—Original—

Objective Colorimetric Evaluation of Aging-related Articular Cartilage Degeneration

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Background: Aging is a primary risk factor for the development of osteoarthritis (OA). Recently, advanced glycation end products (AGEs) have received much attention in relation to aging and OA. Some AGEs are reported to be brownish, but the association between AGE levels and browning in articular cartilage is unknown. The purposes of this study were first, to develop a colorimetric device to evaluate the quality and aging of the articular cartilage, and second, to investigate the relationship between AGE levels and color of articular cartilage by using this device without enzymatic digestion of the articular surface.

Design: Open-label, single-center, prospective study

Methods: Seven patients with OA (1 man, 6 women; mean age, 74.4 years; age range 58–81 years) who underwent primary total knee arthroplasty at our university hospital between July and December 2014 were enrolled in the study. Articular cartilage was harvested from the femur and tibia during surgery. The color and chromaticity of the articular cartilage was assayed by using a newly developed device with high validity. The characteristics of the articular cartilage were examined using the CIE XYZ Color Coordinate System (International Commission on Illumination, Vienna, Austria), and the color indicative of browning of the cartilage was defined on the X- and Y-axes. The brightness was adjusted and the specimen was photographed submerged in distilled water and the color was measured using a commercial luminance and color analyzer. Measurement of X and Y was repeated 3 times per site at 9 different points on the articular cartilage surface, and the mean value per specimen was calculated. Pentosidine (a well characterized biomarker of AGEs) levels and hydroxyproline content in articular cartilage were determined by high-performance liquid chromatography (HPLC). The correlation between age, articular cartilage AGE levels, and browning was analyzed using Spearman's rank correlation coefficient. The association between the degree of macroscopic degeneration and AGE levels was analyzed using one-way analysis of variance.

Results: Age was positively correlated with pentosidine levels in articular cartilage (r_s=0.322) and browning of articular cartilage (r_s=0.261). However, a weak negative correlation was observed between pentosidine levels and browning of articular cartilage (r_s=−0.564, p=0.004).

No positive relationship was observed between pentosidine levels and browning of articular cartilage in the visible spectrum.

Conclusions: This study developed an original colorimetric device with high validity. Browning of articular cartilage increased with age, but this study did not detect pentosidine-caused browning. Further study is needed to clarify the factors associated with browning of cartilage. (J Nippon Med Sch 2018; 85: 157–165)

Key words: advanced glycation end products (AGEs), cartilage, osteoarthritis, color, pentosidine

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Introduction

With the aging of society, musculoskeletal disability is becoming a major public health concern because of the increasing prevalence of aging-related bone and joint disease resulting in shorter healthy life expectancy. A previous study estimated that more than 50% of individuals aged 60 years or older have osteoarthritis (OA)[1], the predominant aging-related bone and joint disease. Japanese individuals aged 60 years or older account for 33% of the population, and at least 20 million people are estimated to have OA when considering the number of individuals who are likely to develop the condition[2]. Therefore, the prevention and treatment of OA are important issues from the perspective of medical economics.

In articular cartilage, chondrocytes and the extracellular matrix (ECM) undergo changes related to aging. Intracellular changes include a decline in chondrocyte metabolism due to enhanced antioxidative stress, a decreased response to humoral factors, and cell death[3]. In the ECM, aging-related changes are characterized by changes in physical properties, such as softening and reduced elasticity, due to structural changes in proteoglycan and type II collagen[4]. These aging-related changes in the matrix, particularly changes in collagen cross-linking, have recently attracted much attention. Collagen fibers form the skeleton of articular cartilage ECM and are cross-linked to maintain the elasticity of the matrix. Physiological cross-linking is important for strengthening the linkages between collagen fibers and retaining water between the fibers. However, as aging progresses, advanced glycation end products (AGEs) accumulate in the matrix because of oxidation or glycation of collagen fibers by glucose and ribose. AGEs are a heterogeneous family of macromolecules produced by non-enzymatic glycation of proteins, lipids, and nucleic acids. The accumulation of aging-associated AGEs occurs because the turnover of collagen is extremely slow compared with that of other proteins, and results in formation of cross-linkages due to glycation in addition to physiological cross-linking. The hypothesis that aging-associated cross-linking leads to OA has recently been investigated because such cross-linking reduces the elasticity of articular cartilage[5,6]. In addition, AGEs trigger intracellular biological processes, including production of various proinflammatory cytokines[7], through activation of specific receptors for AGEs (RAGEs). In OA, many chondrocytes express RAGEs[8] and induce the production of molecules such as matrix metalloproteinases[9], prostaglandin E2, and nitric oxide[10] through the AGE-RAGE pathway. Together with mechanical stress, these humoral factors are involved in the development of OA. Because previous studies have highlighted the causative role of the AGE-RAGE system in the aging of the articular cartilage matrix and subsequent development of OA, many studies are currently investigating prevention of the aging process in articular cartilage by targeting the AGE-RAGE system and its various inhibitors[11].

Previous studies also suggest that by measuring AGE levels, it is possible to diagnose the stiffness of articular cartilage and predict stiffness-related articular cartilage degeneration and subsequent development of OA. *In vitro* measurement of AGEs involves the non-enzymatic reaction of ribose with lysine and arginine residues and the measurement of absorbance at 360 nm. This procedure suggests that the AGE content could increase the intensity of brown coloration. Orthopedic surgeons often observe brownish articular surface in the osteoarthritic knees, whereas white articular cartilage in healthy young patients in surgeries. Therefore, we hypothesized that deposition of AGEs on collagen causes a brownish color[12]. The purposes of this study were first, to develop a colorimetric device to evaluate the quality and aging of the articular cartilage, and second, to investigate the relationship between AGE levels and color of articular cartilage by using this device without enzymatic digestion of the articular surface. This method enables prediction of articular cartilage degeneration based on the color of articular cartilage during arthroscopy.

Materials and Methods

Ethical Considerations

This study was conducted in accordance with the Declaration of Helsinki and was approved by our local institutional review board (No. 25-12-343). All subjects provided written informed consent to participate. This open-label prospective study was performed in a single center.

Patients

Seven patients with OA (1 man, 6 women; mean age, 74.4 years) who underwent primary total knee arthroplasty (TKA) at Nippon Medical School Hospital between July and December 2014 were enrolled in the study. In total, 24 specimens were examined. **Table 1** shows the background data of the patients. Patients with severe OA evaluated as Kellgren-Lawrence grade[13] and having no other inflammatory disease were enrolled according to the clinical and radiographic criteria of the American College of Rheumatology as well as standard exclusion criteria. Articular cartilage was harvested from

Table 1 Characteristics of patients by Kellgren-Laurence grade

Variables	Total (n=7)	KL Grade		
		Grade 2 (n=1)	Grade 3 (n=5)	Grade 4 (n=1)
Age (years)	74.4±8.8	58	76.4±4.2	78
Serum creatinine (mg/dL)	0.77±0.12	0.68	0.81±0.19	0.65
HbA1c (%)	5.9±0.25	6.5	5.8±0.33	5.7

Values are shown as the mean±standard deviation; KL Grade, Kellgren-Laurence grade.

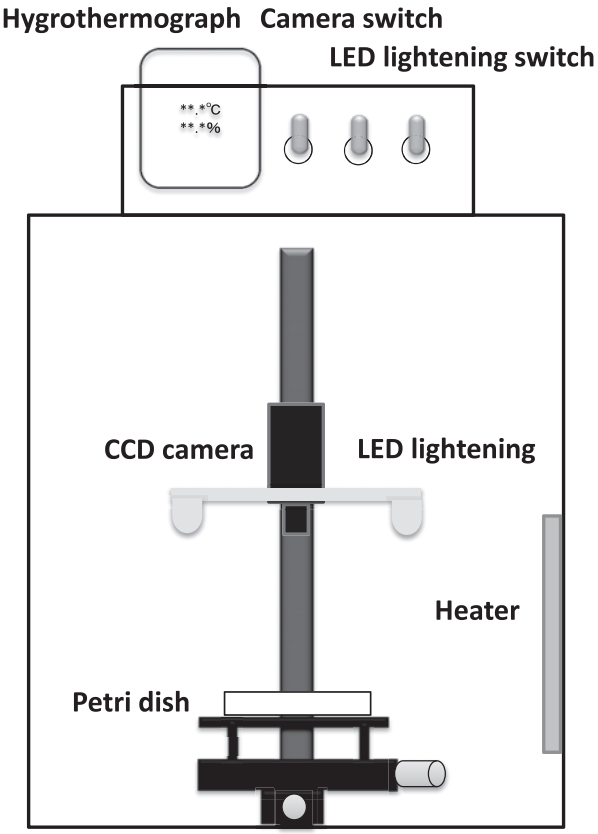

Fig. 1 Overview of the instruments

the femur and tibia during surgery. The cartilage was excised above the subchondral bone and cut into small 4×4 mm pieces.

Assessment of Articular Cartilage Degeneration

The grade of articular cartilage injury associated with degeneration was recorded according to the International Cartilage Repair Society (ICRS) classification[14] as follows: 0, normal intact cartilage; 1, chondral softening and blistering, superficial lesions, fissures and cracks, and soft indentation; 2, fraying, lesions and fissures extending down to less than 50% of the cartilage depth; 3, partial loss of cartilage thickness, cartilage defects extending down to greater than 50% of the cartilage depth as well as down to the calcified layer; and 4, full-thickness cartilage loss with exposure of subchondral bone.

Instruments

The color and chromaticity of articular cartilage was assayed at 25.8°C by using a newly developed tristimulus colorimeter equipped with a CCD camera (CB-030GE; JAI, Kanagawa, Japan) and a dome-shaped indirect lighting system (WKM-150; Nissin Electronics Co., Ltd., Tokyo, Japan) as the reference light source (**Fig. 1**). Data were analyzed using Eyescale-TWL colorimetric analysis software (I System Co., Ltd., Tokyo, Japan).

Evaluation of Validity of the Device

First, the light source was calibrated with a color reference chart containing 30 colors (X-Rite ColorChecker; Edmund Optics, Barrington, NJ) and brightness (L) was set

to 550 using a distance of 80 mm, gain of 1 (the size of the signal increase by the amplifier), and shutter speed of 1/200 s. The following conditions were then set for imaging: distance, 80 mm; gain. 1; shutter speed, 1/400 s; and temperature, 25.8°C. Chromaticity was displayed using the CIE XYZ Color Coordinate System, and the color indicative of browning of articular cartilage was defined on the X- and Y-axes. To determine the range of reliability of the reference light source, 2 specimens were photographed every 5 min for 1 h (12 times in total) at L=400, 500, and 600. Then, the coefficients of variation (CV) for L, X, and Y were calculated, and within-run and between-day reproducibility were analyzed. Human articular cartilage was evaluated every 5 min for 1 h (12 times in total) at L=550. The measurement was repeated twice at the same anatomical site on the same day for a period of 5 days, and for each measurement, the CV of X and Y was calculated. Using the data, we determined the minimum number of measurements per sample.

A commercially available spectrophotometer (CM-700d; Konica Minolta, Tokyo, Japan) was used to photograph 24 different colors on a color reference chart (X-Rite ColorChecker). Measurement values and X and Y coordinates from the two devices were analyzed by using Spearman's rank correlation coefficient (rs) to determine the validity of the colorimetric device.

Colorimetric Evaluation of Articular Cartilage

To prevent drying, articular cartilage specimens were covered with gauze soaked in normal saline and placed on ice immediately after harvesting. Specimens were immersed in distilled water at 37°C for 5 min before measurement, which was performed within 1 h after harvesting. The color reference chart was photographed using the settings described above, and brightness was adjusted to L=550 to calibrate the light source before performing actual measurements. Specimens were photographed submerged in distilled water at a distance of 80 mm, gain of 1, and shutter speed of 1/400 s, and the color was measured using the Eyescale-TWL. The measurement of X and Y was repeated 3 times/site at 9 different points on the articular cartilage surface and the mean value per specimen was calculated.

Biochemical Quantification of AGEs

Pentosidine, a well-characterized fluorescent AGE formed by cross-links between lysine and arginine residues, was used as a representative AGE marker of other AGE measures such as cross-linking[15]. Total AGE levels and hydroxyproline content were determined by our previously reported method[16]. Specimens were hydrolyzed in 6 N HCl at 110°C for 24 h. Hydrolysates were analyzed for hydroxyproline content on a Shimadzu LC9 Liquid Chromatography HPLC instrument fitted with a cation exchange column (0.9×10 cm, Aa pack-Na; Jasco, Ltd., Tokyo, Japan) linked to an inline fluorescence flow monitor (RF10AXL; Shimadzu, Shizuoka, Japan). The weight of collagen was assumed to be 7.5 times the measured weight of hydroxyproline of molecular weight 300,000 Da. The resulting data were used to calculate the cross-link values as mol/mol of collagen. AGE levels were determined using a spectrofluorometer at excitation and emission wavelengths of 370 and 440 nm, respectively (FP6200, Jasco International Co., Ltd., Tokyo, Japan) and were normalized by a quinine sulfate standard.

Statistical Analysis

The association between age, articular cartilage AGE levels, and browning was analyzed using Spearman's rank correlation coefficient. The association between the degree of macroscopic degeneration and AGE levels, and between the degree of articular cartilage degeneration and browning of articular cartilage were analyzed using one-way analysis of variance with Tukey's post hoc test. Additionally, the association between AGE levels and browning of articular cartilage was evaluated by multiple regression analysis with AGE levels as an objective variable and X and Y as explanatory variables. Statistical analyses were performed using SPSS Ver.23 software (IBM, Chicago, IL), with significance set at $p<0.05$ in a two-tailed test.

Results

Evaluation of Validity of the Device

At L=400, 500, or 600, within-day and between-day CV for L, X, and Y were all 1% or less (**Fig. 2**), indicating the reproducibility of the colorimetric measurement at a brightness of $400 \leq L \leq 600$.

Table 2 shows within-run reproducibility and between-day variability at L=550. CV for within-run reproducibility were all 0.2% or less, whereas CV for between-day reproducibility were all 0.5% or less.

The number of repeat measurements was 2.11, 2.23, and 2.32 times for reliability of ≥90%, ≥95%, and ≥99%, respectively. This shows that ≥99% reliability is attained when the measurement is repeated at least 3 times. In addition, Spearman's coefficient was respectively 0.94 and 0.91 for chromaticity coordinates X and Y obtained in spectrophotometric analysis, demonstrating the validity of the measurement device.

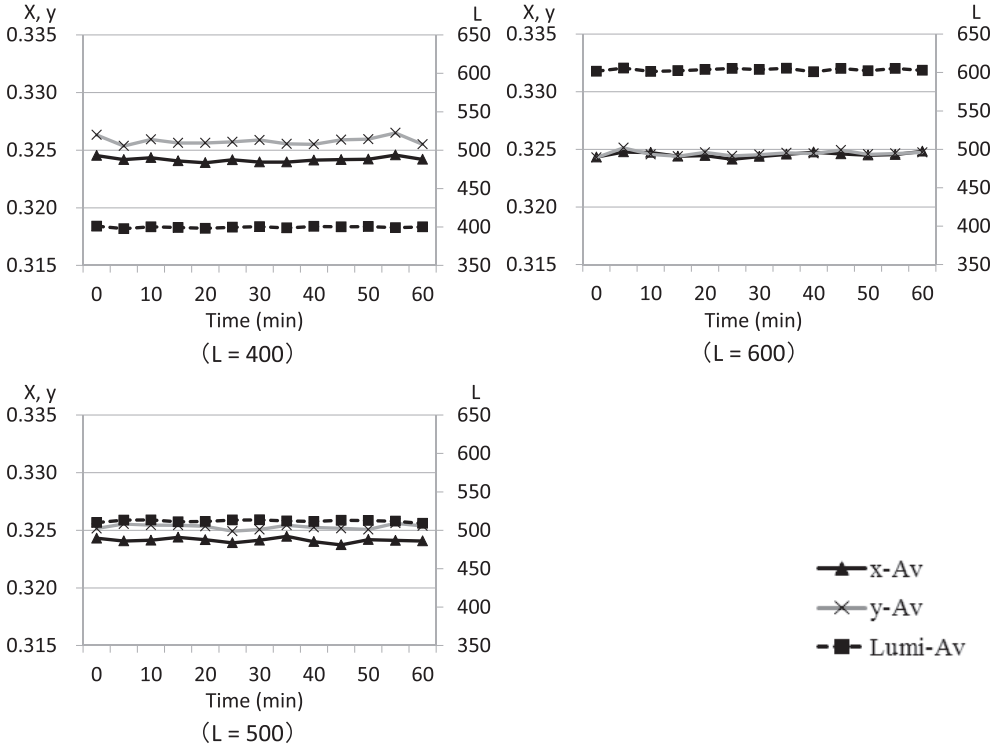

Fig. 2 Range of reliability for the reference light source

For 400 ≤ brightness (L) ≤ 600, within-day coefficients of variation for L, X, and Y were all less than 1%, showing the extremely high reliability of the reference light source for the range. The symbols show ▲ x,×y, and ■ luminance.

Table 2 Reproducibility of measurement values at L=550 in articular cartilage

Variables	Luminance	Color x	Color y
Max	552	0.320	0.306
Min	537	0.325	0.315
Average	545	0.323	0.310
SD	2.58	0.0238	0.00575
CV (within-run)		0.001<(%)	0.13 (%)
CV (between-day)		0.33 (%)	0.34 (%)

Max, maximum; Min, minimum; SD, standard deviation; CV, coefficient of variation.

Table 3 Characteristics of specimens by ICRS grade of knee osteoarthritis

ICRS Grade	Grade 0 (n=0)	Grade 1 (n=3)	Grade 2 (n=11)	Grade 3 (n=11)	Grade 4 (n=0)	P value
Age (years)	-	72–79	58–81	58–81	-	
Mean±SD (years)	-	76.3±3.8	73.0±8.8	72.0±9.2	-	N.S.

Almost all patients (1 man, 6 women; mean age, 74.4 years) had Kellgren-Lawrence grade 3/4 (moderate/severe) OA. Over 85% of the OA specimens had ICRS grade 2 or higher, but none had ICRS grade 0 (early OA).
ICRS, International Cartilage Repair Society; N.S., not significant; SD, standard deviation; OA, osteoarthritis.

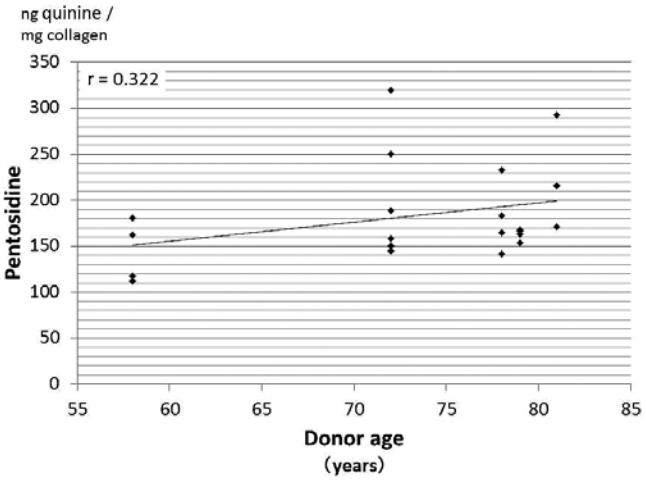

Fig. 3 Association between aging and articular cartilage advanced glycation end product (AGE) levels
AGE levels in articular cartilage had a weak positive correlation with age (rs=0.322).

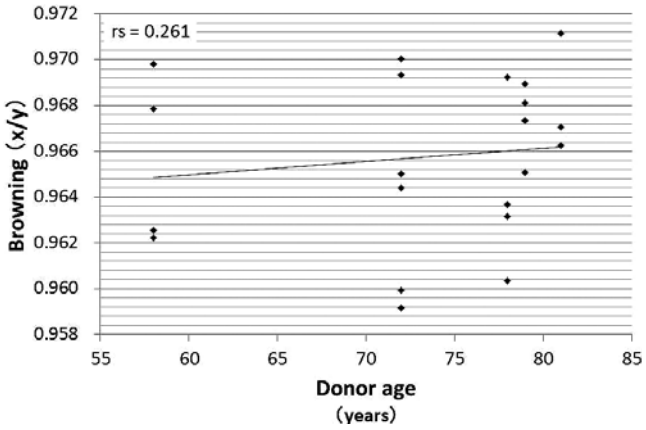

Fig. 4 Association between aging and browning of articular cartilage
Age had a weak positive correlation with browning in articular cartilage age (rs=0.261).

Macroscopic Degeneration

The ICRS grade of the harvested specimens was grade 1 in 3 specimens (mean age, 76.3 years), grade 2 in 11 specimens (mean age, 73.0 years), and grade 3 in 10 specimens (mean age, 72.0 years). No significant correlation was observed between the grade of osteoarthritis and age (**Table 3**).

Association between Aging and Articular Cartilage AGE levels

Pentosidine levels in articular cartilage increased with age. A weak positive correlation was observed between AGE levels in articular cartilage and age (r_s=0.322; **Fig. 3**).

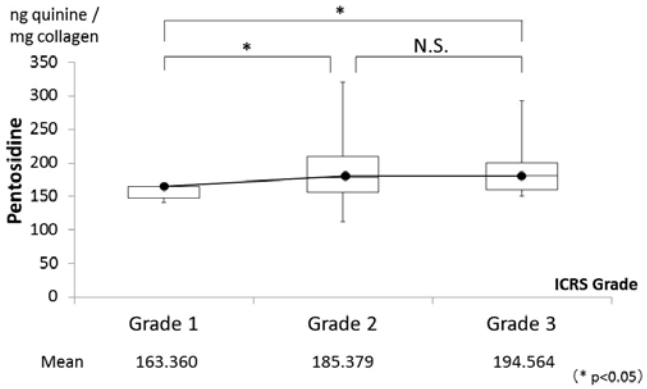

Fig. 5 Association between degree of degeneration and articular cartilage advanced glycation end product (AGE) levels
The amount of AGEs tended to increase as the degenerative changes in the cartilage increased macroscopically. No statistically significant difference was observed between the International Cartilage Repair Society grades.

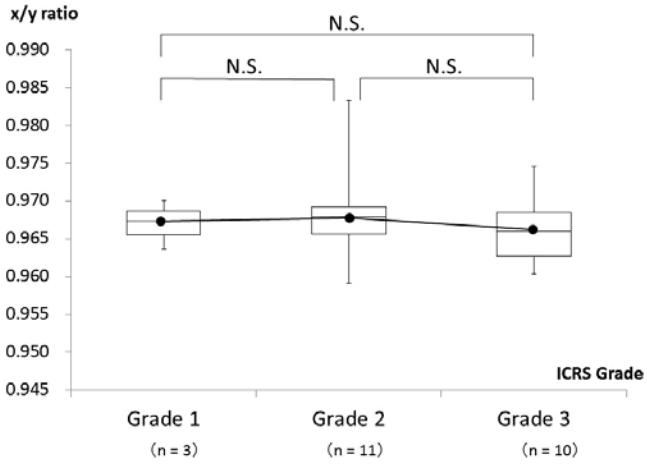

Fig. 6 Association between degree of cartilage degeneration and browning of articular cartilage
Browning increased as macroscopic degeneration progressed to grade 2, but decreased in grade 3. There was no significant difference between the grades.

Association between Aging and Browning of Articular Cartilage

Browning of articular cartilage increased with age. A weak positive correlation was observed between browning and age (r_s=0.261; **Fig. 4**).

Association between Degree of Degeneration and Articular Cartilage AGE Levels

The AGE levels in articular cartilage tended to increase with progression of macroscopic degeneration in cartilage. Pentosidine levels increased from 163.4 for ICRS grade 1 to 185.4 for grade 2 and 194.6 ng quinine/mg

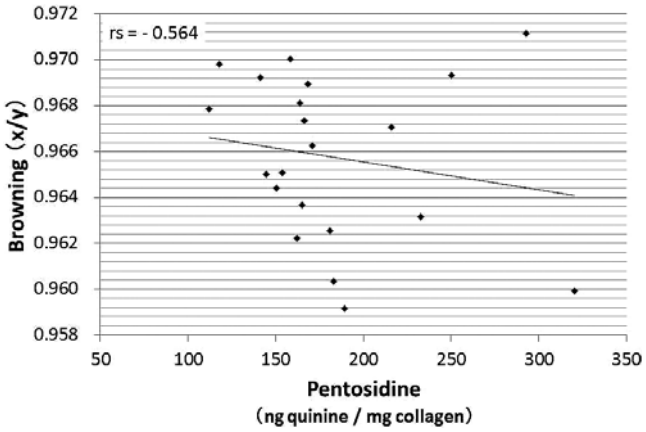

Fig. 7 Association between browning and articular cartilage advanced glycation end product (AGE) levels
A weak negative correlation was observed between browning and AGE levels in articular cartilage (rs=–0.564, p=0.004).

collagen for grade 3, with a significant difference between grades 1 and 2 (p=0.036), but not between grades 2 and 3 (**Fig. 5**).

Association between Degree of Degeneration and Articular Cartilage Browning

Mean browning was 0.967, 0.968, and 0.966 for ICRS grades 1, 2, and 3, respectively, showing that browning increased as macroscopic degeneration progressed to grade 2, but decreased in grade 3 specimens. There was no significant difference between the grades (**Fig. 6**).

Association between AGE Levels and Articular Cartilage Browning

A weak negative correlation was observed between AGE levels and browning of articular cartilage (r_s=–0.564, p=0.004; **Fig. 7**). Multiple regression analysis revealed an extremely weak correlation (r_s=–0.0384, coefficient of determination R^2=0.00149). No positive correlation was observed between AGE levels and browning.

Discussion

This is the first objective quantitative study to show that browning of articular cartilage increases with age. No previous study carefully analyzed the correlation between aging of articular cartilage and the color of the articular surface. This report describes an original colorimetric device that we developed to achieve this.

Accurate color quantification requires determining appropriate lighting conditions, measuring light reflected from and absorbed by the target object, and determining the color space coordinates by using a color stimulus function for normal eye sensitivity. However, measurement values are affected by temperature, humidity, surface conditions of articular cartilage, and other factors, and the same color can be perceived differently depending on viewing conditions such as lighting and viewing angle. Therefore, scientific investigation of colors requires standardized conditions. In a previous study, the reliability of the measurement values was found to be low because the measurement conditions were not consistent throughout the study, despite using a device that was reliable under certain conditions[17]. Therefore, in the present study, we maintained consistent measurement conditions in the colorimetric analysis as much as possible.

Because the optical absorbance of 360 nm light by enzymatically digested articular cartilage reflects AGE levels, we hypothesized that AGE deposition can be quantified based on the color of the superficial layer of cartilage. However, our findings revealed that color does not reflect the level of pentosidine, presumably because of irregular surface conditions such as fibrillation caused by loss of the lamina splendens due to degeneration of articular cartilage. Objects with an irregular surface scatter light due to diffuse reflection, resulting in fluctuating measurement values. To address this problem, we used an integrating sphere to illuminate specimens equally from all directions and to receive light in the surface normal direction of the sample. However, the lighting system was not effective enough, possibly because the surface irregularity of the samples was greater than anticipated.

Our findings showed that pentosidine levels in articular cartilage increase with age. Age-related increases in AGE levels in the matrix of articular cartilage have been demonstrated in many studies[18–20]. Also, the correlation coefficient for aging and the level of pentosidine was 0.88 in a study by Verzijl et al.[15] but 0.493 in a study by Vos et al.[20]. Furthermore, a recent study found a strong correlation between aging and pentosidine levels in articular cartilage (R=0.93)[19]. The discrepancy between the previous studies and our study (R=0.322) could be because specimens were collected from patients in a wide age range in the previous studies.

In this study, no correlation was observed between degeneration of articular cartilage and the level of pentosidine. Earlier studies reported significantly higher levels of pentosidine in articular cartilage with macroscopic osteoarthritis findings compared with normal articular cartilage[21,22]. However, many studies have recently shown no association between the progression of OA and articular cartilage pentosidine levels. The present cartilage specimens were harvested from patients with advanced OA.

In such articular cartilage with severe degeneration, turnover of the ECM surrounding the chondrocytes is enhanced and collagen turnover affects the accumulation of AGEs in collagen[18]. In patients with OA, cartilage AGE levels decrease in the area around chondrocytes but increase on the articular surface[23]. Similarly, Hirose et al. have shown that pentosidine levels decrease in the area surrounding chondrocytes but increase at the superficial layer of knee articular cartilage in humans[24]. As OA progresses, turnover of aggrecan molecules in the area surrounding chondrocytes is also enhanced. Compared with normal articular cartilage, aggrecan in articular cartilage affected by OA does not contain pentosidine[25], suggesting that the levels of pentosidine in the matrix change during the course of degeneration. Consequently, the low levels of pentosidine in the present study might be due to enhanced matrix metabolism in the specimens collected from patients with advanced OA.

The present findings also suggest that pentosidine is not a cause of browning of articular cartilage, and thus further study is needed to clarify the degenerative factors associated with browning of articular cartilage in the future. It is expected that the utility of arthroscopy will be enhanced by the addition of important arthroscopic evaluation items if a method can be established that enables the properties of the articular cartilage matrix to be assessed by simple colorimetric measurements of articular cartilage during arthroscopy.

This study has some limitations. First, the number of samples was small and articular cartilage with a relatively smooth surface was harvested from only a few patients with mild OA. Owing to taking so much time to measure pentosidine in articular cartilage, only seven patients were enrolled in this study. More than 50 specimens are required to show a statistically significant difference with post hoc power analysis. Second, evaluation was performed with tissue samples under water to maintain consistent moisture conditions, and so the measurement values might have been affected by light reflected off the water surface. Changes in the reflection of light at the articular cartilage surface appear as differences in luster, thereby affecting the intensity of specular reflection and the spatial distribution of light diffusion. Furthermore, reflected light modifies color and chromaticity[26], and differences in luster cannot be addressed completely by the use of an integrating sphere alone. In the future, further study with a larger number of specimens is warranted where measurements are performed with both the specimens and camera submerged in water under the conditions used in arthroscopy.

In conclusion, this is the first objective quantitative study to show that browning of articular cartilage increases with age using an original colorimetric device with high validity. A weak negative correlation was observed between AGE levels and browning of articular cartilage, and thus further study is needed to clarify the degenerative factors associated with browning of articular cartilage.

Acknowledgement: This work was supported in part by a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Culture, Sports, Sciences and Technology of Japan (Grant No. 25462386 to S.T.).

Conflict of Interest: The authors declare no conflict of interest.

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(Received, September 13, 2017)
(Accepted, February 5, 2018)