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Intra-articular site-specific distribution of
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37 **Abstract**

38 **Background:** Advanced glycation end products (AGEs) are compounds formed due to
39 aging and diabetes mellitus (DM). They activate NADPH oxidase (NOX) by binding to
40 their receptors, thereby increasing the production of reactive oxygen species (ROS),
41 which cause oxidative stress. In this study, we investigated the effects of AGEs on the
42 tissues of the shoulder joint (such as rotator cuff synovium, and capsule) in patients with
43 DM having rotator cuff tears.

44 **Methods:** This study included eight patients with DM who underwent surgical treatment
45 for rotator cuff tears with contracture. The rotator cuff, synovium, and joint capsule were
46 harvested at the time of surgery and evaluated by hematoxylin-eosin staining.
47 Furthermore, immunostaining was used for evaluating AGEs and receptor for AGEs
48 (RAGE), cell activity, ROS, and apoptosis. Quantitative real-time polymerase chain
49 reaction (qPCR) was employed for the cellular evaluation of NOX, interleukins, RAGE,
50 and collagen.

51 **Results:** The AGEs and RAGE staining as well as the ratio of ROS and apoptosis were
52 in the following order: rotator cuff > joint capsule > synovium. In contrast, the cellular
53 activity was significantly higher in the synovium than in the other regions. The type I
54 collagen expression (as shown by qPCR) as well as the RAGE and NOX expressions

were as follows: rotator cuff > joint capsule > synovium. Conversely, the expression of inflammatory cytokines (i.e., IL-6 and IL-1 β) was higher in the synovium than in the other regions.

Conclusions: Our study is among the first to evaluate the effects of AGEs on each tissue of the shoulder joint in patients with DM having rotator cuff tears and contractures. The accumulation of AGEs in each tissue of the shoulder joint could reveal the locations affected by DM, which can lead to a better understanding of the pathophysiology of DM-related shoulder diseases.

Key words: advanced glycation end products; shoulder capsule; rotator cuff tears; synovium; reactive oxygen species; cell viability; diabetes mellitus

Background

Advanced glycation end products (AGEs) are aging-related compounds and end products of protein glycation.[1] These are the final glycation products produced by the non-enzymatic glycation of proteins in the Maillard reaction,[1,2] in which the ketone groups of reducing sugars react with the free amino groups of proteins, resulting in progressive rearrangement, dehydration, and condensation.[3] AGEs bind to their receptor i.e., receptor for AGEs (RAGE), which activates NADPH oxidase (NOX), thereby increasing the production of reactive oxygen species (ROS), which cause oxidative stress.[4] The production of AGEs increases with age and diabetes mellitus (DM), and AGEs have been reported to cause aging of the skin, internal organs, bones, cartilage, and lens.[5,6] The accumulation of AGEs is associated with coronary artery disease, glomerulosclerosis, and bone fragility, and its effects have been studied in various tissues.[7,8,9,10] AGEs accumulate due to slow metabolism and induce abnormal cross-linking of collagen, leading to tissue fibrosis and inflammation. [11]

The rotator cuff is also rich in collagen, and we have been investigating the relationship between AGEs and the rotator cuff. Using an aging rat model, Mifune et al.[12] reported that increased oxidative stress leads to increased apoptosis, and decreased cell viability and rotator cuff strength. Furthermore, they reported that accumulation of AGEs in human

rotator cuff-derived cells causes a decrease in cell viability and rotator cuff strength, and induces apoptosis due to the production of ROS.[12] In the previous studies using rat models of DM, the expression of AGEs in serum and immunohistochemistry increased in DM group compared with non-DM group, and collagen fibers of rotator cuff were disorganized and ruptured in DM group.[13,14] In clinical practice, rotator cuff tears (RCTs) are associated with joint contractures and synovitis; [15,16] however, there are few studies on the association of AGEs with each of these conditions. The accumulation of AGEs is reported to be associated with the disease periods of hyperglycemia. DM and aging have been reported as risk factors for the accumulation of AGEs.[17,18] In a previous study, Yano et al. compared gene expression level and immunoreactivities of RAGE in coracohumeral ligament and anterior inferior glenohumeral ligament in frozen shoulder group and RCTs group, and those of RAGE were greater in frozen shoulder group, suggesting that AGEs binding to RAGE might play important roles in frozen shoulder.[19] Shinohara et al.[20] compared AGEs deposition, cellular activity, ROS production, and apoptosis in the joint capsule in DM and non-DM patients with RCTs with shoulder joint contractures. Their results showed that the accumulation of AGEs was significantly higher in the DM group, suggesting that deposition of AGEs in the joint capsule may be involved in the shoulder contracture associated with RCTs in DM patients.

Hence, we hypothesized that in DM patients, AGEs are deposited not only in the joint capsule but also in the rotator cuff and synovium and are associated with RCTs and synovitis in addition to shoulder joint contractures. The aim of our study was to evaluate the effects of AGEs on intra-articular shoulder tissues (such as rotator cuff, synovium, and capsule) and compare them to determine the main loci of pathological conditions in which AGEs are deposited in patients with DM having RCTs.

Methods

Selection of study participants

This study was approved by our Ethics Committee (approval number: B200273), and we obtained informed consent from all patients who participated in this study. Patients with limited range of motion (ROM) of the shoulder joint related to degenerative RCT were included in this study. We excluded patients with the following conditions: traumatic RCT, rotator cuff re-tear, calcific tendinopathy, frozen shoulder without RCT, osteoarthritis, rheumatoid arthritis, absence of DM, and well-controlled DM (hemoglobin A1c [HbA1c] < 6.5%). Thus, eight patients with DM (five men and three women; average age: 64.6 years; range 59-72), who were treated surgically for RCT with ROM limitation of the shoulder joint in the 2-year period from April 2019 to January 2021 at our hospital, were

included. DM was defined as the presence of a preoperative HbA1c level $\geq 6.5\%$ based on the World Health Organization criteria for diabetes.[21] We defined ROM limitation of the shoulder joint as restriction of active shoulder joint motion (anterior elevation $< 120^\circ$ and external rotation $< 40^\circ$),[16] with normal radiographic findings.

Isolation of samples from the shoulder joint

Tissue samples of the rotator cuff, synovium, and capsule were harvested intraoperatively. Some of them were fixed in formalin for histological staining, and the remaining samples were minced to roughly 1 mm³ in size and cultured. The minced tissue samples were cultured in monolayer in 100 mm diameter culture dishes which contain Dulbecco's modified Eagle medium (DMEM; HyClone, Logan, UT, USA) mixed with 10 % fetal bovine serum (Cansera, Rexdale, Ontario, Canada), 100 $\mu\text{g/mL}$ streptomycin, and 100 U/mL penicillin. The cultures were incubated at 37°C in a humidified atmosphere of 5 % CO₂/95 % air and were passaged every 1–2 weeks. We used these cells in passages 2–4 for the investigations.

Histological examination

After formalin fixation for 24 h, the tissues were dehydrated and embedded in paraffin

wax. Paraffin specimens were sliced to 5 μm sections and mounted on slides, and hematoxylin and eosin (H&E) staining (Fig. 1) and immunostaining (Fig. 2) were performed. For immunostaining, the sections were deparaffinized, dehydrated, and incubated overnight at 4°C with the primary antibodies, anti-AGE (10 $\mu\text{g/mL}$; Abcam, Cambridge, UK), which is specific for carboxymethyl lysine, and anti-RAGE (10 $\mu\text{g/mL}$; Abcam). Thereafter, the sections were incubated with the secondary antibodies (Histofine® Simple Stain MAX Po; Nichirei Biosciences Inc., Tokyo, Japan) for 1 h at room temperature (25°C) and counterstained with H&E. Digital images of the slides were obtained using the BioZero BZ-8000 microscope (Keyence, Osaka, Japan). For quantifying the stained areas, in accordance with the previous study, [20,22] we used ImageJ (ver. 1.52), a Java-based public domain image processing software developed by the National Institutes of Health. Briefly, the color images were digitally processed and separated into blue, red, and green images. Pixel subtraction was done by subtracting the blue image from the red image, and the mean pixel intensity of the subtracted image was calculated (Fig. 2 (a)). The staining rate of AGEs and RAGE was calculated as the average of four fields of view.

Cell viability (Cell proliferation assay)

Cell viability was evaluated by the water-soluble tetrazolium salt (WST) assay with the Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). After seeding each of the 96 wells with 5,000 cells and incubating them in DMEM for 24 h, 10 μ L of WST was added to the well and incubated at 37°C for 4 h. After incubation, the soluble formazan produced by the viable cells was quantified. Cell viability was assessed by measurement of the absorbance of the reduced formazan product at 450 nm.

ROS expression

Immunofluorescence staining was performed to evaluate the effect of ROS on cells derived from the rotator cuff, synovium, and capsule. In accordance with previous studies, [12,20,23,24] the ROS expression was detected using the ROS-ID® Total ROS/Superoxide Detection Kit (Enzo Life Sciences, Inc., Farmingdale, NY, USA). Cells (5×10^4 /mL) were incubated with the oxidation-sensitive fluorescent probe dichlorodihydrofluorescein diacetate at a final concentration of 10 μ M for 1 h at 37°C in the dark. Thereafter, the cells were rinsed with phosphate buffered saline and then resuspended in trypsin. To quantify, the number of ROS-positive and 4',6-Diamidino-2-phenylindole (DAPI)-positive cells were counted on each slide in the four fields of view (0.75 mm $\times$ 1.0 mm).

174

175 *Apoptosis rate*

176 In accordance with previous studies, [12,20,23,24] the apoptosis rate of the cells was
177 compared using immunofluorescence staining performed two days after incubation. The
178 apoptosis rate was assessed by terminal deoxynucleotidyl transferase dUTP nick end
179 labeling staining using the APO-DIRECT® kit (Phoenix Flow Systems, San Diego, CA,
180 USA) based on the manufacturer's protocol. The ratio of the green-stained apoptosis to
181 DAPI-positive cells was calculated in the four fields of view of each slide.

182

183 *Quantitative real-time polymerase chain reaction (PCR)*

184 1.0×10^5 cells were seeded in each of the 12 wells and cultured in DMEM for 48 h. The
185 total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) based
186 on the manufacturer's protocol. The High Capacity cDNA Transcription Kit (Applied
187 Biosystems, Foster City, CA, USA) was used for synthesizing the oli-
188 go(deoxythymidine)-primed first-strand cDNA. Quantitative real-time PCR was per-
189 formed on an ABI Prism 7500 sequence detection system (Applied Biosystems) with a
190 20-μL reaction mixture using the SYBR Green Master Mix reagent (Applied Biosys-
191 tems). The PCR conditions were as follows: one cycle of 10 min at 95°C, followed by 40

cycles of 15s at 95°C, and 40 cycles of 1min at 60°C.

The messenger RNA expression of type I collagen (COL1), type III collagen (COL3), NOX1, NOX4, RAGE, interleukin-6 (IL-6), and interleukin-1 β (IL-1 β) were determined using the aforementioned method. The primer sequences are shown in Table I. Using glyceraldehyde-3-phosphate dehydrogenase as a reference, the relative gene expression levels were calculated using the delta-delta-Ct method. The expression of each gene was compared between the three regions of the shoulder i.e., rotator cuff, synovium, and capsule.

Table 1

Primers for Real-time PCR

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
<i>NOX1</i>	GGTTTTACCGCTCCCAGCAGAA	CTTCCATGCTGAAGCCACGCTT
<i>NOX4</i>	GCCAGAGTATCACTACCTCCAC	CTCGGAGGTAAGCCAAGAGTGT
<i>RAGE</i>	CACCTTCTCCTGTAGCTTCAGC	AGGAGCTACTGCTCCACCTTCT
<i>COL1</i>	AGGAATTCGGCTTCGACGTT	GGTTCAGTTTGGGTTGCTTG
<i>COL3</i>	GGGAACAACCTTGATGGTGCT	CCTCCTTCAACAGCTTCCTG
<i>IL-6</i>	AGACAGCCACTCACCTCTTCAG	TTCTGCCAGTGCCTCTTTGCTG
<i>IL-1β</i>	TACGAATCTCCGACCACCACTACAG	TGGAGGTGGAGAGCTTTCAGTTCATATG
<i>GAPDH</i>	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA

Abbreviations: NOX: NADPH oxidase; RAGE: receptor for advanced glycation end products; COL1: type I collagen; COL3: type III collagen; IL-6: interleukin-6; IL-1 β : interleukin-1 β ; GAPDH: glyceraldehyde-3-phosphate dehydrogenase

205

206 *Statistical analysis*

207 All data are represented as the mean $\pm$ standard deviation. The cell viability and

208 quantitative real-time PCR were represented as n-fold changes from the baseline control.

209 Significant differences between the three regions i.e., rotator cuff, synovium, and cap-

210 sule were detected using the Kruskal–Wallis test and the Steel–Dwass method. All sta-

211 tistical analyses were performed with Statcel, a Microsoft Excel add-in statistical soft-

212 ware package (Social Survey Research Information Co., Ltd., Tokyo, Japan). Statistical

213 significance was set at $p < 0.05$.

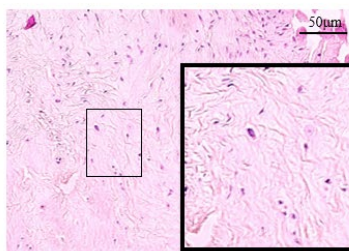
214

Results

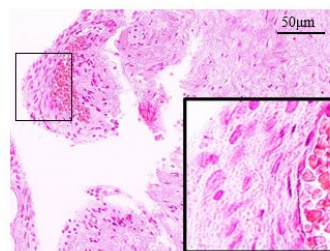
Histology and immunohistochemistry

Histological staining (H&E staining) exhibited disorganized collagen arrangement in the rotator cuff, and multilayered synovial cells were observed in the synovium (Fig. 1). In the capsule, dense collagen fibers and proliferation of fibroblasts with dark blue color and spindle-shaped nuclei were observed (Fig. 1). Brown-colored AGEs were observed mainly in the extracellular matrix. The percentage of the AGEs and RAGE staining was calculated as the average of the four fields of view, and the percentage of staining for both AGEs and RAGE was observed in the following order: rotator cuff > joint capsule > synovium. The RAGE staining revealed the presence of dark red stain around the cell nuclei. The average percentage of AGEs staining was significantly higher in the rotator cuff (23.1 ± 0.25 %), followed by the capsule (7.15 ± 0.4 %) and synovium (1.41 ± 0.03 %) (Fig. 2 (b)). Similarly, the average percentage of RAGE staining was significantly higher in the rotator cuff (8.73 ± 0.41 %), followed by the capsule (2.28 ± 0.21 %) and synovium (0.56 ± 0.07 %) (Fig. 2 (b)).

(a) Rotator cuff



(b) Synovium



(c) Capsule

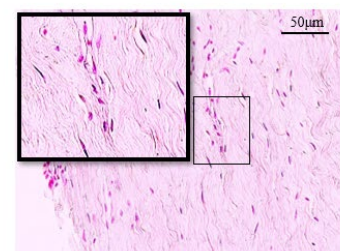
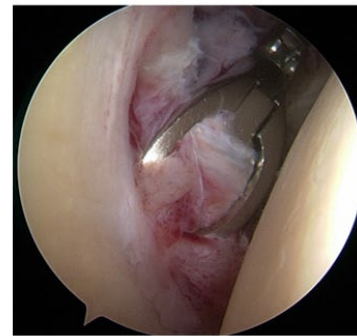
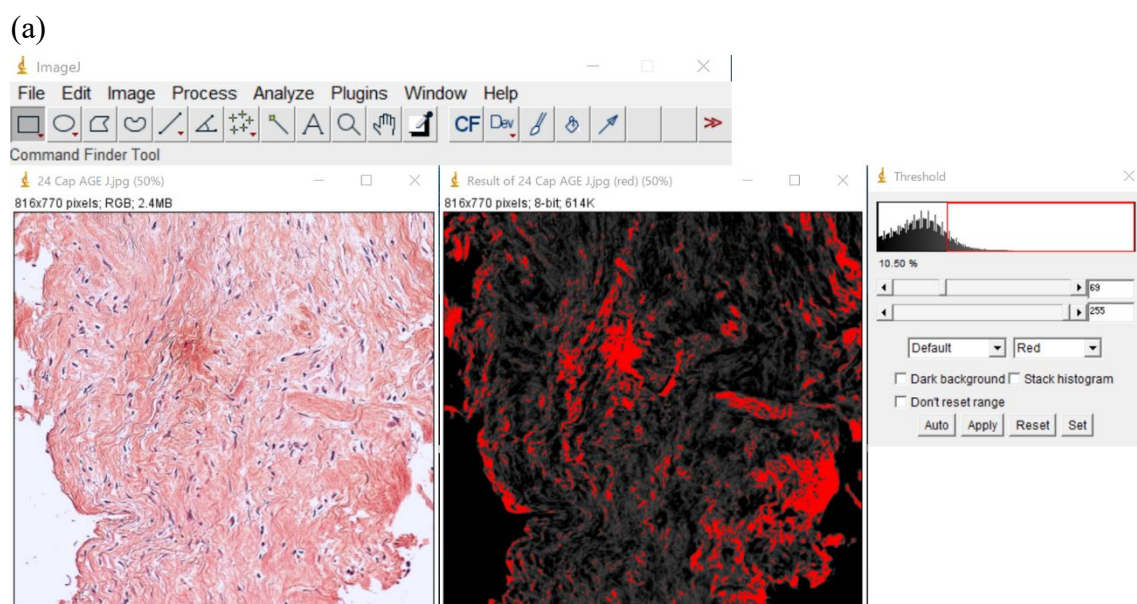
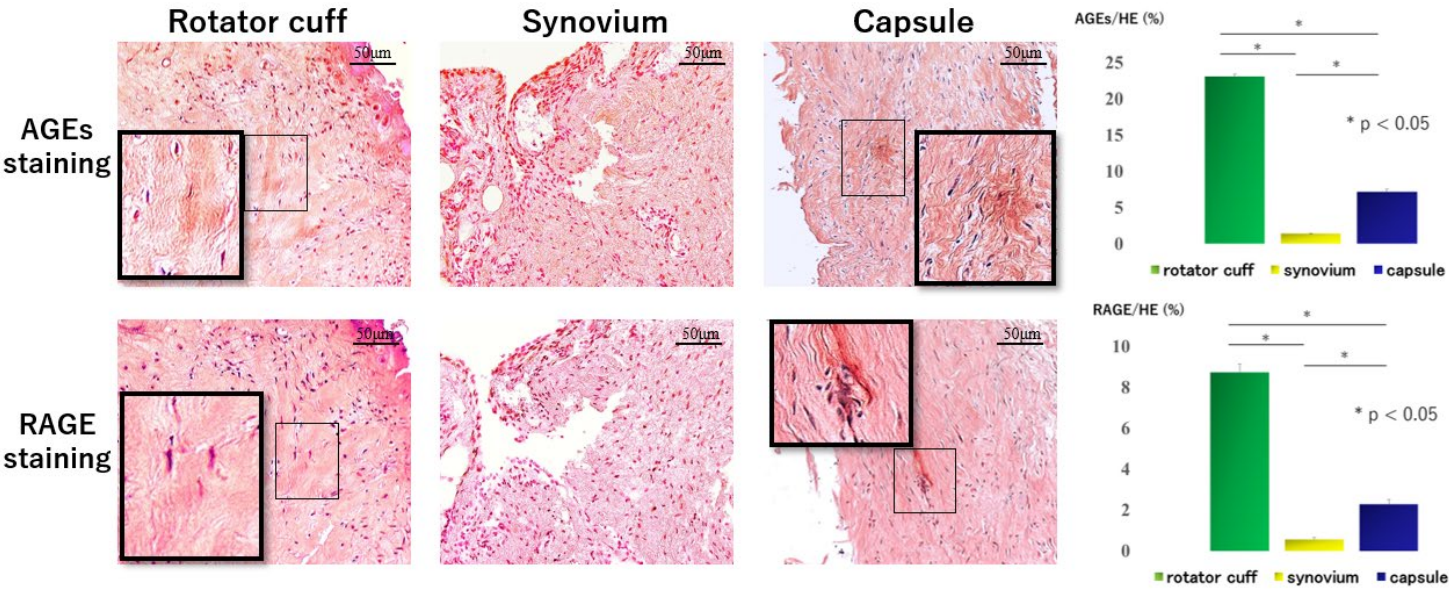


Fig. 1. Hematoxylin and eosin staining. (a) The collagen arrangement of the rotator cuff was disorganized, and (b) the synovium showed multilayered synovial cells. (c) In the capsule, dense collagen fibers and proliferating fibroblasts with dark blue color and spindle-shaped nuclei were observed.





237 Fig. 2. Immunostaining for AGEs and RAGE. (a) Quantification of the percentage of
238 immunostaining to hematoxylin and eosin staining using the ImageJ software. (b) Both
239 AGEs and RAGE staining were significantly higher in the rotator cuff, followed by the
240 capsule and synovium.

241 Abbreviations: AGEs: advanced glycation end products; RAGE: receptor for advanced
242 glycation end products; HE: hematoxylin-eosin staining

244 *Cell viability (Cell proliferation assay)*

245 As observed in Fig. 3, the cell viability, expressed as fold change, was significantly higher
246 in the synovium (1.65 ± 0.024) than in the rotator cuff (1.00 ± 0.041) and capsule ($0.97 \pm$
247 0.038).

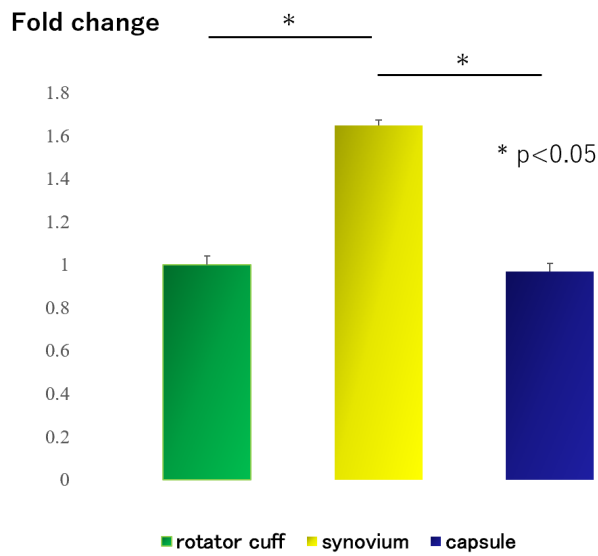


Fig. 3. Cell viability. Cell viability was represented as n-fold changes from the rotator cuff. Cell viability was significantly higher in the synovium than in the rotator cuff and capsule.

ROS expression

ROS staining, which indicates the presence of oxidative stress, was semi-quantified by the percentage of green-stained ROS-positive cells to the DAPI-stained blue cells. The percentage of ROS staining was higher in the rotator cuff (22.7 ± 2.3 %) and capsule (17.4 ± 3.4 %) than in the synovium (4.8 ± 0.9 %) (Fig. 4).

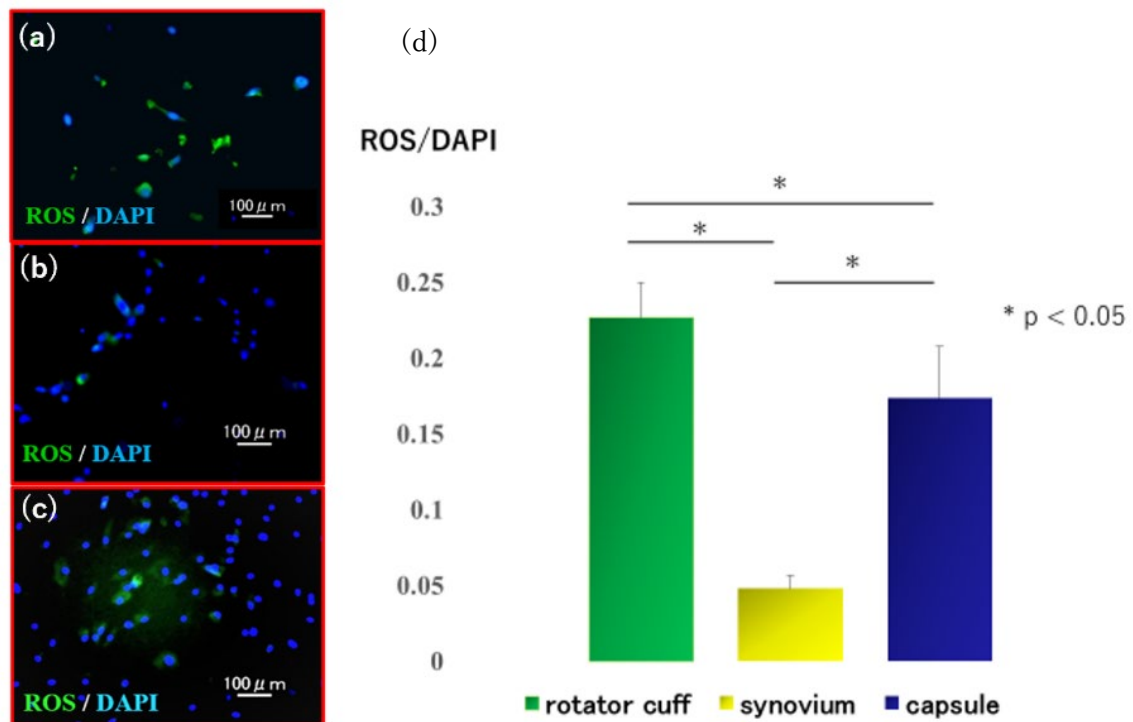


Fig. 4. ROS staining. (a) rotator cuff. (b) synovium. (c) capsule.

(d) The percentage of ROS staining (green-colored cells) was higher in the rotator cuff and capsule than in the synovium, with the highest percentage observed in the rotator cuff.

Abbreviations: ROS: reactive oxygen species; DAPI: 4',6-Diamidino-2-phenylindole

Apoptosis rate

The percentage of cells that underwent apoptosis was semi-quantified as the ratio of green-stained nuclear fragments to DAPI-stained cells. The apoptosis rate was significantly higher in the rotator cuff (14.0 ± 2.3 %) and capsule (8.8 ± 3.4 %) than in the synovium (2.4 ± 0.9 %) (Fig. 5).

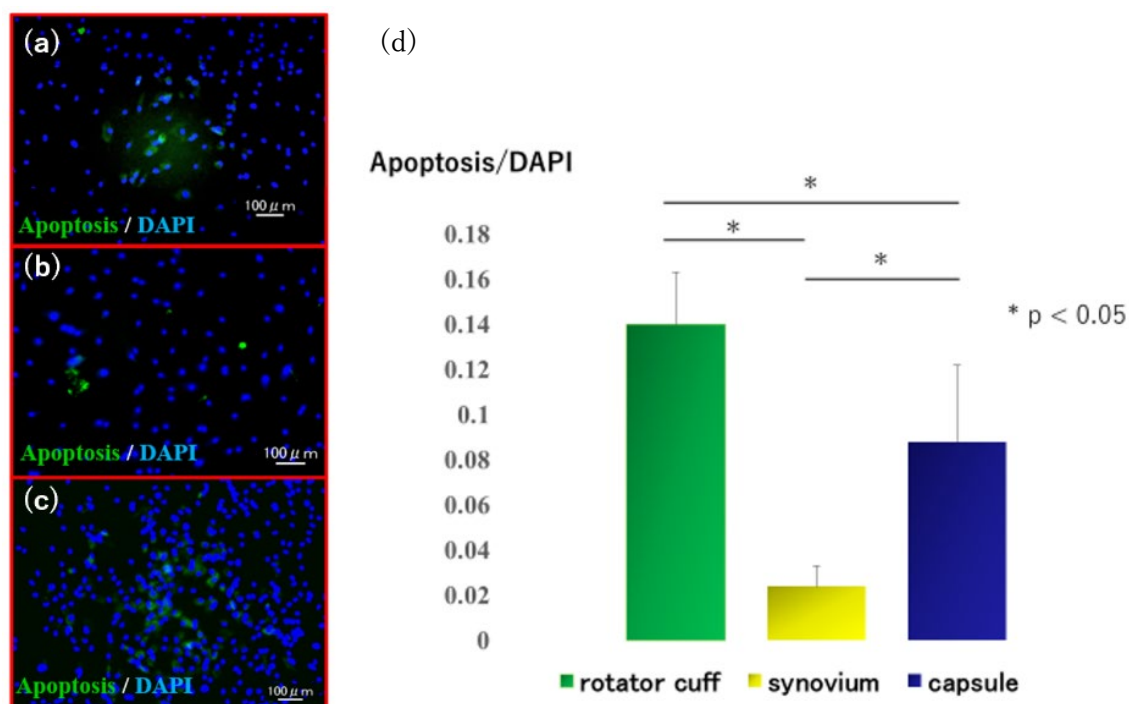


Fig. 5. Apoptosis rate. (a) rotator cuff. (b) synovium. (c) capsule.

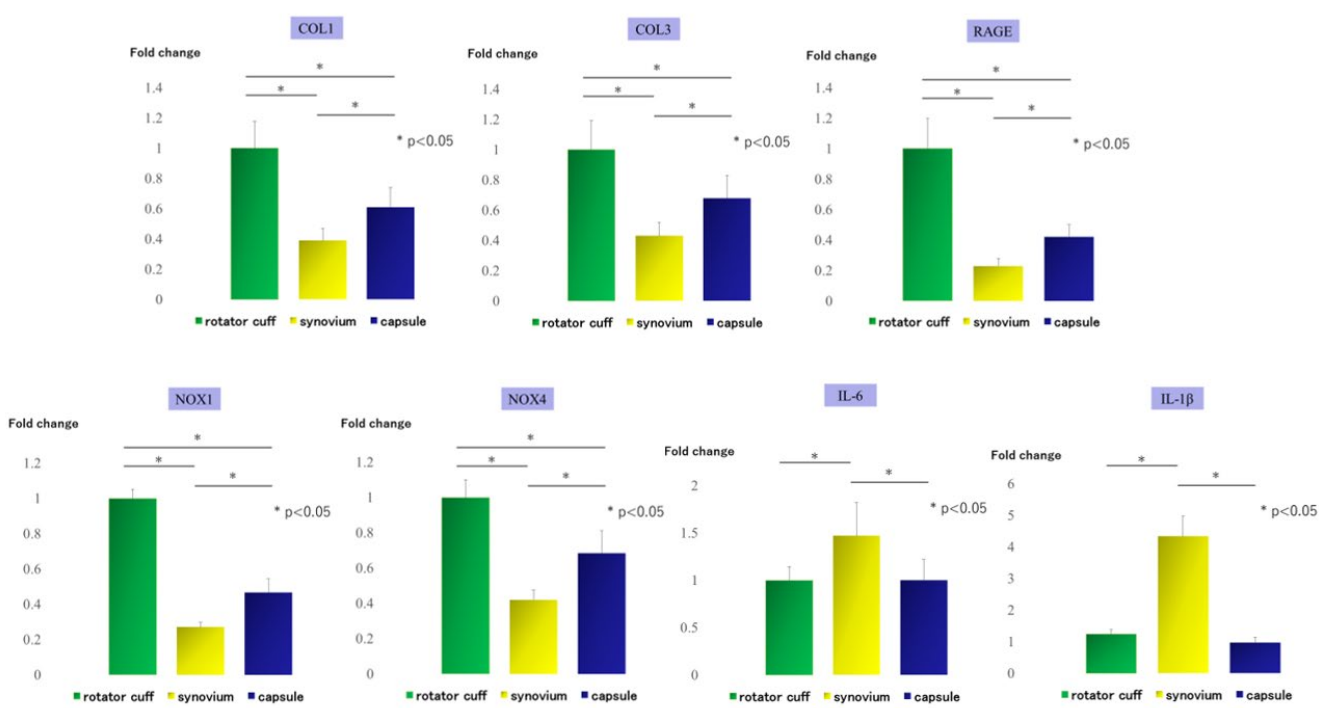
(d) The rate of apoptosis was significantly higher in the rotator cuff and capsule than in the synovium, with the highest rate observed in the rotator cuff.

Abbreviation: DAPI: 4',6-Diamidino-2-phenylindole

Quantitative real-time PCR

Gene expressions of COL1, COL3, RAGE, NOX1, NOX4, IL-6, and IL-1 β were compared between the rotator cuff, synovium, and capsule. In terms of collagen expression, the COL1 and COL3 gene expressions were the highest in the rotator cuff, followed by the capsule and synovium. Similarly, the highest expression of RAGE was

278 in the rotator cuff, followed by capsule and synovium (Fig. 6). The gene expressions of
 279 NOX1 and NOX4, which are involved in increasing the production of ROS, were sig-
 280 nificantly higher in the rotator cuff and capsule than in the synovium (Fig. 6). In contrast,
 281 the expressions of the inflammatory cytokine genes IL-6 and IL-1 β were significantly
 282 lower in the rotator cuff and capsule than in the synovium (Fig. 6).



283 Figure 6. Results from the quantitative real-time polymerase chain reaction. The
 284 quantitative real-time PCR were represented as n-fold changes from the rotator cuff.
 285 Abbreviations: NOX: NADPH oxidase; RAGE: receptor for advanced glycation end
 286 products; COL1: type I collagen; COL3: type III collagen; IL-6: interleukin-6; IL-1 β : in-
 287 terleukin-1 β

Discussion

Many studies have reported aging and DM as risk factors for the accumulation of AGEs. The disease periods of hyperglycemia are reported to play a role in the accumulation of AGEs; additionally, slow-metabolizing AGEs tend to accumulate in DM.[25,9] In tissues, collagen is prone to AGEs accumulation, causing its abnormal cross-linking, which leads to inflammation and tissue fibrosis.[11] Furthermore, AGEs bind to their receptor i.e., RAGE, which activates NOX, thereby causing ROS production.[4] The generation of ROS induces oxidative stress, causing tissue damage and fibrosis in addition to the abnormal cross-linking of collagen.[4,26] The increased ROS levels cause an increase in the production of inflammatory cytokines through the activation of NFκB.[27] In addition, increased ROS levels also induce mitochondrial DNA damage and activate caspases, thereby inducing apoptosis.[28] As a result, the increased inflammatory cytokine levels and apoptosis lead to fibrosis and tissue fragility.[29] Our study findings indicated that increased levels of AGEs are associated with increased gene expressions of RAGE and NOX, as well as increased ROS production. This increase in ROS production was accompanied by increased apoptosis, decreased cell viability, and increased levels of inflammatory cytokines. In cardiovascular and chronic kidney diseases, extracellular AGEs induce oxidative stress, inflammation, and cell apoptosis.[20,30]

In this study, we investigated the pathogenesis of the accumulation of AGEs, cellular activity, ROS production, apoptosis rate, and gene expression (NOX, RAGE, inflammatory cytokines, and collagen) in the rotator cuff, capsule, and synovium of the shoulder joint in patients with DM having RCTs. Tissue samples of various tissues from the shoulder joint (synovium and rotator cuff) were obtained for comparing the cell viability and differentiation ability, and it was found that the cell viability and differentiation ability of the synovial-derived cells were higher than that of cells present in the other tissues.[31] Moreover, the synovial cells also showed the highest cellular activity. These findings are explained by the fact that the synovium is mainly composed of sparse connective tissue, and has a large number of cells and a low accumulation of AGEs. In contrast, the expression of IL-6 and IL-1 β was significantly higher in the synovium, suggesting that the synovium is less directly affected by AGEs; however, it could be the main site of inflammation. Previous studies have reported the following findings: (a) IL-1 β expression in the subacromial bursa is higher in patients with DM,[32] (b) IL-1 β expression in the synovium is higher in patients with massive RCTs,[15] and (c) RCTs are more likely to progress in patients with DM[33]. These findings suggest that IL-1 β is highly expressed in the synovium of patients with DM, which are consistent with the results of this study.

324 In this study, the levels of AGEs, ROS, and apoptosis among patients with DM having
325 RCTs were higher in the rotator cuff and joint capsule than in the synovium. This can be
326 explained by the fact that the rotator cuff is rich in collagen fibers and easily accumulates
327 AGEs. AGEs induce oxidative stress, damage DNA, and cause cell death through mitotic
328 catastrophe.[34] Previous reports indicate that excessive ROS production induces
329 cytotoxicity and apoptotic cascades via phosphorylation of c-Jun N-terminal kinase
330 (JNK) and activation of Bax, and via activation of caspases and regulation of Bcl-2 family
331 protein expression.[35,36] ROS promotes the process of apoptotic overproduction by
332 increasing caspase-3 activity and suppressing Bcl-2 expression, suggesting an interaction
333 between oxidative stress and apoptosis.[28] ROS have been shown to induce apoptosis of
334 rotator cuff tenofibroblasts.[37] These findings suggest that in patients with DM,
335 deposition of AGEs in the rotator cuff can lead to rotator cuff fragility and susceptibility
336 to RCTs. Gotoh et al.[38] highlighted the increased concentration of IL-1 β in the
337 synovium of patients with anterior shoulder instability. This instability, secondary to RCT,
338 could cause an increased production of IL-1 β , which could lead to the onset of
339 synovitis.[39] Our findings indicated that the joint capsule was also rich in collagen fibers
340 and showed the proliferation of fibroblasts. The ROM was limited due to tissue fibrosis
341 and adhesion to the surrounding tissues caused by AGEs. In a study investigating the

presence of AGEs in shoulder tissues, the amount of AGEs accumulated in the joint capsule was higher in patients with a frozen shoulder than in patients with unstable shoulder arthritis or RCTs, indicating that fibroblast proliferation and collagen fibrosis due to increased AGE expression are the causes of a frozen shoulder.[40]

To the best of our knowledge, this study is among the first to evaluate the effects of AGEs on each tissue of the shoulder joint (rotator cuff, synovium, and joint capsule) in patients with DM having RCTs. In patients with DM, AGEs tend to be deposited in the rotator cuff and joint capsule, contributing to rotator cuff fragility and joint capsule fibrosis, which may be pathological conditions that cause RCTs and shoulder joint contractures.

In contrast, patients with DM having RCTs often encounter the complication of synovitis. However, this study suggests that the synovium is not easily affected by AGEs and synovitis is secondary to RCTs.

This study has several limitations. First, the sample size was not large. Hence, further studies with more number of samples are required to confirm the results of this study.

Second, we had no information regarding the administration of steroid injections by the patients prior to the study period; however, we were able to confirm that none of the patients received steroid injections within one month prior to the surgery. Administration of steroids could affect the underlying biologic events in the tendon. Thus, further studies

are required to confirm the results of this study.

Conclusions

Our study is among the first to evaluate the effects of AGEs on each tissue of the shoulder joint i.e., rotator cuff, synovium, and joint capsule in patients with DM having RCTs with contracture. In our study on the patients, the levels of AGEs, ROS, and apoptosis were higher in the rotator cuff and joint capsule than in the synovium. This is due to the higher content of collagen fibers in the rotator cuff and joint capsule, which allows the easy accumulation of AGEs. In contrast, the synovium, composed of sparse connective tissue, had more cells and was less affected by AGEs. The accumulation of AGEs due to aging and DM could possibly contribute to rotator cuff fragility and fibrosis of the joint capsule. Determining the locations of the shoulder in which AGEs accumulate can lead to a better understanding of the pathophysiology of DM-related shoulder diseases.

373 **Declarations**

374 **Ethics approval and consent to participate**

375 The Kobe University Graduate School of Medicine Ethics Committee approved this study
376 (No. B200273). Each author certifies that all investigations were conducted in conformity
377 with ethical principles. All study participants provided informed consent. All procedures
378 performed in this study were in accordance with the ethical standards of the Institutional
379 Review Board of Kobe University Graduate School of Medicine, the 1964 Declaration of
380 Helsinki and its subsequent amendments, or comparable ethical standards.

381 **Consent for publication**

382 Not applicable

383 **Availability of data and materials**

384 The data presented in this study are available on request from the corresponding author.

385 The data are not publicly available because of confidentiality issues.

386 **Competing interests**

387 The authors declare that they have no competing interests.

388 **Funding**

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Authors' contributions

IS, AI, YM, HN, and RK contributed to the study concept and design. IS, and AI contributed to the data acquisition, analysis. TK and YM drafted the article and TK and IS, HN, TY, FT, MK, ST, YH, TM, and RK contributed to the interpretation of the data and critically revised the article for important intellectual content. All authors gave final approval of the version to be submitted for publication and agreed to be held accountable for all aspects of the work.

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