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Influence of advanced glycation end products on rotator cuff

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Background: Most rotator cuff tears are the result of age-related degenerative changes, but the mechanisms underlying these changes have not been reported. Recently, advanced glycation end products (AGEs) have been regarded as an important factor in senescence. Therefore, we hypothesized that AGEs would have detrimental effects on rotator cuff–derived cells. In this study, we investigated the influence of AGEs on rotator cuff–derived cells in vitro and ex vivo.

Methods: Rotator cuff–derived cells were obtained from human supraspinatus tendons. The cells were cultured in the following media: (1) regular medium with 500 μg/mL AGEs (High-AGEs), (2) regular medium with 100 μg/mL AGEs (Low-AGEs), and (3) regular medium alone (Control). Cell viability, secretion of vascular endothelial growth factor, and the expressions of hypoxia-inducible factor-1α, reactive oxygen species, and apoptosis were assessed after cultivation. An ex vivo tissue culture with AGEs was also performed to measure the tensile strength.

Results: Cell viability in the High-AGEs group was significantly suppressed relative to that in the Controls. The amount of vascular endothelial growth factor secretion was significantly greater in the High- and Low-AGEs groups than in the Controls. Immunofluorescence stain demonstrated enhancement of hypoxia-inducible factor-1α and reactive oxygen species expressions and cell apoptosis in the High- and Low-AGEs groups relative to that in the Controls. In ex vivo mechanical testing, tensile strength was significantly higher in the Control group than in the AGEs groups.

Discussion: These results indicated that AGEs caused age-related degenerative rotator cuff changes. The reduction of AGEs might prevent rotator cuff senescence-related degeneration.

Level of evidence: Basic Science Study; Histology and Biomechanics

Keywords: Advanced glycation end products; rotator cuff; reactive oxygen species; apoptosis; degeneration; hypoxia-inducible factor-1α; vascular endothelial growth factor

Rotator cuff tear is one of the most common disorders associated with pain and dysfunction in the shoulder. It has been reported that 20.7% of 1366 shoulders, with a mean age of 57.9 years, had full-thickness rotator cuff tears in medical checkups.[37] Rotator cuff tears can be caused by acute injury,

The study protocols were approved by the Ethics Committee of Kobe University Graduate School of Medicine (No. 1735).

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but most are the result of age-related degenerative changes.[23] Multiple etiologies have been implicated in the pathogenesis of rotator cuff tears, including intrinsic factors, such as aging, diabetes, and hypercholesterolemia,[1,17] and extrinsic factors, such as subacromial and internal impingement, tensile overload, and repetitive stress.[19,23] Pathologic changes in rotator cuff degeneration have been reported to include thinning and disorientation of collagen fibers as well as loss of cellularity, vascularity, and fibrocartilage mass at the site of cuff insertion.[19] However, the mechanisms underlying age-related degenerative changes have not been elucidated.

Recently, advanced glycation end products (AGEs) have been regarded as an important factor related to senescence. AGEs are the result of chemical reaction chains after an initial glycation reaction, and formation and accumulation of AGEs have been implicated in progression of age-related diseases.[27] AGEs are mainly accumulated in collagen, which is the main component of the rotator cuff tendon, because collagen is an extracellular protein exposed to glycemia and has a slow metabolic turnover. AGEs can induce abnormal covalent intermolecular bonds of collagen in the matrix and interfere with cell-matrix interactions as well.[2,4-6,15,20,25] Moreover, AGEs have been reported to alter signal transduction pathways, reduce levels of important cytokines, and directly alter protein function in target tissues during the healing process.[12]

On the other hand, hypoxia-inducible factor-1 (HIF-1) is a key transcription factor involved in the regulation of intracellular metabolism, which induces the expression of downstream gene vascular endothelial growth factor (VEGF).[3] Moreover, reactive oxygen species (ROS) are also known to be critical for senescence, and the relationship between AGEs and ROS was recently reported.[28] However, the mechanism by which AGEs modulate the expressions of VEGF, HIF-1α, and ROS in rotator cuff tissue remains unclear.

Considering this background, we hypothesized that AGEs would have detrimental effects on rotator cuff–derived cells. Therefore, in this study, we investigated the influence of AGEs on rotator cuffs under in vitro and ex vivo conditions.

Materials and methods

Preparation of human rotator cuff tissue and derived cells

Human rotator cuff tissues were obtained from torn edges of supraspinatus tendons during arthroscopic rotator cuff repair. Twelve patients with rotator cuff tears were enrolled, with an average age of 64.8 years (range, 32-72 years). The human adult rotator cuff tissues were transported in sterile saline solution on ice. For isolation of rotator cuff–derived cell, the tissues (approximately 0.3 g each) were cut into small pieces under sterile conditions, followed by 4 hours of digestion in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) supplemented with 30 mg/mL collagenase II (Gibco, Big Cabin, OK, USA) at 37°C, 95% humidity, and 5% CO_2. After digestion, the cells were pelleted, washed in phosphate-buffered saline, and subsequently cultured in 25-cm^2 cell culture flasks with Dulbecco's modified Eagle's medium supplemented with 2% fetal bovine serum (Sigma) and 1% penicillin-streptomycin (Sigma) (regular medium). The cultures obtained from each patient were gathered into one for each examination, and these cells were already analyzed in the previous paper and showed mesenchymal stem cells-related markers and pluripotency.[22] All experiments were performed with 1 or 2 passaged cells, and the same passage of cells was used for each experiment.

Cell culture with AGEs

To assess the effects of AGEs on rotator cuff–derived cell, the cells were plated to a density of 5×10^4 cells/well in 12-well plates and cultured in each treated medium: (1) regular medium supplemented with 500 μg/mL AGEs (High-AGEs group), (2) regular medium supplemented with 100 μg/mL AGEs (Low-AGEs group), and (3) regular medium alone (Control group). The AGE concentrations of 100 and 500 μg/mL used in this study were representative of high and low plasma concentrations of AGEs in the normal human body, respectively.[36]

Cell viability assays

Cell viability was evaluated by performing a water-soluble tetrazolium salt assay using a Cell Counting Kit-8 in the same way as previously reported.[32] The cells in each treatment group were plated in 96-well plates and cultured with regular medium for 3 days; then each well was supplemented with 10 μL/well water-soluble tetrazolium salt stain for 3 hours before spectrophotometric evaluation. The control for AGEs (only media supplemented with AGEs) and a media blank (no cells, just media) were run in parallel with the other groups. This reaction reflects the reductive capacity of the cells, which represented the viability of the cells. Values were obtained by comparing these cells with their respective controls.

VEGF expression in the supernatant of the cultured cell

We estimated the expression of VEGF in the supernatant of the cultured cells in each experimental group. As previously described, the rotator cuff–derived cells (each containing 5×10^4 cells) were incubated with each treated medium in 12-well dishes for 24 hours. The amount of VEGF that was secreted into the supernatant was measured by the enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA) as previously described.[22]

Immunofluorescence staining for HIF-1α

After the cells were cultured for 3 days, the culture solution was removed and the cells were washed 3 times in phosphate-buffered saline. The cells were fixed by using 4% paraformaldehyde for 10 minutes. They were then incubated with a polyclonal antibody to

HIF-1α (anti-HIF-1α antibody; Abcam, Cambridge, MA, USA) at 1:100 dilution at room temperature for 1 hour. The secondary antibody for HIF-1α was Alexa Fluor 488-conjugated goat anti-mouse IgG (Abcam) used at a 1:500 dilution at room temperature for 2 hours. The diamidino-2-phenylindole (DAPI) solution was applied for 5 minutes for nuclear staining. The number of stained cells was counted in 5 randomly selected fields by using a BZ-8000 confocal microscope (Keyence, Osaka, Japan).

Detection of ROS

ROS is also known to be critical for senescence, and an increase in ROS has an important role in senescence induction.[17] The expression of ROS was assessed by immunofluorescence staining 3 days after cultivation by using the ROS Detection Mix (Sigma) according to the manufacturer's protocol. The nucleus was stained with DAPI, and the numbers of stained cells were counted in 5 randomly selected fields by using a BZ-8000 confocal microscope (Keyence).

Cell apoptosis analysis

To evaluate the effect of AGEs on cell apoptosis, immunofluorescence staining was performed 3 days after cultivation by using an APO-DIRECT Kit (BD Pharmingen, Bedford, MA, USA) according to the manufacturer's protocol. The DAPI solution was also applied for nuclear staining. After staining, we assessed the number of stained cells in 5 randomly selected fields by using a BZ-8000 confocal microscope (Keyence).

Tissue culture and biomechanical test

An ex vivo tissue culture was performed to confirm the effect of AGEs on rotator cuff tissue by using upper limbs from Sprague-Dawley rats, because an animal model with rotator cuff repair in rat has been established and used in lots of studies.[11,13,30] Four Sprague-Dawley rats (10-week-old) were euthanized with CO_2 overdose. Both the upper limbs were harvested, and then the right shoulders were cultured in the regular medium supplemented with 500 μg/mL AGEs (AGEs group) for 7 days, and the left ones in the regular medium alone (Control group) for the same days. After tissue cultivation, 4 shoulders from each group were biomechanically tested according to the protocol previously reported.[13] Before the biomechanical tests, the forelimbs were completely freed of all soft tissue except for the infraspinatus muscle, which was carefully dissected. The prepared upper limb was mounted in a conventional tensile tester, model AGIS 5kN (Shimadzu, Tokyo, Japan). The applied load was directed along the median axis of the infraspinatus muscle. Before testing, we applied 10 cycles of 1-N longitudinal loads to the shoulder specimen for preconditioning. Then, each upper limb was stretched at a cross-head speed of 0.25 mm/s until gross failure of the infraspinatus tendon occurred.

Statistical analysis

All data are expressed as mean values ± standard deviations. Experiments using rotator cuff–derived cells isolated from 12 different donors were repeated on separate occasions, and the data are presented as an average of all donors except where otherwise indicated. All conditions are expressed as an n-fold difference from the baseline control at the corresponding time point. Comparisons among multiple groups were made by performing the analysis of variance test followed by a Kruskal-Wallis test. A value of $P < .05$ was considered as indicating statistical significance.

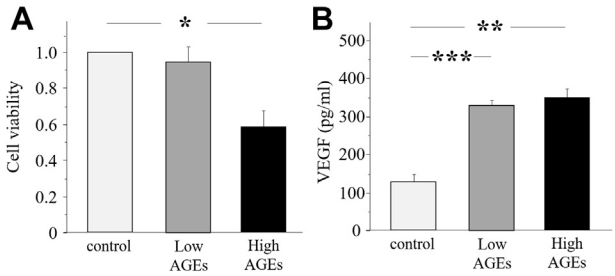

Figure 1 (**A**) Cell viability in the High-AGEs group was significantly suppressed relative to that in the Control group, whereas there was no significant difference between the Low-AGEs group and the Control group (*$P = .006$). (**B**) The estimated concentrations of VEGF in the supernatant from cultured cells were significantly greater in the High- and Low-AGEs groups than in the Control group (**$P < .001$, ***$P = .002$). *AGE*, advanced glycation end product; *VEGF*, vascular endothelial growth factor.

Results

Cell viability assays

Cell viability in the High-AGEs group was significantly suppressed relative to that in the Control group, whereas there was no significant difference between the Low-AGEs group and the Control group (High-AGEs, 0.59 ± 0.08; Low-AGEs, 0.92 ± 0.09; Control, 1.00 ± 0.12; $P = .006$ for the High-AGEs group vs. the Control group) (Fig. 1, *A*).

Enzyme-linked immunosorbent assay for VEGF secretion

The estimated concentrations of VEGF in the supernatant from cultured cells were significantly greater in the High- and Low-AGEs groups than in the Control group (High-AGEs, 354.3 ± 26.0; Low-AGEs, 321.5 ± 12.4; Control, 139.0 ± 17.3 [$\times 10^5$ pg/cell/24 h for all]). $P < .001$ and $P = .002$ for the Control vs. the High- and Low-AGEs groups, respectively (Fig. 1, *B*).

HIF-1α expression

HIF-1α expressions were significantly greater in the High- and Low-AGE groups than in the Control group, but the difference was not significant between the High- and Low-AGEs groups (High-AGEs, 65.3 ± 15.1/mm^2; Low-AGEs,

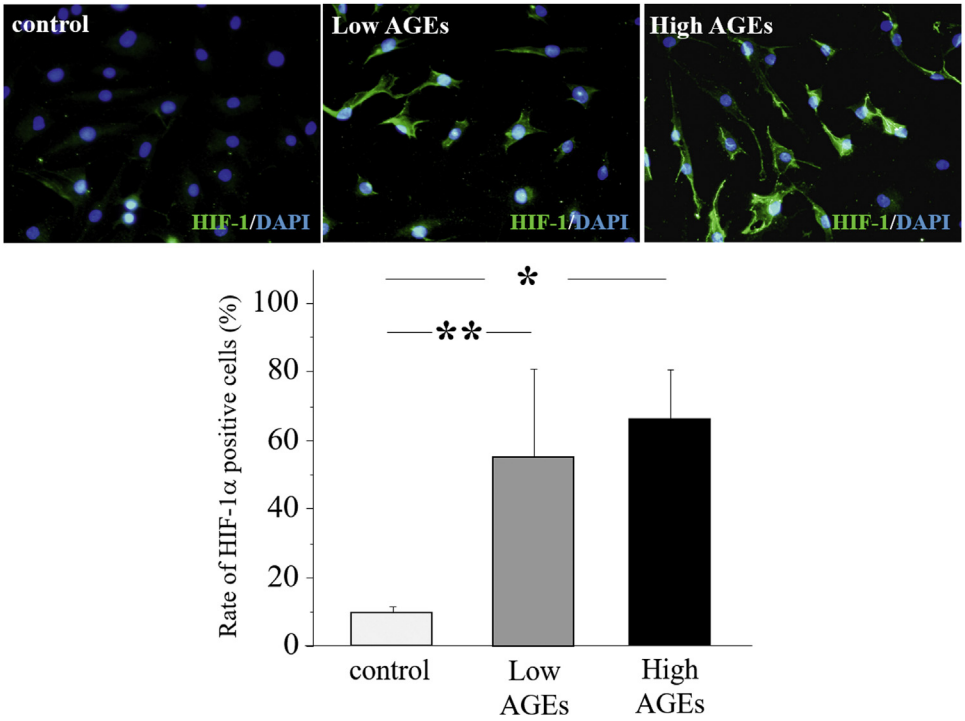

Figure 2 HIF-1α protein expressions were significantly greater in the high- and low-AGEs groups than in the control group (*$P = .006$, **$P = .008$). *HIF-1α*, hypoxia-inducible factor-1α; *AGE*, advanced glycation end product; *DAPI*, diamidino-2-phenylindole.

57.5 ± 22.8/mm^2; Control, 10.7 ± 2.2/mm^2). $P = .006$ and $P = .008$ for the Control vs. the High- and Low-AGEs groups, respectively (Fig. 2).

Expression of ROS

Immunofluorescence staining of ROS demonstrated enhancement of ROS expression in the High-AGEs group relative to that in the Control group. The numbers of ROS-positive cells were significantly higher in the High- and Low-AGEs groups than in the Control group and was significantly higher in the High-AGEs group than in the Low-AGEs group (High-AGEs, 83.7 ± 2.8/mm^2; Low-AGEs, 71.8 ± 1.7/mm^2; Control, 27.4 ± 1.8/mm^2; $P = .007$ and $P = .009$ for the High- and Low-AGEs groups vs. the Control group, respectively; $P = .043$ for the High-AGEs vs. Low-AGEs groups) (Fig. 3, *A*).

Apoptotic cell analysis

Immunofluorescence staining for apoptotic cells showed induction of cell apoptosis in the High-AGEs group that was not observed in the Control group. The numbers of apoptotic cells were significantly higher in the High- and Low-AGEs groups than in the Control group and in the High-AGEs group than in the Low-AGEs group (High-AGEs, 20.4 ± 1.8/mm^2; Low-AGEs, 8.2 ± 0.7/mm^2; Control, 3.2 ± 1.5/mm^2; $P = .009$ and $P = .007$ for the High-AGEs vs. the Low-AGEs group and the Control group, respectively; $P = .033$ for the Low-AGEs vs. the Control group) (Fig. 3, *B*).

Ex vivo effect of AGEs on biomechanical strength in rotator cuff tissue

After cultivation for 7 days, mechanical testing demonstrated significantly higher tensile strength in the Control group than in the AGEs group (AGEs, 13.5 ± 0.4; Control, 15.0 ± 0.2; $P = .038$ for the AGEs group vs. the Control group) (Fig. 4).

Discussion

In the present study, our results showed that AGEs had detrimental effects on rotator cuff–derived cells in vitro, including decreased cell viability, enhancement of VEGF and HIF-1α expressions, and increased ROS expression and cell apoptosis. Moreover, AGEs decreased the biomechanical strength of the tendon tissue ex vivo.

AGEs are produced by post-translational modification of proteins via nonenzymatic glycation, accumulate with age, and abundantly increase in the case of diabetes. They have been shown to be deposited in atherosclerotic lesions and to promote diabetes-accelerated atherosclerosis.[16,21] Moreover, AGEs impair cell-matrix interactions and growth factor depletion.[31] Recent studies have also shown that AGEs promote endothelial progenitor cell (EPC) apoptosis, inhibit

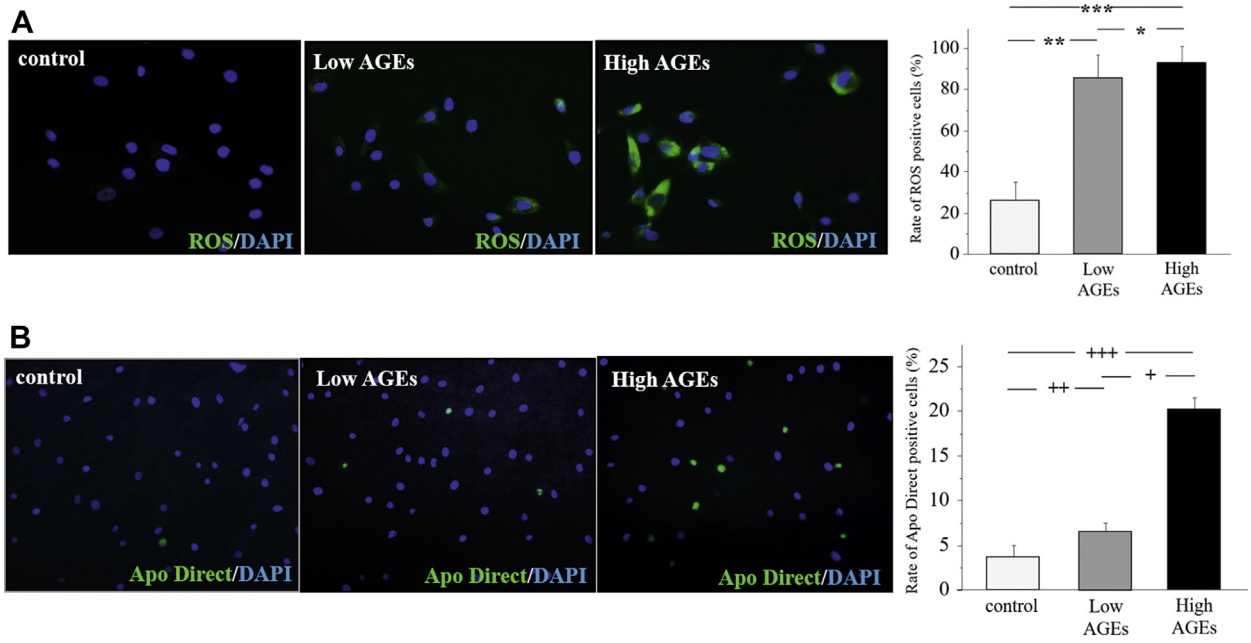

Figure 3 (**A**) Immunofluorescence staining of ROS demonstrated enhancement of ROS expression in the High-AGEs groups. The numbers of ROS-positive cells were significantly higher in the High- and Low-AGEs groups than in the Control group, and were significantly higher in the High-AGEs group than in the Low-AGEs group (*** $P = .007$, ** $P = .009$, *$P = .043$). (**B**) Immunofluorescence staining for apoptotic cells showed induction of cell apoptosis in the High-AGEs group that was not observed in the Control group. The numbers of apoptotic cells were significantly higher in the High- and Low-AGEs groups than in the Control group and in High-AGEs group than in the Low-AGEs group (+++ $P = .007$, ++$P = .009$, + $P = .013$). *ROS*, reactive oxygen species; *AGE*, advanced glycation end product; *DAPI*, diamidino-2-phenylindole.

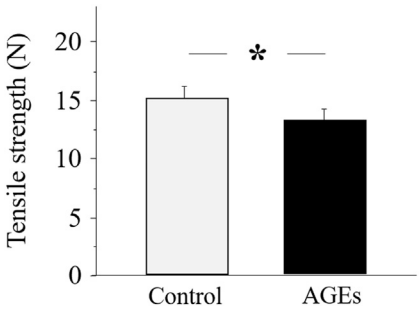

Figure 4 After ex vivo tissue culture for 7 days, mechanical testing demonstrated significantly higher tensile strength in the Control group than in the advanced glycation end products (AGEs) groups (*$P = .038$).

EPC proliferation, and impair EPC functions, such as migration, adhesion, and tube-forming ability.[7,9] This evidence suggests that increased AGEs contribute, at least in part, to the vascular damage in aged patients and patients with diabetes. On the other hand, AGEs upregulate the expression of VEGF protein in vascular endothelial cells by increasing the levels of HIF-1α protein.[42] A recent paper reported that HIF-1α was found to have a role in degeneration of rotator cuff and that HIF and VEGF expressions were correlated with fatty infiltration and muscle atrophy in the rotator cuff.[18] In the present study, our results indicated that AGEs promoted VEGF secretion and increased HIF-1α expression in rotator cuff–derived cells. Therefore, we think that the accumulation of AGEs could promote HIF-1α expression by upregulating VEGF expression resulting in degeneration of the rotator cuff.

ROS are also considered to be critical for oxidative stress and senescence and have been shown to induce apoptosis in rotator cuff tenofibroblasts.[10,24,41] Apoptosis of rotator cuff tenofibroblasts is known to be related to rotator cuff tendon rupture.[8,12] Oxidative stress involves an imbalance between oxidation caused by ROS and reduction elicited by antioxidant systems, leading to the initiation and progression of age-related diseases, such as diabetes, hypertension, atherosclerosis, osteoporosis, and neurodegenerative diseases.[29] However, the relationship between AGEs and ROS in rotator cuff tissue remains unclear. In chondrocytes, AGEs-induced apoptosis occurs primarily via ROS production and mitochondria-mediated caspase-3 activation.[38] AGEs have also been reported to increase the apoptosis of rat bone marrow stem cells via activation of caspases and involve Tumor Necrosis Factor α (TNFα) production/secretion, p38 Mitogen-activated Protein Kinase (MAPK) signaling, and oxidative stress.[35] The present study revealed that AGEs increased ROS expression and the apoptotic rate in rotator cuff–derived cells. Wang et al[34] stated that an antioxidant enzyme, peroxiredoxin 5, was significantly upregulated in the degenerative supraspinatus

tendon relative to that in the normal subscapularis tendon, which suggested a compensated response to oxidative stress in the degenerative tendon. Other studies have also shown that exogenous oxidative stress by H_2O_2 loading induced cellular apoptosis in human tendon fibroblasts.[39,40] Therefore, it is thought that AGEs enhance ROS expression, which results in an increase in the apoptosis rate of rotator cuff–derived cells.

Disruption of collagen metabolism is known to be associated with age-related changes in connective tissues.[14,26,33] Our results indicated that AGEs have detrimental effects on the rotator cuff because of decreased cell viability, enhancement of VEGF and HIF-1α expressions, and increased ROS expression and cell apoptosis, which could result in degeneration of the rotator cuff.

There were several limitations in this study. First, our results were obtained in vitro only; therefore, an in vivo study using aging models or overuse models, which are accepted in current practice for the investigation of rotator cuff degeneration, is needed. Second, 2 AGE concentrations were used in the experiments, 100 and 500 μg/mL, which were representative of the plasma concentrations of AGEs described in a referenced paper.[35] To determine optimal concentration ranges, other concentrations of AGEs should be examined. Finally, for clinical applications, the accumulation of AGEs in human rotator cuff and the AGE concentration in the synovial fluid of the human shoulder should be determined. These limitations of the current study suggest avenues to be explored in further research.

Conclusion

This study demonstrated that AGEs had detrimental effects on rotator cuff–derived cells, including decreased cell viability, enhancement of VEGF and HIF-1α expressions, and increased ROS expression and cell apoptosis. These results indicate that AGEs could cause age-related degenerative changes in the rotator cuff, and reduction of AGEs might prevent rotator cuff degeneration caused by senescence.

Disclaimer

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