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Decrease in Glycosaminoglycan with Aging in Normal Rat Articular Cartilage Is Greater in Females than in Males

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Abstract

Objective. Osteoarthritis (OA) is more prevalent in females. We hypothesized that changes in articular cartilage (AC) constituents with aging may cause differences. Herein, we aimed to compare the changes in AC constituents with aging in male and female normal rats. *Design.* The glycosaminoglycan (GAG) and collagen (COL) contents of the AC in knee, hip, and shoulder joints of male and female rats were quantified and compared between age groups and sexes. *Results.* The amount of GAG was decreased in multiple joints in both males and females with aging. In females, it had a significant decrease in all joints measured. The decrease in GAG with aging was more severe in females than in males. Even in young rats, the amount of knee joint GAG was significantly less in females than in males. The amount of COL in the AC was unchanged with aging in both sexes. *Conclusions.* The drastic GAG decrease with aging in female normal rats may explain the higher prevalence and more severe OA in females.

Keywords

articular cartilage, glycosaminoglycan, aging, female

Introduction

The regeneration disability of the articular cartilage (AC) commonly causes the widespread of osteoarthritis (OA) among elderly people. Patients with OA are increasing globally with aging and are estimated to occur in >10 million by research on osteoarthritis/osteoporosis against disability (ROAD) study in Japan.[1] The OA onset is earlier in females than in males according to the National Institute for Longevity Sciences-Longitudinal Study of Aging (NILS-LSA) study,[2] and the incidence steeply increases in females aged >50 years.[3,4] From a meta-analysis of OA prevalence and incidence, the severity is higher in females than in males.[5,6] The OA prevalence is also higher in females in various countries,[7-9] indicating that its incidence is higher in females than in males regardless of the lifestyle. Therefore, we supposed that the constitutional changes in AC with aging have intrinsic differences between sexes.

The AC is hyaline cartilage and one of the richest extracellular matrix (ECM) tissues with fewer chondrocytes. Chondrocytes are embedded in the abundant ECM with 60%-80% of water content.[10] The ECM is mainly composed of glycosaminoglycans (GAGs) and collagen (COL) fibers. The AC constituents are continuously changing with aging. The difference in amount and ratio of constituents corresponds with the difference in mechanical properties.[11-13] In normal AC with aging, the amount of GAG is significantly decreasing, while the amount of COL is stable in human knee[14] and horse joints.[15] As GAG decreasing is seen as one of the OA characteristics, the decrease in GAG with aging in normal AC facilitates the development of OA in elderly people.[16] That is, sex differences in the prevalence and severity of OA may be due to differences in the decrease in GAG with aging between the sexes. However, there are few comparison data between the sexes. We hypothesized that the amount of GAG is more decreasing in females than in males with aging even in normal animals. In this study, we compared the amounts of GAG and hydroxyproline (Hyp)

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as an indicator of COL between young and old normal male and female rats. We also compared these amounts between sexes and between the protrusion side and the fossa side in the knee, hip, and shoulder joints.

Methods

Animals

Wistar/ST rats were purchased from Shimizu Laboratory Supplies Co. (Kyoto, Japan) and dissected at young (7-9 weeks; male, 4; female, 5), and old (>1 year; male, 7; female, 7), respectively. They were anesthetized using CO_2. Dissection was started after the termination of respiration was confirmed. ACs from the femur (femur) and tibia (tibia) in the knee, from the femoral head (head) and lunate articular of the acetabular fossa (acetabulum) in the hip, and from the humerus head (humerus) and the glenoid fossa of the scapula (scapula) in the shoulder joints were harvested carefully.

Ethical Approval

This study was performed with permission from the Animal Research Committee at Shimane University (IZ24-98 and IZ27-125).

Solubilization of AC

The harvested tissues were freeze-dried and solubilized with thermolysin from *Bacillus thermoproteolyticus* (Code No. 3504; Lot. No. 650201; Peptide Institute, Inc, Osaka, Japan; 900 U) at 70 °C for 24 hours in 200 mM ammonium acetate (pH 8.0), 6 mM $CaCl_2$, 20 mM sodium acetate, and 0.88 mg/mL bovine serum albumin in the total volume up to 100 μL per 1 mg dry tissue weight which was modified from the previous methods.[17] Then, the samples were centrifuged at 20,000*g* for 15 minutes. The supernatant was collected as the analytes.

Measurement of GAG

A part of the analytes was added to 18 times volume of chilled ethanol, was kept overnight at 4 °C, and centrifuged at 20,000*g* for 15 minutes. Precipitation was reconstituted with distilled water. The total GAG of the sample was quantified with 1, 9-dimethyl methylene blue using the Blyscan GAG assay kit (Biocolor, Carrickfergus, UK).

Measurement of Hyp

The proportion of Hyp in COL fibers is about 13%-14% in various mammal tissues.[18] The modification of Pro to Hyp quickly occurred after translation, and the location of Hyp was almost determined.[19,20] Five microliters of the analyte was added to 45 μL of 10 N HCl. The solution was heated at 100 °C overnight for COL hydrolysis. The Hyp content of the solution was quantified with chloramine T and dimethylamino benzaldehyde, following Woessner.[21]

Statistical Analysis

The data were analyzed statistically using R ver.3.6.3 free software. The amount of GAG and Hyp and the GAG/Hyp ratio, at each AC between the sexes and between young and old, was compared using the Mann-Whitney test. The data were also analyzed using Friedman's test to clarify the variation of ACs in the same individual rat.

Results

The amounts of GAG between the young and old rats were compared (**Fig. 1A** and **B**). In the old males, at the knee joint, the amount of GAG was significantly decreased in the femur ($P = 0.042$) but not in the tibia ($P = 0.41$). At the hip joint, the amount in the head was significantly decreased ($P = 0.012$), but not in the acetabulum ($P = 1.00$). At the shoulder joint, no significant difference was observed on both sides (humerus $P = 0.11$, scapula $P = 0.23$). In the old females, the amounts of GAG were significantly decreased at all ACs we measured ($P = 0.0025$, 0.010, 0.0025, 0.0025, 0.0025, and 0.048 at the femur, tibia, head, acetabulum, humerus, and scapula, respectively) (**Fig. 1B**). The amount of Hyp was compared between young and old rats (**Fig. 1C** and **D**). The amount in all ACs did not change in either males ($P = 0.93$, 0.11, 0.11, 0.79, 0.16, and 0.23 at the femur, tibia, head, acetabulum, humerus, and scapula, respectively; **Fig. 1C**) or females ($P = 0.76$, 0.15, 0.15, 0.53, 0.27, and 0.88 at the femur, tibia, head, acetabulum, humerus, and scapula, respectively; **Fig. 1D**).

The decrease in GAG content with aging in all ACs was more pronounced in females than in males (**Fig. 2A**). In males, the amount of GAG did not decrease in the tibia and acetabulum, and it decreased by approximately 40% in the femur and humerus. In females, the decrease was approximately 60% in the femur, tibia, humerus, and scapula. The decrease at the head was approximately 75% and 70% in females and males, respectively. In contrast, the amount of Hyp did not decrease by more than 35% in males and females (**Fig. 2B**).

The amounts of GAG and Hyp were compared between the sexes (**Fig. 3**). In young knee joints, the amounts of GAG were significantly less in females than in males (femur $P = 0.024$, tibia $P = 0.024$) (**Fig. 3A**). In other young joints, the amounts of GAG were not significantly different between the sexes (head $P = 0.53$, acetabulum $P = 0.42$, humerus $P = 0.53$, and scapula $P = 0.93$) (**Fig. 3A**). In the old joints, significant differences were observed at the femur, tibia, and

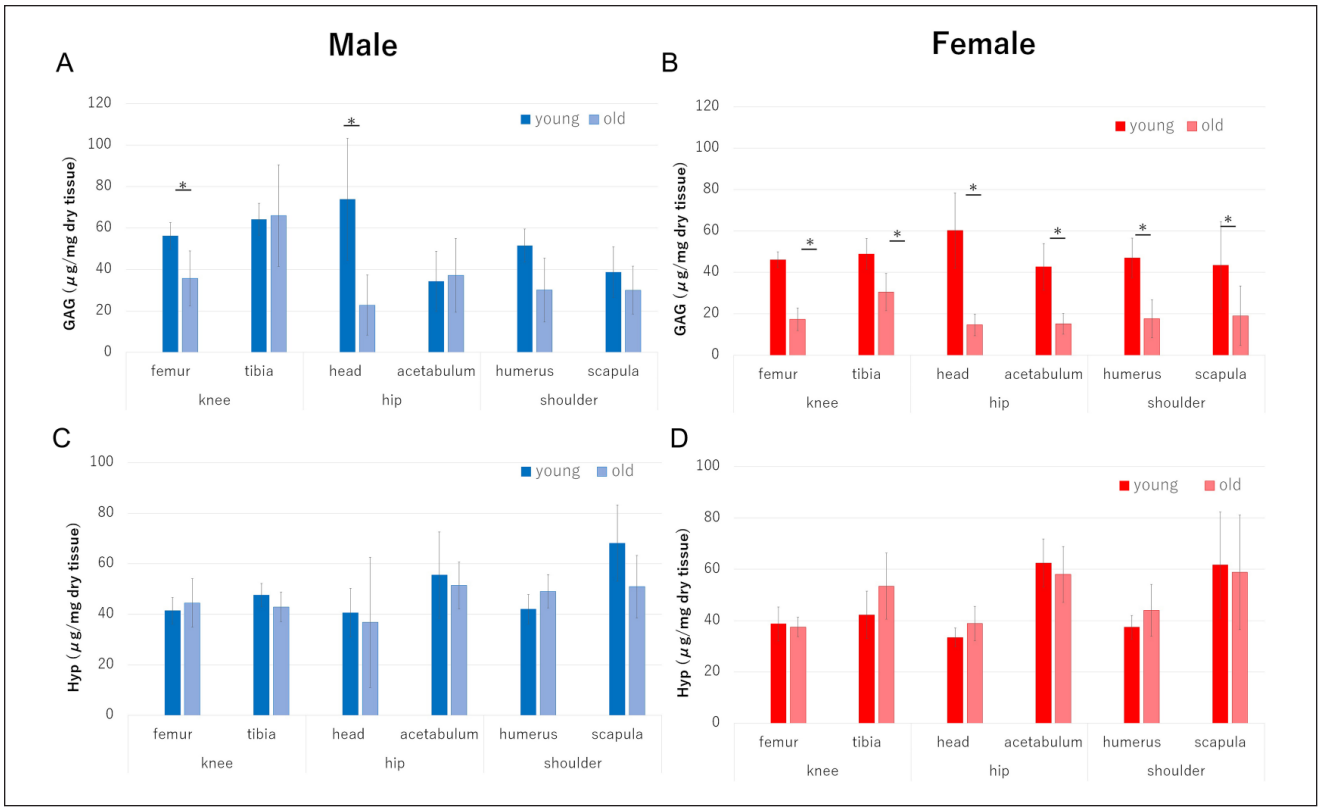

Figure 1. Comparison of multiple articular cartilage constituents (GAG and Hyp) between young and old rats. (**A**) GAG in male, (**B**) GAG in female, (**C**) Hyp in male, and (**D**) Hyp in female. The data show means ± SD. GAG = glycosaminoglycan; Hyp = hydroxyproline.
*There is a significant difference between young and old rats ($P < 0.05$).

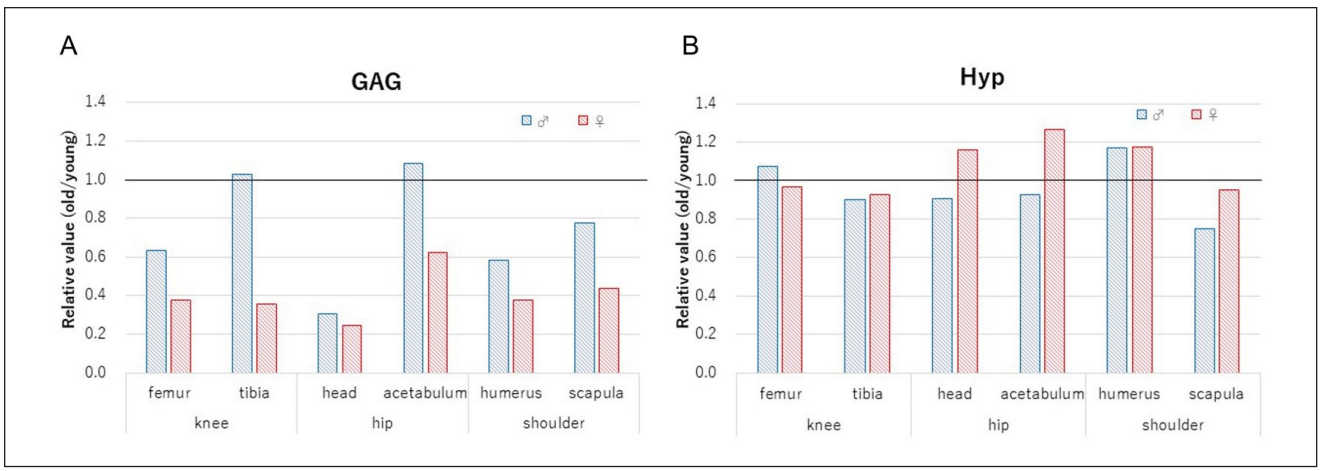

Figure 2. Relative value of the amount in the old based on the amount in the young. The relative value (old/young) was shown in both males and females. (**A**) GAG and (**B**) Hyp. GAG = glycosaminoglycan; Hyp = hydroxyproline.

acetabular ACs ($P = 0.010$, 0.0051, and 0.0025, respectively) (**Fig. 3B**). In the other old ACs, no significant differences were observed (head $P = 0.34$, humerus $P = 0.11$, scapula $P = 0.11$) (**Fig. 3B**). In all the ACs, no significant differences in the amount of Hyp between sexes were observed in the femur (young, $P = 0.78$; old, $P = 0.27$),

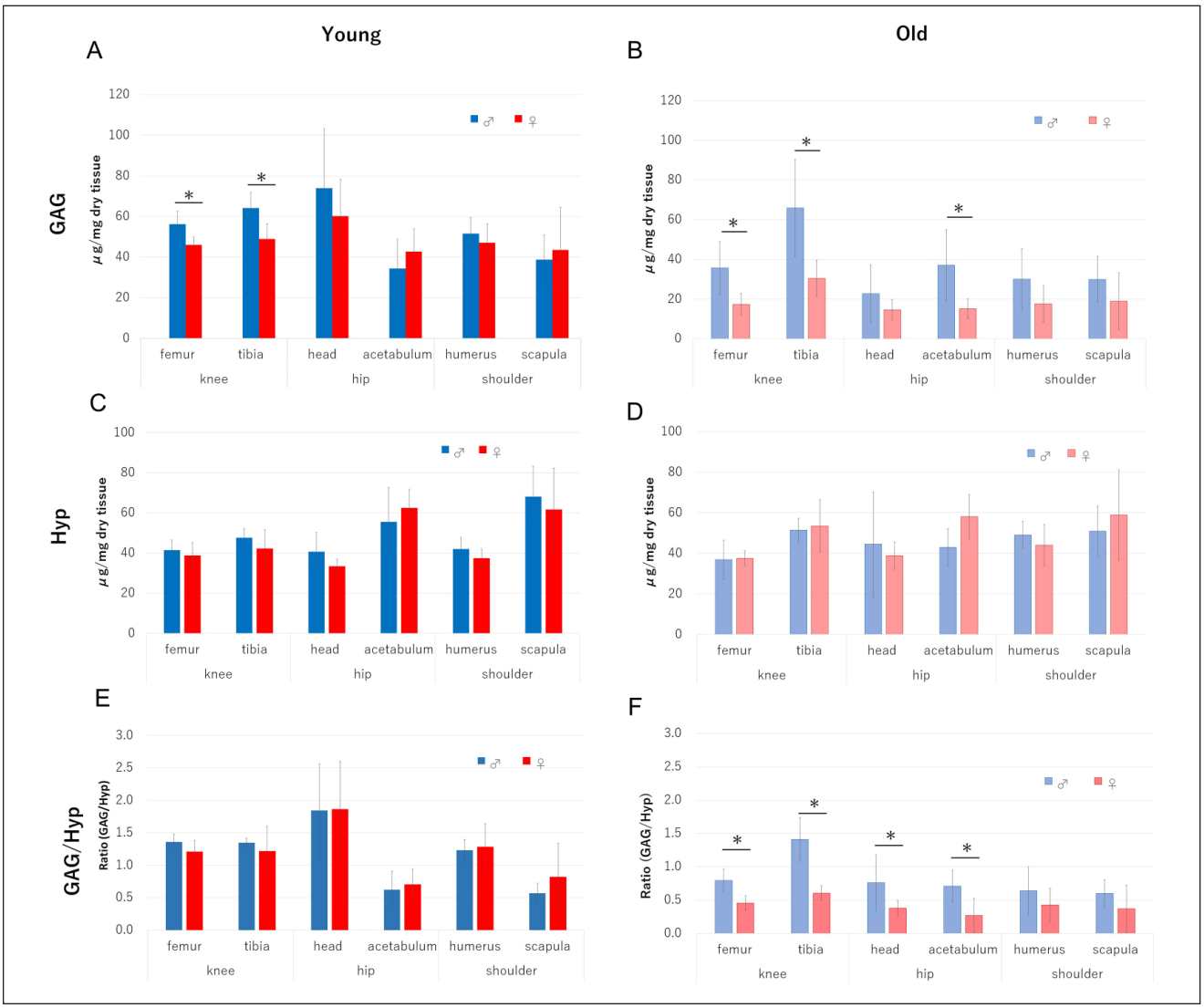

Figure 3. Comparison of multiple articular cartilage constituents (GAG and Hyp) between male and female rats. (**A**) GAG in young, (**B**) GAG in old, (**C**) Hyp in young, (**D**) Hyp in old, (**E**) GAG/Hyp ratio in young, and (**F**) GAG/Hyp ratio in old. The data show means ± SD. GAG = glycosaminoglycan; Hyp = hydroxyproline.
*There is a significant difference between male and female rats ($P < 0.05$).

tibia (young, $P = 0.32$; old, $P = 0.15$), head (young, $P = 0.32$; old, $P = 0.11$), acetabulum (young $P = 0.53$, old $P = 0.64$), humerus (young, $P = 0.16$; old, $P = 0.64$), and scapula (young, $P = 0.41$; old, $P = 0.64$) (**Fig. 3C** and **D**). The GAG/Hyp ratio, as an indicator of the constituent proportion, was also compared between the sexes at each young AC (**Fig. 3E**). In the young ACs, no difference was observed at any AC between the sexes. In the old ACs, a significant difference between the sexes was observed at the knee (femur $P = 0.015$, tibia $P = 0.0025$) and hip joints (head $P = 0.048$, acetabulum $P = 0.0073$), but not in the shoulder joint (humerus $P = 0.20$, scapula $P = 0.11$) (**Fig. 3F**).

The amounts of GAG and Hyp in the ACs of the shoulder, hip, and knee joints of the young and old rats are shown in Table 1. Among the ACs from the protrusion and fossa sides in these joints of the same individual, the amounts of GAG and Hyp, as well as the GAG/Hyp ratio, were compared. Even in the young group, the amount of GAG varied among the ACs in the same male, and the amount of Hyp varied among the ACs in the same female. The GAG/Hyp ratio also varied significantly among the ACs in both young males and females.

Discussion

We indicated the decrease in GAG at multiple ACs with aging (**Figs. 1A** and **B** and **2A**). The proportion of GAG constituent of the femoral condyle is decreased among fetal,

Table. 1. The Amounts of GAG and Hyp, and the Ratio of GAG/Hyp in Young and Old, and Male and Female Rats.

	Femur (M ± SD) (μg/mg Dry Tissue)	Tibia (M ± SD) (μg/mg Dry Tissue)	Head (M ± SD) (μg/mg Dry Tissue)	Acetabulum (M ± SD) (μg/mg Dry Tissue)	Humerus (M ± SD) (μg/mg Dry Tissue)	Scapular (M ± SD) (μg/mg Dry Tissue)	*P* Value
GAG							
Young							
Male*	56.23 ± 6.43	64.19 ± 7.75	73.86 ± 29.41	34.30 ± 14.52	51.54 ± 7.94	38.68 ± 12.20	0.017
Female	46.02 ± 3.83	48.91 ± 7.46	60.19 ± 18.17	42.72 ± 11.17	47.01 ± 9.58	43.48 ± 20.99	0.182
Old							
Male*	35.73 ± 13.26	65.94 ± 24.51	22.76 ± 14.51	37.16 ± 17.88	30.01 ± 15.41	29.99 ± 11.60	0.002
Female	17.31 ± 5.43	30.47 ± 9.02	14.62 ± 5.22	15.16 ± 5.03	17.61 ± 9.22	19.00 ± 14.37	0.109
Hyp							
Young							
Male	41.45 ± 5.22	47.59 ± 4.61	40.65 ± 9.54	55.50 ± 17.06	42.05 ± 5.81	68.10 ± 15.04	0.051
Female*	38.73 ± 6.62	42.25 ± 9.30	33.42 ± 3.63	62.47 ± 9.32	37.45 ± 4.59	61.68 ± 20.36	<0.001
Old							
Male*	44.51 ± 9.60	42.94 ± 5.83	36.81 ± 25.78	51.41 ± 9.27	49.08 ± 6.58	50.93 ± 12.38	0.046
Female*	37.49 ± 3.73	53.44 ± 12.92	38.83 ± 6.73	58.01 ± 10.88	44.03 ± 10.12	58.84 ± 22.29	0.022
GAG/Hyp ratio							
Young							
Male*	1.36 ± 0.12	1.35 ± 0.07	1.85 ± 0.72	0.62 ± 0.28	1.23 ± 0.17	0.57 ± 0.16	0.012
Female*	1.21 ± 0.18	1.22 ± 0.38	1.87 ± 0.74	0.70 ± 0.24	1.29 ± 0.35	0.82 ± 0.52	0.004
Old							
Male*	0.79 ± 0.17	1.41 ± 0.32	0.76 ± 0.42	0.71 ± 0.24	0.64 ± 0.36	0.60 ± 0.21	0.012
Female	0.46 ± 0.11	0.61 ± 0.26	0.38 ± 0.11	0.27 ± 0.11	0.43 ± 0.25	0.37 ± 0.35	0.080

GAG = glycosaminoglycan; Hyp = hydroxyproline.
*Friedman's test $P < 0.05$.

calf, and steer in normal bovine.[22] Elliott and Gardner[23] show that the amount of GAG in human femoral AC peaks at birth and then continuously decreases with aging; these correspond with our results. In contrast, the COL amount, estimated using the Hyp amount, did not decrease with aging at any ACs of the joints and in both sexes (**Figs. 1C** and **D** and **2B**). The Hyp amount did not significantly change with aging in the bovine femoral condyle AC[14] and human ankle AC.[24] It would be considered common among normal animals that GAGs may be more easily declined than the COL with aging. Here, we indicated that the decrease in GAG with aging was greater in females than in males **(Figs. 1A** and **B** and **2A**).

Generally, the effect of sex hormones is assumed to be the most likely cause of sex difference. As the estrogen treatment after menopause decreases the OA incidence,[25] estrogens may have a protective effect on AC to the progression to OA in elderly people. In contrast, a clear relationship between OA and estrogen use was not found in the Framingham knee OA study[26] and in a systematic analysis from an epidemiological database.[4] Thus far, it is unclear whether estrogen and OA incidence have a relationship. Therefore, the relationship between hormones and the decrease in GAG with aging is unclear.

The GAG decrease with aging occurred in various ACs (**Fig. 1A** and **B**) and the magnitude of GAG decrease was different among ACs (**Fig. 2A**). In the male joint, the GAG had a trend that the decrease was more severe in the protrusion side than in the fossa side (**Fig. 2A**). The decrease is different between locations on the same right metacarpophalangeal joint in the equine.[15] It is feasible that the shape of the surface produces the side-specific loading difference, and it may cause a different vulnerability in each side. In normal human nasal cartilage, which is also hyaline cartilage, the amount of GAG remains unchanged with aging.[27] As the nasal cartilage must be less loading than ACs, loading may be one of the important factors for GAG decrease with aging. In contrast, the side difference was no longer clear in females (**Fig. 2A**). This may imply that GAG decreases on the protrusion side and then on the fossa side. As the decrease in AC GAGs is one of the characteristics of patients with OA,[16] the decrease in GAGs at both sides of the joint may hasten the progression of OA. Therefore, the decrease in GAGs at both sides in normal female joints may explain the higher OA prevalence in females.

As the OA prevalence is also different in each joint,[28-30] the constituents (GAG and COL) in the young group between the sexes were compared (**Fig. 3A** and **C**). In the young group, the amount of knee joint GAGs was significantly different between the sexes. It is possible that females develop OA more easily depending on the amount of GAGs in a young joint. There were variations in the amounts of

constituents within the same individual among ACs (**Table 1**). The maturing processes of the bone and growth plate during endochondral ossification are deeply associated with AC maturation. The closed timing of the growth plate in the bone is also different on each AC even in the same joint.[31] Therefore, the AC characteristics may be different at each AC of each joint. The GAG/Hyp ratio was not different between the sexes (**Fig. 3E**) in the young. As the decrease in GAGs with aging was more pronounced in females than in males, the GAG and GAG/Hyp ratio between the old sexes were significantly different in the knee and hip joints (**Fig. 3B** and **F**). As the constituents have a role in deciding the mechanical characteristics,[12,13] the variation of ACs may indicate the difference in the mechanical property. In the human ankle AC, neither GAG nor COL changes with aging,[32] and the ankle AC maintains the tissue for earlier turnover of constituents than the knee AC.[33] Thus, the different effects of aging depending on the AC locations may be caused by the intrinsic differences of each location.

In this study, we indicated that the decrease in GAG with aging in normal rats was greater in females than in males at all joints measured (**Figs. 1A** and **B** and **2A**), and that may explain the higher OA prevalence in females. The decrease in GAG may be also related to the loading and location. However, it remains unclear why the amount of GAG was severely decreasing in female AC at any joint even under normal conditions. Therefore, further detailed research is warranted for clarifying the question.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

This study was performed with permission from the Animal Research Committee at Shimane University (IZ24-98 and IZ27-125).

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