Osteoblastic response to collagen scaffolds varied in freezing temperature and glutaraldehyde crosslinking

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Abstract: Collagen sponges are widely used scaffolds in bone engineering. To form bone, the osteoblastic cells undergo proliferation, differentiation, and mineralization stages in the scaffold. Crosslinking and freezing temperature are two important variables in fabricating collagen sponges. The purpose of this study was to examine the osteoblastic responses to collagen sponges prepared with or without glutaraldehyde crosslinking at different freezing temperatures (−20°C or −80°C). MC3T3-E1 osteoblastic cells were cultured in differently prepared sponges. Osteoblastic responses examined included cell numbers, osteocalcin expression, and calcium deposition. Cell numbers were measured by DNA content. Osteocalcin expression was determined by RT-PCR and real-time RT-PCR. Calcium deposition was assayed by ortho-cresophthalein complexone method and von Kossa stain. The osteoblastic cells grown in all collagen sponges did not show apparent signs of cytotoxicity. Collagen sponges differed in freezing temperatures resulted in similar osteoblastic responses. Glutaraldehyde-crosslinked sponges demonstrated less cell-mediated contraction and more cell numbers at day 7 ($p < 0.005$). However, they showed lower osteocalcin expression at day 7 ($p < 0.05$) and less calcium deposition at day 21 ($p < 0.001$). In summary, different freezing temperatures played a minor role in osteoblastic responses. Glutaraldehyde crosslinking process, though improved the dimensional stability of collagen sponges, might compromise the osteoblastic differentiation and mineralization. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res 80A: 399–409, 2007

Key words: collagen sponges; bone engineering; freezing temperature; glutaraldehyde crosslinking; osteoblastic responses

INTRODUCTION

Tissue engineering involves using scaffolds, cells, and factors to regenerate organs and tissues. The application of tissue engineering strategies to bone regenerative therapies has received increasing interest in recent years. Autogenous bone has been the most preferred bone grafting material; however, its use is often limited by the availability of a source and morbidity at the donor site. Therefore, bone engineering has emerged as an alternative approach to regenerate bone.[1,2]

Collagen is a widely used biomaterial for porous scaffolds in tissue engineering due to its excellent biocompatibility and biodegradability. Collagen sponges have been used in bone engineering as a scaffold for bone regeneration[3] and as carriers for the delivery of cells,[4] growth factors,[5] or plasmid DNA into osseous defects.[6,7]

The properties of the scaffold may affect the behavior of ingrowing cells during healing and eventually the regenerative results of tissue engineering. For example, the pore size of the scaffold affects cell ingrowth[8,9] as well as cellular activities such as adhesion and matrix deposition.[10–12] Pore shape can affect cell morphology,[13] while scaffold heterogeneity can modulate cellular responses *in vitro*.[10] The architecture of collagen scaffolds can be controlled through freezing process during preparation.[14]

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Mechanical strength and biodegradability are two other important properties of scaffolds. Scaffolds with higher strength can resist cell contraction during healing,[9] withstand greater external pressure, and maintain space during regeneration.[15,16] Biodegradability determines resorption time and the durability of the scaffold. In addition, when served as a carrier, it also affects the release kinetics of the incorporated molecules.[5] Crosslinking increases both mechanical strength and biodegradation time. However, it may also alter cellular responses to the scaffold.[17] Glutaraldehyde (GA) is a common chemical crosslinking agent for collagen,[18–20] which does not compromise its biocompatibility in previous reports.[18,20]

During bone engineering, the osteoblastic cells grown in the scaffold undergo proliferation, differentiation, and mineralization stages to form bone. The freezing temperature and crosslinking process may alter osteoblastic responses through their modification of collagen scaffolds. The purpose of this study was to investigate the effects of freezing temperature and GA crosslinking on osteoblastic responses to the collagen sponges. Collagen sponges differed in freezing temperature and GA crosslinking were fabricated. The effects of different preparations on proliferation, differentiation, and mineralization of osteoblastic cells were determined *in vitro*.

MATERIALS AND METHODS

Materials

Type I atellocollagen was extracted from Sprague–Dawley rat tails with pepsin treatment and salt precipitation.[21] The purity of the extracted collagen was analyzed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis with Coomassie blue staining. The collagen solution was dialyzed against 0.02*M* phosphate buffer, frozen at −80°C, lyophilized, and then ground in liquid nitrogen using a freezer mill (Model 6750, Spex Certiprep, Metuchen, NJ); the resulting collagen powder was stored in a desiccator at 4°C until use.

Preparation of collagen sponges

Four groups of collagen sponges (−20UL, −20L, −80UL, −80L), varied in freezing temperature and glutaraldehyde (GA) crosslinking, were fabricated (Table I). Round Teflon molds, 10 mm in diameter and 2.5 mm in height, were fixed on Petri dishes.[22] Collagen powder was dissolved in 0.02*M* neutral phosphate-buffered saline (PBS) at 4°C to a final concentration of 2.0% by weight. The collagen solution was then homogenized with a homogenizer (X10/20-750, Ystral, Dottingen, Germany) at 20,000 rpm for 15 min on ice. Collagen solution (200 μL) was added into each Teflon mold and frozen at either −20°C or −80°C for 2 h. To avoid collagen skin formation on surface of collagen sponge, a piece of moist folded tissue paper (Kimwipes, Kimberly-Clark, Roswell, GA) was placed on top of the frozen collagen in molds.[22] The solidified collagen was frozen at −20°C or −80°C again for another 4 h and then freeze-dried for at least 18 h to form porous collagen sponges. Finally, additional GA crosslinking was performed on the −20L and −80L collagen sponges.[18] The collagen sponges were immersed in 0.2% GA in 0.1*M* phosphate buffer at 37°C for 12 h. Residual GA was removed from sponges by washing with 0.1*M* glycine for 12 h and distilled water for 24 h. The collagen sponges were then freeze-dried again using the same conditions as described above.

TABLE I
Groups of Collagen Sponges

Group	Freezing Temperature	GA Crosslinking
−20UL	−20°C	−
−20L	−20°C	+
−80UL	−80°C	−
−80L	−80°C	+

Round collagen disks, 6 mm in diameter, were punched from all sponges and UV irradiated for 12 h on each side in a tissue culture hood.[22–24] Finally, the collagen sponges were wetted with culture medium before cell seeding.

Determination of collagen sponge architecture

The architecture of various collagen sponges were examined using a scanning electron microscope (SEM) (S-2700, Hitachi, Tokyo, Japan). The cross-sectional surfaces were revealed after cutting collagen sponges with a microtome blade in a liquid nitrogen environment. In addition, collagen sponges were embedded in methyl methacrylate, sectioned longitudinally using a Polycut E microtome (Reichert-Jung, Nussloch, Germany) set at 7 μm thickness, and stained with aniline blue. The images were captured under a light microscope and analyzed using ImagePro Plus analysis software (Media Cybernetics, Silver Spring, MD). Ellipses were fit to pores manually and mean diameters, which were the average diameters measured at 2° intervals and passing through object's centroid, were calculated automatically by software.

Cell seeding, cell morphology observation, and dimensional stability analysis

MC3T3-E1 subclone 4 (MC4) cells (Bioresource Collection and Research Center, Hsinchu, Taiwan) were maintained in ascorbic acid-free α minimum essential medium (Gibco, Grand Island, NY) containing 10% heat-inactivated fetal calf serum (Biological Industries, Kibbutz Beit Haemek, Israel), 100 units/mL penicillin G sodium, 100 μg/mL streptomycin sulfate, and 0.25 μg/mL amphotericin B (Gibco) at 37°C in a 5% CO_2 humidified atmosphere. The MC4 cells undergo differentiation and mineralization when cultured in differentiating medium containing 50 μg/mL ascorbic acid and 3.0 m*M* β-glycerophosphate.[25] Cells were used before passage 15. MC4 cells, 1.4×10^5

cells in 15 μL culture medium, were seeded onto collagen sponges placed in 96-well culture plates. Three hours later, the collagen sponges with the cells were transferred to 24-well culture plates containing 1 mL culture medium. After 24 h of culture, medium was changed into differentiating medium. The differentiating medium was changed every other day thereafter. At day 7, the collagen sponges were fixed with 2.5% GA in 0.1*M* phosphate buffer overnight and prepared for cell morphology observation under SEM. To determine the dimensional stability of various collagen sponges over time, collagen sponges were photographed by a digital camera (Coolpix 4500, Nikon, Tokyo, Japan) with a fixed distance and a reference ruler at various time points. The diameter of collagen sponges was measured by analyzing the photographs using ImagePro Plus analysis software.

DNA quantification

DNA was determined by a method modified from Ishaug-Riley.[26] After 1, 3, and 7 days in culture, the cells in sponges and control plate were washed three times with PBS and stored at −80°C until assay. All specimens were homogenized by a tissue homogenizer (TH115, Omni International, Warrento, VA) in 10 m*M* EDTA for 5 min. Calf thymus DNA (Sigma, St Louis, MO) was used as the DNA standard. A series of cell solutions with known cell concentrations were used as the cell standard. After adding Hoechst 33258 dye, fluorescent emission at 460 nm was read after excitation at 355 nm using a fluorescence spectrophotometer (Wallac Victor,[2] PerkinElmer Life Sciences, Boston, MA). Cell number and DNA content in the sponges were determined by comparing the fluorescent readings of the samples to the DNA and cell standard curves.

Differentiation assay

Osteocalcin expression of MC4 cells was determined after 7 days of culture in collagen sponges. Total RNA was isolated using TRI reagent (Molecular Research Center, Cincinnati, OH) according to manufacturer's protocol. The RNA obtained was treated with DNase I (Ambion, Austin, TX) containing ribonuclease inhibitor (RNasin®, Promega, Madison, WI) at 37°C for 30 min to remove contaminating genomic DNA. The concentrations of purified RNA were measured using a UV spectrophotometer (DU 640, Beckman Coulter, Fullerton, CA). The integrity of RNA was verified by 1% agarose gel electrophoresis. Two micrograms of total RNA was reverse transcribed using reverse transcriptase (SuperScript II, Invitrogen, Carlsbad, CA).

Complimentary DNA (cDNA) was amplified by Tag polymerase (Protech Technology, Taipei, Taiwan) in a PCR Sprint Thermal Cycler (Thermo Hybaid, Ashford, UK). The thermocycling conditions were as follows: initial denaturation at 95°C for 30 s followed by cycles (17 cycles for osteocalcin and 19 cycles for β-actin) of 94°C for 15 s (denaturation), 55°C for 30 s (annealing), 72°C for 30 s (extension), and final extension at 72°C for 5 min. The sequences of primers used were as follows: osteocalcin 5′-GCT TAA CCC TGC TTG TG-3′ (forward), 5′-TGC TGT GAC ATC CAT ACT T-3′ (reverse), amplicon size 191 bp; β-actin 5′-TAT GCC AAC ACA GTG CT-3′ (forward), 5′-GCT GAT CCA CAT CTG CT-3′ (reverse), amplicon size 195 bp. The final products were run on a 2% agarose gel and stained with ethidium bromide. Semi-quantitative analysis of osteocalcin expression was performed by ImageQuant analysis software (Molecular Dynamics, Sunnyvale, CA) and normalized with the β-actin expression control.

Real-time PCR was performed in a LightCycler (Roche Molecular Biochemicals, Mannheim, Germany) using a Lightcycler FastStart DNA Master SYBR Green I kit (Roche). The final products were confirmed by melting curve analysis. In addition, their specificity was further verified by running on a 2% agarose gel. Standard coefficient curves were made from serial dilutions of osteocalcin and β-actin cDNA. Data was analyzed by the coefficient file and duplicate data analysis program of the Relative Quantification software (RelQuant, Roche).

Mineralization assays

Calcium deposition of 3-week cultures of MC4 cells in collagen sponges was determined using an ortho-cresolphthalein complexone method. Homogenized specimens were incubated in 0.6*N* HCl with shaking for 24 h to dissolve the deposited calcium. Aliquots of 10 μL calcium extract were transferred into microtubes with 200 μL of 0.1 mg/mL ortho-cresolphthalein complexone. 2-Amino-2-methyl-1-propanol (1.5*M*, 200 μL) was added to each tube and incubated at 30°C for 15 min. Sample data were calibrated against a calcium/carbonate standard curve. The amount of deposited calcium per sponge was expressed as micrograms of calcium equivalent. In addition, cultured sponges were fixed in 10% neutral buffered formalin, dehydrated with serial graded ethanol, and then embedded in paraffin. Sections were cut at 5 μm. von Kossa staining with nuclear fast red counter stain was used for light microscopic examination.[19]

Statistical analysis

The differences among various collagen groups were analyzed by one-way analysis of variance (ANOVA) and pairwise multiple comparison (Tukey tests). A probability value of 95% ($p < 0.05$) was used to determine statistical significance.

RESULTS

Architecture of collagen sponges

Scanning electron microscopic (SEM) and light microscopic examinations were performed to compare the structure of the collagen sponges. SEM micrographs of the −20UL and −20L collagen sponges demonstrated a more polygonal and homogenous pore structure on both the surface facing Petri dish

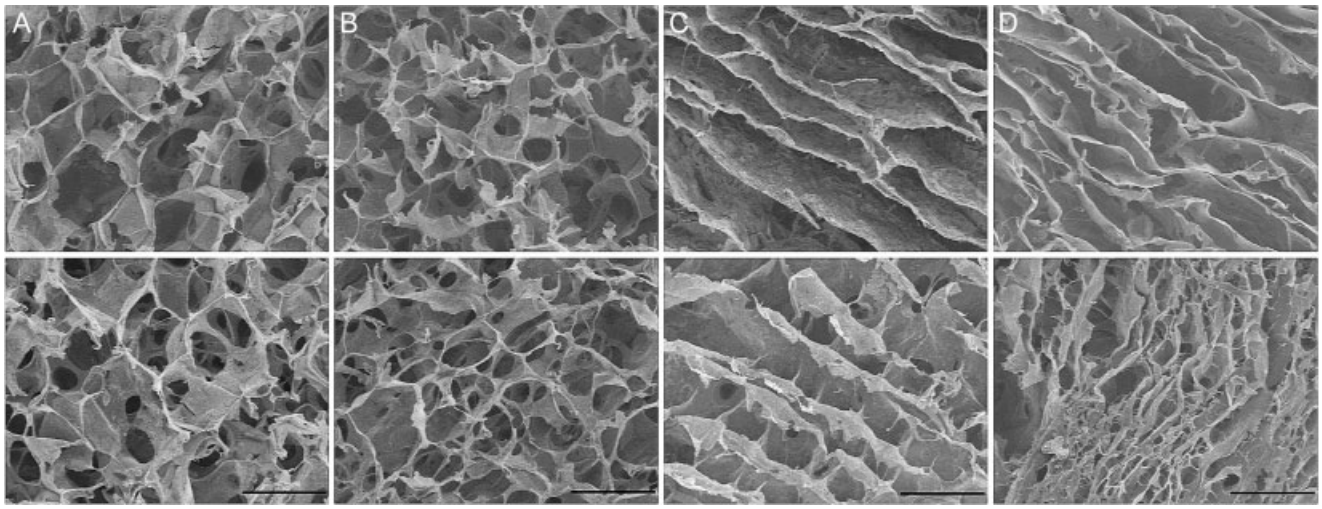

Figure 1. Images of various collagen sponges under SEM. A, −20UL; B, −20L; C, −80UL; D, −80L. Upper, longitudinal section; bottom, surface facing Petri dish. Bars = 150 μm.

and the longitudinal section surface. In contrast, the −80UL and −80L collagen sponges were characterized by more pores that were elongated and inhomogeneous (Fig. 1). The mean diameters on the longitudinal section surfaces calculated from light microscopic images (Fig. 2) were 148 ± 33 μm (standard deviation), 135 ± 27 μm, 238 ± 135 μm, and 202 ± 110 μm for −20UL, −20L, −80UL, and −80L, respectively. The results indicated that a lower freezing temperature (−80°C) created significantly larger (−20UL *vs.* −80UL, $p < 0.001$, −20L *vs.* −80L, $p < 0.001$) and more heterogeneous pores (as evidenced by larger standard deviations) than the higher freezing temperature (−20°C). In contrast, GA crosslinking did not alter the pore size of the collagen sponges made at −20°C (−20UL *vs.* −20L, $p = 0.346$); however, it decreased (with a smaller magnitude) the pore size of those sponges fabricated at −80°C (−80UL *vs.* −80L, $p < 0.005$).

Cell culture, cell morphology, and dimensional stability of collagen sponges

Collagen sponges without UV irradiation or GA crosslinking were very fragile and disintegrated easily in culture medium. Therefore, UV irradiation was carried out on all collagen sponges before testing to facilitate cell culture experiments. No apparent signs of cytotoxicity were found during cell morphology observation of MC4 cells after 1, 3, and 7 days of culture on surface of various collagen sponges under SEM (Fig. 3). The cells increased over time and became fully confluent at day 7 in all groups.

The dimensional stability of various collagen sponges was determined by comparing the diameter of sponges at various time points in culture (with or without cell seeding) to their respective diameter before wetting with culture medium (Fig. 4). Immediately after being wetted with culture medium, the uncrosslinked collagen sponges collapsed, while the crosslinked collagen sponges expanded slightly. In cell-seeded groups [Fig. 4(A)], uncrosslinked collagen sponges reduced in diameter over time until about 58% at day 7. In contrast, the crosslinked collagen sponges expanded slightly in culture and maintained their dimension thereafter. There was no statistic difference between uncrosslinked (−20UL *vs.* −80UL) or crosslinked (−20L *vs.* −80L) groups. However, the crosslinked groups showed significantly larger diameter than uncrosslinked group with the same freezing temperature at all time points in culture. In cell-free groups [Fig. 4(B)], the pattern among groups was

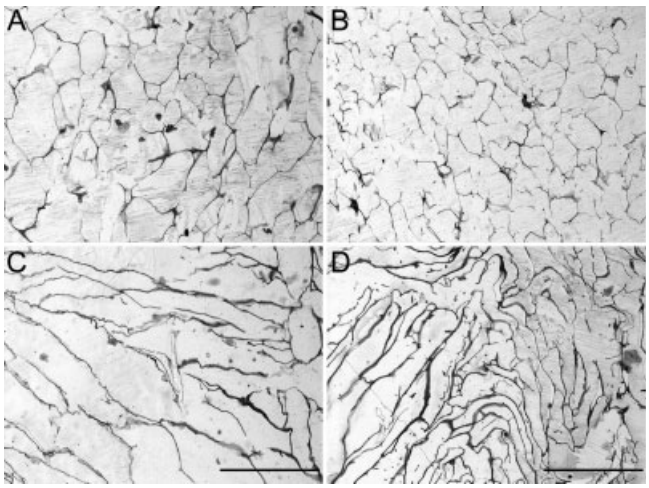

Figure 2. Images of various collagen sponges under light microscope. A, −20UL; B, −20L; C, −80UL; D, −80L; longitudinal section. Bars = 400 μm.

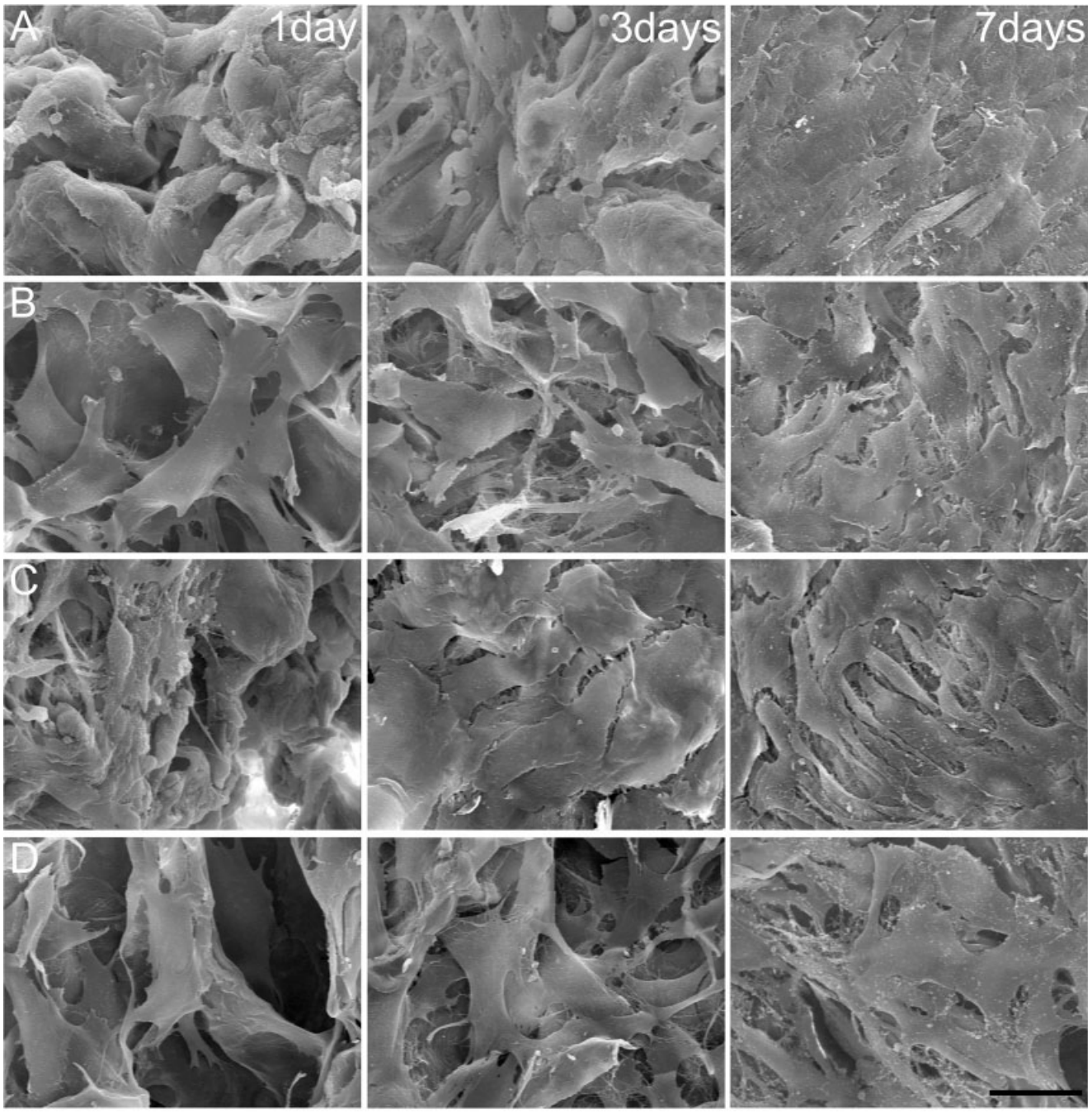

Figure 3. Scanning electron micrographs of MC4 cells grown on various collagen sponges for 1, 3, and 7 days. A, −20UL; B, −20L; C, −80UL; D, −80L. Bar = 30 μm.

similar to cell-seeded groups. However, the diameter of uncrosslinked collagen sponges only decreased slightly and remained about 94% of initial diameter at day 7. No statistic difference between uncrosslinked (−20UL *vs.* −80UL) or crosslinked (−20L *vs.* −80L) groups was found. In addition, the crosslinked groups showed significantly larger diameter than uncrosslinked group with the same freezing temperature at all time points in culture. To reveal the effect of cell-seeding on dimensional stability, the results from cell-seeded groups and cell-free groups were compared. The cell-seeding did not significantly alter the dimension of crosslinked collagen sponges. In contrast, the cell-seeded uncrosslinked sponges (either −20UL or −80UL) showed a smaller diameter than cell-free uncrosslinked sponges with the same freezing temperature at 3 h ($p < 0.05$) and 1, 3, and 7 days ($p < 0.001$). The results indicated that cell-seeding resulted in significant dimensional reduction in uncrosslinked collagen sponges but not in crosslinked ones.

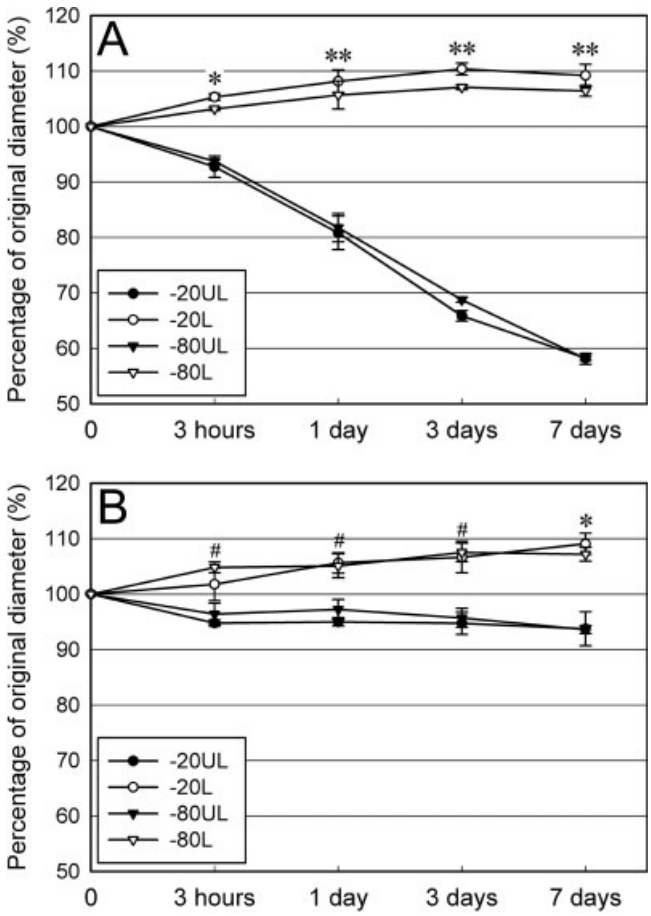

Figure 4. Diameter of various collagen sponges with or without MC4 cell-seeding after 3 h and 1, 3, and 7 days. A, cell-seeded groups; B, cell-free groups. Accumulated data from three independent experiments were presented as mean ± SE. #, $p < 0.05$; *, $p < 0.01$; **, $p < 0.001$ *versus* respective uncrosslinked counterparts at the same time point.

The effect of different collagen preparations on cell numbers

DNA content was measured to determine MC4 cell numbers in the collagen sponges after 1, 3, and 7 days of culture (Fig. 5). For all groups, cell numbers increased with time. The crosslinked groups showed significantly higher cell numbers than the uncrosslinked groups at day 7 (−20L *vs.* −20UL, $p < 0.005$; −80L *vs.* −80UL, $p < 0.005$). The GA crosslinking resulted in about a 3-fold increase in cell numbers. In contrast, the sponges only differed in freezing temperature showed similar cell numbers (−20UL *vs.* −80UL, $p = 0.952$; −20L *vs.* −80L, $p = 0.783$). These data indicated that different freezing temperatures did not significantly affect the cell numbers, while additional GA crosslinking increased cell numbers in the culture system used.

The effect of different collagen preparations on cell differentiation

Osteocalcin is a specific late marker of osteoblastic differentiation. Both semi-quantitative and quantitative RT-PCR analyses were used to determine the expression of osteocalcin of MC4 cells after 7-day culture in collagen sponges with differentiation medium. The results were normalized against the expression of β-actin and expressed as ratio to the plate control (Fig. 6). The groups differed in GA crosslinking showed significant differences in osteocalcin expression (−20UL *vs.* −20L, $p < 0.05$; −80UL *vs.* −80L, $p < 0.01$). The addition of GA treatment resulted in a 3- to 4-fold reduction in osteocalcin expression. In contrast, the groups differed in freezing temperature showed similar osteocalcin expression levels (−20UL *vs.* −80UL, $p = 1.0$; −20L *vs.* −80L, $p = 0.996$).

Osteocalcin mRNA expression levels were further confirmed by real-time PCR analysis. The results revealed a similar pattern across the groups (Fig. 7). The variation in freezing temperature did not significantly affect osteocalcin expression (−20UL *vs.* −80UL, $p = 1.0$; −20L *vs.* −80L, $p = 1.0$), while the additional GA crosslinking lead to a 6- to 8-fold reduction in osteocalcin expression (−20UL *vs.* −20L, $p < 0.005$; −80UL *vs.* −80L, $p < 0.005$). These results suggested that GA crosslinking may inhibit osteoblastic differentiation.

The effect of different collagen preparations on calcium deposition

The ability of MC4 cells to mineralize after 3-week culture in differentiating medium was verified by

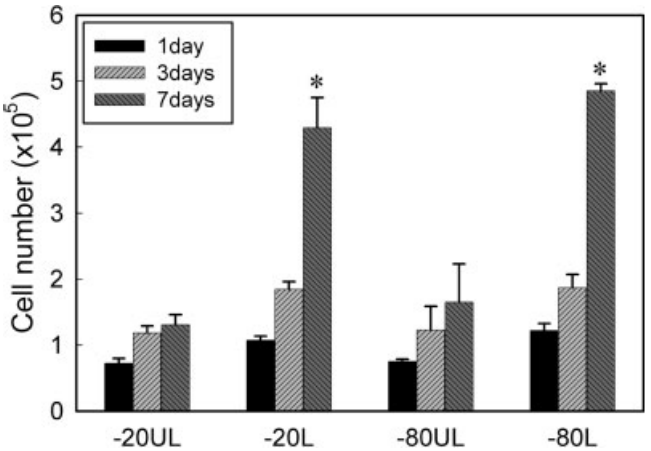

Figure 5. Cell numbers of MC4 cells cultured in various collagen sponges for 1, 3, and 7 days determined by DNA quantification. Accumulated data from three independent experiments were presented as mean ± SE. *, $p < 0.005$ *versus* uncrosslinked groups with the same freezing temperature after 7 days.

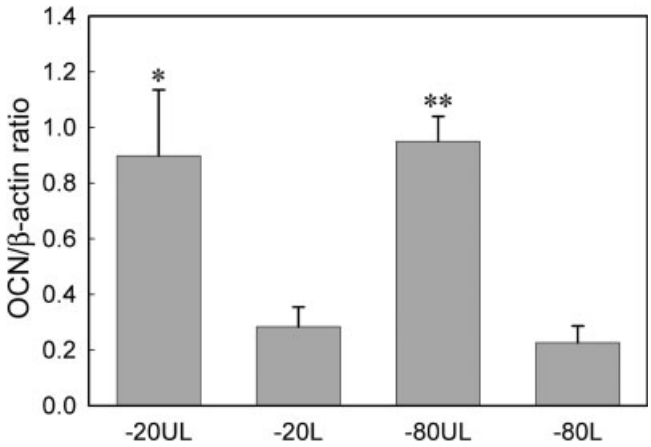

Figure 6. Osteocalcin expression levels of MC4 cells cultured in various collagen sponges for 7 days quantified by RT-PCR. Accumulated data of osteocalcin to β-actin ratio from three independent experiments were presented as mean ± SE. All groups were normalized against the accompanied control group cultured on Petri dishes. *, $p < 0.05$; **, $p < 0.01$ *versus* crosslinked groups with the same freezing temperature.

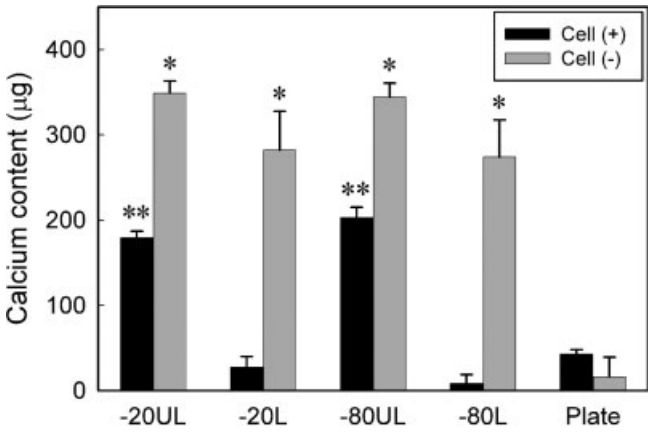

Figure 8. Calcium content of collagen sponges after 3-week culture with or without MC4 cells determined by an ortho-cresolphthalein complexone method. Accumulated data from three independent experiments were presented as mean ± SE. "Plate" is the calcium content of cells cultured on plates served as the control. *, $p < 0.05$ *versus* respective cell-seeded groups **, $p < 0.001$ *versus* crosslinked groups with the same freezing temperature.

von Kossa stain (data not shown). The calcium content of various collagen sponges, with or without seeding of MC4 cells, was determined after 3 weeks of culture using the ortho-cresolphthalein complexone method and von Kossa staining. Figure 8 revealed similar amount of calcium deposition among cell-free groups. Results from the cell-seeded groups demonstrated that GA crosslinking, but not freezing temperature, affected calcium deposition. GA crosslinking reduced calcium deposition by at least 6 folds (−20L *vs.* −20UL, 6-fold, $p < 0.001$; −80L *vs.* −80UL, 25-fold, $p < 0.001$). Interestingly, the calcium content of cell-free groups was higher than their respective cell-seeded counterparts ($p < 0.05$).

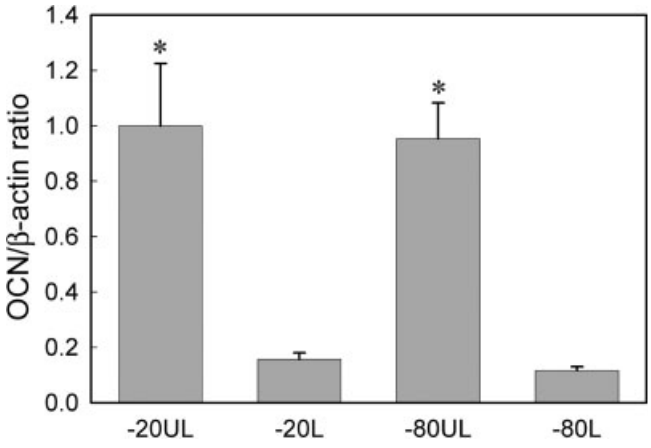

Figure 7. Osteocalcin expression levels of MC4 cells cultured in various collagen sponges for 7 days determined by real-time PCR. Accumulated data of osteocalcin to β-actin ratio from three independent experiments were presented as mean ± SE. All groups were normalized against the same calibrator. *, $p < 0.005$ *versus* crosslinked groups with the same freezing temperature.

Collagen sponges, with or without seeding of MC4 cells, were stained with von Kossa stain after 3 weeks of culture (Fig. 9). As expected, the lower freezing temperature resulted in more elongated pores among the cell-free groups. Uncrosslinked collagen sponges (−20UL and −80UL), but not crosslinked sponges (−20L and −80L), showed reduced porosity near the surface after cell culture. In agreement with the results of calcium content assay, the staining of the deposited calcium was more apparent in groups without cells than their counterparts with cells. Among the cell-free groups, the uncrosslinked specimens revealed darker staining than the crosslinked specimens. Among the cell-seeded groups, GA crosslinking reduced calcium deposition, while freezing temperature did not affect the deposition. These data indicated that the GA crosslinking of collagen sponge exerted an adverse effect in mineralization of osteoblastic cells.

DISCUSSION

The purpose of this study was to determine the effects of different freezing temperatures and GA crosslinking on the properties of collagen sponges as a scaffold for bone engineering. The osteoblastic responses to collagen sponges including proliferation, differentiation, and mineralization were determined *in vitro*.

Collagen sponges without UV irradiation or GA crosslinking were very fragile and disintegrated eas-

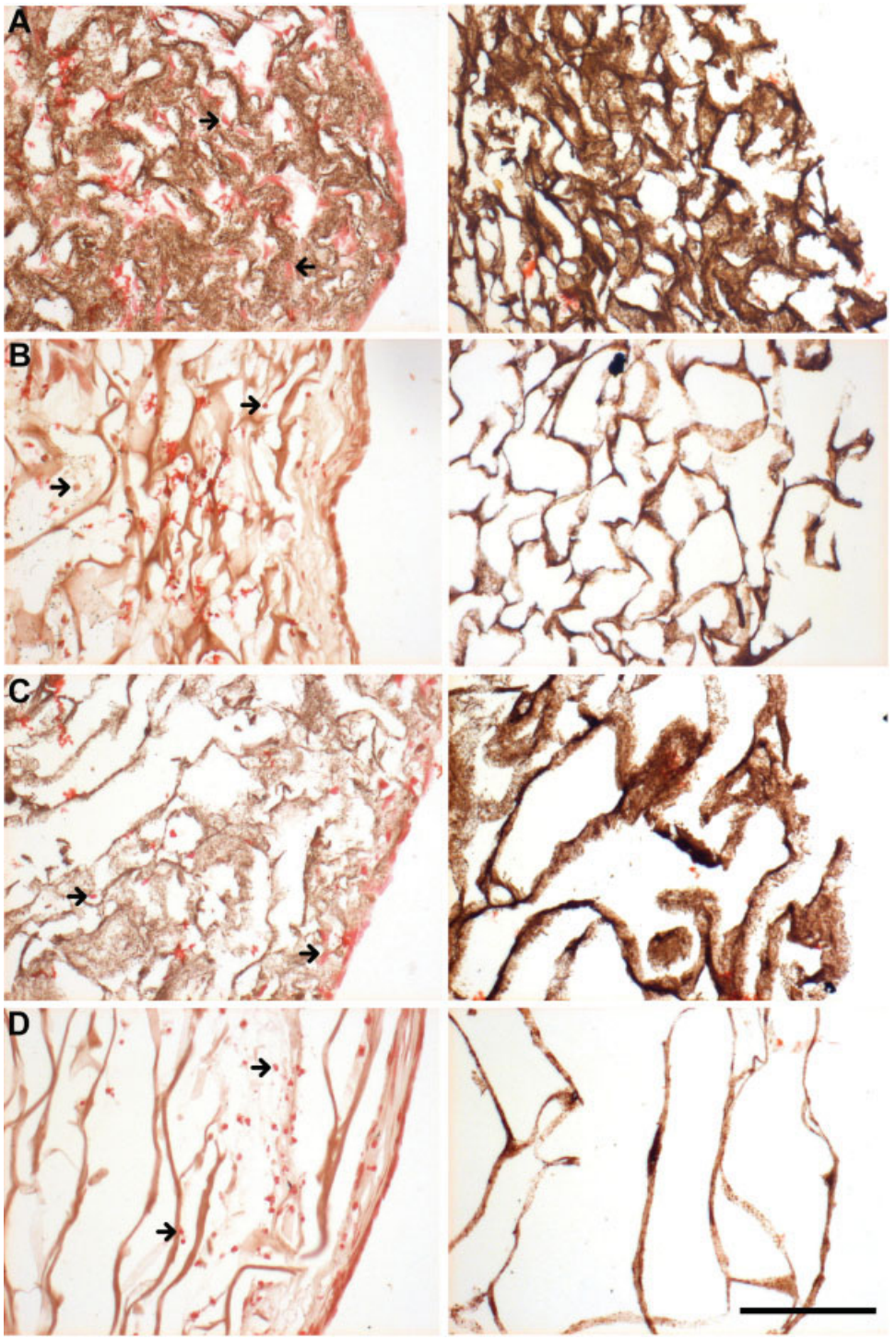

Figure 9. Deposited calcium in various collagen sponges, with or without 3-week MC4 cell culture, revealed by von Kossa stain with nuclear fast red counter stain. A, −20UL; B, −20L; C, −80UL; D, −80L. Left, cell-seeded collagen sponges; right, cell-free collagen sponges. In the cell-seeded groups, most cells were found on the surface of collagen sponges. The arrow points indicate cells penetrating into the collagen sponges. Bar = 100 μm.

ily in culture medium, which incurred difficulties for cell culture studies. Thus, UV irradiation was used to stabilize all collagen sponges in this study. The UV irradiation allows the collagen fibrils to retain a relative native structure without apparent denaturing effects.[24] The effects of UV irradiation are less prominent when compared to the chemical crosslinking on collagen sponges.[27] Thus, it was assumed that the minor effect of UV irradiation would not mask the differences resulting from the GA crosslinking process.

The architecture of collagen sponges was significantly affected by freezing process as revealed in previous reports.[13,14,28] Fixed freezing temperature is convenient to apply for altering porous architectures of collagen sponges. The effects of two commonly used freezing temperatures, −20°C and −80°C, were determined in this study. Higher freezing temperature (−20°C) produced polygonal homogenous pores similar to reported results in alginate scaffolds,[13] while the lower freezing temperature (−80°C) led to inhomogeneous elongated pores.

Type I collagen is the most abundant extracellular protein in bone. Collagen sponge meets many properties of an ideal scaffold including porosity, biocompatibility, hydrophicility, biodegradability, and cell recognition. However, collagen sponge often cannot withstand external pressure and maintain space in bone regenerative therapy. Its poor mechanical properties become a major limitation for its use in bone engineering. Thus, different strategies were developed to reinforce the collagen-based scaffolds, such as forming composites,[29–33] and crosslinking the collagen.[5,18,34] GA is a commonly used chemical crosslinker for collagen graft materials that may dose-dependently improve the mechanical properties and longevity of collagen materials without compromising their excellent biocompatibility.[18,20] In accordance with previous reports, the GA-crosslinked sponges in this research demonstrated signs of superior mechanical properties including less fragility during manipulation and better resistance to cell-mediated contraction force during culture. In addition, osteoblastic cells were able to grow into the GA-crosslinked collagen sponges without apparent signs of cytotoxicity.

Cell numbers after osteoblastic culture in collagen sponges were significantly affected by the GA crosslinking. Significant fewer cells were found in the uncrosslinked sponges than their GA-crosslinked counterparts after 7 days of culture. This result may be explained, at least in part, by the increased mechanical properties of collagen sponges after GA crosslinking. Uncrosslinked collagen sponges contracted progressively during cell culture, which has been reported in previous studies.[17,20] In contrast, the contraction was not observed in GA-crosslinked collagen sponges that gained mechanical strength from the crosslinking process. Previous studies have indicated that uncrosslinked collagen sponges show contraction and lower cell numbers.[17,20] Cell-mediated contraction of collagen sponges may confine and frustrate the cell proliferation that occurs in uncrosslinked collagen sponges. In addition, the effect of contact inhibition may be more pronounced in uncrosslinked collagen sponges due to their reduction in size over time. Furthermore, the reduction in pore sizes resulting from cell-mediated contraction may hinder the necessary nutritional exchanges in uncrosslinked collagen sponges. Different freezing temperatures resulted in different architectures for the collagen sponges but did not have a statistical significant effect on cell numbers. The pore diameter of all prepared collagen sponges in this study are above 100 μm, which was sufficient for cells to penetrate and grow.[9] It has also been shown that variation in pore shape has no effect on albumin diffusion, which suggests that its effect on nutritional exchange during cell growth may be minimal.[13] Therefore, it is reasonable that collagen sponges with different architectures did not significantly alter cell numbers in this study.

Osteocalcin is a specific marker of differentiated osteoblasts. The expression level of osteocalcin was lowered by GA crosslinking but was not affected by the different freezing temperatures. At least two possible underlying mechanisms may account for the effects of GA crosslinking. First, GA crosslinking may affect osteoblastic differentiation through the collagen–integrin interaction. Extracellular matrix (ECM) can modify cell activity via integrins that help attach cells to the matrix and mediate mechanical and chemical signals from it.[35–37] Type I collagen is the major component of ECM and plays an important role in osteoblastic differentiation.[38,39] For examples, type I collagen upregulates osteocalcin gene expression of odontoblasts through collagen binding to the integrin receptors,[38] while BMP-7 and collagen showed synergistic effects on the regulation of osteocalcin through the mitogen-activated protein kinase pathway.[37] The GA-induced mechanical or chemical changes of collagen sponges may affect the osteocalcin expression via influence on the collagen-integrin interaction. In contrast, the different freezing temperatures resulted in different architectures of the collagen sponge, but it had little effect on the chemical or mechanical properties of the collagen, which may explain why no significant difference in osteocalcin expression was detected between the different temperature-processed collagen sponges. The other possible mechanism that influences osteocalcin expression may be related to the calcium-phosphate apatite deposition on the collagen sponges. Reports have demonstrated that a substrate containing calcium-phosphate apatite enhances osteoblastic cell differentiation.[40,41] In this study, calcium deposition on collagen-based materials incubated in cell culture medium was found as reported previously.[31] In cell-free collagen sponges, the average calcium deposition in GA-crosslinked groups is somewhat lower than the uncrosslinked groups. The deposited calcium on collagen sponges may feedback positively on osteoblastic differentiation and contributed to the different levels of osteocalcin expression between the cell-seeded collagen sponges with and without GA crosslinking.

The calcium content in this study was altered by both cell seeding and GA crosslinking. In agreement

with previous reports, collagen sponges seeded with osteoblasts or fibroblasts showed reduced mineral deposition compared to cell-free conditions.[31] In addition, calcification was significantly inhibited in the crosslinked sponges as has been found in previous report[17] although a different cell type and chemical crosslinking reagent were used. The total calcium deposition in this study may be affected by cells and by acellular calcification in collagen sponges. However, compared to the control cell culture on plates, the calcium contents of both −20L and −80L were lower. It is highly possible that both osteoblastic mineralization and acellular calcification were inhibited by GA crosslinking. Among the collagen sponges without cell seeding, although the total amount of calcium contents was not statistically different, von Kossa staining revealed darker stain in uncrosslinked groups than the GA-crosslinked groups. The variation in volume after the collagen sponges incubated in culture medium may be responsible for obtaining a similar total content of calcium with different calcium densities presented based on von Kossa staining. Further studies to clarify the unexpected result are needed.

CONCLUSIONS

Different freezing temperatures (−20°C or −80°C) affected the architecture of the collagen sponges, but did not significantly affect osteoblastic responses to them. The GA crosslinking only slightly changed the architecture of collagen sponges, but significantly affected osteoblastic responses to them *in vitro*. When compared to the uncrosslinked collagen sponges, the GA-crosslinked ones resulted in higher cell numbers, but compromised osteoblastic differentiation and mineralization. Within the limits of this study, GA crosslinking, but not freezing temperature, significantly affected collagen sponge as a scaffold for bone engineering. GA crosslinking may provide superior dimensional stability against cell-mediated contraction, but it may compromise osteoblastic differentiation and mineralization.

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