing-editing the manuscript.

Declaration of Competing Interest

Authors have declared that there is no conflict of interest.

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Data Availability

Data will be made available on request.

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