Society For Biomaterials

PGA-incorporated collagen: Toward a biodegradable composite scaffold for bone-tissue engineering

Shirin Toosi,[1] Hojjat Naderi-Meshkin,[2] Fatemeh Kalalinia,[1,3] Mohammad Taghi Peivandi,[4] Hossein HosseinKhani,[5] Ahmad Reza Bahrami,[2] Asieh Heirani-Tabasi,[2] Mahdi Mirahmadi,[2] Javad Behravan[1]

[1]Biotechnology Research Center, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran
[2]Stem Cell and Regenerative Medicine Research Group, Iranian Academic Center for Education, Culture and Research (ACECR), Khorasan Razavi Branch, Mashhad, Iran
[3]Medical Genetic Research Center, Medical School, Mashhad University of Medical Sciences, Mashhad, Iran
[4]Department of Orthopedic Surgery, Orthopedic and Trauma Research Center, Mashhad University of Medical Sciences, Mashhad, Iran
[5]Graduate Institute of Biomedical Engineering National Taiwan University of Science and Technology (NTUST), Taiwan

Received 9 February 2016; accepted 25 March 2016
Published online 00 Month 2016 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jbm.a.35736

Abstract: Nowadays composite scaffolds based on synthetic and natural biomaterials have got attention to increase healing of non-union bone fractures. To this end, different aspects of collagen sponge incorporated with poly(glycolic acid) (PGA) fiber were investigated in this study. Collagen solution (6.33 mg/mL) with PGA fibers (collagen/fiber ratio [w/w]: 4.22, 2.11, 1.06, 0.52) was freeze-dried, followed by dehydrothermal cross-linking to obtain collagen sponge incorporating PGA fibers. Properties of scaffold for cell viability, proliferation, and differentiation of mesenchymal stem cells (MSCs) were evaluated. Scanning electron microscopy showed that collagen sponge exhibited an interconnected pore structure with an average pore size of 190 μm, irrespective of PGA fiber incorporation. The collagen-PGA sponge was superior to the original collagen sponge in terms of the initial attachment, proliferation rate, and osteogenic differentiation of the bone marrow-MSCs (BM-MSC). The shrinkage of sponges during cell culture was significantly suppressed by fiber incorporation. Incorporation of PGA fiber is a simple and promising way to reinforce collagen sponge without impairing biocompatibility. © 2016 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 00A:000–000, 2016.

Key Words: collagen sponge, fiber reinforcement, poly(glycolic acid), sponge fabrication, osteogenic differentiation

How to cite this article: Toosi S, Naderi-Meshkin H, Kalalinia F, Pievandi MT, HosseinKhani H, Bahrami AR, Heirani-Tabasi A, Mirahmadi M, Behravan J. 2016. PGA-incorporated collagen: Toward a biodegradable composite scaffold for bone-tissue engineering. J Biomed Mater Res Part A 2016:00A:000–000.

INTRODUCTION

Despite surgical or non-surgical treatment for bone fractures, some of them fail to heal, and become non-unions. Non-union can be defined as the failure of a fracture to heal after 6 months, without further progress toward healing.[1] However, the most advanced available treatments are limited in effectiveness and often result in some complications. Thus, an alternative strategy is important in order to treat a severe non-union fracture. The field of tissue engineering aims to combine engineering technology and the principles of biological science to develop plans for the regeneration and repair of lost or damaged tissue.[2] The most important factor to enhance regeneration by tissue engineering is that the scaffold material should be suitable for cell proliferation and differentiation.[3]

The biocompatibility and biodegradability properties of the scaffold materials are very important to provide suitable conditions for cell seeding. Among many materials currently available as cell scaffolds, collagen has been extensively used and has *in vivo* safety proven through long-term applications in clinical trials, cosmetics, and the food industries.[4–6] Collagen is the major protein of bone structure and the natural scaffold for osteoblast migration.[7] Some collagen advantages are the low immunogenic response across species,[8,9] affluence in nature, hemostatic promotion, and ease manipulation into different forms.[10,11] However, association with extracellular components,[11], such as proteoglycans,[12] seeding of osteogenic cells,[13] and chemical modifications[14] could enhance its applicability as a biomaterial.[15] Collagen sponges are highly porous with interconnected pore structure which is effective in infiltration of cells, supplying of nutrients and oxygen to cells and excluding cell waste. However, according to the previous studies, collagen sponge as a scaffold for cell proliferation and differentiation in hard

Correspondence to: J. Behravan; e-mail: Behravanj@mums.ac.ir
Contract grant sponsor: Research Council of Mashhad University of Medical Sciences, Iran

tissue has poor mechanical strength and is not suitable for use in bone tissue engineering.[16] In order to overcome this problem with collagen sponge, combination with other materials should be attempted.[17]

The materials that are used for combination should be bioabsorbable and it is preferable to select material that has been clinically used. Some biodegradable synthetic polymers, such as poly(glycolic acid) (PGA) and its copolymers with L-lactic acid and D-lactic acid, have been combined into collagen sponges for tissue engineering. PGA has US FDA approval for clinical use in humans in a variety of medical applications. Studies have shown that incorporation of PGA fibers enables collagen sponge to increase its resistance to compression *in vitro*.[18]

In this study, we aimed to produce a collagen sponges with incorporation of PGA fibers in order to use the advantages of each other. In addition, we investigated the behavior of MSC extract from bone marrow (BM) in the collagen-PGA sponge.

MATERIALS AND METHODS

Preparation and culture of MSCs

Mesenchymal stem cells (MSCs) from reamer-irrigator-aspirate (RIA) were isolated from three patients with long bone fracture. About 5–20 mL, RIA were collected in a syringe containing 10,000 IU heparin to prevent coagulation. Cell extraction was performed according to the lympholyte-H (Cedarlane, Canada) protocol. Briefly, after centrifugation with Ficoll, mononuclear cells was collected from the milky interface layer and washed twice in phosphate-buffered saline (PBS). The cell pellets were resuspended in Dulbecco's modified Eagle's medium (DMEM) with low glucose content, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum (FBS) (all reagents from Gibco) and cultured in T75 flasks at 37°C in a 5% CO_2 atmosphere. Non-adherent cells were removed after 3 days by medium exchange, and adherent cells were allowed to grow with medium replacement every 2 days. After 10 days in culture, isolated colonies of MSC became apparent. They were harvested by trypsinization with aqueous solution of 0.05% trypsin and 1 mM ethylenediaminetetraacetic acids (EDTA) in 0.1 M PBS, pH 7.4 for 3–5 min at 37°C and reseeded in new flasks at a density of 5–10 $\times$ 10^3 cells/cm^2. The cells used at P3.

Fabrication of collagen sponge

An aqueous solution of type I collagen, prepared from porcine tendon by pepsin treatment (6.33 mg/mL, pH 3.0) in HCl, was purchased from Nitta Gelatin Inc. The non-woven fabric of PGA fiber, 20 mm in diameter (0.5 mm in thickness, 200–210 g/m^2) was gifted by Dr. Hossein Hosseinkhani (Taipei, Taiwan). DMEM and FBS were purchased from Gibco.

Collagen sponges with different amounts of PGA fiber were fabricated using the dehydrothermal method. In order to remove oils and fats, PGA non-woven fabric was immersed in acetone for 1 h and washed three times with double-distilled water for 10 min at 25°C. PGA fiber was loosened by using tweezers to obtain the PGA component. Then, different collagen/fiber ratios (w/w) 4.22, 2.11, 1.06, and 0.52 (1.5, 3, 6, and 12 mg) were homogeneously were placed into a 24-well tissue culture flask (Biofill) and 1 mL of collagen solution was poured into each well. The resulting collagen solution was frozen at −20°C for 24 h to obtain collagen sponge incorporating PGA fiber. The freeze-dried sponge was dehydrothermally cross-linked at 140°C for 12 h under 0.1 torr vacuum conditions. This cross-linking method was selected because it is toxicologically more acceptable than the chemical cross-linking alternative. A similar preparation procedure was performed using only the collagen solution, to obtain collagen sponge without PGA fiber incorporation. Scaffolds were sterilized with ethylene oxide gas at 40°C.

Morphological observation

The appearance and infrastructure of collagen sponges, with and without PGA fiber incorporation, were observed with a scanning electron microscope (SEM; Leo, 1450 VP, Germany). The collagen sponges were cut with a razor blade. Sponge cross sections were coated with gold on an ion sputterer (E-1010; Hitachi) at 50 mtorr and 5 mA for 30 s and viewed by SEM at a voltage of 15 kV. The pore size of collagen sponges was calculated as the geometric mean of the major and minor diameters of the pores from the pictures of cross sections of the sponges.[19–21]

Sponge characterization

The porosity of the sponge was measured by a liquid substitution method. Isopropanol with density ρ_i was used as the displacement liquid and operated at 4°C.[22] In this method, a density bottle was used to measure the porosity of the scaffold. A density bottle filled with isopropanol was weighed (W_1). A sponge sample of weight W_s was immersed in the density bottle and then weighed (W_2). The sponge saturated with isopropanol was taken removed and then the density bottle was weighed (W_3).

The following scaffold parameters, including the volume of the sponge pore (V_p), the density of the sponge skeleton (V_s), and the porosity (ε), were calculated[23]

$$V_p = (W_2 - W_3 - W_s)/\rho_i$$

$$V_s = (W1 - W2 + W_{s)/}\rho_i$$

$$\varepsilon = V_p/(Vp + V_s)$$

Maximum water absorption of sponges was checked after shaking samples in PBS at 300 rpm on an orbital shaker (VISION, model: VS-8480, Korea) at 37°C for 24 h. The equation for the maximum water uptake is[24]

$$\text{Water absorption (\%)} = (\text{[Wet weight} - \text{Initial dried weight]}/\text{Initial dried weight}) \times 100$$

The diameter of sponges with and without PGA was checked in MSC culture after 14 days. The equation for the contraction (shrinkage) is[24]

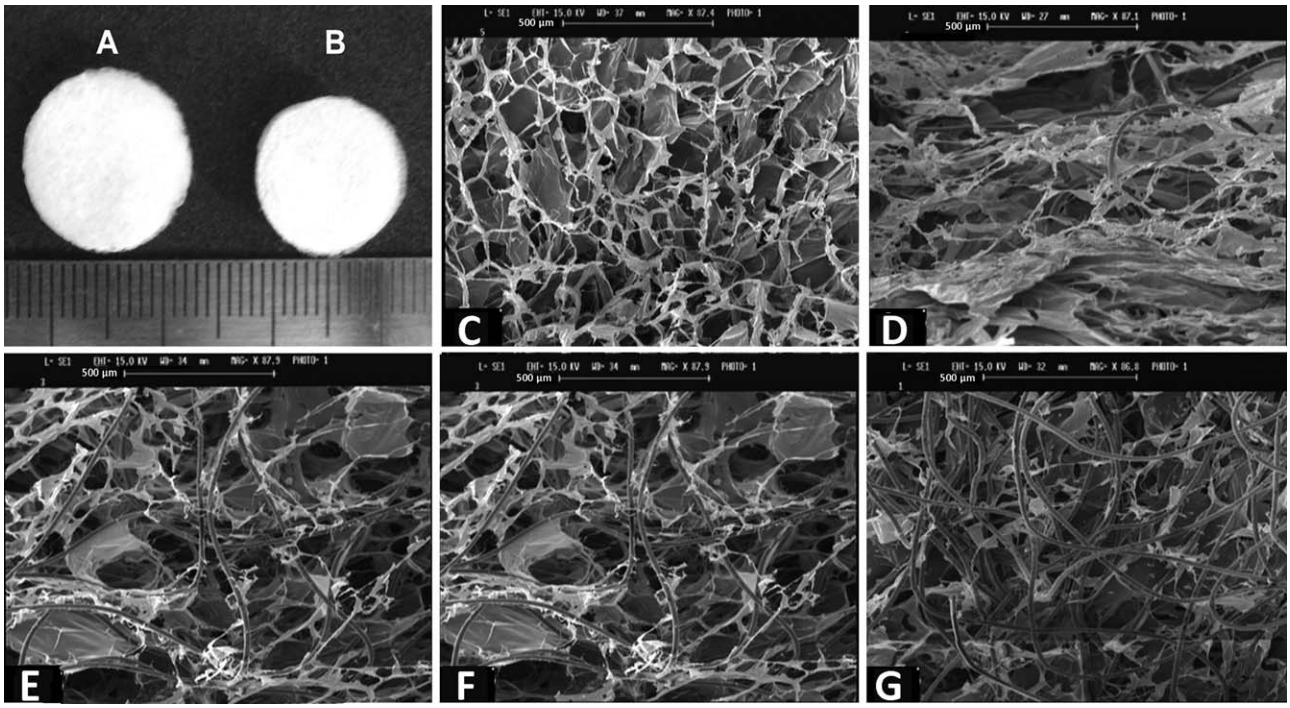

FIGURE 1. Frame structure and scanning electron microscopy photographs of a collagen sponge with and without PGA fibers. (A) with PGA fiber incorporation (B) without PGA fiber incorporation. (C) Cross-sectional scanning electron microscopy photographs of a collagen sponge without PGA fiber incorporation (collagen/fiber ratio [w/w]: 6.33) and collagen sponges incorporating PGA fibers with different collagen/fiber ratio (w/w) (D) 4.22, (E) 2.11, (F) 1.06, and (G) 0.52.

$$\text{Shrinkage } (\%) = ([\text{Initial diameter} - \text{Diameter after 14 days}]/\text{Initial diameter}) \times 100$$

The sample number was three for each experimental group.

Fourier transforms infrared spectroscopy

Infrared (IR) spectra were recorded using a PERKIN EIMER FT-IR spectrophotometer PARAGON 1000 in the wave number region 400–4000 cm^{-1}.

Cell seeding into collagen sponge

MSCs were seeded into sponges by the agitated seeding method. Prewetted sponge was placed into a tube (15 mL/sterile tube), and then 0.5 mL of cell suspension containing 1×10^6 cells was added to the tube, followed by culturing by shaking the sponge–cell mixture at 300 rpm on an orbital shaker (VISION, model: VS-8480, Korea) for 6 h, then incubated for 2 h at 37°C in a 5% CO_2 in 24-well plate. After that, 1 mL of DMEM supplemented with 15% FBS and 1× of 100 U/mL penicillin and 100 μ/mL streptomycin (Gibco) was added to each sponge.

DNA assay

The number of cells attached to each sponge was determined by fluorometric quantification of cell DNA assay.[25] Briefly, the cell-seeded sponge after 48 h was washed with PBS and cells present in the sponge were lysed in a buffer solution (pH 7.4) containing proteinase K (0.5 mg/mL), sodium dodecyl sulfate (0.2 mg/mL) and 30 mM saline-sodium citrate (SSC) at 55°C for 12 h, with occasional mixing. The cell lysate (100 μL) was mixed with SSC buffer (400 μL) in a glass tube. After mixing with a dye solution (500 μL) (composition: 30 mM SSC and Hoechst 33258 dye [1 μg/mL]), the fluorescence intensity of the mixed solution was measured with a fluorescence spectrometer (synergy H4 Hybrid Reader; Biotek) (excitation, 355 nm; emission, 460 nm). A calibration curve between DNA and cell number was prepared by using cell suspensions with different cell densities. The DNA assay was performed three times independently for every experimental sample.

MTT assay

Proliferation of MSCs on sponges was determined by the MTT 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyl-2H-tetrazolium bromide (atocell) assay. The sponges were transferred into a new 24-well plate, and 1 mL of MTT solution (0.5 mg/mL) was added to each well. After incubation at 37°C for 4 h in a 5% CO_2, MTT was taken up by the active cells and reduced in the mitochondria to insoluble purple

TABLE I. Characterization of the Collagen Sponge With and Without PGA Fiber Incorporation

Collagen/fiber ratio (w/w)	6.33	4.22	2.11	1.06	0.52
Porosity (%)	84.12	87.75	88.34	90.15	91.38
Average pore size (μm)	184	188	190	194	198

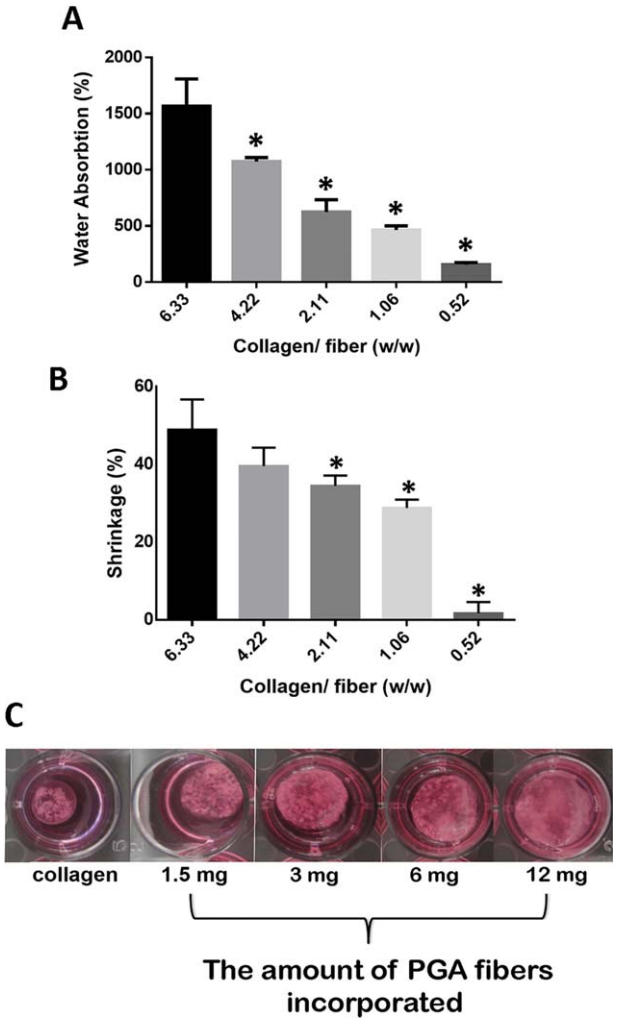

FIGURE 2. Water absorption and shrinkage of collagen sponges. (A) Sponges were immersed in PBS at 300 rpm for 24 h at 37°C. The results were expressed as the Water absorption (%) = ([Wet weight − Initial dried weight]/Initial dried weight) × 100. Each experiment was repeated independently three times in triplicate tests and data are shown as mean ± SD. *$p < 0.05$; significant against the water absorption of collagen sponge without PGA fiber incorporation (collagen/fiber ratio [w/w]: 6.33). (B) Sponges were immersed in MSC culture for 14 days at 37°C. The result were expressed as the Shrinkage (%) = ([Initial diameter − Diameter after 14 days]/Initial diameter) × 100. Each experiment was repeated independently three times in triplicate tests and data are shown as mean ± SD. *$p < 0.05$; significant against the shrinkage of collagen sponge without PGA fiber incorporation (collagen/fiber ratio [w/w]: 6.33). (C) Photographs of collagen and collagen-PGA sponges 14 days after culturing at 37°C.

formazan granules. Subsequently, the medium was discarded and the precipitated formazan was dissolved in DMSO (150 μL/well). The optical density of the solution was evaluated using a microplate spectrophotometer after subtraction of OD 570 nm. The viable cell number was determined using a linear calibration curve between OD and predetermined cell concentration.

Cell attachment

SEM observation was performed to evaluate the morphology of MSCs attached to collagen sponges in normal medium. In this method, the culture medium was discarded and sponges were washed with PBS for three times. Adhered cells on the sponges were fixed with 4 mL of 4% formaldehyde for 90 min at 4°C. After washing with PBS for three times, adhered cells were dehydrated in an ethanol-graded series (30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100%) for 1 h and allowed to dry in a clean bench at room temperature and observed with an SEM (Leo, 1450 VP, Germany).

Live/dead staining

To demonstrate cell viability over the whole period of 3D culture, propidium iodide/fluorescein diacetate (PI/FDA) staining (PI; Sigma, 81845 and FDA; Sigma, F73378) was performed. The grafts were rinsed three times with PBS and incubated for 15 min at 37°C with 2 μg/mL FDA solution. After incubation, the grafts were rinsed again three times with PBS and incubated with 0.1 mg/mL PI solution for 2 min at room temperature. After an additional washing step, the grafts were analyzed under a fluorescent microscope (Olympus BX51).[26]

Evaluation of osteogenic differentiation

To evaluate the osteogenic differentiation of the MSCs, their intracellular alkaline phosphatase (ALP) activity and bone osteocalcin content were measured. For this purpose, cell-seeded collagen sponge incorporating PGA was cultured by a procedure similar to that described above, but using DMEM with low glucose content supplemented with 10% FBS, 10 nM dexamethasone, 50 μg/mL ascorbic acid, and 10 mM β-glycerophosphate (differentiation medium). Normal medium was used as a control. The ALP activity was determined using an ALP assay kit (Lot. No. APF; Sigma). Cultured sponge was washed three times with PBS, minced with scissors, and homogenized in lysis buffer solution (0.2% triton X-100, 10 mM Tris–HCl, 1 mM $MgCl_2$, and pH 7.5). Sample lysate (2 mL) was centrifuged at 12,000 rpm for 10 min at 4°C and the ALP activity of the supernatant was assayed using *p*-nitrophenyl phosphate as a substrate. To determine calcium deposition, cultured sponges were washed three times with PBS. The MSCs were then detached from the scaffold by treatment with an aqueous solution of 0.05% trypsin and 1 mM EDTA in 0.1 M PBS, pH 7.4 (Gibco, 25200) for 20 min at 37°C and after 24 h in low glucose DMEM with 10% FBS. MSCs attached on the plate and fixed with 3% formaldehyde for 10 min at room temperature. To stain the differentiated cells, the samples were immersed in alizarin red at pH 4 for 10 min at room temperature, and washed with PBS three times. The alizarin red was then removed and replaced by PBS, and the pictures were taken by Eyepiece camera (AM4023; Dino-lab digital microscope, USA).

Statistical analysis

Results (mean ± SD) were reported as three independent stages. Statistical analyses were performed by GraphPad Prisme 6 using ANOVA, with Tukey's post-hoc to reveal significant differences between the data and p values < 0.05 were considered significant.

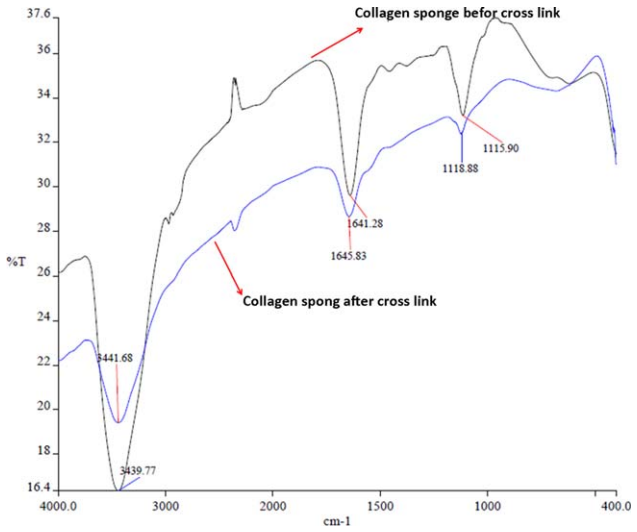

FIGURE 3. FTIR spectra of collagen sponge, before and after dehydrothermal cross-link. Different peak in 1640 cm^{-1} indicates the differences in the functional groups before and after cross-linking.

RESULTS

Morphological and physical properties of collagen-PGA sponges

Morphology of collagen sponges with or without PGA fiber incorporation and SEM photographs of cross sections of collagen sponges incorporating various amounts of PGA fibers (Fig. 1) shows irrespective of the amount of fiber incorporated, every collagen sponge possessed an average pore size of 190 μm (Table I). The morphological characteristics are summarized in Table I and their intrastructural appearance is similar. Although some PGA fibers were exposed in the pores of PGA-incorporated sponges, the internal structure is similar to that of sponges without PGA fiber incorporation.

Evaluation the maximum water absorption of sponges showed decreasing water absorption rate of collagen sponges with increasing amounts of PGA fiber weight [Fig. 2(A)]. Figure 2(B,C) shows the diameter change of collagen sponges with and without PGA fibers incorporation in MSC culture after 14 days.

Fourier transforms infrared spectroscopy analysis

Figure 3 shows the Fourier transforms infrared spectroscopy (FTIR) peak of collagen sponge before and after dehydrothermal cross-link. The result showed different peaks at 1640 cm^{-1}, indicates the differences in the functional groups before and after cross-linking.

Cell attachment and proliferation of collagen sponges

Cell attachment on the surface of fiber in normal media shows in [Fig. 4(A)]. As shown, cells have a tendency on the surface of fiber. Figure 4(B) shows the number of cells attached in collagen sponges with or without PGA fiber incorporation 6 h after seeding with agitation. There was a

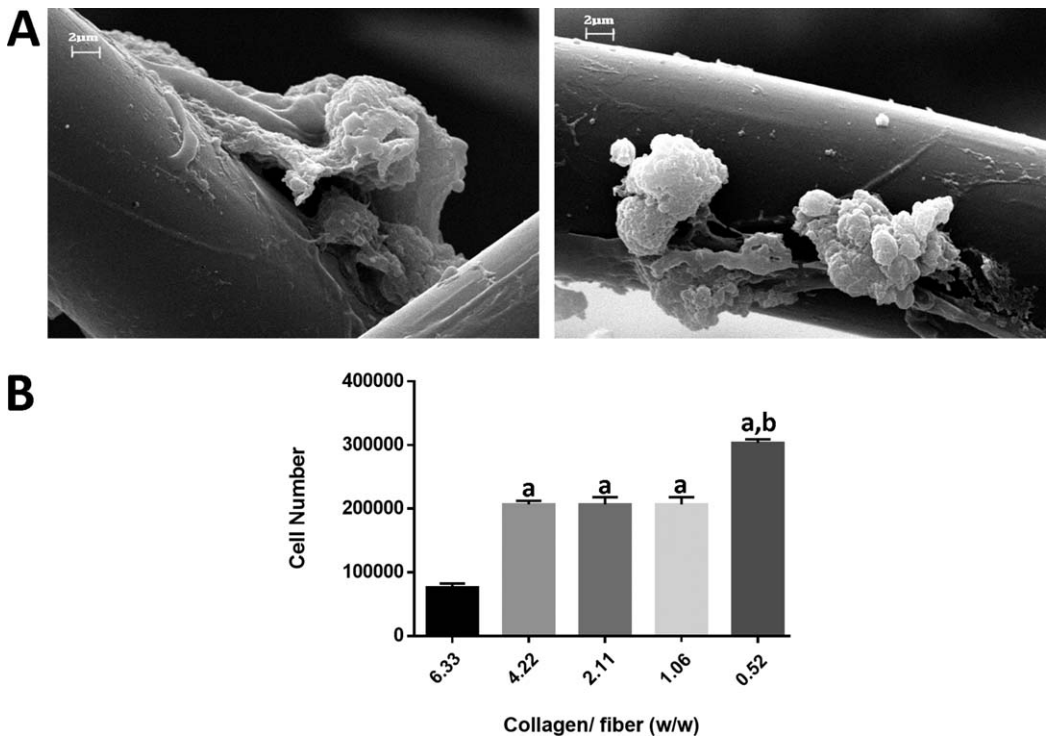

FIGURE 4. The number of MSCs attached to collagen sponges. (A) Scanning electron microscopy photographs of cell adhesion on surface of fibers in normal media at 37°C in a 5% CO_2. (B) The number of MSCs attached to collagen sponges in normal media at 37°C in a 5% CO_2. The results were expressed as the DNA assay method. Each experiment was repeated independently three times in triplicate tests and data are shown as mean ± SD. (a) $*p < 0.05$; significant against the cell number attached to collagen sponge without PGA fiber incorporation (collagen/fiber ratio [w/w]: 6.33), (b) $*p < 0.05$; Significant against the cell number attached to collagen sponges with PGA fiber (collagen/fiber ratio [w/w]: 0.52) to (collagen/fiber ratio [w/w]: 6.33, 4.22, 2.11, 1.06).

significant difference in cell number between the original collagen sponges and the collagen-PGA sponges at collagen/fiber ratio of 0.52.

Cell viability

MTT assay was used to compare cell viability on the collagen scaffolds with and without PGA. PGA scaffold grows with the most viable cells [Fig. 5(A)]. The results showed higher rate of viable cells in collagen-PGA sponges at collagen/fiber ratio of 0.52 in compassion with other tested scaffold. Also, cell viability within the scaffold was determined by using live/dead staining (FDA/PI) after three-dimensional arrangement of expanded MSC in PGA fibers and cultured them for 3 weeks [Fig. 5(B)]. The results indicated that MSCs are distributed homogeneously. The green staining cell proliferation of collagen sponges, with and without different weights of PGA, shows the MSCs to be viable and partly round-shaped cells.

Osteogenic differentiation

Figure 6(A,B) shows MSCs differentiated to osteocytes. Calcium deposits of differentiated cells were qualified by alizarin red after 21 days. Figure 6(C) shows ALP activity of MSCs cultured on collagen sponge, and on collagen-PGA sponges at collagen/fiber ratio of 0.52. ALP activity of MSC cultured in the bone differentiation medium was always high for the collagen sponge reinforced with PGA fiber incorporation, as compared to collagen sponge without PGA fiber incorporation. In induction medium, incorporation of PGA fibers enhanced ALP activity.

DISCUSSION

When a bone fracture is not healed after 6 months, it can be clinically defined as non-union.[27] This decrease is often found in regions complicated by infection or tissue defects. This phenomenon may be the results of decrease in the population of progenitor cells (like MSCs) in local tissue. The field of tissue engineering can use for healing non-union bone fractures. Biomaterial scaffolds, such as biodegradable polymers, have been used in tissue engineering. Collagen is the principle component of extra cellular matrix (ECM) and one of most important materials to fabricate an artificial ECM in tissue engineering.

Non-woven polyglycolide fabrics have been extensively used as scaffolding matrices for tissue regeneration due to their excellent degradability, good initial mechanical properties and protective cell viability on the matrices. The biodegradation of PGA is appropriate for orthopedic use, such as fracture fixation. In *in vivo*, PGA degrades to glycine which enters the tricarboxylic acid cycle and breaks up into carbon dioxide and water and finally excreted by the kidneys.[28]

In this work, we have attempted to reach on an optimum concentration of PGA fibers and investigated their role in the osteogenic differentiation.

SEM observation has revealed that PGA fiber incorporation has no influence on the inter-pore structure of collagen sponge (Fig. 1). The pore structure and the interconnected features of collagen sponge could be reserved by incorporating PGA fiber. Based on this finding, it is possible to compare the efficiency of sponges in the cell scaffold only from the viewpoint of sponge reinforcement. The inner-pore structure and the interconnected feature of collagen sponge would give cells a three-dimensional space suitable for proliferation.

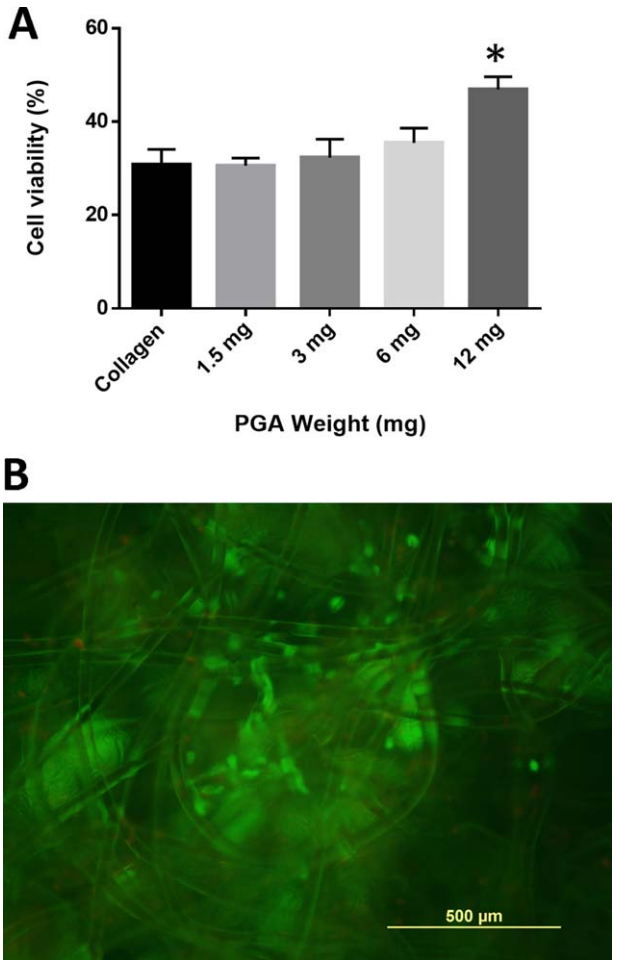

FIGURE 5. Viability of MSCs in sponges. (A) Cell viability on the collagen scaffolds with and without PGA. The results were expressed as proliferation of MSCs on sponges. Each experiment was repeated independently three times in triplicate tests and data are shown as mean ± SD. *$p < 0.05$; significant against the viability of cells on collagen sponge without PGA fiber incorporation (collagen/fiber ratio [w/w]: 6.33). (B) Cell viability staining with PI/FDA. The staining shows a homogeneous cell distribution of viable cells within the scaffold. (Magnification 10×.)

Water absorption behavior [Fig. 2(A)] showed that the hydrophobic property of PGA fibers has a major influence on decreasing the tendency of sponge to an aqueous medium. By decreasing water absorption and providing a physical network between fibers and collagen, the shrinkage of collagen sponge decreases [Fig. 2(B,C)]. Shrinkage is one of the most important deficiencies of collagen sponges.[16] The space generated by the fiber-based suppression of sponge shrinkage is necessary for the oxygen and nutrients supply in the sponge and excretion of the waste products.[29] Incorporation of PGA fibers suppressed the shrinkage of collagen sponges with increasing amount of PGA fibers

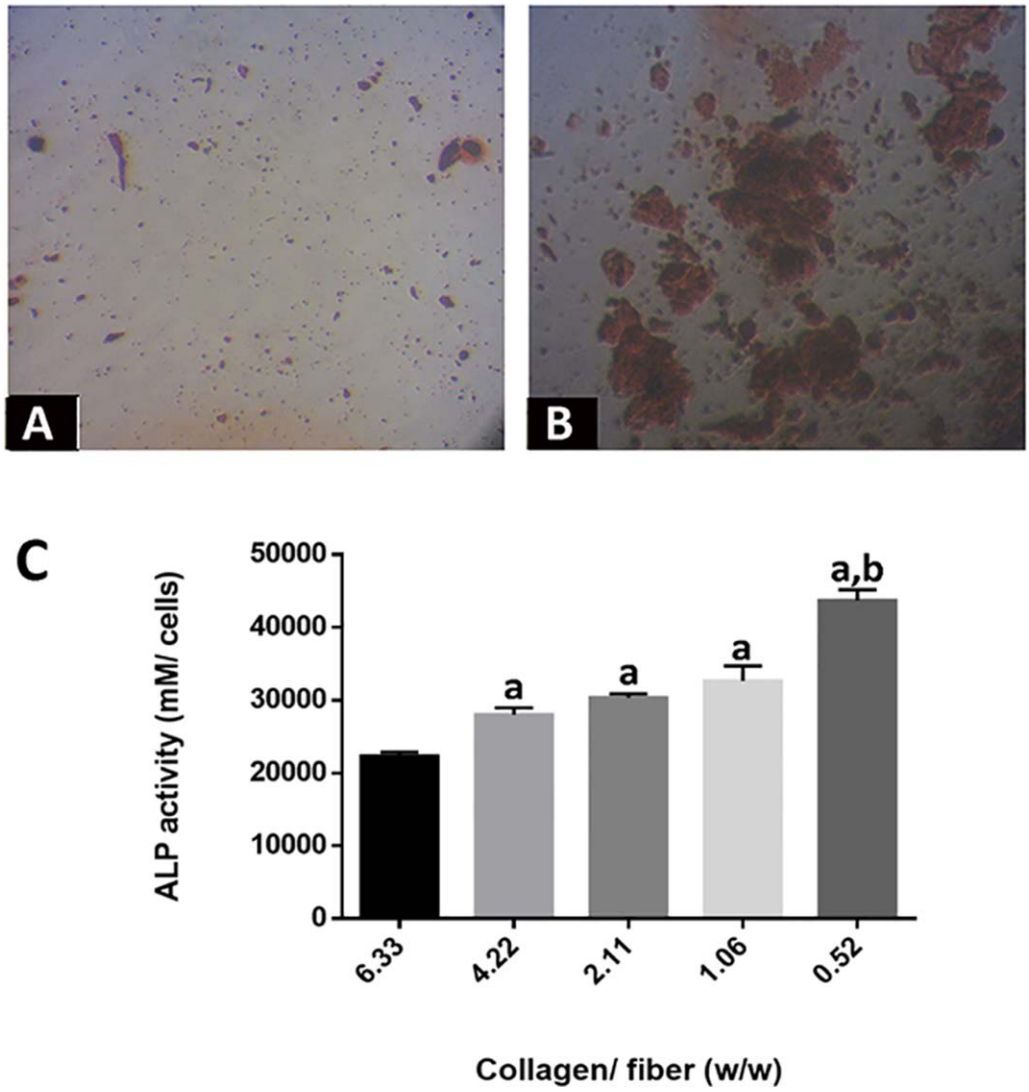

FIGURE 6. Differentiation capacities of MSCs toward osteoblast in sponges. (A and B) Calcium deposition photographs of collagen sponges with PGA fiber (collagen/fiber ratio [w/w]: 0.52) in bone differentiation media after 21 days at 37°C in a 5% CO_2. (C) ALP activity in bone differentiation media at 37°C in a 5% CO_2. The results were expressed as the ALP assay kit. Each experiment was repeated independently three times in triplicate tests and data are shown as mean ± SD. (a) $*p < 0.05$; significant against the ALP activity of collagen sponge without PGA fiber incorporation (collagen/fiber ratio [w/w]: 6.33). (b) $*p < 0.05$; Significant against the ALP activity of collagen sponges with PGA fiber (collagen/fiber ratio [w/w]: 0.52) to (collagen/fiber ratio [w/w]: 6.33, 4.22, 2.11, 1.06).

incorporated. It is likely that the shrinkage of collagen sponge without fiber reinforcement collapses the inner-pore structure, resulting in worse conditions of cell proliferation and the consequent cell death for a long culture period. Takamoto et al. and Mohajer et al. have reported that collagen sponge shrinkage was suppressed with increasing amount of PET and PP/PET fibers incorporated.[16,29]

FTIR provides information on protein structure. Among them, the amide I band (peptide bond C═O stretch; 1650 cm^{-1}) is especially sensitive to secondary structures.[30] Thus, information on protein structure is obtained from analysis of broad peaks consisting of overlapping component bands. This is accomplished by applying such techniques as FTIR. Our result indicates the differences in the functional groups before and after cross-linking (Fig. 3). Because by physical cross-linking, such as the dehydrothermal method, some free radicals, such as COOH or NH_2 in collagen the molecule can bind and cross-link the collagen molecules to each other.

For the MSC attachment experiment, the agitation culture was used because MSC could be homogeneously seeded into the collagen sponge.[18,31–33] Some research has shown that the agitation method was more efficient in cell attachment than static method.[32,33] The increased number of cells attached with the incorporation of PGA fibers was significantly higher than that of the original collagen sponge. Because PGA fiber incorporation suppresses the shrinkage of collagen sponge, it is possible that the pores available for cells entering the inside space of sponges is maintained, resulting in a larger number of cells entered and attached (Fig. 4). Takamoto et al. have reported that shrinkage of collagen sponge without fiber reinforcement collapses the

inner-pore structure, resulting in worse conditions of cell proliferation and the consequent cell death for a long culture period.[29]

For treatment of non-union fractures, we need scaffolds that can provide the facility of diffusion of various medium and factors inside the sponges and give MSC better differentiation to osteoblast. In this study (Fig. 6), alizarin red staining confirmed MSCs differentiation to osteoblast and ALP activity increased in scaffold reinforced with PGA than in the original collagen scaffold. According to the chemical structure of PGA, incorporation fibers do not have any interaction with cells under differentiation media. The improvement in cellular activity on the collagen-PGA sponges at collagen/fiber ratio of 0.52 may originate from the stability of sponge in differentiation media and the initial higher level of cell attachment and proliferation. Hosseikhani et al. have reported that PA nanofibers enhanced *in vitro* differentiation of MSCs.[34] The osteogenic differentiation also depended of the fiber amount was observed. It is highly conceivable that the reinforcement of collagen sponge facilitated the diffusion of various osteogenic differentiation factors like into the inside of sponge and consequently gave MSC better differentiation conditions. Also, another studies showed that the initial cell attachment directly affects such other cellular responses as movement, proliferation, and phenotype expression of cells through the internal signal transduction.[35] Therefore, the improvement in cellular activity on the sponge with collagen/fiber ratio (w/w) of 0.52 may be originated from the stability of sponge in differentiation media and initial higher level of cell attachment and proliferation.

CONCLUSIONS

Fabrication of collagen sponge reinforced with biodegradable PGA fibers as bone tissue-engineered scaffold was studied in this work. The *in vitro* investigation has shown that incorporation of PGA fibers had an impressive effect on physical properties as well as increasing cell attachment, proliferation, and differentiation. These results suggest that PGA fiber incorporation is a suitable method to enhance physical stability without using toxic materials, and for use as treatment of non-union fractures. Further, *in vivo* investigation is necessary to find the reactions of biodegradable fibers under real conditions.

ACKNOWLEDGMENT

The authors are indebted to the Research Council of Mashhad University of Medical Sciences, Iran, for approval and financial support of this research as part of PhD thesis of Shirin Hamedakbari Toosi.

REFERENCES

1. Tseng SS, Lee MA, Reddi AH. Nonunions and the potential of stem cells in fracture-healing. J Bone Joint Surg 2008;90:92–98.
2. Mistry AS, Mikos AG. Tissue engineering strategies for bone regeneration. Regener Med. II 2005;94:1–22.
3. Hosseinkhani H, Inatsugu Y, Hiraoka Y, Inoue S, Tabata Y. Perfusion culture enhances osteogenic differentiation of rat mesenchymal stem cells in collagen sponge reinforced with poly (glycolic acid) fiber. Tissue Eng 2005;11:1476–1488.
4. Stenzel KH, Miyata T, Rubin AL. Collagen as a biomaterial. Annu Rev Biophys Bioeng 1974;3:231–253.
5. Rault I, Frei V, Herbage D, Abdul-Malak N, Huc A. Evaluation of different chemical methods for cross-linking collagen gel, films and sponges. J Mater Sci Mater Med 1996;7:215–221.
6. Friess W. Collagen–biomaterial for drug delivery. Eur. J Pharm Biopharm 1998;45:113–136.
7. Rocha LB, Goissis G, Rossi MA. Biocompatibility of anionic collagen matrix as scaffold for bone healing. Biomaterials 2002;23: 449–456.
8. DeLustro F, Smith ST, Sundsmo J, Salem G, Kincaid S, Ellingsworth L. Reaction to injectable collagen: Results in animal models and clinical use. Plast Reconstr Surg 1987;79:581–592.
9. Kamer FM, Churukian MM. Clinical use of injectable collagen: A three-year retrospective review. Arch Otolaryngol 1984;110:93–98.
10. Watson SP. Collagen receptor signaling in platelets and megakaryocytes. Thromb Haemost 1999;82:365–376.
11. Chiang TM. Collagen-platelet interaction: Platelet non-integrin receptors. Histol Histopathol 1999;14:579–585.
12. Pohunkova H, Stehí J, Vachal J, Adam M. Morphological features of bone healing under the effect of collagen-graft—glycosaminoglycan copolymer supplemented with the tripeptide Gly-His-Lys. Biomaterials 1996;17:1567–1574.
13. Toung JS, Ogle RC, Morgan RF, Lindsey WH. Repair of a rodent nasal critical-size osseous defect with osteoblast augmented collagen gel. Laryngoscope 1999;109:1580–1584.
14. Radhika M, Sehgal P. Studies on the desamidation of bovine collagen. J Biomed Mater Res 1997;35:497–503.
15. Radhika M, Babu M, Sehgal P. Cellular proliferation on desamidated collagen matrices. Comp Biochem Physiol Part C 1999;124: 131–139.
16. Mohajeri S, Hosseinkhani H, Golshan Ebrahimi N, Nikfarjam L, Soleimani M, Kajbafzadeh A-M. Proliferation and differentiation of mesenchymal stem cell on collagen sponge reinforced with polypropylene/polyethylene terephthalate blend fibers. Tissue Eng Part A 2010;16:3821–3830.
17. Chen G, Ushida T, Tateishi T. Fabrication of PLGA-collagen hybrid sponge. Chem Lett 1999;1999:561–562.
18. Hiraoka Y, Kimura Y, Ueda H, Tabata Y. Fabrication and biocompatibility of collagen sponge reinforced with poly (glycolic acid) fiber. Tissue Eng 2003;9:1101–1112.
19. Mandal BB, Kundu SC. Cell proliferation and migration in silk fibroin 3D scaffolds. Biomaterials 2009;30:2956–2965.
20. Hu K, Cui F, Lv Q, Ma J, Feng Q, Xu L, Fan D. Preparation of fibroin/recombinant human-like collagen scaffold to promote fibroblasts compatibility. J Biomed Mater Res Part A 2008;84:483–490.
21. Lv Q, Feng Q. Preparation of 3-D regenerated fibroin scaffolds with freeze drying method and freeze drying/foaming technique. J Mater Sci Mater Med 2006;17:1349–1356.
22. Li X, Feng Q, Cui F. In vitro degradation of porous nano-hydroxyapatite/collagen/PLLA scaffold reinforced by chitin fibres. Mater Sci Eng C 2006;26:716–720.
23. Tu C, Cai Q, Yang J, Wan Y, Bei J, Wang S. The fabrication and characterization of poly (lactic acid) scaffolds for tissue engineering by improved solid-liquid phase separation. Polym Adv Technol 2003;14:565–573.
24. Li X-k, Cai S-x, Liu B, Xu Z-l, Dai X-z, Ma K-w, Li S-q, Yang L, Sung KP, Fu X-b. Characteristics of PLGA–gelatin complex as potential artificial nerve scaffold. Colloids Surf B 2007;57:198–203.
25. Rao J, Otto WR. Fluorimetric DNA assay for cell growth estimation. Anal Biochem 1992;207:186–192.
26. Endres M, Neumann K, Schröder S, Vetterlein S, Morawietz L, Ringe J, Sittinger M, Kaps C. Human polymer-based cartilage grafts for the regeneration of articular cartilage defects. Tissue Cell 2007;39:293–301.
27. Hernigou P, Beaujean F. Bone marrow in patients with pseudarthrosis. A study of progenitor cells by in vitro cloning. Rev Chir Orthop Reparatrice Appar Mot 1996;83:33–40.
28. Blasier R, Bucholz R, Cole W, Johnson L, Mäkelä E. Bioresorbable implants: Applications in orthopaedic surgery. Instruct Course Lect 1996;46:531–546.

29. Takamoto T, Hiraoka Y, Tabata Y. Enhanced proliferation and osteogenic differentiation of rat mesenchymal stem cells in collagen sponge reinforced with different poly (ethylene terephthalate) fibers. J Biomater Sci Polym Ed 2007;18:865–881.
30. Torii H, Tasumi M. Theoretical analysis of the amide I infrared bands of globular proteins. In: Mantsch HH, Chapman D, eds. Infrared Spectroscopy of Biomolecules. New York: Wiley-Liss, Inc., 1996:1–18.
31. Takahashi Y, Tabata Y. Homogeneous seeding of mesenchymal stem cells into nonwoven fabric for tissue engineering. Tissue Eng 2003;9:931–938.
32. Carrier RL, Papadaki M, Rupnick M, Schoen FJ, Bursac N, Langer R, Freed LE, Vunjak-Novakovic G. Cardiac tissue engineering: Cell seeding, cultivation parameters, and tissue construct characterization. Biotechnol Bioeng 1999;64:580–589.
33. Kim B-S, Putnam AJ, Kulik TJ, Mooney DJ. Optimizing seeding and culture methods to engineer smooth muscle tissue on biodegradable polymer matrices. Biotechnol Bioeng 1998;57:46–54.
34. Hosseinkhani H, Hosseinkhani M, Tian F, Kobayashi H, Tabata Y. Bone regeneration on a collagen sponge self-assembled peptide-amphiphile nanofiber hybrid scaffold. Tissue Eng 2007; 13:11–19.
35. Anselme K. Osteoblast adhesion on biomaterials. Biomaterials 2000;21:667–681.