Effectively improved 3-dimensional structural stability of atelocollagen-gelatin sponge biomaterial by heat treatment

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Atelocollagen-gelatin (ACG) sponge was fabricated from atelocollagen and gelatin by lyophilization without introducing toxic substances. This study aimed to investigate the effects of heat treatment on the 3-dimensional structural stability of ACG sponge biomaterial. ACG sponge samples were fabricated and heat treated at 125°C for 12 h in the vacuum. The results revealed that heat treatment did not affect porosity, pore size and mechanical compressive strength. Heat-treated ACG sponge showed decreased absorbance and peak shift of amid I (C=O) stretches, slightly higher water uptake degree and significantly decreased in vitro degradation rate. Moreover, heat-treated ACG sponge maintained good 3-dimensional surface morphology and porous microstructure throughout 7 days, while non-heat-treated ACG sponge collapsed in less than 24 h. The human mesenchymal stromal cells (hMSCs) were shown to adhere and grow well on heat-treated ACG sponges. These results indicate that heat treatment is effective and safe to stabilize 3-dimensional ACG sponge biomaterial for tissue engineering.

Keywords: Collagen, Gelatin, Heat treatment, Sponge biomaterial, Stability

INTRODUCTION

Tissue engineering has been proven as a useful method to cure tissue defects such as bone, skin and cartilage in recent two decades1-3. The design of biomaterial scaffolds for mechanical functions and nutrients transportation plays a vital role in tissue engineering4,5. Porous scaffolds were shown to have several superior properties, especially to promote the exchange of nutrients and metabolites, and cell growth and differentiation on scaffolds6-9. Lyophilization was reported to be one of the effective methods to fabricate porous scaffolds of collagen and gelatin biomaterials6,10-13.

Collagen is an abundant structural protein in the extracellular matrix, comprising almost one third of the total protein content in human14,15. Collagen consists of amino acids bound together to form a triple helix of elongated fibrils15. Moreover, collagen is particularly an attractive tissue engineering biomaterial due to its excellent biocompatibility, physiological metabolic end-products, and suitable interactions with cells and macromolecules14,16. For these reasons, collagen and collagen-based biomaterials have been widely used to fabricate bioactive scaffolds for regenerative medicins12,17,18. Collagen powder is generally water-insoluble, but its solubility can be increased by lowering the pH level of solutions. Thus, acidic solutions like acetic acid were usually used to dissolve collagen powder12,17,19-21. Interestingly, collagen powder was found to be easily dissolved into gelatin solution at room temperature without adjusting the pH level22. As gelatin is the irreversibly hydrolyzed form of collagen, this method makes it much easier to obtain collagen solution without using any acidic solution or neutralization reaction to adjust the pH level. In addition, crosslinking treatment is critically important to stabilize collagen and collagen-based biomaterials.

Crosslinking treatment mainly includes two main categories, chemical and biophysical crosslinkings20. Representative examples of chemical crosslinking are glutaraldehyde23,24 and carbodiimides18,19,25, whereas examples of biophysical crosslinking are UV light26,27) and dehydrothermal (DHT) crosslinking17,21,22,28,29. The major disadvantage of chemical crosslinking was potential toxic residues left in the scaffolds30-32. Removal of toxic residues from scaffolds was time consuming18) and might compromise the original 3-dimensional structure of porous scaffolds. As for collagen and collagen-based biomaterials, crosslinks are bonds between the side chains of amino acids in collagen molecules23,33. DHT treatment is a common biophysical crosslinking method to stabilize collagen and collagen-based materials20,21,34,35, which involves subjecting collagen to increased temperature (>90°C) in the vacuum. Formation of crosslinks during DHT treatment depends on the exhaustive removal of bound water from collagen molecules, resulting in the formation of intermolecular crosslinks through condensation reactions between the carboxyl and amino groups by either esterification or amide formation27,36. DHT treatment is preferable to other chemical crosslinking methods as no cytotoxic reagents are employed. Crosslink density was found to increase with DHT temperature instead of exposure period, while denaturation was increased with DHT.
temperature and exposure period, ranging from 25% at 105°C to 60% at 180°C. A certain amount of denaturation caused by DHT treatment was supposed to partially compensate the loss of cell binding sites after crosslinking.

Our previous study reported an easy method to fabricate porous atelocollagen-gelatin (ACG) sponge from gelatin and water-insoluble atelocollagen with lyophilization and heat treatment. The water-insoluble atelocollagen powder was shown to be dissolved into gelatin solution at room temperature without using other acid solutions. Heat treatment at 125°C in the vacuum was employed to crosslink ACG sponge. This method was highly reproducible without introducing foreign toxic substances into ACG sponge. However, how heat treatment affects the porous microstructure and 3-dimensional structural stability of ACG sponge biomaterial remains elusive. This study aimed to investigate the effects of heat treatment on the 3-dimensional structural stability of ACG sponge biomaterial, which was critical for ACG sponge to maintain its 3-dimensional porous microstructure and promote efficient nutrients exchange for tissue engineering.

MATERIALS AND METHODS

Preparation of 0.6%AC+2.4%G sponge samples
In this study, ACG sponge samples containing 0.6% atelocollagen and 2.4% gelatin (abbreviated as 0.6%AC+2.4%G) was fabricated according to the protocol as described previously. Briefly, the 2.4% (wt/v) gelatin solution was first prepared by dissolving 960 mg of gelatin powder (Nitta Gelatin, Osaka, Japan) into 40 mL of Milli-Q water. Then 180 mg of water-insoluble atelocollagen powder (KOKEN, Tokyo, Japan) was dissolved into 30 mL of 2.4% gelatin solution under constant stirring at room temperature. After the 0.6%AC+2.4%G composite solution became transparent, all air bubbles were carefully removed. The composite solution was added into 96-well plates at 150 μL/well or 24-well plates at 1,000 μL/well, and frozen at −30°C for 12 h. The frozen samples were freeze-dried at −50°C for 24 h in a vacuum freeze dryer (FREEZONE 2.5 freeze dryer, LABCONCO, Kansas City, MO, USA). Finally, the 0.6%AC+2.4%G sponge samples in cylindrical shape were obtained without any crosslinking treatment.

Heat treatment of 0.6%AC+2.4%G sponge samples
Half of 0.6%AC+2.4%G sponge samples were heat-treated at 125°C for 12 h in the vacuum (Vacuum dryer, AVO-200NB, ASONE, Osaka, Japan), and used as the experimental group (ACG-HT group). The other half were not heat-treated and used as the control group (ACG-NHT group). All samples were stored in the same dry condition for further experiments.

Scanning electron microscope (SEM) examination of porous microstructure
Gross observation of ACG sponge samples was first performed before SEM (HITACHI SU6600, Hitachi, Tokyo, Japan) examination. SEM was then used to evaluate the effect of heat treatment on the porous microstructure of ACG sponge samples. Samples were carefully prepared to expose fresh cross-sectional surface and in the same size (6.0 mm diameter, 3.0 mm height). Sputter coating with gold-palladium (Au-Pd) alloy was performed in a sputter coater (SC500A, Elminet, Tokyo, Japan). The cross-sectional surfaces were examined with SEM at an accelerating voltage of 15 kV. Five SEM fields (×150) of each sample were randomly captured to evaluate porous microstructure.

Image analysis of porosity and pore size
The porosity and pore size of samples were analyzed with ImageJ 1.50g software (NIH, Bethesda, MD, USA). As pores were not regular circles, Feret diameter and pore area were chosen for a more precise evaluation of pore size. Three independent samples of each group were randomly selected. Five SEM fields (×150) of each selected sample were measured for porosity and pore size.

Fourier transform infrared spectroscopy (FTIR) assay
FTIR spectra were used to evaluate the crosslinking of ACG sponges by heat treatment. Potassium bromide (KBr) pellets were prepared as follows. Briefly, approximately 1 mg of sample was well mixed into 150 mg of KBr powder, finely pulverized and added into a pellet-forming die. A force of 70 kN was applied for 3 min to form a transparent pellet. Degassing was performed to eliminate air from KBr powder. FTIR analysis was performed with Fourier transform infrared spectrometer 430 (JASCO, Tokyo, Japan) at room temperature. Background measurements with KBr pellets were first performed to correct for infrared light scattering losses in the pellet and moisture absorbed on the KBr. Then samples of gelatin powder, atelocollagen powder, ACG-HT and ACG-NHT groups were measured in triplicate. The FTIR spectra were recorded in the range of 4,000–400 cm⁻¹ at a resolution of 4.0 cm⁻¹. The collected data were analyzed with KnowItAll® Software (Bio-Rad, Hercules, CA, USA).

Mechanical compressive strength test
The compressive moduli of ACG-HT and ACG-NHT groups were measured on a universal mechanical testing machine equipped with 100 N sensor (AG-I 20 kN, SHIMADZU, Kyoto, Japan). The machine was set and calibrated according to the manufacturer’s protocol. Samples of each group were prepared in the same size (8.0 mm diameter, 4.0 mm height). Samples with flat surfaces were selected as test specimens. A solid compression plate was used for mechanical compressive strength test. The small tare load of 0.010 N was applied to ensure the same degree of initial compression. The compression ratio was preset at 65% for all samples based on the preliminary study results. When the compression ratio was over 65%, porous structures were fully compressed and compressive force increased.
significantly before breakdown of samples. The samples were compressed at a speed of 0.2 mm/min. All data were collected and analyzed with Trapezium X software (SHIMADZU).

**Water uptake degree test**
To evaluate effects of heat treatment on water uptake degree, samples of ACG-HT and ACG-NHT groups in the same size were precisely weighted (dry weight, $W_0$) and fully immersed in 10 mL of 1× Dulbecco's phosphate-buffered saline (for cell culture, pH 7.0 to 7.3, Gibco, Grand Island, NY, USA) at room temperature for 30, 60 and 120 min, respectively. The samples of each time point were taken out, carefully removed excessive water from sample surfaces and weighted (wet weight, $W_w$). The water uptake degree was calculated with the following formula: water uptake degree ($\%$) = ($W_w - W_d$) / $W_d$ × 100%

**In vitro degradation test**
Samples of ACG-HT and ACG-NHT groups in the same size were precisely weighted (dry weight, $W_0$) and immersed in 5 mL of Milli-Q water at room temperature for 3 h, 6 h, 24 h, 3 d, 5 d and 7 d, respectively. All samples were placed on a rocker to ensure samples being fully immersed in Milli-Q water throughout the test. Samples of each time point were taken out and freeze-dried at −50°C for 24 h in a vacuum dryer. The freeze-dried samples were weighted ($W_f$). The degradation rate was calculated with the following formula: degradation rate ($\%$) = ($W_f - W_d$) / $W_d$ × 100%.

**Evaluation of 3-dimensional structural changes**
Gross observation of samples in Milli-Q water was first performed at each time point during the in vitro degradation test. Standard pictures were captured to evaluate gross changes of 3-dimensional surface morphology in Milli-Q water. Then samples of each time point were taken out and freeze-dried at −50°C for 24 h in a vacuum dryer. Pictures of freeze-dried samples were captured to evaluate the changes of 3-dimensional surface morphology. Finally, the freeze-dried samples were carefully prepared at the height of 3.0 mm. SEM were performed to evaluate the changes of porous microstructure. The same protocol for SEM examination was followed in this study.

**Cell culture of human mesenchymal stromal cells (hMSCs)**
hMSCs (Cell Applications, San Diego, CA, USA) were cultured with MEM alpha (Gibco), containing 10% Fetal bovine serum (FBS; BioWest, Nuaille, France) and 1% streptomycin/penicillin (Gibco). The hMSCs were cultured in a humidified atmosphere of 5% CO$_2$ at 37°C. The culture medium was refreshed every two days. The hMSCs at passage 7 were used for cell adhesion test of ACG-HT sponge.

**Cell adhesin test on ACG-HT sponge**
To evaluate the cell adhesion on heat-treated ACG sponge, samples of ACG-HT group were prepared into discs (6.0 mm diameter, 3.0 mm height) in a sterilized condition, and placed in each well of 96-well plates. According to our previous protocol22), 20 μL of hMSCs P7 suspension at 1.25×10$^5$ cells/mL was added onto each ACG-HT sponge disc for experimental group (2,500 cells per sample). For control group, 20 μL of culture medium without cells were added onto each ACG-HT sponge disc. The 96-well plates were incubated in a humidified atmosphere of 5% CO$_2$ at 37°C for 90 min. Then 130 μL of culture medium was added to make the final volume of 150 μL. Samples were harvested on day 3, and fixed with 10% neutral buffered formalin for 30 min at 4°C. Fixed samples were dehydrated in each concentration of ethanol series (50, 70, 90, and 99.5%) for 15 min and then pre-moistened with 150 μL of culture medium for 30 min in the incubator. After that, 20 μL of hMSCs P7 cell suspension at 1.25×10$^5$ cells/mL was seeded into each well or onto ACG sponge disc. The cell number was 2,500 cells per well. Each group had 5 replicas. All 96-well plates were pre-incubated for 30 min in a humidified atmosphere of 5% CO$_2$ at 37°C. Finally, 130 μL of fresh culture medium was added to make the final volume of 150 μL per well. At each time point of day 1, 3, 5 and 7, 15 μL of Cell Counting Kit-8 solution (CCK-8, Dojindo Laboratories, Kumamoto, Japan) was added to each well. After incubation at 37°C for 90 min, 110 μL of reaction solution was transferred to each well in a new 96-well plate. The optical absorbance at 450 nm was measured using a micro-plate reader (iMark Microplate Reader, Bio-Rad). The tests were repeated 3 times with independent 96-well plates.

**Statistical analysis**
All data were expressed as mean±standard deviation (SD). Statistical significance was determined using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). Analysis of variance by Student's t-test was used to determine the significant difference. The $p$ value less than 0.05 was considered statistically significant.

**RESULTS**

**Characterization of porous microstructure**
Heat-treated 0.6%AC+2.4%G (ACG-HT) and non-heat treated 0.6%AC+2.4%G (ACG-NHT) groups both showed
similar sponge structures from superior and lateral views. Heat treatment at 125°C for 12 h in the vacuum did not change the gross surface morphology of ACG sponge (Fig. 1). SEM results showed that ACG-HT and ACG-NHT groups shared regular porous microstructure (Fig. 2a). As for porosity, ACG-HT group was 70.15% and ACG-NHT group was 68.28%. No statistical difference was observed (Fig. 2b). In this study, Feret diameter and pore area were used to better evaluate the pore size as pores were not regular circles. The pore diameter was 99.99 μm for ACG-NH group, and 102.83 μm for ACG-NHT group. The ACG-NHT group showed a slightly bigger diameter than ACG-HT group \( (p=0.0361) \) (Fig. 2c). The pore area was 4,994.47 μm² for ACG-HT group, and 5,104.84 μm² for ACG-NHT group. The pore area showed no statistical significance between the two groups (Fig. 2d).

**FTIR assay of heat treatment on ACG sponge**

The characterization of chemical structures of heat-treated ACG sponge was revealed by FTIR assay. Representative FTIR results (Fig. 3) showed that atelocollagen powder presented characteristic absorbance peaks at 1,652 cm\(^{-1}\) for amid I (C=O) stretching vibration, 1,541 cm\(^{-1}\) for amid II (N-H) bending vibration, 1,455 cm\(^{-1}\) for CH\(_2\) bending, and 1,236 cm\(^{-1}\) for C-N stretching. ACG-HT group shared similar absorbance peaks with ACG-NHT group, but had relatively lower absorbance. The absorbance peak of amid I (C=O) shifted from 1,646 cm\(^{-1}\) to 1,637 cm\(^{-1}\) after heat treatment.

**Mechanical compressive strength analysis**

Compressive strength test was performed to investigate the effect of heat treatment on mechanical compressive strength. The results showed that the mean compressive strength of samples in the porous condition between 0 and 8 N was 0.89 MPa \((n=10)\) for ACG-HT group and 0.81 MPa \((n=10)\) for ACG-NHT group (Fig. 4a). The maximum compressive strength of samples at 65% compression ratio was 17.55 MPa for ACG-HT group and 17.02 MPa for ACG-NHT group (Fig. 4b). No statistical significance was found between the two groups. Heat treatment did not affect the mechanical compressive strength.
Fig. 4 Mechanical compressive strength test of ACG sponge with heat treatment.
(a) Compressive strength of ACG sponge samples in porous condition between 0 to 8 N, (b) Maximum compressive strength of ACG sponge samples at the compression ratio of 65% (n=10, ns: no significance).

Water uptake degree analysis
The water uptake degree test was performed to evaluate the water absorption of ACG sponge. The results were shown in Fig. 5. At 30 min, the water uptake degree of ACG-HT group was 1,122.69% and ACG-NHT group was 849.23% (n=15, p<0.01). The ACG-HT group was 1.3 times higher than ACG-NHT group. At 120 min, the water uptake degree was 1,592.93% for ACG-HT group, and 1,249.25% for ACG-NHT group. Although the water uptake degree of both groups increased at 60 min and 120 min respectively, the ACG-HT group remained about 1.3 times higher than ACG-NHT group. Heat treatment slightly increased the water uptake degree of ACG sponge.

Decreased degradation rate of ACG sponge by heat treatment
The in vitro degradation rate results (Fig. 6a) showed that at 3 h, the weight loss was 46.94% for ACG-NHT group, while only 14.7% for ACG-HT group (n=5, p<0.0001). At 24 h, ACG-NHT group lost 58.69%, and ACG-HT group lost only 22.40% (n=5, p<0.0001). On day 7, ACG-NHT group had 73.24% of weight loss, and ACG-HT group lost 30.32% % (n=5, p<0.0001). Over the period of 7 days, the weight loss rate of ACG-NHT group was 2.42 times faster than that of ACG-HT group. According to the semi-logarithmic regression model (Fig. 6b), the ACG-NHT group (y=6.5178ln(x)+40.601, R²=0.9731) showed a faster in vitro degradation rate than ACG-HT group (y=3.956ln(x)+10.304, R²=0.9586).

Evaluation of 3-dimensional structural changes of ACG sponge
The effect of heat treatment on the stability of 3-dimensional macro- and micro- structures was investigated during the in vitro degradation test. It was found that heat treatment significantly stabilized the 3-dimensional surface morphology and porous microstructures (Fig. 7). From the gross observation (Figs. 7a1–a6), ACG-HT group maintained its good 3-dimensional surface morphology of cylindrical shape in Milli-Q water throughout 7 days. On the contrary, the 3-dimensional surface morphology of ACG-NHT group remained almost intact at 3 h (Fig. 7a1), but began to collapse at 6 h (Fig. 7a2). After that, the 3-dimensional surface morphology almost collapsed at 24 h and no cylindrical shape remained (Fig. 7a3). From day 3 on,
only the remnant of ACG-NHT samples and air bubbles caused by the pores were present (Figs. 7a4–a6). As for freeze-dried samples (Figs. 7b1–b6, d1–d2), the 3-dimensional surface morphology of ACG-HT group was well maintained in cylindrical shape throughout 7 days (Figs. 7b1–b6). The 3-dimensional surface morphology of ACG-NHT group was slightly changed at 3 h (Fig. 7d1), and no regular cylindrical shape was present at 6 h (Fig. 7d2). As the 3-dimensional surface morphology of ACG-NHT group completely collapsed after 24 h, no representative images of the rest samples were captured. SEM results showed that the porous microstructure of ACG-HT group was well maintained throughout 7 days (Figs. 7c1–c6). No significant changes of porous microstructure were identified between the time points 3 h and 7 d (Figs. 7c1, c6). As for ACG-NHT group, the porous microstructure did not change at 3 h and 6 h (Figs. 7e1, e2). The SEM evaluation of the rest ACG-NHT samples after 6 h was not performed due to the significantly collapsed 3-dimensional surface morphology.

**Cell adhesion and growth on ACG-HT sponge**

As for ACG-HT group without hMSCs, regular porous microstructure was well maintained in the culture medium after 3 days in the incubator and the shape of pores did not collapse (Fig. 8a). When observed at a higher magnification (15.0 kV ×500), ACG-HT sponge was degraded to some extent as small holes were present in the walls of pores (Fig. 8b). For ACG-HT group seeded with hMSCs, the hMSCs were evenly distributed and well attached onto the surface of ACG-HT sponge on day 3 (Fig. 8c). The hMSCs were shown to fully extend to attach to the surface of ACG-HT sponge at a higher magnification (15.0 kV ×500). The connections between cells were also found (Fig. 8d). The cell proliferation assay (Fig. 9) revealed that hMSCs on ACG-HT sponge had a bit lower OD value than control group at each time point of day 1, 3, 5 and 7. However, hMSCs on ACG-HT sponge showed a similar growth curve as control group over the period of 7 days. The results showed that hMSCs grew and proliferated on ACG-HT sponge normally, although the cell number was relatively lower than control group.

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**Fig. 7** Evaluation of 3-dimensional structural changes of ACG sponge after heat treatment.

Representative images shown as follows: (a1–a6) gross observation of surface morphology changes in Milli-Q water, ACG-HT group (red arrows), ACG-NHT group (green arrows). (b1–b6) gross observation of freeze-dried ACG-HT group. (c1–c6) SEM images of freeze-dried ACG-HT group. (d1–d2) gross observation of freeze-dried ACG-NHT group. (e1–e2) SEM images of freeze-dried ACG-NHT group. As the ACG-NHT samples collapsed in less than 24 h, no images after 6 h were not shown. Scale bar represent 200 μm in c1–c6, e1–e2.
DISCUSSION

Acidic solutions and chemical crosslinking reagents were used in the fabrication process of ACG sponge. The cell growth and proliferation on the ACG sponge was not compromised as reported\textsuperscript{29}. Based on the previous study, lyophilization and heat treatment were employed to fabricate porous ACG sponge in this study. The effects of heat treatment on the 3-dimensional structural stability of ACG sponge were investigated. Heat treatment was shown not to change the porous microstructure including porosity and pore size. The porous microstructure of ACG sponge was consistent with previous results\textsuperscript{22}. The porosity remained the same, while the pore size of 0.6%AC+2.4%G sponges was bigger as higher freezing temperature (−30°C vs. −80°C) was used. This had been proven that higher freezing temperature did not change the porosity, but increased pore size.

DHT treatment did not involve the use of cytotoxic reagents\textsuperscript{21,28}. A further advantage was the sterilization provided by high temperature and exposure periods\textsuperscript{21}. Strong links could also be formed between the ceramic phase and organic phase of collagen/β-TCP scaffold via DHT processing\textsuperscript{27}. The key issue of DHT treatment is the control of temperature for crosslinking reaction. Higher temperature may cause enhanced crosslinking but more denaturation of collagen, while lower temperature may lead to incomplete crosslinking but less denaturation\textsuperscript{21}. The biological performance of collagen-based scaffolds was affected by the balance between crosslinking and denaturation. Crosslinking gave rise to improved mechanical and enzymatic stability, and reduced cell binding sites. On the contrary, denaturation reduced the mechanical and enzymatic stability, but exposed more cell binding domains for cell growth. The extent of denaturation of collagen were reported to maintain under 25% after DHT treatment at temperatures up to 120°C\textsuperscript{28}. In this way, mild DHT treatment combined with other crosslinking methods may provide a useful strategy to control the crosslink density and cause a certain amount of denaturation to expose more cell binding sites which were compromised by crosslinking.

Although it was reported that heat treatment with increasing temperature improved the compressive modulus\textsuperscript{21}, heat treatment did not affect the compressive strength of ACG sponge in this study. This might be attributed to different biomaterials and duration of heat treatment. Moreover, whether heat treatment will affect the compressive strength of ACG sponge in wet condition will be investigated in further study. While DHT processing improves crosslink density, it simultaneously leads to the denaturation of collagen. Heat treatment increased the water uptake capacity of ACG sponge which might be attributed to the partial denaturation of atelocollagen. The water uptake capacity of the scaffolds shows greater importance for tissue engineering in terms of cell initial attachment, growth and differentiation. Another important feature of biomaterial scaffold is the capacity to maintain 3-dimensional morphology structures after transplantation. Heat treatment was shown to significantly reduce the in vitro degradation rate. Moreover, heat treatment effectively stabilized
the 3-dimensional surface morphology and porous microstructure throughout 7 days. These supported the usefulness of heat treatment as a crosslinking treatment for ACG sponge.

The hMSCs were shown to attach and grow well on the ACG-HT sponge in this study. Connections between cells were also found. Cell proliferation assay revealed that hMSCs on ACG-HT sponge showed a similar growth curve as control group, although hMSCs on ACG-HT sponge had a relatively lower cell number at each time point. This might be attributed to partial loss or death of cells when hMSCs were initially seeded onto ACG-HT sponge. After initial attachment, hMSCs grew and proliferated well on ACG-HT sponge as shown by the typical growth curve. These indicated that heat-treated ACG sponge was suitable for cell initial attachment, growth and proliferation. ACG sponge consisted of atelocollagen and gelatin, an irreversible hydrolyzed form of collagen. Gelatin was used to facilitate the dissolution of water-insoluble atelocollagen powder. The existence of gelatin in the ACG sponge may reduce the negative effects of crosslinking by providing more cell binding sites as mentioned above. The increased cell binding sites of ACG sponge may improve cell attachment, growth and proliferation. Moreover, this may shorten the heat treatment period, thus reducing the denaturation of atelocollagen.

CONCLUSION

Heat treatment effectively improved the 3-dimensional structural stability of ACG sponge biomaterial, while did not compromise its porous microstructure and mechanical compressive strength. No cytotoxic substances were introduced in the whole fabrication process. Heat-treated ACG sponge was suitable for cell initial attachment and growth. These results indicate that heat treatment could be an effective and safe method to crosslink and stabilize ACG sponge biomaterial for tissue engineering. The capacity of ACG sponge biomaterial to facilitate tissue regeneration will be investigated in further study.

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