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Original Article

Inhibitory effect of taurine on rotator cuff degeneration via mitochondrial protection

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Abstract: Objectives: Degenerative rotator cuff tears do not heal spontaneously, necessitating surgical intervention. This makes prevention crucial, but effective prophylactic measures are currently lacking. Oxidative stress has recently been implicated as a cause of degenerative rotator cuff tears, while mitochondrial injury has been reported in the development of age-related rotator cuff degeneration. Taurine, which has antioxidant properties, has been found to be effective in the treatment of various mitochondrial abnormalities. This prompted us to investigate the inhibitory effect of taurine and some other antioxidants against rotator cuff degeneration using tenocytes. Methods: Hydrogen peroxide (H_2O_2, 2 mM) was added to tenocytes in medium with 0.8 μM taurine (Group TAU), medium with 100 μM α-tocopherol (Group E), and medium with 150 μM ascorbic acid (Group C), then each medium was cultured for 24 h. Tenocytes supplemented with 2 mM H_2O_2 alone were similarly cultured for 24 h (Group H_2O_2). In each group, immunostaining was performed for the oxidative stress marker 8-hydroxy-2'-deoxyguanosine and advanced glycation end products (AGE), which contribute to the development of age-related rotator cuff degeneration. In addition, levels of reactive oxygen species were measured using a cell-based assay kit, and results were compared. Immunostaining was also performed for indices of apoptosis (caspase-9, cleaved caspase-3 and Bcl-2), and Western blotting was used to quantify activation of caspase-9 at an early stage in each group. Results: Oxidative stress and AGE levels were decreased in the E and C groups. Levels of all parameters were reduced in the TAU group. Conclusions: Taurine showed preventative effects against rotator cuff degeneration. The simple method of administration and paucity of side effects make clinical application easy, and the clear potential as a novel prophylactic strategy against degenerative rotator cuff tear warrants further study.

Keywords: Degenerative non-traumatic rotator cuff tears, taurine, oxidative injury, advanced glycation end products (AGE), reactive oxygen species (ROS), mitochondria-mediated apoptosis

Introduction

Rotator cuff tears are caused by trauma and rotator cuff degeneration. Such injuries greatly disrupt daily life through major decline in the ability to conduct activities of daily living (ADL). Once a rotator cuff has ruptured, spontaneous repair does not occur and surgical intervention becomes necessary. Since there is unclear timing on the onset of degenerative, non-traumatic rotator cuff tears, it makes investigation of the underlying pathophysiology difficult, and detailed studies into the causes of degenerative rotator cuff tear have not yet been conducted. Oxidative stress has recently been implicated as one underlying cause [1, 2]. Apoptosis has also been reported to play a role [3]. In an *in vitro* model in which hydrogen peroxide (H_2O_2) was added to tenocytes to induce rotator cuff degeneration [4], exposure of the tenocytes supplemented with H_2O_2 to hyaluronic acid was reported to inhibit tenocyte cytotoxicity and inflammation by reducing oxidative stress [5, 6]. However, the lack of information on the etiology and pathophysiology of degenerative rotator cuff tears has impeded the establishment of clearly effective preventive methods.

Levels of mitochondrial transcription factor A (TFAM) in the rotator cuff have been documented to decrease with age in some animal models, with a role for mitochondrial injury reported

in the development of age-related rotator cuff degeneration [7]. Mitochondria have also been implicated in the pathogenesis of oxidative injury [8, 9]. Various therapeutic and prophylactic strategies based on mitochondria-associated factors are now being devised for disorders of the cardiovascular system and various other systems [10]. Of these, taurine (2-aminoethanesulfonic acid) has recently attracted attention as a therapeutic agent useful against some mitochondrial abnormalities. Taurine is easy to ingest and does not appear to be associated with significant side effects. This substance also exhibits anti-inflammatory and antioxidant actions that contribute to mitochondrial functional repair and protection, and its efficacy as a therapeutic agent has been documented for some mitochondrial diseases such as mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) syndrome [11-16]. Moreover in an osteonecrosis model in which oxidative stress plays a role, the administration of taurine inhibited mitochondrial injury and in turn the development of osteonecrosis [8, 17-19]. Meanwhile, α-tocopherol and ascorbic acid exert potent antioxidant actions that are known to exert inhibitory effects on various conditions. Against this background, we undertook the present study using tenocytes supplemented with H_2O_2, to compare the inhibitory effect of taurine against rotator cuff degeneration with that of the antioxidants α-tocopherol and ascorbic acid, and to clarify their preventative effects against rotator cuff degeneration.

Materials and methods

Cell culture

Human tenocytes (Zen-bio, Durham, NC) were maintained as a subconfluent monolayer culture in tenocyte growth medium (Zen-bio) supplemented with 10% fetal calf serum. When the culture reached 70% confluency in culture at 37°C under 20% O_2 and 5% CO_2, human tenocytes were treated with 2 mM H_2O_2 (WAKO, Tokyo, Japan) for 24 h. Next, 0.8 μM taurine (WAKO) (Group TAU), 100 μM α-tocopherol (WAKO) (Group E) or 150 μM ascorbic acid (WAKO) (Group C) was added to the medium. As control groups, cells were cultured under 20% O_2 in culture medium without any H_2O_2, taurine, α-tocopherol or ascorbic acid (control group) or in culture medium containing only H_2O_2 (Group H_2O_2). Three independent experiments with each were carried out.

Immunostaining for 8-hydroxy-2'-deoxyguanosine (8-OHdG), advanced glycation end products (AGE), caspase-9, cleaved caspase-3, and Bcl-2

Immunostaining was performed for 8-OHdG, AGE, caspase-9, cleaved caspase-3, and Bcl-2 as markers of degenerative rotator cuff tear. Levels of the oxidative stress marker 8-OHdG, apoptosis initiator caspase-9, apoptosis effector caspase, cleaved caspase-3, and apoptosis inhibition factor Bcl-2 were also determined. AGE is produced during the process of age-related rotator cuff degeneration and therefore was also investigated.

Cultured cells were fixed in 4% paraformaldehyde, washed in phosphate-buffered saline (PBS), and permeabilized with 0.3% Triton X-100 in PBS. Non-specific binding was blocked by incubating sections with 10% bovine serum albumin (Dako Cytomation, Santa Clara, CA) in PBS for 15 min. Cells were incubated with anti-Bcl-2 (Proteintech, Rosemont, IL), anti-8-OHdG (Abcam, Cambridge, UK), anti-AGE (Abcam), anti-caspase-9 (Proteintech), or anti-cleaved caspase-3 (Cell signaling technology, Danvers, MA) antibody for 2 hours at concentrations of 2.0, 10.0, 5.0, 5.0, or 5.0 μg/ml, respectively, followed by fluorescence-labeled secondary antibody (for Bcl-2: Alexa 488; Thermo Fisher Scientific, Waltham, MA, and for 8-OHdG, AGE, caspase-9, and cleaved caspase-3: Alexa 594; Thermo Fisher Scientific) and by 4',6-diamidino-2-phenylindole (DAPI) for 30 min. After washing, a ProLong Diamond antifade mountant (Thermo Fisher Scientific) was added, and cover slips were mounted. Images were taken using a confocal microscope (LSM710; Carl Zeiss, Oberkochen, Germany). Fluorescence microscopy (470 nm and 530 nm LED modules) was performed at low magnification using a BZ-X700 fluorescence microscope (Keyence, Tokyo, Japan).

Determination of reactive oxygen species (ROS) level

Quantification of cell fluorescent intensity was done using ROS, a marker of oxidative injury. The widely used cell-permeable redox-sensitive

fluorescent probe 2,7-dichlorofluoroscin diacetate (DCFDA) becomes highly fluorescent 2,7-dichlorofluoroscein when oxidized by ROS and a specific reactive nitrogen species. The ROS Detection Cell-Based Assay Kit (Cayman Chemical, Ann Arbor, MI) uses DCFDA as a fluorescent probe to detect ROS production.

Tenocytes were seeded at a density of 3,000 cells per well in a 96-well plate. ROS measurements were conducted using the ROS Detection Cell-Based Assay Kit. Briefly, control cells to which nothing had been added, and cells of groups treated with 2 mM H_2O_2, 2 mM H_2O_2 and 0.8 μM taurine, 100 μM α-tocopherol or 150 μM ascorbic acid were incubated at 37°C, in 20% O_2 and 5% CO_2 for 24 h. After this period, cells were rinsed in PBS and incubated with 5 μM ROS staining buffer for 1.5 h. Next, the 5 μM ROS staining buffer was aspirated and exchanged for the cell-based assay buffer. As a positive control, 10 mM pyocyanin was used. Cell fluorescent intensity was measured with a plate reader (Fluoroscan Ascent; Thermo Fisher Scientific, Waltham, MA) able to measure at an excitation wavelength of 480-520 nm and an emission wavelength of 570-600 nm. In each group, 5 wells were assayed 3 times.

Western blotting

Immunoblotting was performed for quantification in each group for 2 h of H_2O_2 treatment. Protein was extracted using a protein extraction solution (M-PER Mammalian Protein Extraction Reagent; Thermo Fisher Scientific, Rockford, IL). Twenty micrograms of the protein was electrophoresed on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane (Atoh, Tokyo, Japan). Membranes were reacted overnight at 4°C with the primary antibodies. The primary antibody applied was anti-caspase-9 (Proteintech, Rosemont, IL) at a concentration of 1.0 μg/ml. After incubation with peroxidase-labeled goat anti-mouse or anti-rabbit immunoglobulin G antibody (Dako Cytomation) for 1 h at room temperature and vigorous washing, the nitrocellulose membrane was incubated with Chemiluminescence Luminol Reagent (Immuno Star LD; Wako, Tokyo, Japan) and photographed digitally using ImageQuant LAS 4000 mini (GE Healthcare Japan, Tokyo, Japan). Immunoblot using anti-actin monoclonal antibody (Sigma Chemical Co., St. Louis, MO) was used for standardization. Intensity was measured using the Multi Gauge v3.1 (Fujifilm, Tokyo, Japan). Experiments were repeated at least three times.

Statistical analysis

All quantified results were expressed as mean ± standard deviation. Statistical significance in comparisons of ROS levels between the control and each experimental group was analyzed using Dunnett's multiple comparison test. Significance was defined at the level of $P < 0.05$. All statistical analysis were performed using Stat View J-5.0 software (SAS Institute, Cary, NC).

Results

Inhibition of oxidative stress and rotator cuff degeneration by taurine, α-tocopherol, and ascorbic acid in tenocytes supplemented with H_2O_2

With the addition of H_2O_2 to tenocytes for 24 h (H_2O_2 group), expression of the oxidative stress marker 8-OHdG and AGE which are generated in age-related rotator cuff degeneration was confirmed. The TAU (taurine), E (α-tocopherol), and C (ascorbic acid) groups, as compared with the H_2O_2 group, showed decreases in expression of both 8-OHdG and AGE (**Figure 1**). That is, taurine, α-tocopherol, and ascorbic acid showed H_2O_2 preventative effects against oxidative stress and rotator cuff degeneration.

ROS inhibition due to taurine, α-tocopherol, and ascorbic acid in tenocytes supplemented with H_2O_2

In the H_2O_2 group, expression of ROS was confirmed as a marker of oxidative injury. In the E and C groups, ROS expression was decreased (*$P < 0.01$ vs. H_2O_2 group). The TAU group, as compared with the H_2O_2, E, and C groups, showed significant decreases in ROS (*$P < 0.01$) (**Figure 2**). These results confirmed that taurine, α-tocopherol, and ascorbic acid all exerted inhibitory effects on oxidative injury, and that the most potent antioxidant action among the three groups was exhibited by taurine.

Inhibitory effect against apoptosis induction by taurine, α-tocopherol and ascorbic acid in tenocytes supplemented with H_2O_2

Immunostaining of the H_2O_2 group confirmed expression of caspase-9, cleaved caspase-3,

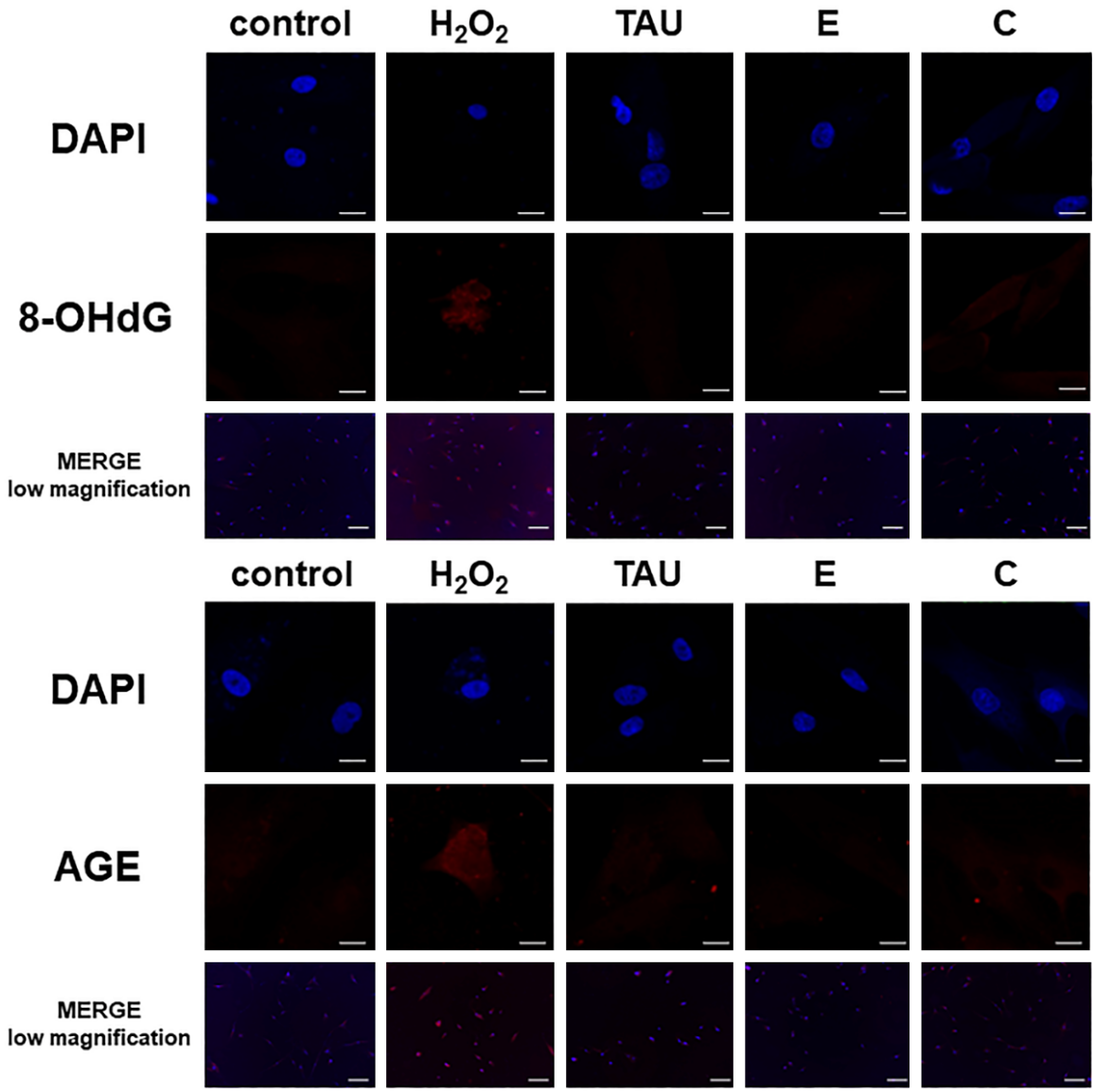


Figure 1. Inhibition of 8-hydroxy-2'-deoxyguanosine (8-OHdG) and advanced glycation end products (AGE) expression following exposure to taurine, α-tocopherol, and ascorbic acid in tenocytes supplemented with H_2O_2. The H_2O_2 group showed expression of 8-OHdG and AGE. In the TAU, E, and C groups, expressions of 8-OHdG and AGE were decreased. Scale bar: 20 μm, 200 μm (low magnification). Magnification: ×63, ×10 (low magnification).

and Bcl-2. The C group displayed shrunken nuclei and caspase-9 and cleaved caspase-3 expression. In the E group, despite the finding of caspase-9 expression, cleaved caspase-3 expression was absent, and Bcl-2 expression was identified. In the TAU group, neither caspase-9 nor cleaved caspase-3 expression was found, and Bcl-2 did not show any marked change as compared with the control group (**Figure 3A**, **3B**). The finding of cleaved caspase-3 expression in the H_2O_2 and C groups confirmed the induction of apoptosis in both these groups. These results confirmed that taurine inhibited the induction of apoptosis. Western blotting was also examined at 2 h after H_2O_2 treatment to verify the presence of caspase-9 activation at an early stage. With Western blotting and immunostaining, expression of caspace-9 was found in the H_2O_2 and C groups, while the E group showed only slight

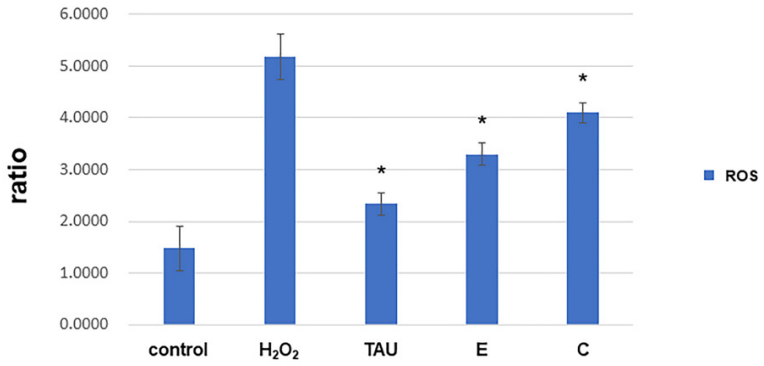


Figure 2. Reactive oxygen species (ROS) levels in tenocytes supplemented with H_2O_2 and exposed to taurine, α-tocopherol, and ascorbic acid. Using an ROS Detection Cell-Based Assay Kit, levels of ROS were measured in each group, as shown in the graphs (n = 5 each). Each graph indicates the ratio of ROS under the indicated conditions. Columns and bars indicate means and standard deviation. The H_2O_2 group showed ROS expression. In the E and C groups, ROS was decreased as compared with the H_2O_2 group (*P < 0.01). In the TAU group, ROS was significantly decreased as compared with the H_2O_2, E, and C groups (*P < 0.01).

expression. However, in the TAU group, activation of caspase-9 was suppressed even at the early stage (**Figure 4**).

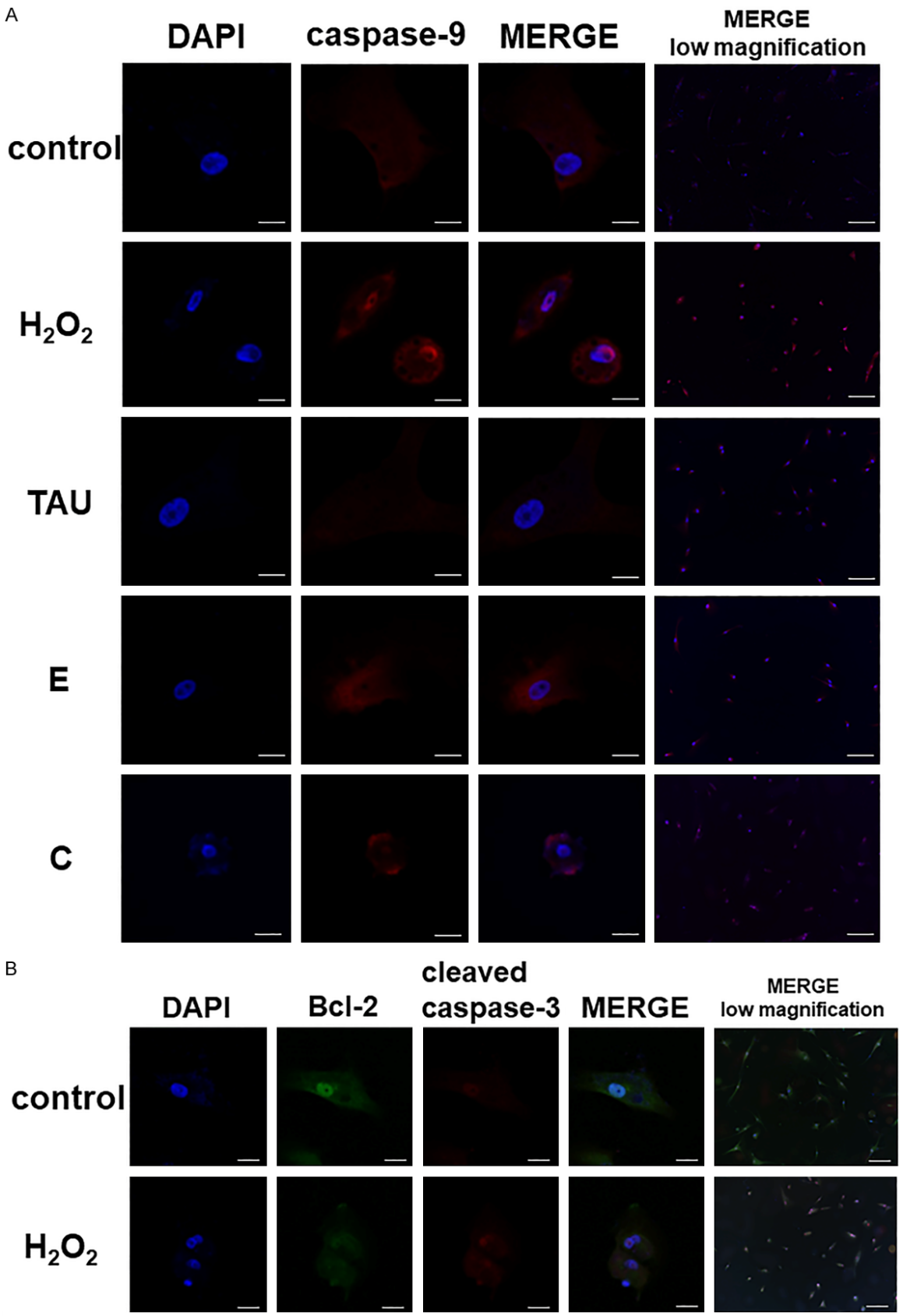
Discussion

When rotator cuff tears are due to degeneration, no treatment other than surgical intervention is available, and osteoarthritis progresses over time, culminating in cuff tear arthropathy. To treat the associated pain, highly invasive shoulder replacement arthroplasty eventually becomes necessary. Furthermore, rotator cuff degeneration is irreversible, and the only clinically available preventative countermeasure is injection of hyaluronic acid into the subacromial bursa. However, even this intervention only achieves pain relief, with no preventative effects on further rotator cuff degeneration.

Meanwhile, therapies for oxidative and mitochondrial injuries are being investigated in various fields. Strategies to treat mitochondrial injury with taurine have been proposed, and preventative effects have been documented [11, 12, 14, 15, 19]. The antioxidant action and efficacy of α-tocopherol and ascorbic acid have been described in both *in vivo* and *in vitro* studies, and validation of their usefulness in diverse disorders is anticipated [20-23]. Moreover, taurine and vitamin agents are easily ingested and have almost no side effects, making their clinical application trouble-free.

This research focused on the preventative effects of taurine and vitamin agents against oxidative stress, which is considered a major cause of rotator cuff degeneration. Taurine, α-tocopherol, and ascorbic acid have all demonstrated inhibition of oxidative stress. AGE, another factor thought to promote age-related rotator cuff degeneration, has been reported to induce apoptosis via increases in ROS production and mitochondria-mediated caspase-3 activity [24]. In this experiment, confirmation of the inhibition of AGE accumulation allowed us to indirectly confirm the inhibition of degeneration itself. Moreover, while cells exposed to H_2O_2 develop mitochondria-mediated apoptosis [25], cytochrome C is released in the pathway of caspase activation (intrinsic pathway) due to mitochondrial damage, and caspase-9 is activated. Activated caspace-9 then cleaves caspase-3, and apoptosis is induced by this activation of cleaved caspase-3 [26, 27]. In the present study as well, cleaved caspase-3 was expressed when tenocytes were exposed to H_2O_2, confirming induction of apoptosis.

This result suggested the possibility that rotator cuff degeneration might be clinically preventable. However, in the case of ascorbic acid, cleaved caspase-3 was expressed, nuclei were shrunken, and apoptosis was already in progress, implying weak inhibitory effects on rotator cuff degeneration as compared with the other two agents. A total preventative effect likewise cannot be claimed for α-tocopherol, because expression of the initiator caspase, caspase-9, was found. As compared with the vitamin groups, taurine inhibited both oxidative stress and degeneration, and also suppressed activation of caspase-9 and cleaved caspase-3, so taurine was considered the most effective of the three supplements for prevention. In this research, a preventative effect against rotator cuff degeneration was found in each of the three groups, with taurine in particular demonstrating a potent inhibitory effect against oxidative stress, degeneration, and apoptosis.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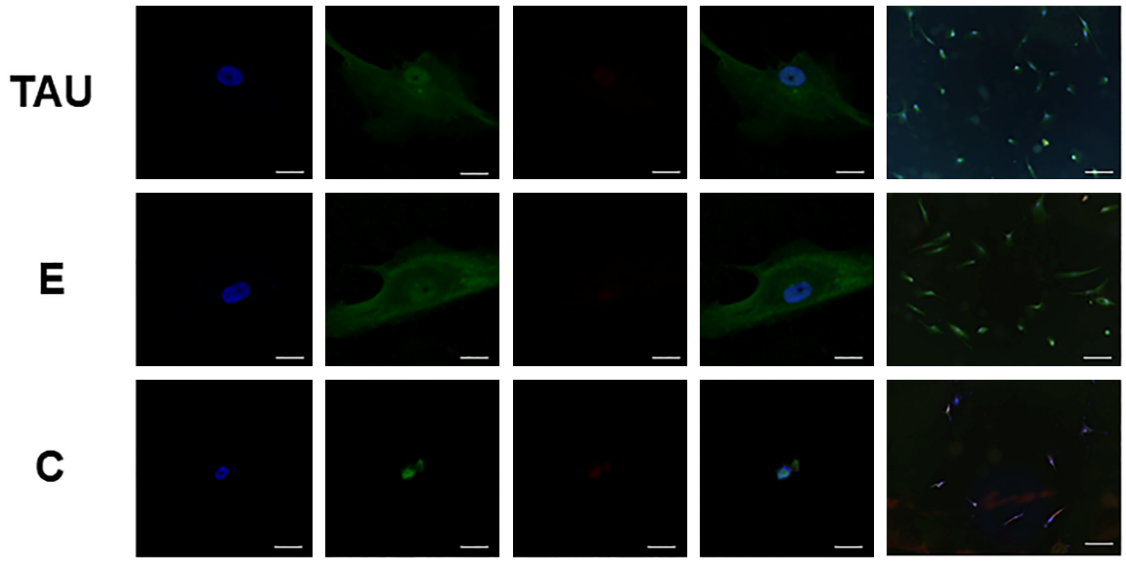

Figure 3. Caspase activation by exposure to taurine, α-tocopherol, and ascorbic acid in tenocytes supplemented with H_2O_2. A. Immunocytochemical study of apoptosis initiator caspase-9. B. Immunocytochemical studies of apoptosis inhibition factor Bcl-2 and the effector caspase cleaved caspase-3. With immunostaining, the H_2O_2 group showed expression of initiator caspase, caspase-9, and cleaved caspase-3, along with expression of the inhibition factor Bcl-2. In the C group, expression of caspase-9, cleaved caspase-3, Bcl-2 and nuclear shrinkage was found. In the E group, caspase-9 and Bcl-2 expression was present. In the TAU group, caspase-9 and cleaved caspase-3 expression was decreased, and Bcl-2 expression was found. Inhibition of apoptosis induction by taurine was evident, as was inhibition of caspase-9 activation. Scale bar: 20 μm, 200 μm (low magnification). Magnification: ×63, ×10 (low magnification).

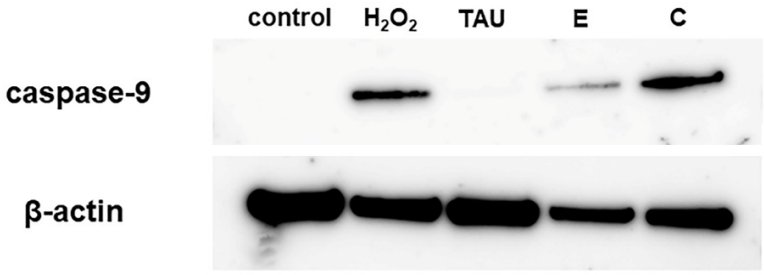

Figure 4. Caspase-9 activation by exposure to taurine, α-tocopherol, and ascorbic acid in tenocytes supplemented with H_2O_2 at an early stage. Western blotting for caspase-9 (35 kDa) with 2 h of H_2O_2 treatment. With Western blotting, caspase-9 expression was found in the H_2O_2 and C groups, while the E group showed only slight expression of caspase-9. No caspase-9 expression was found in the TAU group. Inhibition of caspase-9 activation by taurine was found at the early stage.

Caspase-9 is known to regulate mitochondria-mediated apoptosis and production of ROS [28-30]. Inhibition of caspase-9 has also been reported to prevent mitochondrial oxidative stress and apoptosis [31]. Furthermore, inhibition of oxidative stress and mitochondria-mediated apoptosis has been documented as effective against traumatic brain injury and alcoholic-induced liver injury [32, 33]. This study therefore examined the early activity of caspase-9 not only as an indicator of apoptosis, but also to confirm whether taurine can inhibit mitochondrial oxidative stress at an early stage. The results showed that taurine inhibited caspase-9 activity early after H_2O_2 treatment. This suggests that taurine, which exerts a mitochondrial protective effect, can prevent mitochondrial damage at an early stage. Taurine may therefore prevent degeneration to the rotator cuff by inhibiting oxidative stress and mitochondria-mediated apoptosis at an early stage. We thus conclude that taurine shows the most promise for preventing rotator cuff degeneration and rupture, particularly since clinical application is easy.

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Disclosure of conflict of interest

None.

Abbreviations

H_2O_2, hydrogen peroxide; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; AGE, advanced glycation end products; ROS, reactive oxygen species; ADL, activities of daily living; TFAM, mitochondrial transcription factor A; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis, stroke-like episodes; PBS, phosphate-buffered saline; DAPI, 4',6-diamidino-2-phenylindole; DCFDA, 2,7-dichlorofluoroscin diacetate.

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