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Evaluation of the Chondrogenic Differentiation of Mesenchymal Stem Cells on Hybrid Biomimetic Scaffolds

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ABSTRACT: Hybrid materials are widely and promisingly used as scaffolds in cartilage tissue remodeling. In this study, hybrid scaffolds consist of polycaprolactone (PCL), poly(vinyl alcohol) (PVA) with/without gelatin (GEL) to mimic natural cartilage extracellular matrix (ECM) were investigated. Scaffolds were prepared by freeze drying and characterized by scanning electron microscopy and compressive mechanical testing. Biological assays of mesenchymal stem cell (MSC) cultures, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, and dimethyl methylene blue were performed, and real-time polymerization chain reaction analysis of the cartilage-specific ECM gene marker expression was done. The results show an open interconnected porous structure with a compression modulus of 1.27 ± 0.04 MPa. The surface of the scaffolds showed an excellent efficiency in the adhesion and proliferation of MSCs. A significant increase in the proteoglycan content from 3.70 ± 0.96 to 5.4 ± 1.13 μg/mL was observed after 14 days in the PCL–PVA–GEL scaffolds. The expression amount of the sex-determining region Y–Box 9 (SOX9) and collagen II (COL2) mRNA levels of the MSCs showed significant increases in SOX9 and COL2, respectively in comparison with PCL–PVA scaffold. The study revealed that the aforementioned scaffold as a blend of natural and synthetic polymers may be a promising substrate in tissue engineering for cartilage repair with MSC transplantation. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 40635.

KEYWORDS: PCL; PVA; gelatin; cartilage tissue engineering; mesenchymal stem cell differentiation; hybrid scaffold

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INTRODUCTION

Loss of cartilage regardless of the cause is problematic because of the extremely limited repair capacity of the tissue. Cartilage transplantation has never met success because of the scarcity of donor sites and the associated morbidity in the harvest procedures.[1,2]

It is known that the functional properties of cartilage are mainly dependent on its extracellular matrix (ECM) components.[3] One common strategy involves the incorporation of different biomaterials, such as synthetic and natural polymers, to mimic the real ECM of this tissue.[4,5] Niche signaling influences the mesenchymal stem cell (MSC) differentiation to chondrocytes. This microenvironment, or niche, could be established by a well-designed polymeric scaffold together with the appropriate growth factor.[6] Because cartilage is composed mainly of water, devices intended for its substitution or tissue regeneration should be made of a material that can hold large amounts of water and should also have good handling properties to withstand loads imparted by the cells and ECM during *in vitro* culturing. Moreover, recent efforts also have focused on biomimetic approaches for incorporating biologically active macromolecules to generate specific cellular responses, such as the incorporation of peptides that mediate cellular adhesion, for example, Arginine-glycine-aspartic acid (RGD).[7] To achieve these goals, poly(vinyl alcohol) (PVA), polycaprolactone (PCL), and gelatin (GEL) were selected. PVA is a polymer with a large number of hydroxyl groups that can be fully hydrated; PCL is a semicrystalline material with good mechanical properties and a slow degradation rate,[8] and GEL, a partial derivative of collagen that has antigenicity because of its animal origin, has a relatively low antigenicity compared to its precursor. It still retains some of the information signals that may promote cell adhesion,

Additional Supporting Information may be found in the online version of this article.

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Table I. Compositions of the Four Kinds of Scaffolds

Scaffold	PCL (w/w %)	PVA (w/w %)	GEL (w/w %)
PCL–PVA	50	50	—
PCL–PVA–GEL	50	25	25
PCL–PVA(C)	50	50	—
PCL–PVA–GEL(C)	50	25	25

C, crosslinked with GTA (0.5% v/v).

differentiation, and proliferation, such as the RGD sequence of collagen. Thus, the purpose of this study was to evaluate the potential of PCL–PVA scaffolds for cartilage tissue engineering and to determine the enhancing effect of GEL incorporation on cell adhesion, direction of proliferation, and also differentiation.

EXPERIMENTAL

Scaffold Preparation

Semi-interpenetrating polymer networks (semi-IPNs) of hydrophilic PVA (M_w = 12,000–23,000, Sigma-Aldrich, St. Louis, MO), hydrophobic PCL (M_w = 80,000; Sigma-Aldrich), and GEL type A (ca. 300 Bloom, Sigma, St. Louis, MO) from porcine skin in powder form were prepared as hybrid scaffolds in four groups (Table I). Briefly, PCL was dissolved in chloroform (10% w/v) at room temperature, and both aqueous solutions of PVA and GEL were prepared by the addition of 10% w/v of each polymer in distilled water (DW) at 100 and 40°C with a mechanical stirrer for 1 h, respectively. A foamlike mixture was formed with the high-speed mixture of the aqueous and nonaqueous solutions at 6000 rpm for 5 min at room temperature. Then, the mixture was immediately transferred to a vial, cooled down to liquid nitrogen temperature, and lyophilized for 24 h to prepare a three-dimensional scaffold with a highly porous structure. The scaffold then was immersed in a 0.5% v/v glutaraldehyde (GTA) solution for 1 h at room temperature for the crosslinking of PVA and GEL to produce a semi-IPN structure. Finally, the scaffolds were removed from the crosslinking solution, rinsed twice by DW, cooled down to liquid nitrogen temperature, and lyophilized for 24 h once again.[9] Samples for all tests were prepared in a circular shape with a diameter of 8 mm and a thickness of 3 mm.

Mechanical Properties of the Scaffolds

The compression modulus of the dried scaffolds was performed to evaluate the mechanical properties of the specimens according to ASTM standards and procedure. These tests were performed with a Zwick/Roell Z050 instrument (Germany) equipped at room temperature. A cylindrical sample with a height-to-diameter ratio of 2:1 was used for testing. Three samples of each composition were compressed at a speed of 0.5 mm/min to half height, and at this point, the maximum load was measured, and the data were expressed.[10]

In Vitro Degradation

For degradation studies, the weight loss of the scaffolds was followed as a function of the incubation time in deionized water at 37°C. After 30 days, the samples were removed from water, dried *in vacuo* for 24 h, and then weighed. The weight loss of each sample was thus calculated from eq. (1):

$$\text{Weight loss } \% = ((W_0 - W_t)/W_0) \times 100 \quad (1)$$

where W_0 is the initial dried weight of the samples and W_t is the weight of dried samples after 30 days of immersion.

GEL Release

The stability of the ternary complex scaffold was evaluated by the examination of the release of GEL with a Bradford assay.[11] Bradford assays rely on the binding of the dye Coomassie Blue G250 to protein. Two different scaffolds (i.e., crosslinked PCL–PVA–GEL and the noncrosslinked one) were incubated in deionized water at 37°C for a duration of 21 days in sterile conditions. After this period of time, the quantity of GEL release was determined by the evaluation of the absorbance of the solution at 595 nm (Start Fax 2100).

3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay

To evaluate the proliferation rate of the rabbit MSCs obtained from the National Cell Bank, Pasteur Institute of Iran, in the presence of the prepared scaffolds, an extraction method was done according to ISO 10993-5. The sterile scaffolds were placed in 12-well plates (each well contained 0.15 g of scaffold per milliliter of culture medium). After 7 and 21 days of incubation, these media were taken out to use in cell proliferation assays. A specified amount of serum-free culture medium, Dulbecco's Modified Eagle's Medium (DMEM), was kept under the same conditions as a negative control. The proliferation rate of the MSCs on the samples were measured with the MTT assay. Briefly, on the first day, the MSCs were plated into 96-well plates at 1×10^4 cells/well. After 24 h, the culture medium of each well was removed and replaced with 90 μL of extract plus 10 μL of Fetal bovine serum (FBS). In the next 24 h, the medium was eliminated, and 100 μL of a 0.5 mg/mL solution of MTT (Sigma) was added to each well. This was followed by incubation for 5 h at 37°C. The purple formazan crystals (formed in the mitochondria of the cells) were detected and later dissolved by the addition of 100 μL of isopropyl alcohol (Sigma) per well. The plates were then incubated at 37°C for 15 min before the absorbance measurements. The absorbance of the solution was measured at 545 nm with an ELISA reader (Start Fax 2100). The viability (%) was calculated from eq. (2):

$$\text{Viability } \% = (\text{OD}_s/\text{OD}_c) \times 100 \quad (2)$$

where OD_s and OD_c are the average optical densities of the sample and the control, respectively.

In Vitro Cell Culture

The scaffolds were prewetted with DMEM/F12 (Dulbecco's Modified Eagle Medium/Ham's Nutrient Mixture F_12) cell culture medium overnight to make their surfaces more efficient for cell attachment before seeding. Then, the MSCs (passage 2–3) were detached from the culture flasks by the use of trypsin [0.05% containing 1 m*M* ethylenediaminetetraacetic acid (Sigma)] and suspended in DMEM/F12 medium containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma). Then, 100 mL of the cell suspension at a density of 200×10^3 cells/scaffold were seeded on the scaffolds in 24-well

plates (Nunc, Denmark). Afterward, the plates were placed in an incubator for 15 min to allow initial cellular attachment, and subsequently, 1 mL of supplemented medium was added to each well, and the plates were returned to the incubator. The plates were incubated at 37°C in a humidified atmosphere with 5% CO_2, and their culture medium was replaced every 3 days. For morphological studies, the scaffolds were removed from the plates after 3, 14, and 21 days, rinsed three times with phosphate-buffered saline (PBS), fixed with 2.5% v/v GTA solution, dehydrated with the graded ethanol, then covered by a layer of gold, and viewed through a VEGA 2 TESCAN scanning electron microscope (operating at 10 kV).

Because we achieved more cell efficiency for glycosaminoglycan (GAG) and real-time polymerase chain reaction (PCR) assays, the scaffolds were placed in round-bottomed, non-tissue-culture-treated tubes (Nunc CryoTube Vials, Nalge Nunc Intl., Roskilde, Denmark) instead of 24-well plates under the same conditions.

Determination of GAG Expression

The proteoglycan content was determined by the amount of sulfated GAGs released into media with a dimethyl methylene blue assay at pH 6.8. The method of cell culturing was the same as reported in previous sections. After 14 and 21 days, 500 μL of medium of each well was aspirated and transferred to a vial. Then, 1.5 mL of ice-cold acetone (Merck) was added and kept for 24 h at −20°C. The samples were centrifuged at 1800 rpm for 30 min at 4°C. The precipitated pellets were suspended in 100 μL of PBS containing papain (20 μg/mL) and activated with 5 m*M* cysteine; this was followed by incubation at 60°C for 16 h and boiling for 15 min. A working range of the standard solution of a known concentration from chondroitin sulfate C (shark cartilage extract, Sigma) was used. The GAG content was quantified with an ELISA plate reader at 545 nm.[12]

Quantitative Real-Time PCR

Total RNA was extracted from the cells by an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the supplier's data. Briefly, after 21 days of cell culturing with the aforementioned method, the scaffolds were removed from the wells and washed with PBS to remove the loosed cells. In the next step, the adhered cells to the scaffolds were disrupted and lysed with the supplied buffer (Qiagen) and measured by a nanophotometer (Implen GmbH, Munich, Germany). According to the previous study,[13] rabbit articular cartilage tissue (control) was harvested from rabbit proximal humerus, cooled down to liquid nitrogen temperature, and then cut into small pieces. Total RNA was extracted from tissue by an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the supplier's data for tissues. An amount of 1 μg of total RNA was used as a template for complementary DNA synthesis with a QuantiTect reverse transcription kit (Qiagen). Expression of collagen II (COL2), aggrecan (AGG), and sex-determining region Y–Box 9 (SOX9) as chondrogenic markers and Glyceraldehyde 3-phosphate dehydrogenas (GAPDH) as the housekeeping gene were quantified in triplicate with SYBR Green Master Mix and an ABI StepOne real-time PCR instrument (both from Applied Biosystems, Foster City, CA). Quantification of gene expression was performed with the comparative CT method (a method for analyzing data obtained from PCR), in which for each sample, the CT values of each target gene were normalized to those of GAPDH. The result of real-time PCR was represented as a fold increase with respect to the control sample.[14]

Statistical Analysis

At least three replicates of the samples used in all of the experiments were done, and the results are given as the mean plus or minus the standard deviation. One-way analysis of variance was used for all of the statistical analyses with SPSS Software.

RESULTS AND DISCUSSION

Scanning Electron Micrograph of the Scaffold

It is known that the pore size of the scaffold plays an important role in cell binding, migration, and ingrowth. Although nutrient materials, gases, and metabolic waste can be transported more easily via interconnected large pores in the scaffold, large pores can lead to low cell attachment and intracellular signaling. In contrast, small pores can have the opposite effect, in which cell attachment is promoted, but there is poor nutrient and gas delivery.[15] From our results, scanning electron microscopy (SEM) photographs of two different compositions are shown in Figure 1. There was an anisotropic range of interconnected pores, small and large ones (17.42 ± 2.03 and 117.96 ± 28.00 μm in PCL–PVA and 12.91 ± 2.47 and 100.71 ± 24.65 μm in the PCL–PVA–GEL scaffolds, respectively), regardless of their different composition [Figure 1(A,B)]. It is mentioned in the literature that the structure of the scaffold surfaces with topological features having sizes on the order of 100 μm can provide direct tissue development.[16] Therefore, this method of fabrication resulted in scaffolds with a broad distribution of pore structure; this was in a wide range of pore sizes that resembled those of similar anisotropy in native cartilage.[17] Consequently, the construction of these scaffolds containing both macropores and micropores may provide essential physical support for cellular growth.[18]

Mechanical Tests

A comparison of the compressive properties of our four different groups of semi-IPN scaffolds showed that their module increased as a result of crosslinking in both the PCL–PVA and PCL–PVA–GEL scaffolds in comparison with the noncrosslinked ones [Figure S1 (in the Supporting Information) and Table II]. From the mechanical point of view, cartilage can be considered as two phases: one is collagen, which supplies the mechanical support, and the other is proteoglycan, which reserves the water in the tissue and gives flexibility to it. Actually, collagen and proteoglycans form about 30% of the cartilage tissue, and the remaining 70% is water. This is a good reason that the scaffolds that are prepared for cartilage should be porous and have a high ability to reserve the body fluid into their pores. Compression tests have often been performed to assess the biomechanical properties of cartilage. As mentioned earlier, the selection of polymers in this study was made to mimic the properties of native components of the cartilage. PCL in the scaffolds was expected to provide the mechanical strength (resembling the role of collagen), whereas the PVA/GEL, which had a high water-holding capacity, was supposed to mimic the proteoglycans. As a matter of fact, PCL was retained as a linear polymer,

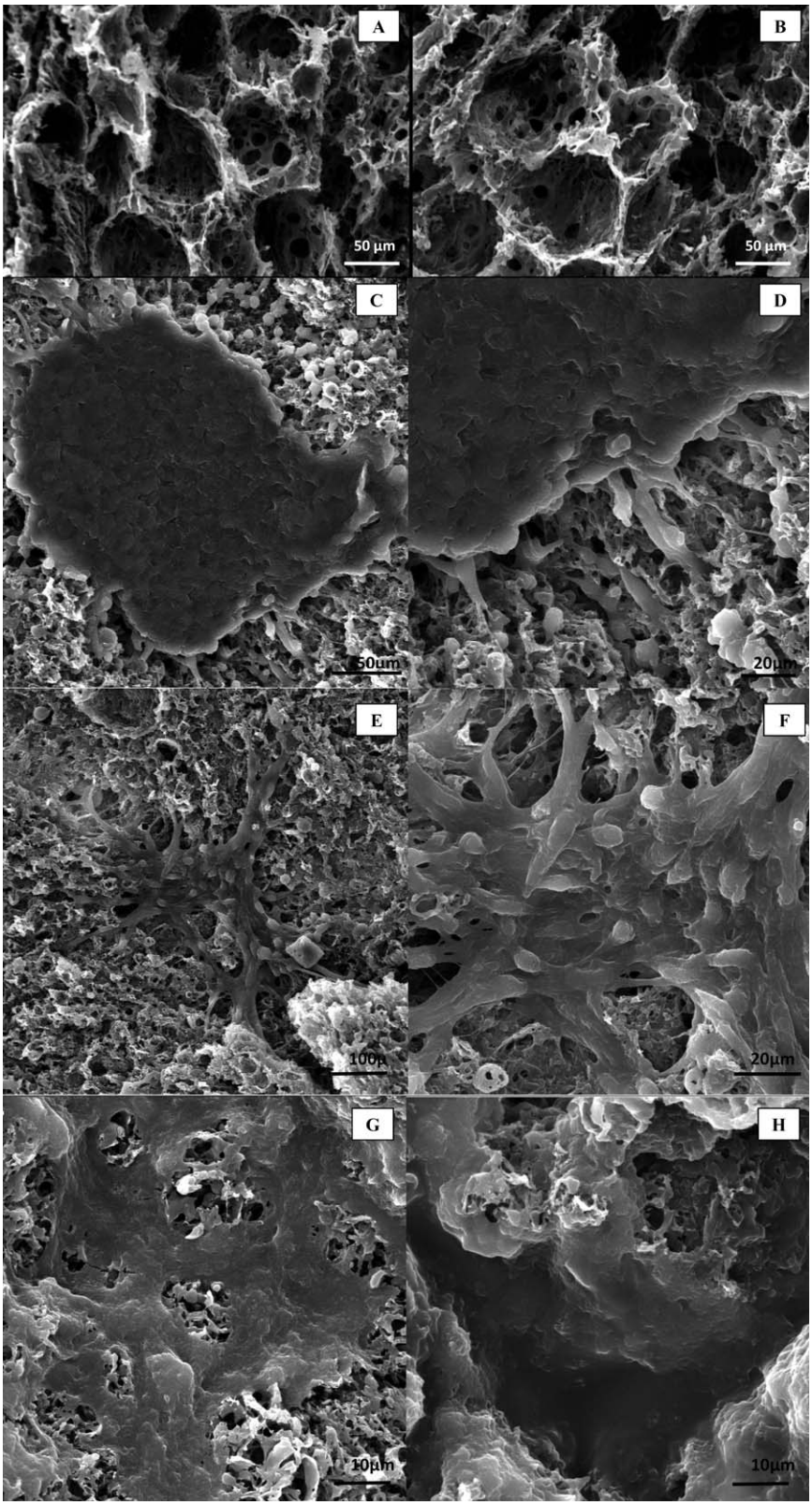

Figure 1. Morphological observations by SEM: (A) PCL–PVA (250×) and (B) PCL–PVA–GEL (250×). Scanning electron micrographs of scaffolds seeded with MSCs: (C) PCL–PVA (500×), (D) PCL–PVA (1000×), (E) PCL–PVA–GEL (400×), and (F) PCL–PVA–GEL (1200×) after 3 days of culturing; (G) PCL–PVA–GEL after 14 days of culturing (2000×); and (H) PCL–PVA–GEL after 21 days of culturing (2000×).

Table II. Compressive Properties of the Scaffolds

Composition	PCL–PVA	PCL–PVA–GEL	PCL–PVA(C)	PCL–PVA–GEL(C)
Modulus (MPa)	1.07 ± 0.04	0.728 ± 0.02	2.34 ± 0.05	1.27 ± 0.04

C, crosslinked.

whereas the PVA/GEL chains were partially crosslinked with GTA. The crosslinking resulted in interchain and intrachain bonding by the covalent linkage of PVA/GEL, within which the PCL chains were entangled; this resulted in a semi-IPN. This entangled network of all of the polymers resembled the ECM of cartilage and enhanced the mechanical stability of the system.[19] The crosslinking also prevented the phase separation of the three polymers.[19] These were the reasons for the increase in the compressive modulus of the crosslinked scaffolds (from 1.07 ± 0.04 to 2.34 ± 0.05 MPa for PCL–PVA and 0.72 ± 0.02 to 1.27 ± 0.04 MPa for PCL–PVA–GEL) in comparison to noncrosslinked ones. Moreover, according to Table II, the addition of GEL decreased the compressive modulus. It has been reported in the literature that samples with greater water uptakes have a lower compressive modulus.[18] Therefore, the lower compressive modulus of the PCL–PVA–GEL (0.72 ± 0.02 MPa) compared to that of PCL–PVA (1.07 ± 0.04 MPa) was probably due to its higher water uptake.[20] Human articular cartilage has been shown to have a compressive modulus that ranges from 0.53 to 1.34 MPa.[21] On the basis of this fact and according to our test conditions, the crosslinked PCL–PVA–GEL (1.27 ± 0.04 MPa) scaffold could be considered as the best choice for cartilage tissue engineering among all of the other samples. It has been mentioned in the literature that a higher modulus can direct stem cells to osteogenesity.[22]

Degradation and Erosion

It is known that porous scaffolds for cell culturing should be biodegradable and provide enough space for cell growth and tissue formation. The erosion behavior of a scaffold has a crucial impact on the long-term performance of cell/scaffold constructs. Figure 2 compares the weight loss or erosion of the scaffolds on the basis of their composition and structure for a duration of 30 days in deionized water at 37°C. Degradation and/or dissolution are mechanisms that can lead to the erosion of the scaffolds. The dominant mechanism for the erosion of the noncrosslinked scaffolds was dissolution. GEL was soluble at low temperatures and may have leached out of the scaffolds more rapidly than PVA, which was soluble at high temperatures and had a solubility at low temperatures that was negligible. Therefore, we expected that the PCL–PVA–GEL scaffold eroded more rapidly than the PCL–PVA one, but our results showed no significant differences between these two scaffolds ($p > 0.05$) because of physical crosslinking among the PVA and GEL chains and also their physical entanglements.[23] On the other hand, in crosslinked scaffolds, cleavage of the chemical crosslinkers (degradation) and then dissolution led to erosion. Because degradation is slower than dissolution, the erosion of the scaffolds decreased after crosslinking.[24]

GEL Release

The GEL was added to the scaffold composition to enhance the attachment and proliferation of cells by providing cell-recognition sites.[25] Therefore, it was essential to retain it inside the scaffold structure during its application time. For this reason, it was important to investigate the success of the crosslinking process in reserving the GEL within the scaffold backbone. As presented in Figure 3, the amounts of released GEL of the noncrosslinked and crosslinked samples were determined via the Bradford method within 21 days.[10] We observed that crosslinking resulted in a significant decrease in the amount of released GEL (from 166.31± 31.41 to 98.08± 17.27 μg/mL). The reason for this might have been the formation of chemical bonds between the functional groups of GTA and GEL; this resulted in the prevention of the ability of GEL to be dissolved and come out from the scaffold.[26]

MTT Assay

The MTT assay was carried out to evaluate the proliferation of MSCs on PCL, PCL–PVA, PCL–PVA–GEL (with a composition of 50% PCL, 25% PVA, and 25% GEL), and GEL-PCL–PVA (with a composition of 50% GEL, 25% PCL, and 25% PVA) after 7 and 21 days (Figure 4). According to our results, a significant decrease in the amount of cell proliferation in the PCL and PCL–PVA samples compared to the control ($p < 0.05$) after 7 days was observed. Cell proliferation was significantly higher on both the GEL containing scaffolds ($p < 0.05$) in comparison to the PCL and PCL–PVA ones. These results indicated that GEL has accelerated the proliferation and differentiation of MSCs cells. This positive effect of GEL on cell proliferation has been reported in the literature.[27] Another cause might be an RGD sequence of GEL, as a denatured collagen, that improved cell adhesion.[28] Another possible cause was that cell behavior, such as migration, proliferation, and differentiation, may have been mediated by the physicochemical properties of the

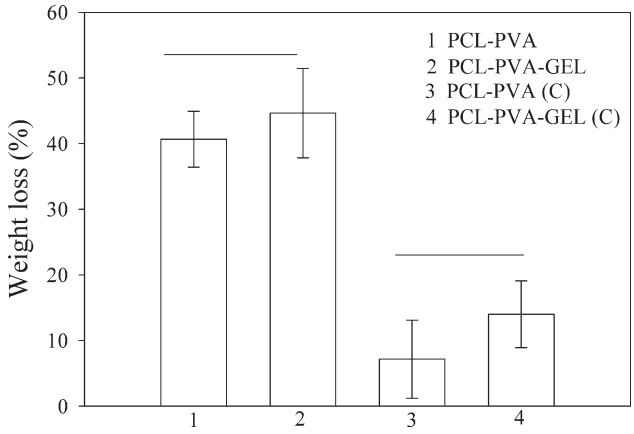

Figure 2. Weight loss percentage in DW after 30 days: (1) PCL–PVA, (2) PCL–PVA–GEL, (3) PCL–PVA(C), and (4) PCL–PVA–GEL(C) ($n = 3$). C stands for crosslinked; the data are presented as means and standard deviations.

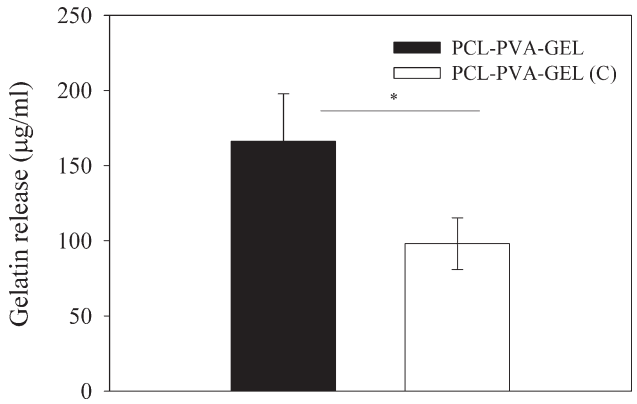

Figure 3. GEL release from PCL–PVA–GEL and PCL–PVA–GEL(C) scaffolds ($n = 3$). C stands for crosslinked; the data are presented as means and standard deviations. $^{*}p < 0.001$ was considered very significant.

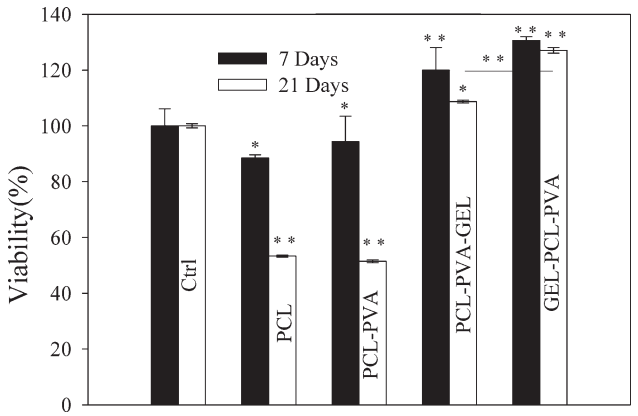

Figure 4. Viability of the MSCs after exposure for 7 and 21 days ($n = 3$). The data are presented as means and standard deviations. $^{*}p < 0.05$ was considered significant. $^{**}p < 0.001$ was considered very significant. GEL–PCL–PVA indicates 50% GEL/25% PCL/25% PVA, and PCL–PVA–GEL indicates 50% PCL/25% PVA/25% GEL.

matrices.[29] As mentioned earlier in the Experimental section, the extraction media were prepared from the porous scaffold after either 7 or 21 days. After 21 days, unlike 7 days, the extraction that was taken from PCL and PCL–PVA decreased the cell numbers significantly in comparison with the control. It might have been because of the nature of PCL as a synthetic material that, in a long time, did not support cell proliferation effectively. This was true about the other two groups, but the positive effect of GEL presence overcame those reasons and resulted in a decrease in the metabolic activities of the cells. Another reason for this assumption was that increasing the percentage of GEL from 25 to 50% enhanced the cell viability ($p < 0.01$).

In Vitro Cell Culturing

The cells attached and proliferated on the scaffold surfaces were monitored with SEM, as shown in Figure 1. Figure 1(C,E) compares the results of PCL–PVA and PCL–PVA–GEL. The increase of cell proliferation and the secretion of ECM proteins was achieved through the affinity of cells possibility toward the GEL-containing matrix. The SEM images illustrated that presence of GEL in the composition of the scaffold improved its function and caused the spreading of cells and the formation of cell colonies, which were observed on the surface of the GEL-containing scaffold. This was likely due to the lack of enough cell-recognition signals of PCL–PVA, and this impeded cell adhesion under these conditions. In contrast, the GEL contained a large number of glycine, proline, and hydroxyproline residues. Therefore, the incorporation of GEL into synthetic hydrogels provided various kinds of ligands, which bonded to the receptors on the cell surfaces and provided enhanced adhesion. Lots of cell-recognition sites could promote cell attachment and growth. This was observed in closer views of the pictures as well [1000× in Figure 1(D) and 1200× in Figure 1(F)]. Therefore, it might be said that on the basis of the results of the direct and indirect cellular behavior study (SEM and MTT), the porous PCL–PVA–GEL composite might be a suitable biomaterial for cell proliferation and growth.[18]

Determination of the GAG Expression

As shown in Figure 5, the amount of released proteoglycan by the cells that were cultured in the PCL–PVA and PCL–PVA–GEL scaffolds after 14 days was higher in comparison with that of the negative control (tissue culture polystyrene, $p < 0.01$). The cause of this might have been the interest of the chondrocytes toward anisotropic pore size (large and small pores) rather than the uniform ordered structure. Therefore, this kind of surface topography might have caused the cells to feel as though they were in their natural environment and may have resulted in the secretion of more ECM.[19] This showed that the superior properties of the both scaffolds in the stimulation of the cells for the secretion of more GAGs in comparison with tissue culture polystyrene.[30] Furthermore, a significant increase in the proteoglycan content could also be observed with the addition of GEL to the scaffold composition ($p < 0.01$). It is known that most synthetic polymers, such as PCL and PVA, are known to prevent cellular adhesion for long periods of time because most cells do not find proper ligands on such synthetic polymers for their attachment.[31] However, GEL, as a natural polymer, provided various kinds of ligands that bonded to the receptors on cell surfaces, facilitated a significantly higher cell attachment,

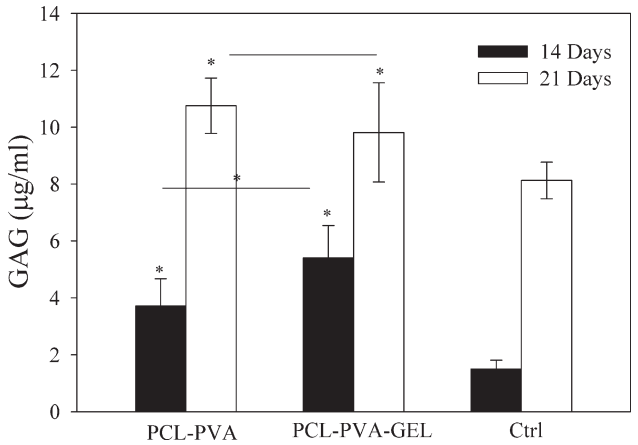

Figure 5. Total GAG contents in the scaffolds after 14 and 21 days ($n = 3$). The data are presented as means and standard deviations. $^{*}p < 0.001$ was considered very significant.

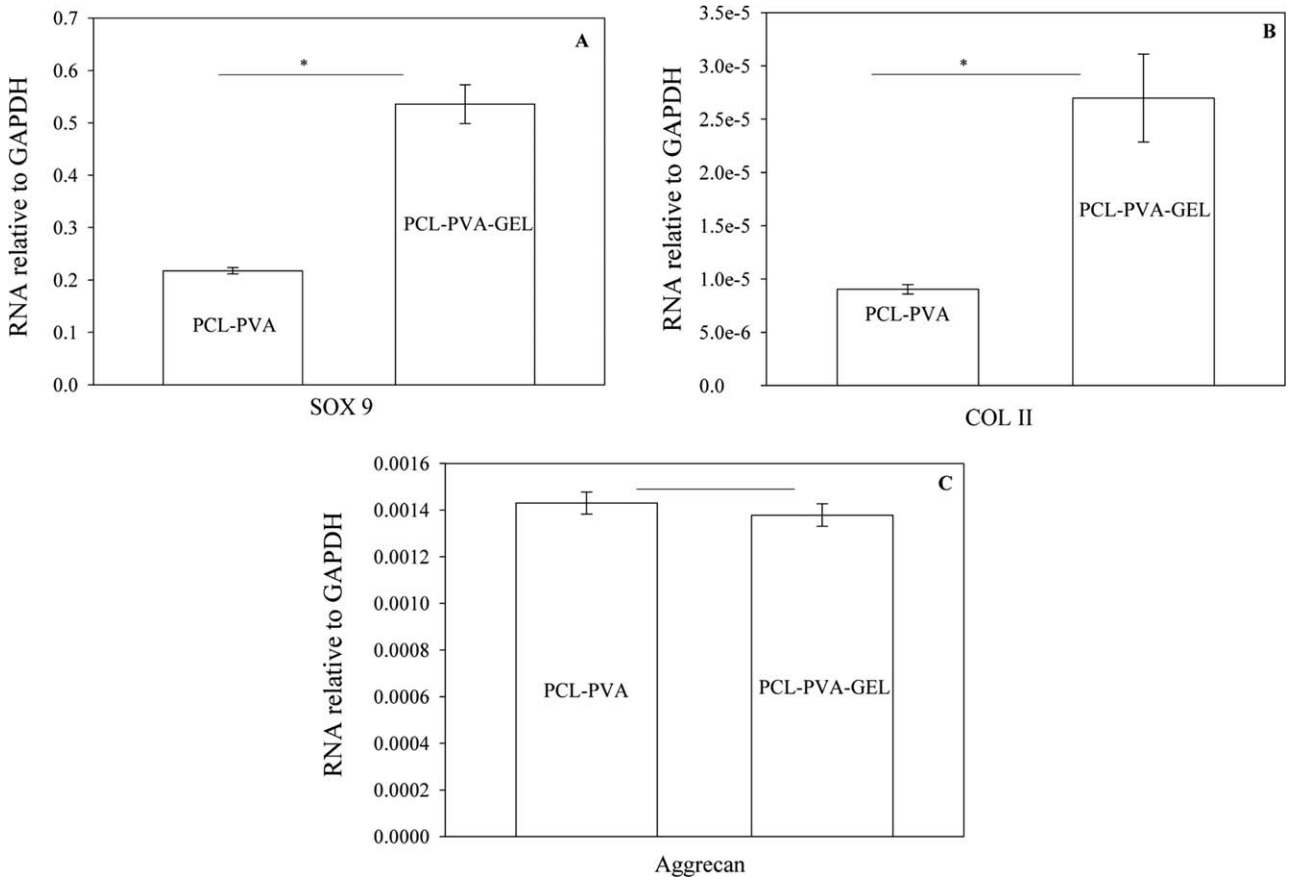

Figure 6. Real-time PCR results for mRNA expression of (A) SOX9, (B) COL2, and (C) AGG of MSC cultures in scaffolds after 21 days of culturing. The data were normalized to the GAPDH value. The values are expressed as fold decreases versus the control (rabbit cartilage, $n = 3$). The data are presented as means and standard deviations. $^*p < 0.001$ was considered very significant.

and promoted cell adhesion.[29] A higher cell number resulted in an increase in the ECM components, as it is known that intercellular contacts exert the extreme importance of chondrocytes to begin ECM deposition. The fact that the cells were able to proliferate at higher rates in GEL containing scaffolds may have justified the higher GAG synthesis that was observed.[32] After 21 days, the amount of GAG was increased from 3.70 ± 0.96 to 10.75 ± 0.97 μg/mL for PCL–PVA; and from 5.4 ± 1.13 to 9.81 ± 1.74 μg/mL for PCL–PVA–GEL. On the other hand, the amount of GAG expression was nearly equal for both scaffolds. The cell adhesion to the surface of a biomaterial can be investigated from two different points of view. We supposed that most GEL releases from the pore walls to the culture medium and, consequently, the number of cell adhesion ligands on the pore walls on the PCL–PVA–GEL scaffold decreased after 21 days (Figure 3). In other words, both of the scaffolds (PCL–PVA and PCL–PVA–GEL) presented rather similar chemical compositions in their pore walls. This might have been the reason for the near equality between the GAG amounts after 21 days.

Gene Expressions from Real-Time PCR

After total RNA isolation and complementary DNA synthesis, the PCL–PVA, PCL–PVA–GEL samples from test groups, and also rabbit cartilage tissue were analyzed for expression of SOX9, AGG, COL2, and GAPDH with a real-time PCR method. The quantification of the SOX9, COL2, and AGG mRNA levels of the MSCs cultured on two different scaffolds without any kind of growth factor was done, and the results were compared. As shown in Figure 6(A,B), the expression amounts of the SOX9 and COL2 mRNA levels of the MSC cells that were cultured onto PCL–PVA–GEL scaffolds showed a significant increase from 0.21 ± 0.01 times to 0.53 ± 0.03 times and from 9.03×10^{-6} $\pm 4.42 \times 10^{-7}$ times to $2.69 \times 10^{-}5 \pm 4.11 \times 10^{-6}$ times for SOX9 and COL2, respectively, in comparison with the PCL–PVA scaffold ($p < 0.05$). Although the amounts of COL2 for both scaffolds were much lower than that of the control, a significant increase in the amount of COL2 was obvious in the PCL–PVA–GEL scaffold in comparison with the PCL–PVA one. It is known that SOX9 is a master transcription part for differentiation into chondrocytes. It binds to enhancer sequences in the promoter region of a series of cartilage-specific genes such as COL2 and, therefore, improves their expression in chondrocytes.[33] It can be presumed that this transient overexpression of SOX9 may be sufficient enough to enhance cartilage repair because it can promote MSC condensation within the defect or nearby in the initial step of chondrogenesis and also increase the cartilage-specific gene and matrix production.[34] As presented in Figure 6(C), the addition of the GEL to the composition of the scaffold did not

influence the AGG expression. This result also was certified by GAG content study after 21 days.[35] However, the difference in ECM production such as COL2 and SOX9 could have been related to difference in cell numbers between the two groups after a duration of 21 days (the primary number of cells was equal) as the MTT results indicated. It was mentioned in the literature that COL2 and SOX9 are transcription factors; their expression happens in the preliminary steps of the differentiation process unlike the AGG. Therefore, a possible reason for the increases in the COL2 and SOX9 expression amounts and the lack of an increase for AGG might have been the presence of GEL at the initial time and its removal from the scaffold after 21 days. In other words, after 21 days in which the AGG was going to express, there was not a sufficient amount of GEL within the scaffold structure (as the Bradford assay also implied) to influence and increase its expression level; as a result, we observed the same amounts for both the PCL–PVA–GEL and PCL–PVA scaffolds. Our results strengthened the argument for the development of biomimetic scaffolds, with GEL in this case, to control cellular functions and direct cell-cell interactions toward the formation of a specific tissue.[36] We noticed in other studies that the development of biomimetic scaffolds to enhance the chondrogenic differentiation of MSCs has largely focused on the alteration of the chemical composition rather than the structural characteristics of scaffolds. For example, the incorporation of collagen I into PCL was found to enhance the chondrogenic differentiation of MSCs. It seems that the expression of the AGG mRNA levels of the cultured MSCs was not considerably related to the chemical composition of the scaffold, but the signaling factors, including chemical (i.e., growth factors) or mechanical (hydrostatic pressure) factors, could be more important, as observed in other research works. Biomaterials, such as PCL, PVA, and GEL, are used due to their similarity with the GAGs normally present in native cartilage to promote chondrogenesis according to the same rationale. In striving toward this goal, we showed in this study that PCL–PVA–GEL blended scaffolds provide a suitable environment for the chondrogenic proliferation and differentiation of MSCs, even in the absence of growth factors in the culture medium.[37]

CONCLUSIONS

In this study, we demonstrated successful the chondrogenic proliferation and differentiation of MSCs on PCL–PVA–GEL blended scaffolds. The PCL–PVA–GEL scaffolds supported the attachment, proliferation, and differentiation of MSCs well with the enhanced functional deposition of ECM compared to the PCL–PVA scaffold. These findings supported the fact that the PCL–PVA–GEL blended scaffold could be used as a suitable substrate for MSC transplantation in tissue-engineering-based cartilage repair.

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