Tissue Eng Regen Med (2018) 15(3):311–319
https://doi.org/10.1007/s13770-018-0117-y

Online ISSN 2212-5469
Print ISSN 1738-2696

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ORIGINAL ARTICLE

Nondestructive Assessment of Glycosaminoglycans in Engineered Cartilages Using Hexabrix-Enhanced Micro-Computed Tomography

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Received: 11 January 2018 / Revised: 12 March 2018 / Accepted: 29 March 2018 / Published online: 25 April 2018
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Abstract It is very useful to evaluate the content and 3D distribution of extracellular matrix non-destructively in tissue engineering. This study evaluated the feasibility of using micro-computed tomography (μCT) with Hexabrix to measure quantitatively sulfated glycosaminoglycans (GAGs) of engineered cartilage. Rabbit chondrocytes at passage 2 were used to produce artificial cartilages in polyglycolic acid scaffolds *in vitro*. Engineered cartilages were incubated with Hexabrix 320 for 20 min and analyzed via μCT scanning. The number of voxels in the 2D and 3D scanning images were counted to estimate the amount of sulfated GAGs. The optimal threshold value for quantification was determined by regression analysis. The 2D μCT images of an engineered cartilage showed positive correlation with the histological image of Safranin-O staining. Quantitative data obtained with the 3D μCT images of 14 engineered cartilages showed strong correlation with sulfated GAGs contents obtained by biochemical analysis ($R^2 = 0.883$, $p < 0.001$). Repeated exposure of engineered cartilages to Hexabrix 320 and μCT scanning did not significantly affect cell viability, total DNA content, or the total content of sulfated GAGs. We conclude that μCT imaging using Hexabrix 320 provides high spatial resolution and sensitivity to assess the content and 3D distribution of sulfated GAGs in engineered cartilages. It is expected to be a valuable tool to evaluate the quality of engineered cartilage for commercial development in the future.

Keywords Micro-CT (μCT) · Glycosaminoglycans (GAGs) · Hexabrix 320 · Cartilage · Tissue engineering

1 Introduction

Recent technological development has made it possible to culture cartilage-like tissues *in vitro* and to transplant them for the recovery of cartilage loss [1, 2]. However, artificial cartilages hardly mimic the histological structure and chemical composition of the natural cartilage. We found previously that engineered cartilages show various outcome in the cartilage regeneration *in vivo* depending on their physicochemical properties *in vitro* [3, 4]. It was particularly true when engineered cartilages were implanted *in vivo* at an immature state. Therefore, it is required to determine the maturity and physicochemical properties of an engineered cartilage *in vitro* before implanting it *in vivo*.

Degeneration of articular cartilage commonly involve loss of the extracellular matrix (ECM) and it is important to measure their composition for proper diagnosis and

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prognosis [5, 6]. Just like native cartilages, the information about the quantity and distribution of ECM in an engineered cartilage is very helpful in understanding its histologic feature and quality. Histological examination and biochemical measure are the most common methods to evaluate the ECM of an engineered cartilage. However, they necessitate damaging the test subject thereby limits their use in a longitudinal analysis or when screening many parameters, particularly with small amount of human subjects.

Imaging techniques, such as computed tomography (CT) and magnetic resonance imaging (MRI) are non-destructive substitutes to evaluate the quality of native and engineered cartilages. Current MRI techniques, including delayed gadolinium-enhanced MRI of cartilage, can produce 3D information of proteoglycan concentration and distribution in cartilage tissues [7–9]. However, these techniques have limitations of high price and may return false positive or false negative results in the detection of patellofemoral lesions [10, 11]. In addition, these CT and MRI techniques have not yet been applied to cultured artificial cartilage. μCT is an X-ray-based and high-resolution imaging modality that enables 3D and quantitative morphological analysis at a micrometer-level voxel resolution. It has been widely used in many animal studies, including those on osteoporosis, rheumatoid arthritis, and osteoarthritis. Nonetheless, researchers in such studies were mostly observing bone changes, while μCT imaging of the cartilage tissues has been reported only after contrast agents have become available recently [9, 11–13].

Equilibrium partitioning of an ionic contrast agent via micro-CT (μCT), a nondestructive imaging technique combining μCT with a charged X-ray-absorbing contrast agent, was shown to provide direct in situ visualization of articular cartilage morphology in rabbit femur [12]. Hexabrix, a non-toxic immune-free reagent, is a representative contrast medium with high density for μCT imaging. The mechanism of its action is known that negative charge of Hexabrix and sulfated GAGs repulse each other, and thus GAG-rich regions of articular cartilage are labeled with Hexabrix less intensively and distinguished from the surrounding area in the μCT images. Although μCT imaging using Hexabrix has been used to measure loss of sulfated GAGs during degradation of native cartilage *in vitro* and *in vivo*, there is no report about its use to measure sulfated GAGs formation in engineered cartilages [14, 15]. Engineered cartilages are commonly not fully mature and contain significantly less amount of sulfated GAGs than the native cartilage. Therefore it is not simply expected that μCT imaging with Hexabrix could be a reliable tool to assess the quality of engineered cartilages in an accurate and reproducible manner.

In this study, we tried to evaluate the feasibility and optimize the protocol of Hexabrix-enhanced μCT imaging to nondestructively assess the content and distribution of sulfated GAGs in engineered cartilage.

2 Materials and methods

2.1 Specimen preparation

Use of animals in this experiment was approved by the Institutional Animal Care and Use Committee (IACUC) of Ajou University (2016–0014). Articular cartilage explants and chondrocytes were isolated from the knee articular cartilage of 3-week-old New Zealand white rabbits. The femora were harvested, dissected free of surrounding tissues, and stored in phosphate buffered saline. Chondrocytes were isolated using collagenase, as previously described [4]. To prepare engineered cartilage tissues, rabbit chondrocytes at passage 2 were seeded on polyglycolic acid (PGA) scaffolds (Mansfield, MA, USA) at a cell density of 5.0×10^5 per ml [3]. The PGA scaffold was in a sheet form and cut into 2 mm diameter and 2 mm thickness using a biopsy punch. The cell-seeded scaffolds were incubated in a chondrogenic medium (DMEM supplemented with 10% FBS and 50 μg/ml L-ascorbic acid-2-phosphate, hereafter referred to as a basal medium), and cultivated for 7, 14, 21, and 28 days *in vitro*.

2.2 μCT scanning and analysis

All specimens were immersed in 20 μl undiluted Hexabrix 320 contrast agent (Guerbet, France) at 37 °C for 20 min, except when the incubation time of Hexabrix 320 was titrated. The excess amount of Hexabrix 320 solution was removed by gently patting the samples on a paper tissue before scanning. Samples were consistently secured in a 15 ml polypropylene conical tube (BD Falcon™, USA) such that the diaphyseal axis was aligned with the vertical axis of the μCT gantry. To avoid dehydration, the tube was sealed tightly with a sealing film. μCT scanning was performed using a Skyscan 1076 *in vivo* μCT scanner (Skyscan, Belgium). The scanning duration was 50 min per sample at an isotropic voxel size of 9 μm (40 kV, 250 mA) with a 25 μm titanium filter. The Skyscan software was used for imaging and analysis of data (Skyscan).

2.3 Optimization of Hexabrix 320 treatment

To optimize the incubation time of Hexabrix 320 contrast agent, the native cartilage tissue of New Zealand white rabbit was first used as a reference. The cartilage blocks including subchondral bone tissue (2 mm diameter and

2 mm thickness) were obtained and immersed in 20 μl of undiluted Hexabrix 320 solution (Guerbet, France) at 37 °C for 0, 10, 20, 30, 60 and 120 min for μCT analysis. After μCT scanning, the cartilage tissues were immersed in a chondrogenic medium to wash off the Hexabrix 320, the removal of which was confirmed by repeating μCT scanning and media change at a 10-min interval. The μCT images were analyzed via Skyscan software to determine signal intensities.

2.4 Optimization of the threshold value

To find a threshold value that can best represent the amount of sulfated GAGs, 14 tissue-engineered cartilage samples were analyzed via μCT scanning. For each of 14 samples, the number of voxels at increasing threshold values from 160 to 255 at a 5-value interval were obtained. Then, the entire data set was compared with the sulfated GAGs content obtained by chemical analysis by using regression analysis to calculate the regression coefficient (R^2). The threshold value with the highest R^2 was set as the optimal threshold value.

2.5 Histology and analysis

Engineered cartilages were fixed in 4% neutral buffered formaldehyde for 24 h, dehydrated, and then embedded in paraffin wax. One transversal section of 4 μm thickness from the middle part of the sample was obtained. Sections were stained for GAGs by using a 0.5% Safranin-O solution with a 0.2% aqueous solution of fast green used as a counterstain.

2.6 Cytotoxicity assay

The percentage of viable and dead cells was measured using a Live/Dead Viability/Cytotoxicity assay (Molecular Probe, Eugene, OR, USA) according to the manufacturer's instructions. Engineered cartilages were incubated in a solution containing 2 mM ethidium homodimer-1 and 4 mM calcein AM for 30 min. After washing in phosphate buffered saline, the construct was placed on a glass slide and pressed flat carefully using a glass cover. The fluorescence image was visualized using a laser-scanning confocal microscope (Zeiss LSM510 Meta; Carl Zeiss, Jena, Germany). The number of viable (green fluorescence) or dead (red fluorescence) cells was quantified by using Image-Pro Plus 4.0 image analysis software (Media Cybernetics, Silver Spring, MD, USA). The viability of cells was determined by dividing the number of green cells (viable cells) by that of the total cells (green cells + red cells).

2.7 Biochemical analysis

Engineered cartilages were dried and digested in papain solution (5 mM L-cysteine, 100 mM Na_2HPO_4, pH 6.4, 5 mM EDTA, and 125 μg/ml papain type III) at 60 °C for 24 h and then centrifuged at 12,000 g for 10 min to obtain solubilized extracts. To measure the sulfated GAGs contents, the supernatant was subjected to a colorimetric assay suing 1,9-dimethylmethylene blue. The DNA content was measured using a Qubit Fluorometer with the protocol given by the supplier (Invitrogen, Eugene, OR, USA).

2.8 Statistical analysis

A statistical analysis of the experimental data was performed with the binary classification task to determine the correlation between voxel number, sulfated GAGs contents, and DNA content of the samples. Data of the Hexabrix 320 optimization was analyzed with one-way ANOVA, and specific inter-data differences between mean values were identified using the Tukey-HSD test. $^*p < 0.05$. Each group's mean value was analyzed with both coefficients of correlation and student T test. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

3 Results

3.1 Optimization of Hexabrix 320 treatment

The incubation time of Hexabrix 320 before μCT analysis was first optimized using rabbit cartilage tissue. Samples were incubated with undiluted Hexabrix 320 and retrieved at 0, 10, 20, 30, 60, and 120 min to measure tissue density via μCT scanning (Fig. 1A). The result showed that tissue density of the engineered cartilage showed a plateau between 20 and 30 min of the Hexabrix 320 incubation, but rose again after 30 min. The phenomenon was observed reproducibly in more than 4 independent experiments ($^*p < 0.05$ vs 20 min and $^{\#}p < 0.05$ vs 30 min samples). To follow changes in tissue density of engineered cartilage samples, the tissue samples should be scanned repeatedly, and the Hexabrix 320 in the construct should be removed successfully after the first round of μCT scanning. Therefore, we tried to optimize the washing conditions of the engineered cartilage. After incubation with Hexabrix 320 for 20 min, the engineered cartilage was washed with chondrogenic medium for 30 min, with the medium changed every 10 min. In the μCT analysis, the tissue density of the construct decreased again by 86% and showed no statistical difference when compared to that of the non-treatment control (Fig. 1B). Following the optimization result, we used 20 min treatment and 30 min

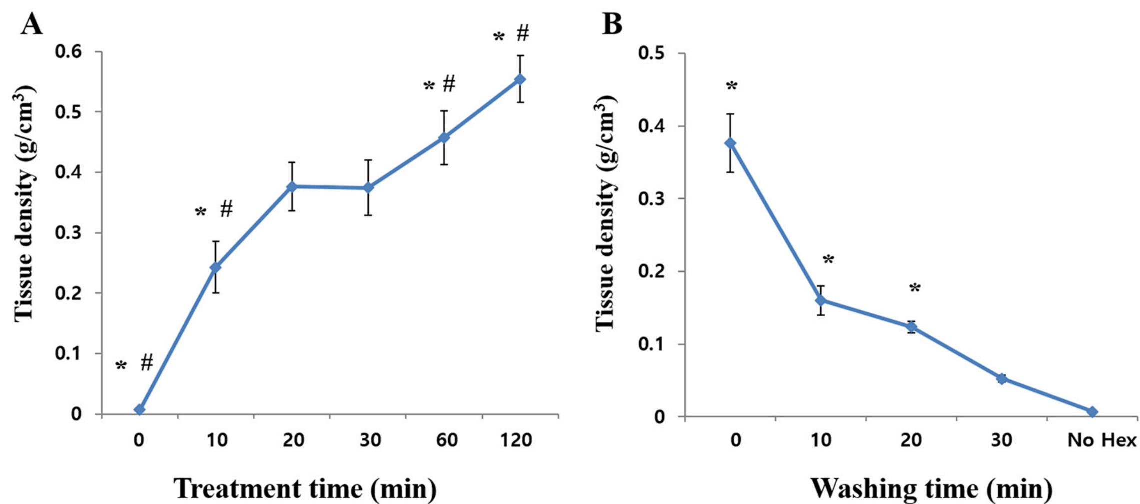

Fig. 1 Optimization of the incubation and washing time of Hexabrix 320 for engineered cartilages. **A** Tissue density of a cartilage tissue was measured using µCT analysis at indicated time points until 120 min after incubation with Hexabrix 320 (n = 4, mean ± SD). The result indicated that 20–30 min of Hexabrix 320 incubation was sufficient to reach an equilibration ($^*p < 0.05$ vs 20 min and $^\#p < 0.05$ vs 30 min samples). **B** After incubation with Hexabrix 320 for 20 min, the cartilage tissue was incubated with washing medium until 30 min. The tissue density was measured using µCT at 0, 10, 20 and 30 min after washing with the washing medium changed every time. Decline of tissue density by 86% was observed at 30 min ($^*p < 0.05$ vs 30 min samples). No Hex represents a tissue intensity without Hexabrix treatment

washing for Hexabrix 320 treatment in the subsequent experiments.

3.2 Comparison between sulfated GAGs distribution and µCT images

Rabbit chondrocytes were seeded in a PGA scaffold and cultured for 2 weeks *in vitro* to construct engineered cartilages of 2 mm in diameter and 2 mm in thickness. The samples were first subjected to µCT scanning for 20 min and then tissue sections were prepared for Safranin-O staining to observe the accumulation of sulfated GAGs (Fig. 2). When the µCT images were compared with histological images, the areas with strong Safranin-O stains (large sulfated GAG accumulation) showed relatively dark signals in the black-and-white images and low attenuations in color images of the µCT analysis (ROI 1). In contrast, the ROI 2 with less or no Safranin-O stains showed brighter signals in black-and-white images and high attenuations in color images of the µCT analysis. Thus, it was confirmed that there is a qualitative correlation between Safranin-O staining images and µCT images in terms of overall distribution and intensity of signals (Fig. 2).

3.3 Quantitative measurement of sulfated GAGs content in the 3D µCT images

Next, we evaluated whether µCT data obtained using Hexabrix 320 correlated quantitatively well with the total content of the sulfated GAGs in the engineered cartilage. Fourteen engineered cartilages cultured for 2, 3 or 4 weeks were subjected to µCT scanning, and the 3D images obtained were analyzed to determine the cartilage area and quantitates the volume by the number of voxels. The outcome of the analysis varied highly depending on the threshold values. The critical low limit of threshold was first fixed at 66 because it was the best to distinguish the engineered cartilage from the surrounding air space. To optimize the critical upper limit of the threshold, the numbers of voxels from the 3D images of all 14 samples were calculated along with the threshold values from 160 to 235, using an interval of 5, and the regression coefficient (R^2) was determined between the calculated voxel numbers at each threshold value and the total content of sulfated GAGs (Fig. 3A). The results revealed that the highest regression coefficient was 0.883 at the threshold value of 200. The result of regression analysis for the 14 individual samples at the optimized threshold value is shown in Fig. 3B ($R^2 = 0.883$, $p < 0.001$). The representative histological sections and their corresponding 2D µCT images of engineered cartilages at 2, 3 and 4 weeks were shown in Fig. 4A–F. In the 3D µCT images the percentage of cartilaginous area at the threshold value of 200 was calculated to be 67.0, 88.1 and 97.7%, respectively (Fig. 4G–I). In the 3D images, the outer gray color indicates a highly cartilaginous area with a larger amount of sulfated GAGs deposition while the purple color in the inner area indicates relatively poor cartilage formation.

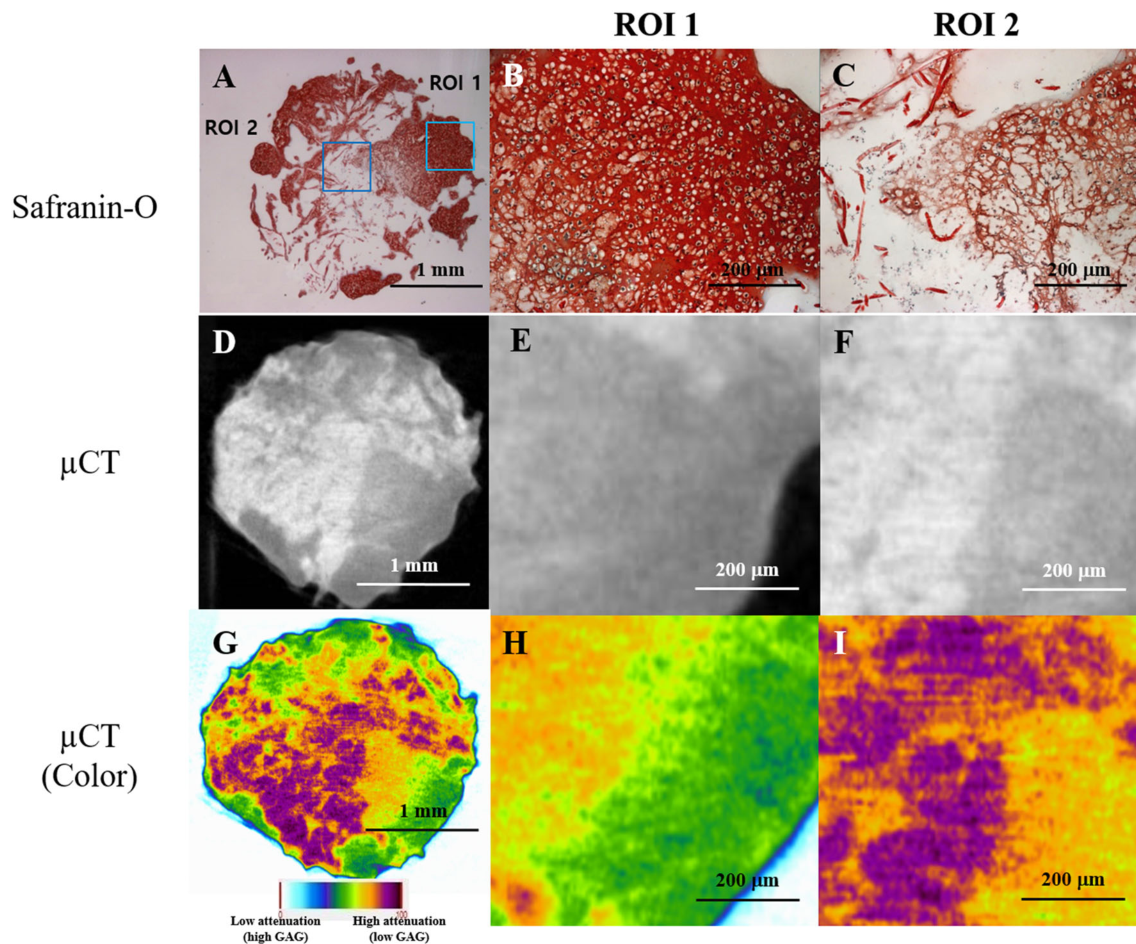

Fig. 2 Representative histological sections and their corresponding μCT images of an engineered cartilage. **A–C** Histological sections were stained with Safranin-O to observe sulfated GAGs in the engineered cartilages. Two ROIs (blue squares) were presented at higher magnification. **D–F** Representative coronal sections of undigested μCT image were shown for the whole construct and two ROIs. **G–I** Color images represent attenuation patterns increasing from cyan to dark purple colors. Scale bars = 1 mm for A, D and G. Scale bars = 200 μm for the other images

3.4 Effect of Hexabrix 320 and μCT radiation on engineered cartilage

The cytotoxicity of Hexabrix 320 and μCT radiation on the engineered cartilage was examined 2 weeks after culture. At 1 and 24 h after the Hexabrix 320 treatment and the μCT scan, samples were subjected to a live/dead cytotoxicity assay (Fig. 5A, B). At both 1 and 24 h, most of the cells were alive, as shown by green fluorescence (a), while some of the cells were dead shown in red (b). Some of the PGA fibers were also observed to be remaining (c). Therefore, the Hexabrix 320 and μCT scanning process appeared to have no adverse effects on the cell viability of the engineered cartilage. The changes in the total content of the sulfated GAGs and DNA of the engineered cartilages were then examined over 4 weeks, during which the engineered cartilages were subjected to Hexabrix 320 treatment and μCT scanning at 2 and 3 weeks. When compared with the untreated control group (no Hexabrix 320 treatment or μCT scan), the experimental group showed no statistically significant difference in both the contents of the sulfated GAGs and the DNA (Fig. 5C, D). These results suggest that Hexabrix 320 and μCT radiation had little cytotoxicity and did not significantly affect cartilage tissue formation, indicating that they might be used as a non-destructive evaluation method for tissue engineered constructs of clinical purpose.

4 Discussion

This study showed that μCT analysis using Hexabrix 320 successfully measured the absolute volume, relative amount, and distribution of sulfated GAGs in tissue-engineered cartilages. In particular, there was a strong positive correlation between the content of the sulfated GAGs of the

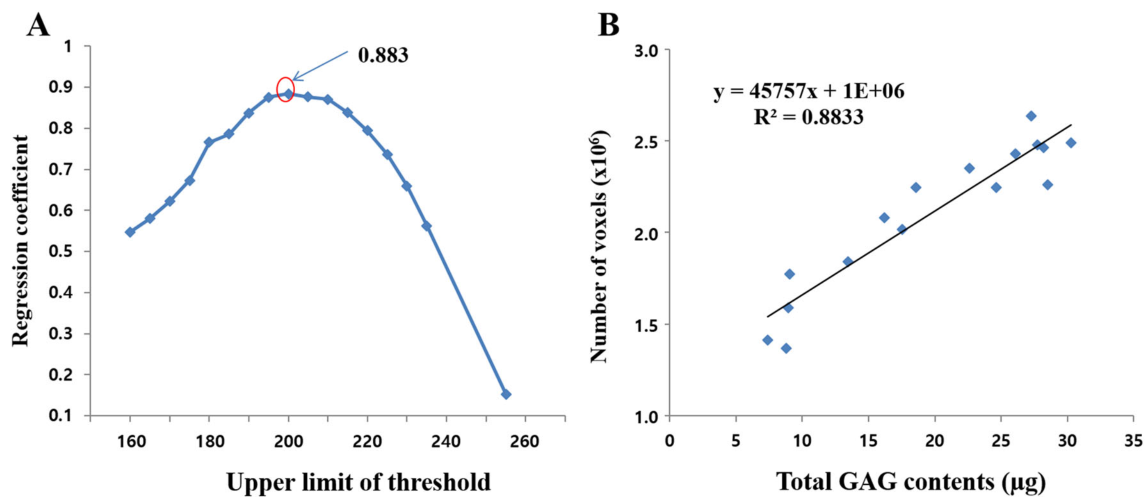

Fig. 3 Quantitative analysis of the µCT images. **A** The threshold values were screened from 160 to 255 with the interval of 5, and the regression coefficient (R^2) was calculated between the GAGs contents and the numbers of voxels in the µCT data. The regression coefficient was the most highest ($R^2 = 0.883$), when the threshold value was 66–200. **B** Correlation between sulfated GAGs contents (µg) and the numbers of voxels (counts) in the µCT data was presented for 14 individual engineered cartilages measured at 7, 14, 21 and 28 days after culture. The result showed very good correlation between two data ($R^2 = 0.883$, $p < 0.001$)

engineered cartilages examined via biochemical assay and the number of voxels of the 3D µCT image. Previously, µCT has been used to examine an engineered cartilage only without Hexabrix and did not provide specific or accurate information regarding the content and distribution of sulfated GAGs [9]. To our knowledge, this is the first study to prove that Hexabrix can be used to specifically measure the relative amount of sulfated GAGs and quantitatively evaluate the quality of engineered cartilage via µCT images.

Current imaging techniques to analyze sulfated GAGs using the gadolinium-enhanced MRI or µCT measure the negative electrical charges of sulfated GAGs. Gadolinium-enhanced MRI techniques have been used to monitor sulfated GAGs content in a cartilage tissue *in vitro* and in clinics [16, 17]; however, the resolution of current clinical (≥ 300 µm) and high-powered research (≥ 25 µm in-plane) MRI devices may limit their application in small-animal models and for engineered cartilages. The µCT using gadolinium-based contrast agents has also shown the potential to monitor sulfated GAGs content in cartilage explants [18]. Optical coherence tomography [19] and Fourier transform infrared spectroscopy [20] may also be useful in the noninvasive monitoring of cartilage ECM changes, potentially providing information on the collagen and GAG contents. Although the latter methods provide high-resolution planar images, quantitative analysis of 3D distributions and morphology are not currently possible. In contrast, µCT scanning using Hexabrix 320 has enabled measurement of both 3D distribution of sulfated GAGs and quantitation of their relative amount in a cartilage construct. Thus, this technique could be a valuable tool to evaluate tissue engineered cartilages not only in the technology development but also in the quality control for commercial development.

In this study, normal cartilage tissues were first used instead of engineered cartilage to optimize the incubation time of Hexabrix. Normal cartilage is generally more compact in structure than engineered cartilage and expected to uptake Hexabrix more slowly. Therefore, it was assumed that the optimal time period of Hexabrix infiltration in normal cartilage would be sufficient for that in engineered cartilage [15]. Infiltration of Hexabrix 320 reached saturation between 20 and 30 min and thereafter increased gradually again. We speculated that the tissue density was first equilibrated in 20 min, while the second increase after 30 min was caused by forced uptake of Hexabrix 320 due to the concentration gradient. Therefore, a treatment of Hexabrix 320 for 20–30 min would optimally represent the distribution and relative amount of sulfated GAGs while a longer treatment time might produce inaccurate data, showing much lower sulfated GAGs contents than actual values. Over 86% of the Hexabrix labeled in the cartilage tissue was removed after 30 min of washing, and there were no positive signals obtained in the µCT analysis (data not shown). It appears that Hexabrix 320 was taken up reversibly to the engineered cartilage and then successfully removed after washing for 30 min. Taken together with the result showing the low cytotoxicity of Hexabrix 320, this result suggests that our washing protocol after µCT scanning is sufficient to remove Hexabrix 320 without causing any toxic effects on engineered cartilages.

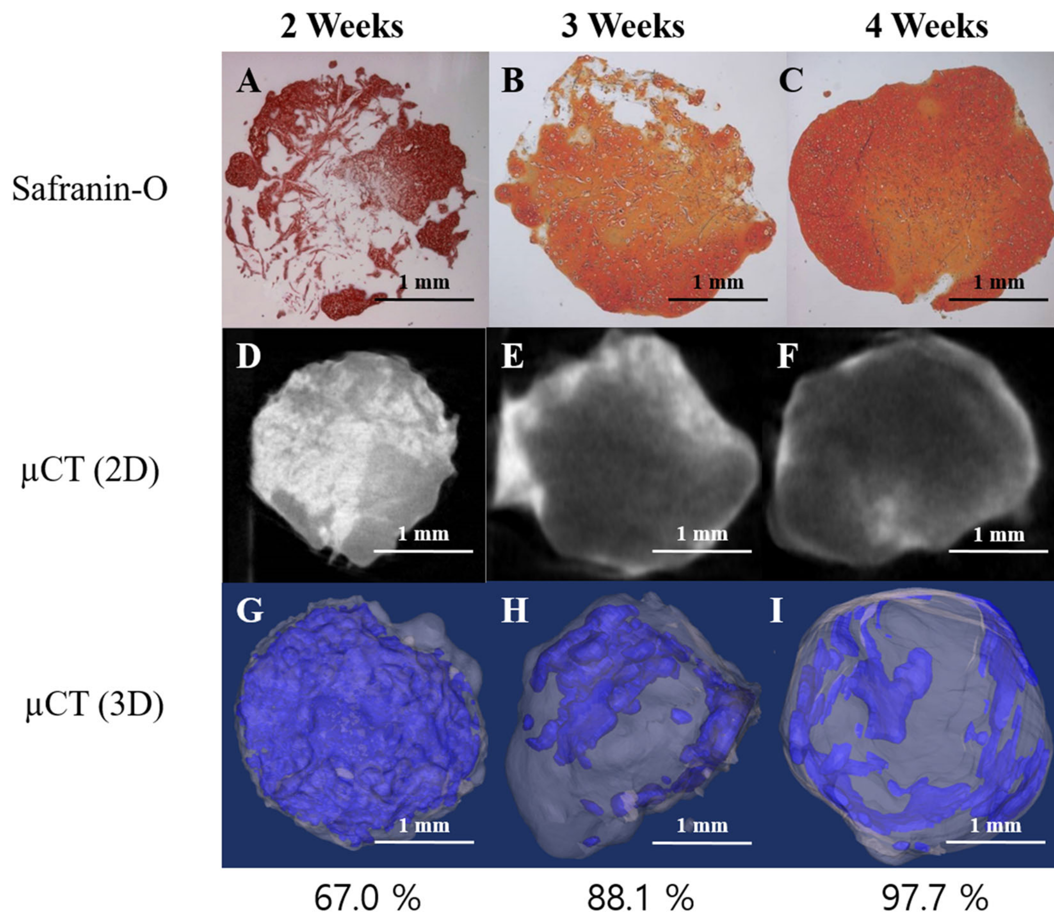

Fig. 4 A representative analysis of 3D μCT images. **A**–**C** Engineered cartilages were cultured *in vitro* for 2, 3 and 4 weeks and representative histological sections are shown after Safranin-O staining. **D**–**F** 2D μCT images using Hexabrix 320 corresponding to the histological sections are shown. **G**–**I** 3D μCT images of the engineered cartilages showing GAG distribution obtained using the optimized threshold value. The grey color in the peripheral region indicates more differentiated area with less Hexabrix 320 incorporation, while the purple inner region indicates less differentiated area with more Hexabrix 320. Percentage of differentiated area with significant GAGs accumulation was calculated by the ratio of the gray area in the 3D images and shown to be 67.0, 88.1 and 97.7% for constructs at 2, 3 and 4 weeks, respectively. Scale bars = 1 mm

When the critical lower limit threshold values were incrementally changed by 5, the voxel numbers showed no significant differences, varying less than 0.5%. Therefore, we chose a value of 66, which showed a clear discrimination of samples from the surrounding background. In contrast, when the upper limit threshold values were incrementally changed by 5, large changes in the voxel numbers of more than 5% were observed, significantly influencing the analysis outcome. In this study, the optimal upper limit threshold value was found to be around 200 for μCT analysis of tissue engineered cartilages with Hexabrix 320 staining. We speculate that this value would be different when other tissue samples or other contrast agents are applied for analysis.

In conclusion, this study found that μCT imaging using Hexabrix 320 provides high spatial resolution and sensitivity to assess the 3D distribution of sulfated GAGs in engineered cartilage. In particular, it enabled quantitation of relative sulfated GAGs content in a cartilage tissue with reliable accuracy and without scarifying samples. There-

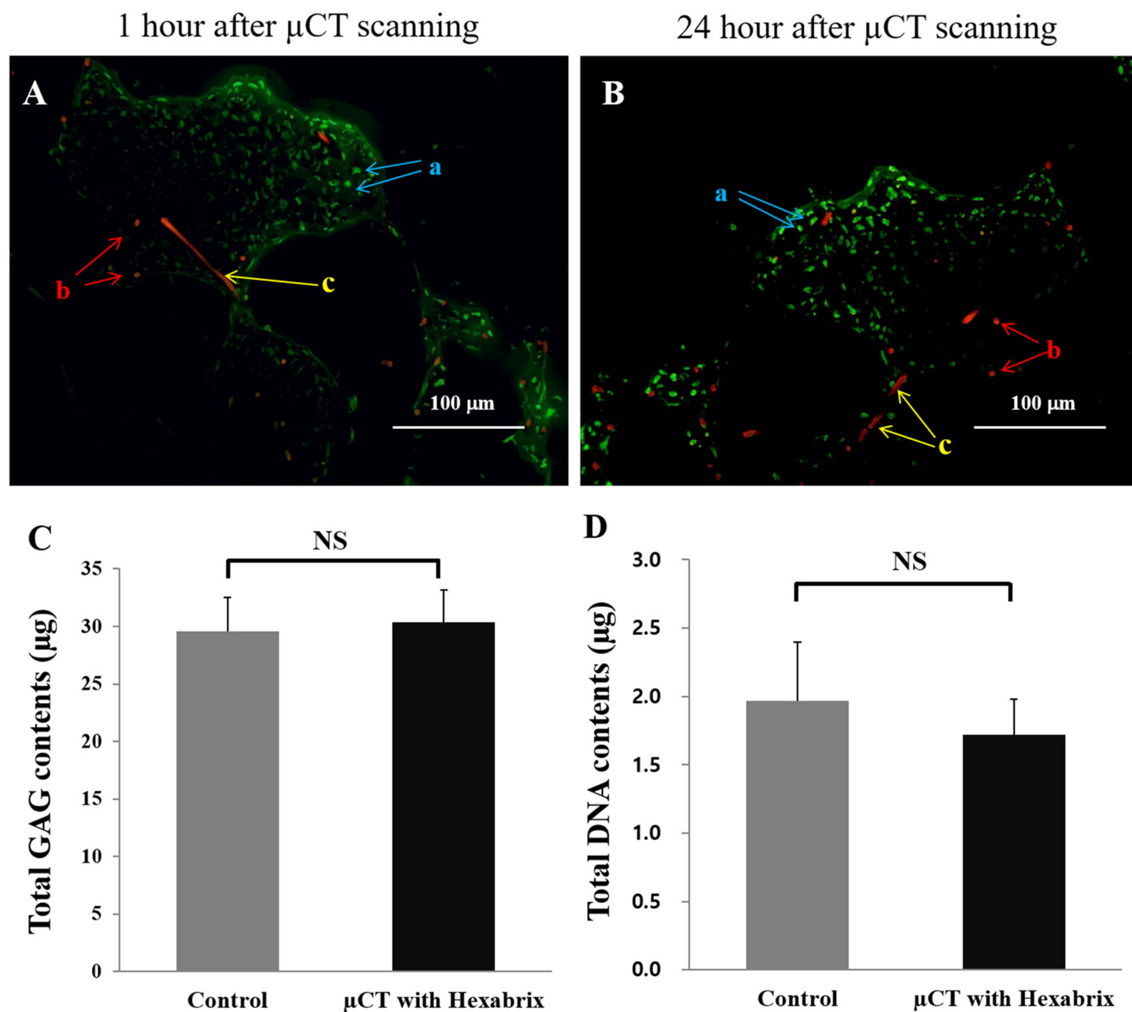

Fig. 5 The cytotoxicity of Hexabrix 320 and µCT irradiation on engineered cartilages. **A**, **B** Engineered cartilages were cultured for 2 weeks *in vitro* and incubated with Hexabrix 320 followed by µCT scan. Then, samples were washed in phosphate buffered saline and analyzed for cell viability after 1 and 24 h using a Live/Dead Cytotoxicity assay kit. In the images, a, b and c indicate live cells in green color, dead cells in red color, and PGA fibers, respectively. Scale bars = 100 µm. **C**, **D** Engineered cartilages were cultured for 4 weeks, during which they were untreated (control group) or treated with Hexabrix 320 and µCT scan at 2 and 3 weeks. Total contents of sulfated GAGs and DNA were compared between two groups and showed no significant statistical difference after 2 or 3 weeks (n = 6)

fore, the presented µCT technique will benefit developing of engineering cartilage constructs *in vitro* and their therapeutic applications *in vivo*.

Acknowledgement This research was supported by a grant of the Korea Health Technology R&D Project funded by the Ministry of Health & Welfare, Republic of Korea (HI17C2191).

Compliance with ethical standards

Conflict of interest The authors indicate no potential conflicts of interest.

Ethical statement Use of animals in this experiment was approved by the Institutional Animal Care and Use Committee (IACUC) of Ajou University (2016-0014).

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