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Enhanced chondrogenic differentiation of bone marrow mesenchymal stem cells on gelatin/glycosaminoglycan electrospun nanofibers with different amount of glycosaminoglycan

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Received 7 January 2018; revised 11 June 2018; accepted 22 June 2018
Published online 00 Month 2018 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jbm.a.36501

Abstract: Tissue engineering is a new technique to help damaged cartilage treatment using cells and scaffolds. In this study we tried to evaluate electrospun scaffolds composed of gelatin/glycosaminoglycan (G/GAG) blend nanofibers in chondrogenesis of bone marrow-derived mesenchymal stem cells (BMMSCs). Scaffolds were fabricated by electrospinning technique with different concentration of glycosaminoglycan (0%, 5%, 10%, and 15%) in gelatin matrix. BMMSCs were cultured on the scaffolds for chondrogenesis process. MTT assay was done for scaffold's biocompatibility and cells viability evaluation. Alcian blue staining was carried out to determine the release of GAG and reverse transcription polymerase chain reaction (RT-PCR) was done for expression of *COL2A1* and also immunocytochemistry assay were used to confirm expression of type II collagen. Scaffold with 15% GAG showed better result for biocompatibility (p =0.02). Scanning electron microscopy (SEM) micrographs showed that MSCs have good attachment to the scaffolds. Alcian blue staining result confirmed that cells produce GAG during differentiation time different from GAG in the scaffolds. Also the results for RT-PCR showed the expression of *COL2A1* marker. Immunocytochemistry assay for type II collagen confirm that this protein expressed. Scaffold comprising 15% GAG is better results for chondrogenesis and it can be a good applicant for cartilage tissue engineering. © 2018 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 00A: 000–000, 2018.

Key Words: Tissue engineering, cartilage, mesenchymal stem cells, electrospun, glycosaminoglycan

How to cite this article: Honarpardaz A, Irani S, Pezeshki-Modaress M, Zandi M, Sadeghi A. 2018. Enhanced chondrogenic differentiation of bone marrow mesenchymal stem cells on gelatin/glycosaminoglycan electrospun nanofibers with different amount of glycosaminoglycan. J Biomed Mater Res Part A. 2018:00:1–11.

INTRODUCTION

Cartilage is avascular and aneural tissue that covers and protects the end of bones and provides bones movement with less tenses. Cartilage tissue has low chondrocyte population and also because does not contain blood vessels to transfer nutrient to the cells it lacks the ability to self-repair.[1,2] Cartilage is a tissue with rich collagen and proteoglycan and in the mature tissue collagen type II with proteoglycans which provide mechanical strength to tolerate impact and load.[3] However, trauma and disease, damage the cartilage and make it dysfunction. Damaged cartilage retain to degraded until pain and impaired mobility outcome, and without any treatment a joint replacement is necessitated.[4] Prevalent clinical treatment utilizes for treating the damaged cartilage such as microfracture, abrasion arthroplasty, mosaicplasty, and autologous chondrocytes implantation which has no complete adequate restoration result yet.[5,6]

Over the previous decade tissue engineering including biocompatible and biodegradable biopolymers combination with stem cells proposed in order to repair or regenerate the injured tissues.[7–9] There are three main parts in tissue engineering: scaffold, cell, and growth factor.

Mesenchymal stem cells (MSCs) are unique population of adult multipotent progenitor cells existed in the bone marrow (BM), which are capable of differentiating to the adipocytes, osteoblasts, chondrocytes, and vascular smooth muscle.[10,11] MSCs derived from BM as a source of cells in cartilage tissue engineering studies[12] because of its differentiation potential, improve angiogenesis, and prevent apoptosis. Different source of stem cells could be used for tissue engineering such as adipose derived stem cell,[13] BM derived stem cell[14] and synovium derived stem cells.[15] BM derived stem cells have proven to be the best stem cells for cartilage tissue regeneration based on the cell surface CD markers.[16,17]

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Contract grant sponsor: Tehran University of Medical Sciences (TUMS); contract grant number: 28747

One of the main objectives in cartilage tissue engineering is to fabricate the scaffolds that mimic the natural cartilage extracellular matrix (ECM) and provides three-dimensional (3D) structure for cell adhesion and proliferation.[18] Researchers tried to use different natural and synthetic biomaterials to fabricate scaffolds that mimic cartilage ECM structure,[19,20] or just synthetized material could be used.[21] Utilizing the natural materials in scaffold preparation could reduce inflammatory concerns and also benefit naturally degradation by body.[22]

Gelatin (G) is a natural biotic material with highest similarity to collagen, which has cell compatibility, low toxicity and has no inflammation.[23] Gelatin is used as one of the favorable biomaterials in cartilage tissue engineering.[24–26]

Glycosaminoglycans (GAGs) such as chondroitin sulfate (CS) which are existing in native cartilage tissue have capacity to provide chondrogenesis cell signaling.[27,28] CS is utilized with many other biomaterials to fabricate scaffolds for cartilage tissue regeneration.[29] Therefore, blend of gelatin and CS is a proper applicant for cartilage tissue engineering. During last decade composition of gelatin and CS was utilized with other materials for cartilage tissue engineering.[30,31]

Electrospinning is a well-organized technique to fabricate nanofiberous scaffolds with interconnected pores.[32] Nanofiberous electrospun scaffolds have shown more suitability for cartilage tissue engineering.[33] Because of advantages in fast, cost efficiency, easy fabrication as well as enhancing cell functions via fiber surface properties, electrospinning became popular for cartilage tissue engineering.[34] Combination of gelatin and GAG were also used to manufacture electrospun scaffold as biocompatible and biodegradable substrate for cell culture,[35] but to the best of our knowledge no studies on G/GAG electrospun nanofiberous scaffold in cartilage tissue engineering applications have been reported.

Growth factors are important to guide cells to differentiate into special tissue but there are concerns about use of growth factors because they have short half-lives and high cost.[36] Therefore, in this study we aimed to explore and evaluate the electrospun gelatin based nanofibers containing different ratio of GAG to enhance differentiation of MSCs to chondrocytes without any growth factor.

EXPERIMENTAL

Materials

Gelatin (bovine skin; Sigma Aldrich, type B, Darmstadt, Germany) and CS (type A) were used. Electrospinning solutions were prepared as previously reported.[35] Briefly gelatin was dissolved in trifluoroethanol (TFE) (Sigma Aldrich) and GAG was dissolved in water. The TFE/water with ratio of 50/50 was used as co-solvent system for dissolving gelatin and GAG at room temperature. Then three different concentration of mixed G/GAG (100: 0, 95:5, 90:10, and 85:15) with total polymer ratio of 13% (w/v) were prepared and homogenized at room temperature for overnight. Then solution has taken in a syringe for prior electrospinning (Co881007 NYI, ANSTCO, Tehran, Iran) process. For cell adhesion and proliferation, the following culture media was acquired and prepared from Gibco, Germany: Dulbecco's Modified Eagle's Medium (DMEM), phosphate buffered saline (PBS) and trypsin. Ascorbic acid and dexamethasone (Sigma Aldrich) were used as differentiation media.

Fabrication of gelatin/glycosaminoglycans nanofibers

Gelatin was dissolved in TFE and GAG was dissolved in water. Then three different concentration ratio of mixed gelatin/GAG (G0: 100:0, G1: 95:5, G2: 90:10, and G3: 85:15) solutions with total polymer concentration of 13% (w/v) were prepared and homogenized at room temperature for overnight. Then solution has taken into a 5 ml syringe prior electrospinning (Co881007 NYI; ANSTCO, Tehran, Iran) process, then horizontal system electrospinning was done by following optimized parameters: applied voltage; 19 kV, feeding rate; 0.6 ml/h, distance between collector and nozzle; 120 mm. Nanofibers were collected by cylindrical collector covered by aluminum foil for 60 min. The nanofiberousrs were chemically cross-linked using glutaraldehyde (GA) vapor 20% (wt/vol) for 4 h at 25°C, followed by immersion in 1% GA solution for 1 h.[35] The nanofiberousrs were chemically cross-linked using GA vapor 20% (wt/vol) for 4 h at 25°C, followed by immersion in 1% GA solution for 1 h.

Physical and chemical characteristics

Scanning electron microscopy. Scanning electron microscopy (SEM; VEGA, TESCAN, and Czech) was done after gold sputter coating to verify G/GAG nanofibers morphology and sizes. For measuring nanofibers diameter, at least 100 different fibers from each scaffold were measured and then analysis was carried out by image analysis software (ImageJ 1.42q; National Institute of Health, Bethesda, Maryland).

Contact angle. Surface hydrophilicity of G/GAG electrospun nanofibers was measured by calculating static contact angle (G10 KRUSS; XXX, Hamburg, Germany). Scaffolds were cut in to 5 × 5 mm and then 2 μl of deionized water was dropped on the solid surface of the scaffolds. Data were captured by charge-coupled device camera and analyzed with software. All data are the mean value of five repetitions.

Fourier transform infrared spectroscopy analysis. The chemical structure of the nanofibers were analyzed using Fourier transform infrared spectroscopy (FTIR) using a FT-IR spectrophotometer (EQUINOX 55; BRUKER, Berlin, Germany). The infrared spectra of the samples were measured over a wavelength range of 4000–400 cm-1.[37]

Sterilization of scaffolds

Sterilization of the scaffolds was done in three stages and each stage done triplicate. In stage one 70% ethanol was utilized to sterilize scaffolds. In second stage sterile water was used to remove ethanol from the scaffolds. At last stage sterile PBS was used to remove remained ethanol from scaffolds.

Cytocompatibility and viability study

Human bone marrow-derived MSCs (hBM-MSCs) were acquired from Boneyakhteh Institute of Iran. Cells were cultured in DMEM culture media (Gibco, Darmstadt, Germany) with 10% FBS (Gibco) and sustained at 37°C in 5% CO_2

humidified incubator. The time that cells confluency reached to the optimum amount, cells were washed by PBS (pH 7.4) and undetached by using trypsin/ethylenediaminetraacetic acid. Cytocompatibility of G/GAG nanofibers were checked by seeding hBM-MSCs onto the scaffolds.

For viability test 3-(4, 5-dimethylthiazol2-yl)-2, 5-diphenyltetrazolium bromide (MTT) powder (Sigma Aldrich) was used. MSCs were seeded in 96-well microplate at a density of 10^4 cell/well for 24, 48 and 72 h. For MTT assay then samples (different percentage of GAG: 5, 10 and 15%) were incubated for 4 h with MTT solution and then dimethyl sulfoxide (Sigma Aldrich) added to samples. Absorbance of solution was measured by Elisa reader (BioTek EL × 800) at wavelengths of 490/630 nm. All tests were done in triplicates.

Cell adhesion study

For cell attachment test, human mesenchymal stem cells (hMSCs) were seeded in 96-well microplate on 5 × 5 mm G/GAG scaffolds with a density of 1×10^4 cell/well. Then after 1, 2, and 6 h MTT assay was used to determine amount of cells that attached to the scaffolds. First scaffolds were washed by PBS three-times and placed on the new wells. During the test, the samples were incubated for 4 h with MTT solution and then dimethyl sulfoxide (Sigma Aldrich) was added to the samples. Absorbance of the solution was measured by Elisa reader (BioTek EL × 800) at wavelengths of 490/630 nm. All tests were done in triplicates. Also the cells present on the scaffolds proved by using SEM. At first step samples were coated by thin layer of gold and then SEM (VEGA, TESCAN, and Czech) was used to study the cells behavior.

4, 6-diamidino 2-phenylindole staining

4, 6-diamidino 2-phenylindole (DAPI) staining was used to show that the cells nuclear were healthy during differentiation tests. Scaffolds were cut to 5 × 5 mm sections and hMSCs were seeded on the scaffolds by density of 1×10^4 cell/well as sample test and also on the tissue culture plate (TCP) as control. After 24 h incubation, samples were washed with PBS (pH 7.4) and then 4% paraformaldehyde was added to them and after 10 min washed with PBS again. For making cells penetrable we used 0.1% triton X-100 and after 2 min washed with PBS. Then DAPI stain (Sigma Aldrich) was used to stain the cells' nuclei for 5 min in the dark. After that samples were washed by PBS for three-times and then cells photography by Nikon fluorescent microscope (Eclips Terminal Emulator 2000-S, Japan).

Chondrogenic differentiation of human mesenchymal stem cells

For chondrogenesis differentiation, hMSCs were seeded on G/GAG scaffold by density of 1×10^4 cell/well (96 well microplate) and also cells with the same density seeded on TCP as a control, in presence of chondrogenesis differentiation medium for 3 weeks. Cells were cultured in growth medium (DMEM with 10% FBS) for 24 h. After that medium was changed with differentiation medium includes: dexamethasone (1×10^{-7} μM), acid ascorbic (0.1 M), and insulin transferrin selenium (ITS 1%), all prepared from Sigma Aldrich, Germany. During the test differentiation medium was refreshed two-times a week for 3 weeks. At certain days MTT assay was used to show viability of differentiated cells during test, culture media was removed from the wells and MTT medium include DMEM and 10% FBS was added and incubated for 4 h. Then dimethyl sulfoxide was used to dissolve dregs. Eliza reader (BioTek EL × 800) was used to measure color absorbance of solution at wavelength of 490/630 nm. Viability of differentiated cells was checked by DAPI staining as previously described, and SEM used to inspect morphology and attachment. For SEM, hMSCs were seeded on G/GAG nanofiber at density of 1×10^4 cell/well in 96-well plate. After 72 h. Scaffolds were washed with PBS three-times and 4.5% glutaraldehyde was used for 2 h to fix cells on scaffolds. PBS was used to wash scaffolds and graded ethanol series (60–100%) were used to dehydrate the scaffolds and replace water. Then scaffolds were dried overnight and sputter coated by gold prior examination by SEM (SEM, VEGA, TESCAN, Czech).

Alcian blue staining

Differentiated cells' expressed GAGs were determined by Alcian blue staining. HMSCs were seeded on 5 × 5 mm G/GAG scaffold by density of 1×10^4 cell/well (96-well microplate) with differentiation media. Tissue culture plate (TCP) with differentiation media and cell seeded on the scaffolds without differentiation media were used as control. Also G/GAG scaffold stained with Alcian blue to insure that GAGs expressed by differentiated cells have taken stain.[38]

For preparing Alcian blue 3 ml of acetic acid was added into 97 ml of distilled water then 1 mg of Alcian blue 8GX was added to prepared 3% glacial acetic acid and adjust pH to 2.5, using acetic acid. The solution filtered and a crystal of thymol was added.

After 1 and 3 week of differentiation test, the scaffolds were fixed by 1% glutaraldehyde for 20 min at room temperature. Then the samples washed three-times with PBS. Scaffolds kept for 30 min in prepared Alcian blue stain, after that scaffolds were washed by hydrochloridric acid (0.1 Mol). Scaffolds then washed by PBS for three-times to remove hydrochloridric acid, and at the end stained scaffolds examined under invert microscope (BEL).

Reverse transcription polymerase chain reaction

Chondrogenesis differentiation was investigated by expression of *COL II* with RT-PCR. The hMSCs were cultured on 1 × 1 cm G/GAG scaffolds in 6-well plate at a density of 1×10^6 cells/well with differentiation media for 2 weeks. Also another set of the prepared scaffolds cultured with the same amount of cell with normal media was used as control. Total RNA was extracted by using RNA extraction kit (Arya Tous, Tehran Iran) as maintained by protocol. Reverse transcriptase enzyme was utilized to convert extracted RNA to c-DNA. The RT-PCR reaction mixture contained 5.5 λ cDNA, 0.5 λ forward primer, 0.5 λ reverse primer, 12.5 λ master mix and 6.5 λ distilled water. Reactions had done with initial

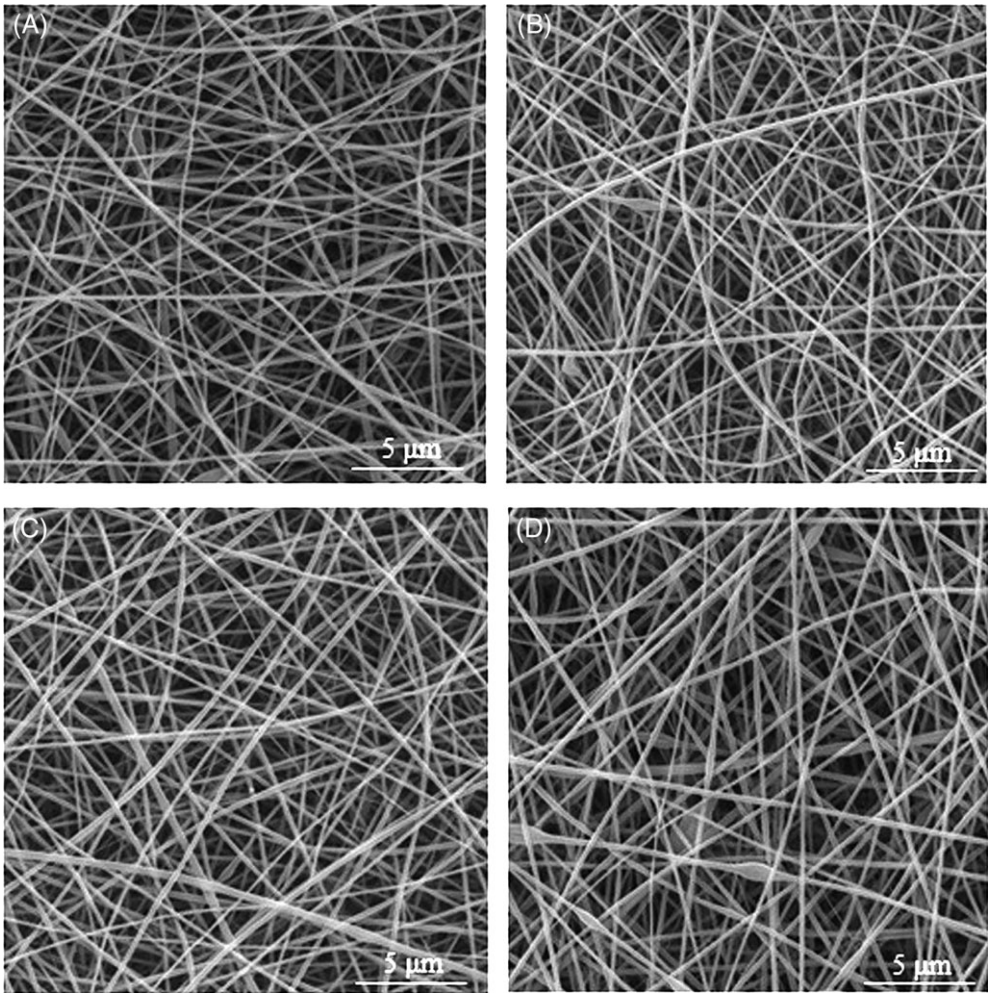

FIGURE 1. SEM micrograph of electrospun G/GAG nanofiber scaffold. Scale bar: 5 μm. (A) gelatin, (B) gelatin/GAG 95/5, (C) gelatin/GAG 90/10,(D) gelatin/GAG 85/15

activation at 95°C for 10 min, then followed by 35 cycles of denaturation at 94°C for 30 s and anneal at 58°C for 30 s, and at 72°C for 30 s for elongation. Gene activity could be shown by determine m-RNA value in cell that means gene expression in the cells. RT-PCR has two phases: produce c-DNA from extracted RNA, and then prepared c-DNA utilized for PCR. For RT-PCR all data were gathered from GenBank (NCBI) and then primer designed by Oligo 7 and Gene Runner software and blasted with Primer Blast (NCBI) software. Designed primers were ordered and purchased from Takapouzis: *COL II*, F: 5'-GGAGAACCTAGAGACAACAAGA-3′, R: 5′- CATCCGGGCAGCACACTTTC-3′, and *GAPDH* cDNA was utilized as a control, F: 5′- CTGGCCAAGGTCATCCATG-3′, R: 5′- GCCATCACGCCACAGTTTC-3′.

TABLE I. Nanofiber Diameter and SD for G/GAG Scaffolds

SD	Nanofiber diameter (nm)	Scaffold code
56.81	189 ±10	G0
34.78	191 ±10	G1
29.99	210 ±10	G2
27.45	230 ±10	G3

Immunocytochemistry

For detecting collagen II (COLII) protein immunocytochemistry was used. Density of 1×10^4 cells/well seeded on 5 × 5 mm G/GAG scaffolds in the 4-well plate, also cells cultured in TCP as control. Differentiation media was refreshed twice a week. After 3 weeks differentiated cells were washed with PBS and then fixed with 4% paraformaldehyde for 20 min at 4°C, wells were washed with PBS for 5 min over again. Goat serum was utilized as blocking serum, for 45 min at room temperature, then 4% Triton used for 5 min. Goat

TABLE II. Contact Angle Property of Gelatin/GAG Scaffolds

Contact angle	Scaffold
34.1° ± 0.5 °	G0
32.9° ± 0.5 °	G1
28.7° ± 0.5 °	G2
25.3° ± 0.5 °	G3

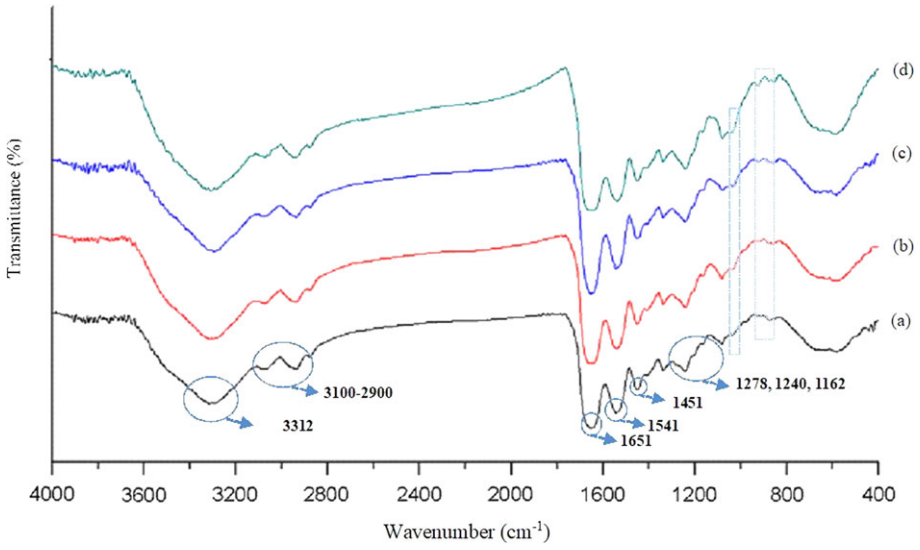

FIGURE 2. The FTIR analysis of cross-linked gelatin/GAG electrospun scaffolds containing different ratio of GAG: (a) GAG 0 (b) GAG 5 (c) GAG 10 (d) GAG 15.

serum was removed and cells incubated with anti-mouse collagen types II antibody overnight at 4°C. Wells washed with PBS and secondary conjugated antibody was added and incubated for 1 h. at 37°C. Thereafter, wells washed with PBS-Tween and then DAPI stain was utilized for 5 min, and finally washed by PBS and examined by fluorescent microscope.

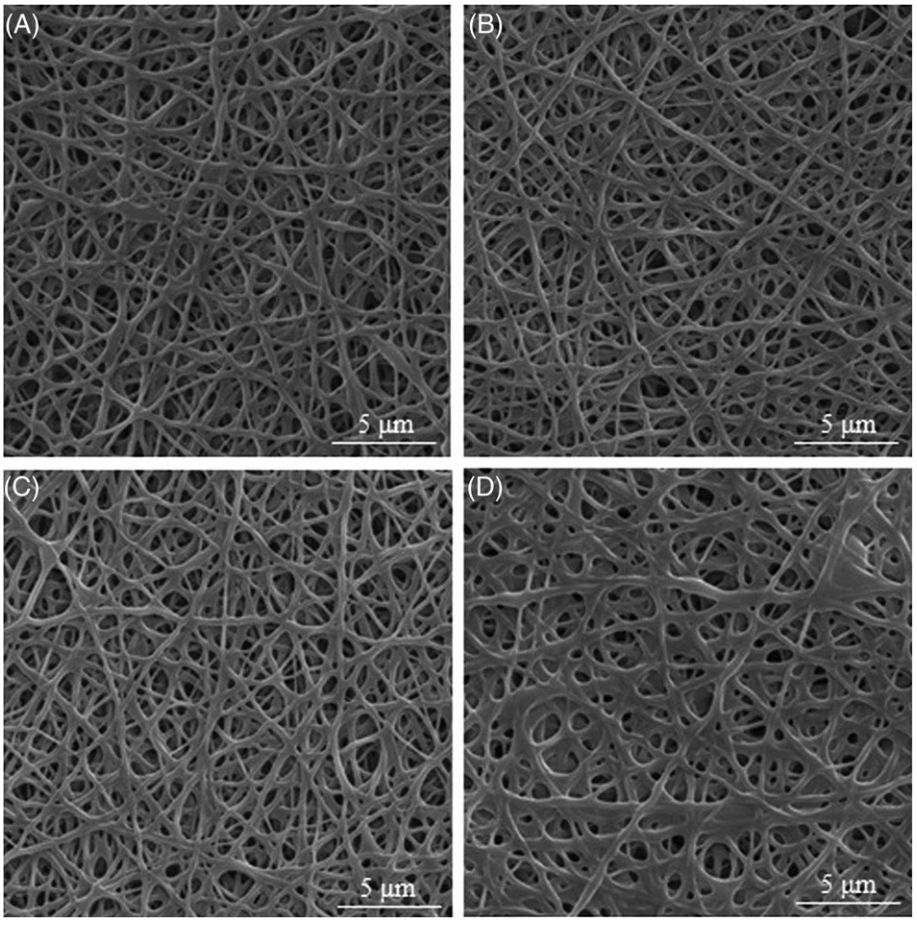

FIGURE 3. SEM images of cross-linked gelatin/GAG electrospun scaffolds containing different ratio of GAG: (A) gelatin, (B) gelatin/GAG 95/5, (C) gelatin/GAG 90/10, (D) gelatin/GAG 85/15.

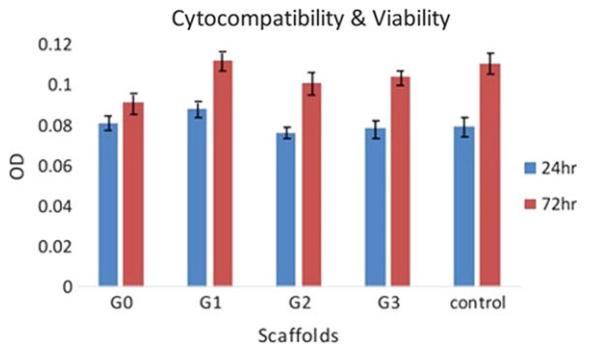

FIGURE 4. Viability results of hMSCs cells for 24 and 72 h by MTT assay. G3 ($p = 0.02$) ($n = 3$), ANOVA, $^{**}p < 0.05$.

Statistical analysis

For statistical analysis all data gathered from tests, statistical software SPSS V.23 was used and one-way ANOVA, difference of $p < 0.05$ was considered to be statistically significant.

RESULTS

Morphology and properties of random electrospun gelatin/glycosaminoglycan nanofibers

Morphology of the G/GAG nanofibers was shown in Figure 1. All scaffolds, reveal bead-free nanofiberous structure fabricated successfully. The mean fiber diameter after cross linking was listed in Table I and the results demonstrated that increasing the amount of GAG lead to decreasing the mean fiber diameter.

Hydrophilicity

Evaluation of hydrophilicity of the scaffolds was achieved by measuring the contact angle as presented in Table II. The obtained results (G0: 34.16°, G1: 32.98°, G2: 28.72°, G3: 25.34°) in gelatin scaffold (34.16°) was higher than the G3 sample (25.34°). The mean values for hydrophilicity were increased with increasing the GAG ratio in the scaffold matrix.

Fourier transform infrared spectroscopyThe characterization of the chemical structure of the cross-linked electrospun scaffolds, was carried out using FTIR spectroscopy. Representative spectra for cross-linked G/GAG electrospun scaffolds containing different ratio of GAG in the wavenumber range of 400–4000 cm^{-1} $cm^{-1}$are shown in Figure 1. The cross-linked electrospun gelatin (GAG 0) spectrum displayed several characteristic absorption bands at 3312 cm^{-1} for —NH_2 and —OH stretching vibration, 3100–2900 cm^{-1} for C—H aliphatic group stretching vibration (3072 cm^{-1} for alkenyl C—H stretch and 2934 cm^{-1} for CH_2 asymmetrical stretching), 1651 cm^{-1} for amid 1 (C═O) stretching vibration, 1541 cm^{-1} for amid 2 (N—H) bending vibration, 1451 cm^{-1} for CH_2 bending, 1278 cm^{-1} for amid 3 (C—N) stretching vibration and 1240 cm^{-1} for -C—N stretching.[39,40]

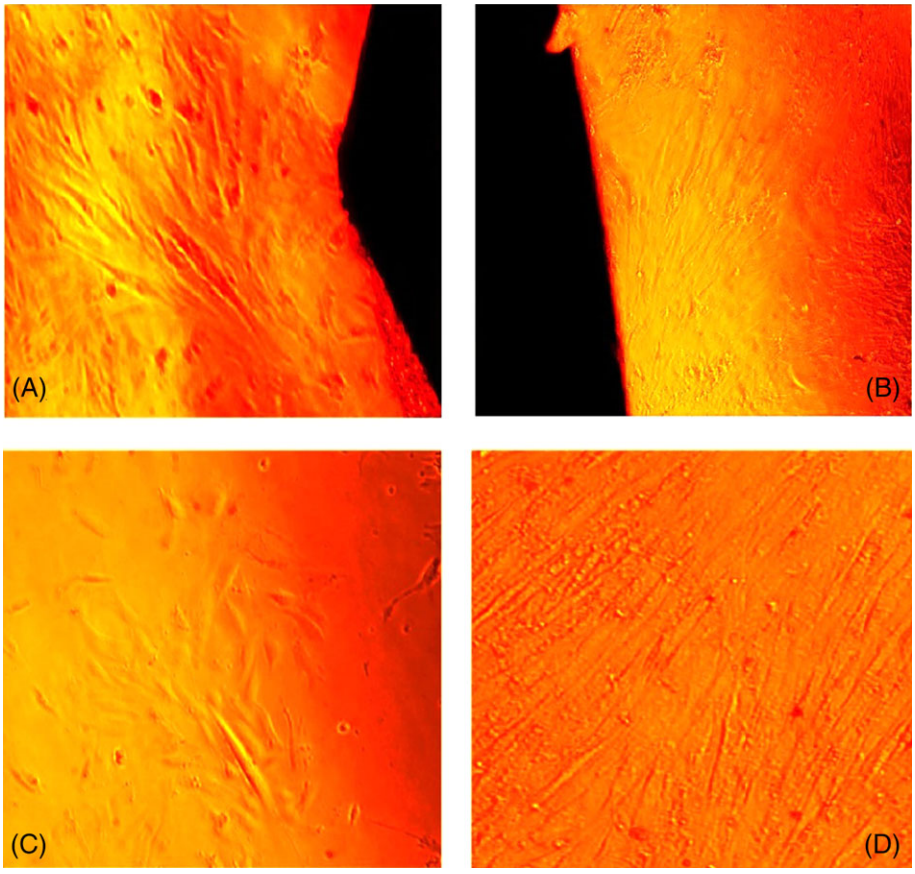

FIGURE 5. Optical microscopy images of cytocompability of scaffolds and TCP control group at 24 and 72 h. (A) G2, (B) G3. The dark areas that were shown by arrows are scaffolds.

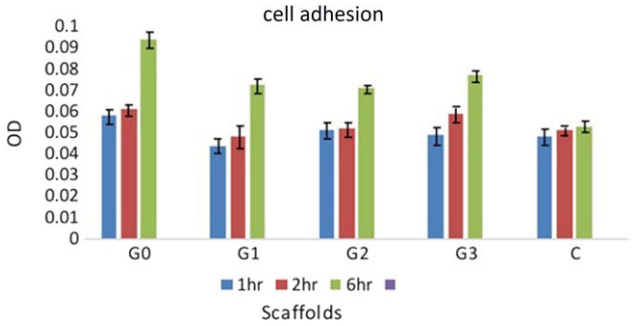

FIGURE 6. MTT assay results for cell attachment during 6 h, G3 showed best attachment compare to other scaffolds and control (p = 0.02). (n = 3), ANOVA, $^{**}p < 0.05$.

Figure 2(B–D) displays the FTIR spectra of the cross-linked G/GAG nanofibrous scaffolds. According to the previous study, raw GAG sample showed characteristic peaks at 1031 cm^{-1} for C—O—C stretching vibration attributed to the saccharide structure, 1274 cm^{-1} attributed to the negatively charged SO_4^{2-} groups of GAG molecules, 875 and 921 cm^{-1} for C—O—S vibration.[5] Considering the presence of sulfated groups in GAG chemical structure and their absence in cross-linked gelatin chemical structure, the sulfide bands could be taken to prove the presence of GAG in the cross-linked G/GAG samples. The representative peak of GAG was observed at 1274 cm^{-1} for S═O stretching vibration but as there is a peak at 1278 cm^{-1} for amid 3 in cross-linked gelatin structure, there is no significant difference in spectra (b), (c), and (d). The peak at 1031 cm^{-1} for C—O—C stretching vibration is attributed to the saccharide structure in GAG spectra. Increasing the GAG content in samples leads to increasing this saccharide band in comparison of peak at 1079. Comparison the spectra of cross-linked gelatin (GAG 0) and G/GAG blends show that the intensity of peaks at 921 and 875 cm – 1 (for C—O—S vibration), which is absent in gelatin, increases by presence of GAG in electrospun scaffolds. These findings confirm the presence of varying levels of GAG in the gelatin/GAG electrospun scaffolds. Figure 3 presents the images of cross-linked G/GAG nanofibrous scaffolds.

Cytocompatibility and viability study

To evaluate that scaffolds were viable for cells, MTT assay was done triplicate for each scaffold by using hMSCs for 72 h. Figure 4 shows the result of MTT assay that evaluate the cell viability on scaffolds. G1 scaffold showed better result (p = 0.01). Also Figure 5 shows that the cells are alive and have tendency to the scaffold.

Cell adhesion

MTT test was done for evaluation hMSCs that attached to the scaffolds after 1, 2, and 6 h. Results in Figure 6 shows that all scaffolds have better adhesion than control and also

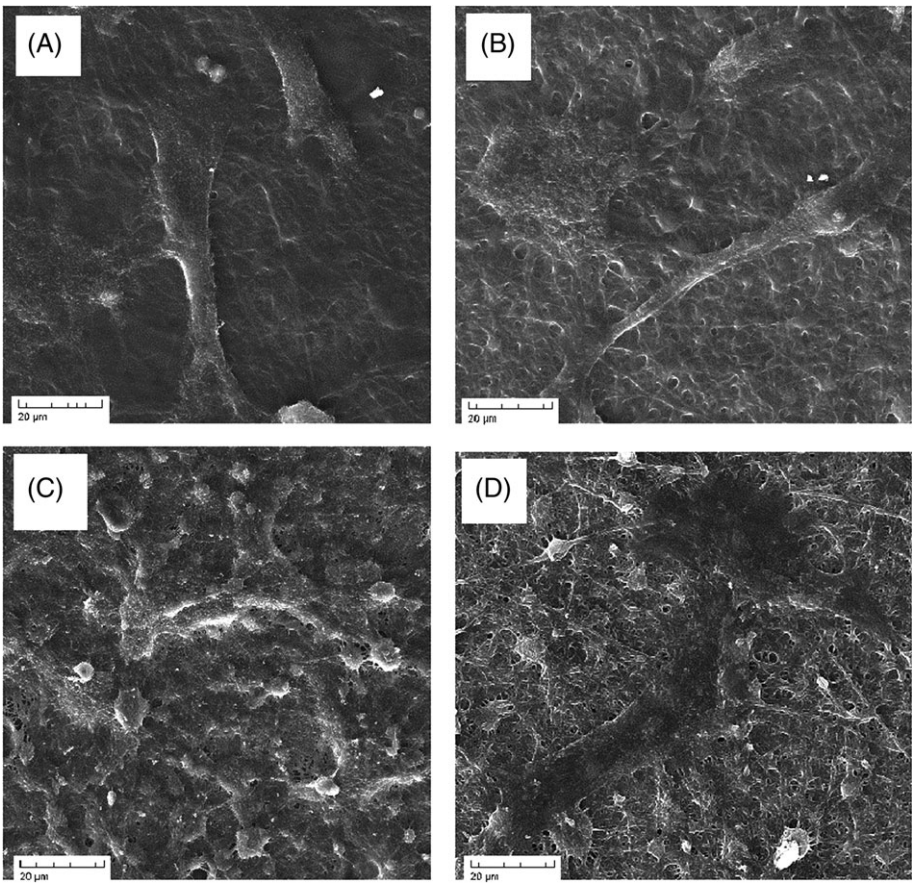

FIGURE 7. SEM micrographs of hMSCs cultured on gelatin/Glycosaminoglycan for 24 h. (A) gelatin, (B) gelatin/GAG 95/5, (C) gelatin/GAG 90/10, (D) gelatin/GAG 85/15, Scale bar 20 µm

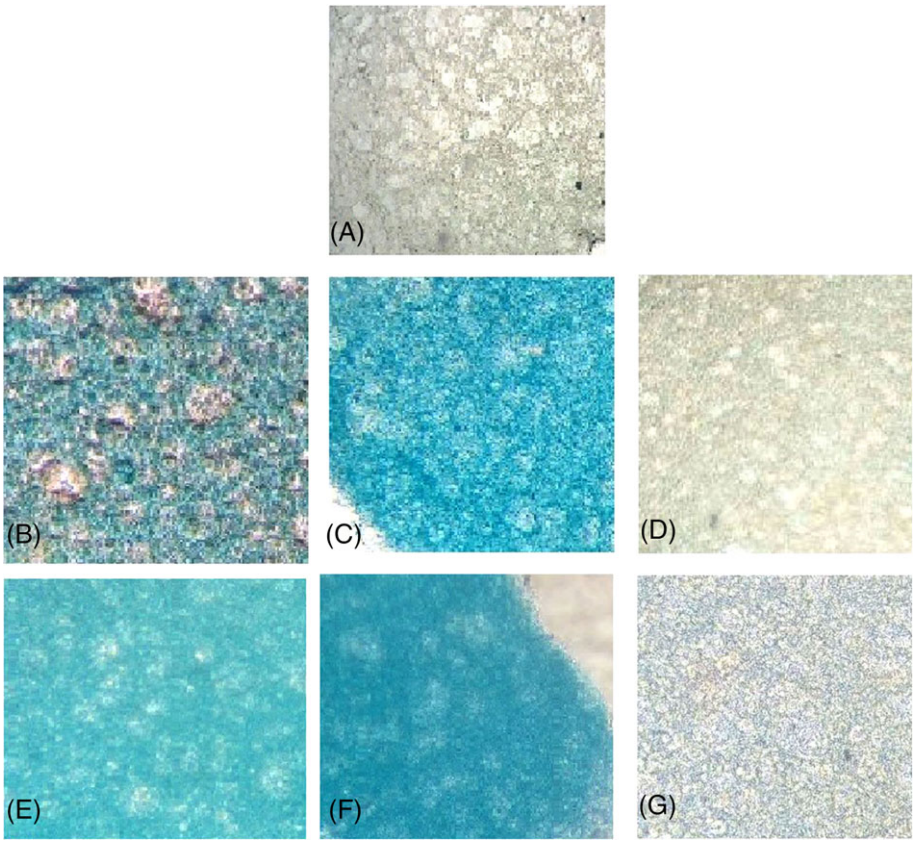

FIGURE 8. Alcian blue staining: (A) scaffold with no cell, (B) G2 scaffold with cell after 1 week, (C) G3 scaffold with cell after 1 week, (D) control scaffold with no GAG after 1 week, (E) G2 scaffold with cell after 3 weeks, (F) G3 scaffold with cell after 3 weeks, (G) control scaffold with no GAG after 3 weeks.

G3 scaffold has the best cell attachment (p = 0.02). Figure 7 shows SEM images that confirmed MTT results.

Chondrogenic differentiation confirmation

Alcian blue staining. Alcian blue staining was used to determined amount of GAGs that produced by differentiated cells during 3 weeks. Based on pH = 2.5, Alcian blue just stain the GAGs that cells produce, GAGs that used in scaffolds was not take any stain in this pH. Figure 8 shows the results for Alcian blue staining during 3 weeks. All scaffolds with cell under chondrogenic media were colored blue that means cells produced GAGs, but controls with cell and also scaffolds without cells got no color. G3 scaffolds have got more color to other scaffolds. Also changes in morphology of the cells were checked by invert microscope during test that confirmed the change in cells morphology.[38] Figure 9 shows the result of MTT test that prove cells proliferation during the differentiation tests.

Reverse transcription polymerase chain reaction. Figure 10 shows expression level of chondrogenic specific gene *Col II* determined after 2 weeks of hMSCs culture under chondrogenic media. Results showed that expression of *Col II* was increased during 2 weeks after cell culture under chondrogenic media compare to control. G3 scaffold showed higher color that means more expression of *Col II* gene compare to G2 scaffold.

Immunocytochemistry. This test was utilized to confirm expression of collagen type II protein that is specific protein during chondrogenesis, for 3 weeks. Our results show that

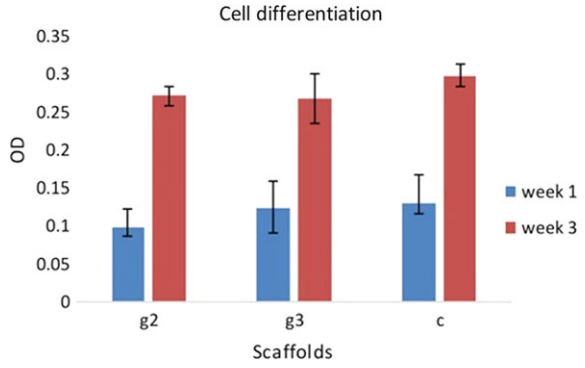

FIGURE 9. Viability of differentiated cells for 3 weeks by MTT assay (n = 3), ANOVA,** $p < 0.05$.

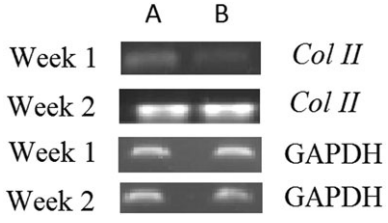

FIGURE 10. RT-PCR results for 2 weeks of cell differentiation test, (A) G2 scaffold, (B) G3 scaffold.

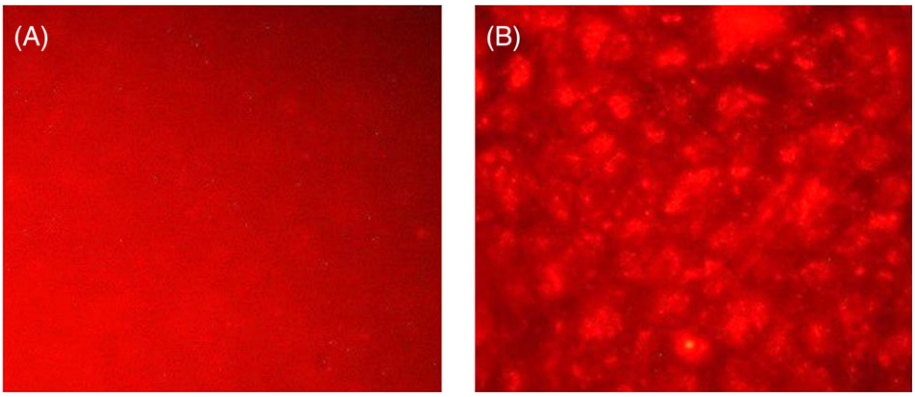

FIGURE 11. ICC result after 3 weeks cell differentiation test, (A) week 1, (B) week 3.

collagen type II protein expressed after 3 weeks of hMSCs culture on G3 scaffold that showed as figure 11.

DISCUSSION

Fabrication of nanofibers depends on the final application of the scaffolds. 3D scaffold should support cell proliferation in the case of damaged tissue replacement. Designed nanofiber should have ability to mimic the natural environment for cell such as cell migration, metabolite transfer, biodegradable, and so forth. The electrospinning technique provides the ways that help to fabricate nanofibers that can mimic the natural ECM structure. Electrospun nanofibers have potential ability to support the cells proliferation and differentiation based on their high porosity and nanofibers diameter. In this study combination of gelatin and CS was used to prepare the scaffolds. The highest concentration for prepare the nanofibers preparation was 15% based on previous study .[35] In this study different ratio of GAG was used to determine effect of GAG on cell differentiation in chondrogenesis process. Gelatin and GAG are both water-soluble. Therefore, the nanofibrous scaffolds were cross-linked using both glutaraldehyde vapor and solution. As shown in Figure 3, it can be observed that with increasing GAG ratio in gelatin/GAG nanofibrous scaffolds, increase in fiber fusing was observed, which related to the lower crosslink density with glutaraldehyde in the scaffolds with higher GAG ratio due to the presence of SO_4^{2-} groups.[41]

MSCs were reported as a good source of cells for repairing of cytoskeletal disorder because of their self-renewal and repair ability.[42] The source of MSCs is the one of concerns for researchers, therefore many scientist try to find the best source of MSCs for specific applications. Cell surface markers play important role in cell differentiation. Many cell surface marker of BM hMSCs are the same as chondrocytes such as CD44.[43] Also CD105 and CD90 are the common markers on MSCs surface that can find on chondrocyte.[44] CD105 has a great role in *COL2A1* and *ACAN* expression.[45] In this study hMSCs from BM was utilized and results show that MSCs extracted from BM have good ability to differentiate into chondrocyte.

Chondrocyte ECM in natural cartilage plays a main role in attachment. For mimic the natural ECM in tissue engineering gelatin is a good choice because it has good performances in many conditions. Gelatin can increase cell attachment.[46] Also gelatin is biocompatible, nontoxic, natural based material with good biodegradation rate. Gelatin plays its role in signal transferring for arginine-glycine-acid aspartic sequence that controls the attachment, proliferation, and differentiation of chondrocyte.[47] In this study by adding the GAG to gelatin in scaffold composition we try to increase the attachment and proliferation. GAG in combination with gelatin in nanofiberous structure and highly porosity can have a great impact on attachment potential of the scaffolds.[48] SEM images approve that G/GAG nanofiber structure is deferent from gelatin nanofibers. Also MTT results shows increasing attachment in scaffolds with high amount of GAG.

Several studies have shown that growth factors such as TGF-β have effect on chondrogenesis which are expensive.[49] Using the scaffold containing specific material can help researchers to omit growth factors.[50] GAGs are polysaccharides that are the major component of cartilage. CS chains bond to serine hydroxyl residue in specific proteins. CS with its negative charge connects to the extra cell matrix that controls many functional pathways in cells, one of these functions is inhibition of inducing translocation of NF-κB by reducing IL-1β in chondrocytes.[51] CS can be work as signal transporter that induces cell potential to differentiate to chondrocyte.[52] Also results show that CS could change poly-L-lysine structure by changing electric charge and increasing the negative charge. It could lead stem cell differentiate to chondrocyte.[53] Results show that increase in amount of CS in scaffold could increase chondrogenesis of hMSCs.

This study shows that electrospun G/GAG nanofibers with 15% GAG ratio can support cell proliferation and chondrogenesis of hBMSCs during 3 weeks culture without using any growth factor. We suggest that these nanofibers have great potential to use for cartilage tissue engineering.

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