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Observation of sGAG content of human hip joint cartilage in different old age groups based on EPIC micro-CT

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Abstract

Objectives—To observe the age-related changes of sulfated glycosaminoglycan (sGAG) content of hip joint cartilage of elderly people based on Equilibrium Partitioning of an Ionic Contrast Agent (EPIC) micro-CT.

Methods—Seventy human hip cartilage–bone samples were collected from hip-fracture patients (ages 51–96) and divided into five groups (10 years in an age group). They were first immersed in 20% concentration of the contrast agent Meglumine Diatrizoate (MD) for 6 h at 37 °C, and then scanned by micro-CT. Following scanning, samples were stained for sGAG with toluidine blue. The X-ray attenuation and sGAG optical density were calculated by image processing. The correlation between X-ray attenuation and sGAG optical density was then analyzed.

Results—The X-ray mean attenuation of the cartilage increased by 18.81% from the 50–80 age groups ($p < 0.01$), but decreased by 7.15% in the 90 age group compared to the 80 age group. The X-ray mean attenuation of the superficial layer and middle layer increased by 31.60 % and 44.68% from the 50–80 age groups, respectively ($p < 0.01$), but reduced by 4.67% and 6.05% separately in the 90 age group. However, the deep layer showed no significant change with aging. The sGAG optical density showed a linear correlation ($r = -0.91$, $p < 0.01$) with the X-ray attenuation.

Conclusion—The sGAG content of hip joint cartilage varied with aging in elderly people. The changes in superficial layer and middle layer were more evident than deep layer.

Keywords

Elderly people; EPIC micro-CT; hip joint cartilage; layered structure; sGAG

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Declaration of interest

The authors have no conflicts of interest to disclose.

Introduction

As a kind of connective tissue, articular cartilage (AC) covers the surfaces of bones within synovial joints, and its primary function is to withstand weight, distribute load, absorb shock and diminish friction. Under normal physiological conditions, these functional properties can persist for many decades. However, factors such as aging and overloading may lead to degeneration of AC and even result in osteoarthritis (OA), one of the most common joint diseases. Patients with OA suffer from joint inflammation and pains, which severely reduce their quality of life. Unfortunately, AC has a low level of metabolic activity and poor regeneration capacities, because it lacks blood vessels, lymphatic vessels and nerves compared with other tissues such as bone and muscle. Thus the nutrients are transported to the chondrocytes by diffusion from the synovial fluid (4–5).

AC is mainly composed of proteoglycans (PGs) (5–10% wet weight), collagen (10–20% wet weight) and water (60–80%) (1–3). PGs primarily exist as aggregates formed by a high density of negatively charged sGAG. The interaction between sGAG and ionic interstitial fluid is responsible for structural integrity of the AC and its mechanical properties (1–3). The sGAG content changes substantially during AC development (6), degeneration and repair (7). Previous studies have suggested that loss in sGAG content precedes other components during AC degeneration, and can be regarded as a monitor of the early AC degeneration (8–11). However, the normal aging process could also lead to the depletion of sGAG in AC (2). Therefore, it is necessary to differentiate between sGAG changes which occur in cartilage due to normal aging and those which are caused by OA in an early diagnosis of OA.

Based on histochemical and biochemical evaluation techniques, Stockwell (12,13), Elliott et al. (14) evaluated changes in content and distribution of sGAG with aging. However, these traditional methods are destructive and time consuming. Nondestructive techniques, including ultrasound (US) and magnetic resonance imaging (MRI), have also been used to study sGAG content in AC (15–19). US, a less expensive imaging technique, has been given much attention in the research of sGAG loss during AC degeneration. The results however are easily affected by the surrounding environment (such as temperature, medium density). Thus acoustic parameters cannot accurately reflect sGAG changes in AC (15–17). MRI, together with delayed gadolinium-enhanced MRI of cartilage (dGEMRIC), can provide information about 3D images of the cartilage, sGAG content *in vitro* and in clinical applications. However, its high cost, relative long imaging time and low resolution limit its application in small specimens in the lab (18,19).

Micro-CT, a 3D X-ray-based imaging device, can provide quantitative morphologic analyses at a micrometer-level voxel resolution over shorter acquisition times compared to MRI. It has become the “gold standard” for bone micro-structural analysis, but has not been widely used for soft tissue because of the low X-ray absorption. Recently, EPIC micro-CT, which combines micro-CT with enhanced X-ray-absorbing contrast agent, has been proposed to compensate for the low X-ray absorption of AC. The main principle of EPIC micro-CT is the balance between the negatively charged sGAG and the ionic contrast agent, Roemer et al. (9) was the first to propose applying the charged X-ray-absorbing contrast

agent into the cartilage scanned by micro-CT. Cockman and Bansal et al. (20,21) have proved the validity of quantitatively analyzing the sGAG content based on micro-CT with various contrast agents such as Gadolinium, Hexabrix and Ioxaglate. Palmer and Xie (10,22,23) have demonstrated that EPIC micro-CT is a powerful, high precision approach to quantitatively assess cartilage composition and 3D morphology for studies of cartilage degeneration and repair.

The aim of this study was to detect the variation of sGAG content of hip joint cartilage in different old age groups based on contrast-agent Meglumine Diatrizoate (MD) enhanced micro-CT, and explore the following research issues: ① the X-ray attenuation of the cartilage in different age groups; ② the layered X-ray attenuation based on the layered structure of the cartilage; ③ the quantitative relationship between X-ray attenuation and sGAG optical density obtained from histological analysis.

Materials and methods

Specimen preparation

Seventy hip-fracture patients between ages 51 and 96 were used in this study. The human femoral heads obtained in operation were divided into five groups (10 years in an age group) and stored at -20°C for further experiments. Prior research indicated that one freeze-thaw cycle could produce minimal deterioration of biochemical and biomechanical properties of articular cartilage (24).

In this study, people with a history of prolonged immobilization or other diseases related to the hip joints, metabolic or endocrine abnormality were excluded. The sex distribution for each age category is approximately 1:1.

In order to compare sGAG content in the same region of femoral heads, all the specimens were harvested from the weight-bearing location of the femoral head (the harvested site of samples is shown in Figure 1). The surfaces of the AC were carefully examined visually, and only areas where the AC was intact were selected for the experiment prior to cutting specimens. The diameter of each specimen was about 12 mm. For this study, ethical approval was given by the Medical Ethical Review Committee of Beihang University.

Micro-CT scanning experiment

In the experiment, all the samples were placed in Phosphate Buffered Saline (PBS) at room temperature 22°C to thaw for 2 h and immersed in the contrast agent (20% MD/80% PBS, Xudong Haipu, Shanghai, China) for 6 h at 37°C . The contrast agent concentration and the incubation time used here have been explored and demonstrated in our previous study (25). After 6 h, the contrast agent saturated the articular cartilage, and the X-ray attenuation showed a negative linear correlation with the GAG optical density. After removal from the contrast agent, samples were patted dry and immediately scanned by micro-CT (SkyScan1076, Bruker Micro-CT NV, Aartselaar, Belgium) at 50 kVp, 170 mA, 200-ms integration time, Al 0.5 mm filtering and a voxel size of $18\text{ }\mu\text{m}$. Each sample scan was 11 min in duration.

During the scan, each sample was placed in a horizon position and consistently secured within a scanning tube so that its axis was aligned with the axis of the micro-CT scanning tube. To reduce dehydration, the tube contained a small amount of PBS at the both sides but did not make contact with the samples. When the scan was finished, the samples were immersed in PBS at 4 °C overnight to get rid of the contrast agent after the scan, and then stored for further experiments.

CT parameters calculation

The X-ray attenuation of human hip joint cartilage was calculated using image processing methods. The X-ray attenuation here referred to the attenuation of the entire cartilage and layered cartilage region.

The images acquired from micro-CT were reconstructed with NRecon software (Bruker Micro-CT NV, Aartselaar, Belgium). Then, the transaxial reconstructed images were imported into Matlab 7.6 (MathWorks, Natick, MA). Afterward, the cartilage region was segmented from the subchondral bone by the region growth methods (21). During the segmentation process, a seed point was placed in the cartilage region and a contour was grown around the seed point until the cartilage region was segmented. As shown in Figure 2, the reconstructed CT image of cartilage–bone sample with pseudo-color representing the range of X-ray attenuation was generated by DataViewer (Bruker Micro-CT NV, Aartselaar, Belgium). There were noticeable X-ray attenuation distinctions between the interface of the cartilage and the bone, allowing the cartilage region to be easily identified against the bone. The X-ray attenuation in this study was reported in Hounsfield Units.

AC has a layered structure and can be divided into four layers: the superficial layer (10–20%), middle layer (40–60%), deep layer (30%) and calcified layer (5%) (26). The main components of the calcified layer are calcium salts deposition without sGAG (27). The X-ray mean attenuation of the calcified layer was not considered in our study, and each proportion of non-calcified layers was chosen as the following proportion: superficial layer (15%), middle layer (55%) and deep layer (30%).

Histological analysis

After the micro-CT scan, all the samples were fixed in 10% neutral buffered formalin solution overnight, and decalcified in 10% EDTA solution. Dehydrated samples were then embedded in paraffin. For each sample, 10 transaxial sections were cut at 5 µm thicknesses, and stained for sGAG with toluidine blue. In optical micrographs, digital images of each section were captured at 0.5 µm resolution under controlled ambient light; a blue content parameter (B_c) representing the sGAG optical density was determined by non-linear weighing of the fractional intensity of the blue component (23,28). The blue content of a region was calculated as the average B_c value for all pixels in that region. The histological section image of hip joint cartilage is shown in Figure 3A.

To make semi-quantitative calculations of the sGAG optical density, the images were first imported into Matlab 7.6 (MathWorks, Natick, MA). The cartilage region was segmented using the region growth method (21). The calcified layer of the histological section images

was not considered for their corresponding CT images. The non-calcified layers were outlined and separated from the deep layer by the tidemark, which was also the boundary between the deep layer and calcified layer (26,27), and distributed by the same proportion in CT images. The sGAG optical density of the entire cartilage and layered cartilage region was then calculated. Figure 3B illustrates the image of non-calcified layer cartilage. Figure 3C shows the percentage image of non-calcified layer cartilage.

Statistical analysis

All data in this study were expressed as mean $\pm$ SD. The X-ray attenuation and sGAG optical density in each age group were evaluated *via* one-way repeated measures analysis of variance (ANOVA) with LSD test for *post hoc* analysis. Additionally, general linear models (two-way repeated measures of ANOVA) were used to evaluate effects of age, layer and age-by-layer on the layered X-ray attenuation and layered sGAG optical density. The relationship between these two parameters was examined *via* linear regression analysis. Statistical significance was set at $p < 0.05$ (SPSS Statistic 20, IBM, Armonk, NY).

Results

X-ray attenuation studies

Figure 4 illustrates the X-ray mean attenuation of the whole cartilage in different age groups. As shown in Figure 4, the X-ray mean attenuation of the entire cartilage had a rising tendency from the 50 age group to the 80 age group (from $1292.35 \pm 84.98\text{HU}$ to $1535.49 \pm 180.76\text{HU}$, increasing about 18.81%, $p < 0.01$), but there was a slight down trend in the 90 age group compared to the 80 age group (from $1535.49 \pm 180.76\text{HU}$ to $1425.66 \pm 90.91\text{HU}$, decreasing about 7.15%).

Figure 5 represents the layered X-ray mean attenuation in different age groups. According to Figure 5, the X-ray mean attenuation of the superficial layer, middle layer and deep layer of the cartilage in the each group declined successively in each age group. With increasing age, there were noticeable increases in the X-ray mean attenuation of the superficial layer and middle layer, but no change in the deep layer was evident. From the 50 age group to the 80 age group, the X-ray mean attenuation of the superficial layer and middle layer had risen (from $1457.20 \pm 127.75\text{HU}$ to $1917.71 \pm 173.44\text{HU}$, increasing about 31.60% and $1247.18 \pm 72.03\text{HU}$ to $1804.42 \pm 203.82\text{HU}$, increasing about 44.68% respectively, $p < 0.01$). However, in line with the X-ray mean attenuation of the whole cartilage in the 90 age group, the superficial layer and middle layer declined slightly when compared to the 80 age group (from $1917.71 \pm 173.44\text{HU}$ to $1828.12 \pm 128.87\text{HU}$, decreasing about 4.67%, and from $1804.42 \pm 203.82\text{HU}$ to $1695.22 \pm 127.88\text{HU}$, decreasing about 6.05% separately). The results of two-way ANOVA illustrated that both the age and layer have significant effects on the layered X-ray attenuation, but the effect of age is more significant than the layer (age: $F(4, 210) = 49.20$, $p < 0.01$; layer: $F(2, 210) = 25.47$, $p < 0.05$; age-layer: $F(8, 210) = 36.99$, $p < 0.01$).

sGAG optical density studies

Figure 6 indicates the sGAG optical density in different age groups. Based on Figure 6, from the 50 age group to the 80 age group, the average sGAG optical density of the entire cartilage decreased from 88.08 ± 4.63 to 69.03 ± 12.61 ($p < 0.01$), while it increased from 69.03 ± 12.61 to 73.49 ± 8.28 in the 90 age.

Figure 7 displays the layered sGAG optical density in different age groups. As shown in Figure 7, the average sGAG optical density of the superficial layer, middle layer and deep layer of the cartilage in each age group increased successively. The superficial layer and middle layer showed obvious decreases with aging, while the deep layer did not. Furthermore, the average sGAG optical density declined in the superficial layer and middle layer from the 50 age group to the 80 age group (from 77.97 ± 6.00 to 45.81 ± 7.31 , and 85.90 ± 3.77 to 51.14 ± 7.92 separately, $p < 0.01$), whereas they rose in the 90 age group (from 45.81 ± 7.31 to 48.83 ± 8.82 , and from 51.14 ± 7.92 to 56.44 ± 9.02 , respectively). The results of two-way ANOVA showed that the effect of age is more significant than the layer (age: $F(4, 210) = 15.67$, $p < 0.01$; layer: $F(2, 210) = 4.26$, $p < 0.05$; age-layer: $F(8, 210) = 10.69$, $p < 0.01$).

Figure 8 shows the relationship between the sGAG optical density and the X-ray mean attenuation. The results shows that the X-ray mean attenuation was linearly and inversely related to the sGAG optical density ($r = -0.91$, $p < 0.01$).

Discussion

This work mainly observes the alteration of sGAG content with aging based on contrast agent MD enhanced micro-CT and builds the relationship between sGAG optical density and X-ray attenuation.

In this study, the 50 age group to the 80 age group displayed an increase in the X-ray mean attenuation of the cartilage (Figure 4), and a decrease in the average sGAG optical density (Figure 6), suggesting that the negatively charged sGAG content decreases with aging. Articular cartilage lacks blood vessels, and nutrients in the synovial fluid are transported to chondrocytes mainly by a pumping action generated by cartilage compression (29). The synovial fluid and pumping action decrease with aging, and less nutrients are transported to chondrocytes (30). In this case inadequate nutrition leads to the degradation of chondrocytes and their capacity to synthesize PG declines, and the content of sGAG, the subunits of PG, also reduces (31). Roughley, Plaas, et al. (32,33) also mention that age could produce changes in content and structure of PGs due to the decline of chondrocyte activity. Once negatively charged sGAG is lost from the matrix, the increased permeability of the matrix leads the contrast agent ions to diffuse into the inner cartilage based on the charge distribution theory. Thus, X-ray absorption increases and the attenuation of cartilage rises.

The gradual variation tendency of the layered cartilage within each group indicates that the sGAG content is different in each layer of cartilage, which is lowest in the superficial layer and highest in the deep layer (Figures 5 and 7). This result is consistent with previous studies observing the sGAG content in different layers of human cartilage (34–36).

Moreover, the variation tendency of X-ray attenuation in the superficial layer and middle layer is more obvious than that in the deep layer; this result indicates that sGAG content in the superficial layer and middle layer is lost more easily than the deep layer with aging. These results could arise from the diverse synthetic capacity of chondrocytes in different layers. Karvonen and Maroudas et al. (37,38) indicate that the synthetic activity of chondrocytes in all articular cartilage layers declines with age. However, the results in our study show that synthetic activity in superficial layer and middle layer declines with age, but the change in deep layer is not obvious. Further research is needed to explore this issue.

The upward trend of the X-ray mean attenuation of hip joint cartilage is not present in the 90 age group. The X-ray mean attenuation in the 90 age group decreased compared to the 80 age group, illustrating that the sGAG content in the 90 age group increased compared with the 80 age group. We speculate the abnormal variations are caused by their better health status and chondrocyte synthetic activity maintenance, which may indicate their longevity in turn. Additionally, there are only five cartilage–bone samples in the 90 age group, so more samples are needed to verify the speculation in our following study.

There were still some limitations in our studies. First, the age cohort is mainly focused on the older individuals, not including the young age group. In future studies, we need to collect samples from a younger age group to enhance the efficiency of the comparative study. Secondly, although the layered X-ray attenuation was calculated and analyzed on the basis of cartilage layer structure in this study, there are also some questions that need to be solved regarding standard cartilage stratification. Present studies about the stratification provide only the range of each layer, especially the superficial layer and middle layer. According to histological section images, the tide mark, the sign of cartilage maturation, moves forward and duplicates with age, thus the proportion of calcified layer and deep layer also varies with age (39,40). The limitation of EPIC micro-CT makes it difficult to clarify the alteration of the calcified layer and tide mark. Therefore, the age-related changes of the calcified layer and tide mark should be considered in future studies, which is significant to establish the protocols for cartilage stratification.

The quantitative relationship between X-ray attenuation and sGAG optical density showed that there was a negative correlation between them (Figure 8), in accordance with the results in previous studies (10,22,23), demonstrating that the X-ray attenuation could not only reflect the variations and distributions of sGAG content with age, but also semi-quantitatively assess sGAG content. The results suggested that EPIC micro-CT can be applied not only to explore the variation of sGAG content in different old age groups, but also to assess sGAG distribution in articular cartilage with high resolution. EPIC micro-CT shows the ability to nondestructively evaluate the alteration and distribution of sGAG content of human hip joint cartilage compared with histological analysis.

In conclusion, age, apart from OA, is another factor that would influence the loss of sGAG content in different layers of cartilage. Future studies about the pathology of OA should distinguish the sGAG changes which occur in cartilage as a result of aging alone from those which are caused by the cartilage degenerative processes during OA development.

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Figure 1.
The harvested site of the cartilage–bone samples.

Figure 2.
(A) The reconstructed CT image of cartilage–bone sample. (B) The cartilage region segmented from bone.

Figure 3.
(A) The histological section image of hip joint cartilage. (B) The image of non-calcified layer cartilage. (C) The percentage image of non-calcified layer cartilage.

Figure 4.

The X-ray mean attenuation in different age groups (mean $\pm$ SD, # p <0.05 versus 50 age groups, * p <0.01 versus 50 age groups).

Figure 5.

The layered X-ray mean attenuation in different age groups (mean $\pm$ SD, # $p<0.05$ versus 50 age groups, * $p<0.01$ versus 50 age groups).

Figure 6.
The sGAG optical density in different age groups (mean $\pm$ SD, # p <0.05 versus 50 age groups, * p <0.01 versus 50 age groups).

Figure 7.

The layered sGAG optical density in different age groups (mean $\pm$ SD, # $p<0.05$ versus 50 age groups, * $p<0.01$ versus 50 age groups).

Figure 8.
The relationship between sGAG optical density and X-ray attenuation.