Spine

SPINE Volume 44, Number 10, pp E585–E595

BASIC SCIENCE

OPEN

Mitochondrial Pathway Is Involved in Advanced Glycation End Products-Induced Apoptosis of Rabbit Annulus Fibrosus Cells

Yiqiang Hu, MD,* Zengwu Shao, MD, PhD,* Xianyi Cai, MD, PhD,* Yunlu Liu, MD,† Min Shen, MD,* Yingtao Yao, MD,‡ Tian Yuan, MD,§ Wentian Wang, MD,* Fan Ding, MD, PhD,¶ and Liming Xiong, MD, PhD*

Study Design. Experimental study.

Objective. The purposes of this study were to evaluate whether advanced glycation end-products (AGEs) induce annulus fibrosus (AF) cell apoptosis and further to explore the mechanism by which this process occurs.

Summary of Background Data. Recent studies revealed that AGEs accumulation is considered an important factor in diabetic intervertebral disc (IVD) degeneration. However, the effect of AGEs on intervertebral disc remains unclear.

Methods. AF cells were treated with various concentrations of AGEs for 3 days. Cell viability and cell proliferation were measured by Cell Counting Kit-8 (CCK-8) and 5-ethynyl-2′-deoxyuridine (EdU) incorporation assays, respectively. Cell apoptosis was examined by Annexin V/PI apoptosis detection kit and Hoechst 33342. The expression of apoptosis-related proteins, including Bax, Bcl-2, cytochrome c, caspase-3, and caspase-9, was detected by western blotting. In addition, Bax and Bcl-2 mRNA expression levels were detected by real-time PCR (RT-PCR). Mitochondrial membrane potential (MMP) and intracellular reactive oxygen species (ROS) production of AF cell were examined by 5,5′,6,6′ -Tetrachloro-1,1′,3,3′- tetraethyl-imidacarbocyanine iodide (JC-1) staining and 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescent probes, respectively.

Results. Our results indicated that AGEs had inhibitory effects on AF cell proliferation and induced AF cell apoptosis. The molecular data showed that AGEs significantly up-regulated Bax expression and inhibited Bcl-2 expression. In addition, AGEs increased the release of cytochrome c into the cytosol and enhanced caspase-9 and caspase-3 activation. Moreover, treatment with AGEs resulted in a decrease in MMP and the accumulation of intracellular ROS in AF cells. The antioxidant N-acetyl-L-cysteine (NAC) significantly reversed AGE-induced MMP decrease and AF cell apoptosis.

Conclusion. These results suggested that AGEs induce rabbit AF cell apoptosis and mitochondrial pathway may be involved in AGEs-mediated cell apoptosis, which may provide a theoretical basis for diabetic IVD degeneration.

Key words: advanced glycation end-products, annulus fibrosus, apoptosis, intervertebral disc degeneration.

Level of Evidence: N/A

Spine 2019;44:E585–E595

From the *Department of Orthopedics, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China; †Department of Orthopaedic Surgery, Yan-Tai-Shan Hospital, Yantai, China; ‡Collaborative Innovation Center for Biomedical Engineering, Wuhan National Laboratory for Optoelectronics-Huazhong University of Science and Technology, Wuhan, China; §Dongfeng Honda Automobile Co., Wuhan, Hubei, China; and ¶Department of Orthopaedic Surgery, The First People's Hospital of Jingmen, Jingmen, Hubei, China.

Acknowledgment date: July 12, 2018. First revision date: September 11, 2018. Acceptance date: October 3, 2018.

YH, ZS, and XC have contributed equally to this article.

The manuscript submitted does not contain information about medical device (s)/drug (s).

National Key Research and Development Program (2016YFC1100100); National Natural Sciences Foundation of China (81401827, 81572204, 81702197, 91649204); Graduates' Innovation Fund, Huazhong University of Science and Technology (5003530002) funds were received in support of this work.

No relevant financial activities outside the submitted work.

Address correspondence and reprint requests to Fan Ding, MD, PhD, Department of Orthopaedic Surgery, The First People's Hospital of Jingmen, Jingmen 448000, Hubei, China;
E-mail: jmyydf@163.com; Liming Xiong, MD, PhD, Department of Orthopedics, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China; E-mail: xiongliming@hust.edu.cn

DOI: 10.1097/BRS.0000000000002930

Intervertebral disc (IVD) degeneration is a primary cause of low back pain (LBP), which has become an expensive global social problem affecting human life.[1–3] It is generally believed that IVD degeneration is associated with many factors, such as age, genetics, excessive mechanical stress, obesity, and diabetes mellitus (DM).[4,5] DM can lead to a reduction in nucleus pulposus matrix stability, a change accompanied by hyalinization and annulus fibrosus (AF) disruption, which accelerate IVD degeneration.[6] In addition, proteoglycan and collagen II levels in the extracellular

matrix are decreased in diabetic nucleus pulposus (NP) cells compared with control NP cells.[7] Interestingly, evidence from prior studies revealed that patients receiving surgical treatment for lumbar IVD herniation had a significant increased incidence of DM compared with similar patients receiving surgical treatment for other reasons.[8] These data showed that DM is closely related to IVD degeneration.

Many studies recently reported that advanced glycation end-product (AGEs) accumulation is involved in diabetes occurrence and development.[9] AGEs are formed through non-enzymatic reactions involving reducing sugars, and their formation results in the oxidation of proteins, nucleic acids, and lipids.[10] Recent studies have reported that AGE expression was significantly higher in diabetic rat discs—particularly in the AF and nucleus pulposus than in nondiabetic rat discs.[11] High glucose, which increases the risk of IVD degeneration, causes AGE accumulation.[12] Abnormal AGE accumulation results in increased AF stiffness and brittleness, which makes the IVD more sensitive to mechanical loading and causes IVD degeneration.[13,14] However, the molecular mechanisms by which AGEs induce IVD degeneration have not been fully elucidated.

Recent researches have reported that IVD degeneration is related to IVD cell death and the loss of extracellular matrix components, including collagen type II and aggrecan.[15,16] A previous study showed that the accumulation of AGEs and their interaction with AGE receptors in the IVD may lead to decreased aggrecan production, which is responsible for IVD degeneration.[17] Tsai *et al*[11] reported that AGEs cause matrix degradation accompanied by high MMP-2 expression in disc cells, which promotes disc degeneration. In addition, many studies suggested that cell apoptosis decreases the numbers of functional and viable disc cells, a phenomenon that plays an important role in IVD degeneration.[16,18,19] AF cells maintain the structural properties of IVD discs, and AF cell apoptosis contributes to IVD degeneration.[20] We also reported that compression-induced NP cell apoptosis contributes to IVD degeneration.[4] Recently, many studies reported that AGEs have cytotoxic effects in other tissues including glomerulus,[21] pancreas islet,[22] and myocardium.[23] However, whether AGEs have cytotoxic effects to AF cells remains unclear. Studies have reported that AGEs induce chondrocyte apoptosis, which plays an important role in osteoarthritis progression.[24,25] Because IVD cells have a phenotype similar to that of chondrocytes, it is essential to explore whether cell apoptosis is involved in AGE-induced IVD degeneration.

Apoptosis plays an essential role in the elimination of damaged or non-essential cells and occurs via the following two main signaling pathways: the extrinsic pathway and the intrinsic pathway, which are mediated by death receptors and mitochondria, respectively.[16,20] The extrinsic pathway, which is activated by death receptors, leads to the formation of the death-inducing signaling complex, resulting in caspase-3 cleavage and cell apoptosis. The intrinsic or mitochondrial pathway is induced mainly by various apoptotic signals, including growth factor deprivation, DNA damage, and oxidative stress. Death-inducing stimuli directly increase the permeability of the outer mitochondrial membrane, which results in the release of cytochrome c into the cytosol, where it binds with apoptotic protease activating factor-1, leading to the formation of the apoptosome. The apoptosome promotes caspase-9 activation, which then leads to caspase-3 activation and triggers apoptosis.[7,20,26]

In this study, we used different methods to investigate the effect of AGEs on cell proliferation and to determine whether AGEs induce rabbit AF cell apoptosis. In addition, we evaluated the role of the mitochondrial pathway in AGE-induced AF cell apoptosis by evaluating apoptosis-related markers and mitochondrial function.

MATERIALS AND METHODS

Primary AF Cell Culture and Treatment

All experiments that were performed in the present study were approved by the Animal Care and Ethics Committee of Huazhong University of Science and Technology. Primary rabbit AF cells were collected and cultured as described previously.[27] The AF tissues of the IVDs of Japanese white rabbits (age 3 month) were harvested immediately after air embolization. The tissues were cut into small pieces, digested by trypsin without phenol red for 15 minutes and then digested by 0.25% type II collagenase (Biosharp, China) for 4 hours. The digested AF tissues were suspended in Dulbecco modified Eagle medium/ha F-12 (DMEM/F-12, Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS, Gibco, Grand Island, NY) and 1% penicillin/streptomycin (Beyotime, China) at 37 °C and 5% CO_2 as described previously.[27] When second-passage AF cells reached 80% to 90% confluence, they were seeded in the appropriate culture plates with DMEM/F-12 containing 10% FBS. To determine the effects of AGEs (Abcam, Cambridge, United Kingdom) on AF cells, we treated the cells with AGEs at various concentrations (0, 25, 50, 100, 150, and 200 μg/mL) in DMEM/F-12 containing 10% FBS for 3d and then used them for subsequent experiments. 0 μg/mL is considered as control group (con).

CCK-8 Assay

Cell viability was detected by Cell Counting Kit-8 (CCK-8, Dojindo, Japan), as described previously.[28] The cell densities were 5×10^3 cells/well for CCK-8. After being seeded in 96-well culture plates, AF cells were treated with AGEs at various concentrations for 3d. After the cells were treated, 100 milliliters (mL) of DMEM/F-12 containing 10 mL of CCK-8 solution was added to each well, and the plates were incubated for 4 hours at 37 °C. The absorbance at 450 nm was determined with a spectrophotometer (BioTek, Winooski, VT).

EdU Incorporation Assay

The effect of AGEs on AF cell proliferation was also detected by 5-ethynyl-2′-deoxyuridine (EdU) incorporation assay (Ribobio, China) as we described previously.[29] After being treated with AGEs various concentrations for 3 days, AF cells were subjected to the indicated assay, which was performed

according to the manufacturer's instructions. After the cells were stained, fluorescence images were obtained by a fluorescence microscope (Evos, life technology, Carlsbad, CA).

Live/Dead Assay

The toxic effects of AGEs on AF cells were quantified by Calcein-AM/PI (Dojindo, Japan). After being treated with AGEs, the cells were rinsed with phosphate-buffered saline (PBS) thrice and incubated in PBS containing 2 μmol/L Calcein-AM and 4 μmol/L propidium iodide (PI) in the dark for 15 minutes at 37 °C. The live/dead cells were then observed in the dark by a fluorescence microscope (Olympus, Japan). Live cells displayed green fluorescence (Calcein-AM positive), while the nuclei of dead cells displayed red fluorescence under the fluorescence microscope (PI positive).

Hoechst 33342 Staining

Cell apoptosis was quantified by Hoechst 33342 staining (Beyotime, China). After being treated with AGEs at various concentrations, the cells were washed with PBS three times and then incubated with 1 mL of Hoechst 33342 dye in the dark for 20 minutes. The morphologic changes in the apoptotic cell nuclei were detected by a fluorescence microscope (Olympus, Japan).

Annexin V/PI Double-Staining

The AF cells from each treatment group were harvested and washed with PBS twice before being labeled by Annexin V/PI double-staining (KeyGen Biotech, China) in the dark, as described previously. All samples were analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, NJ) using CellQuest analysis software (BD).

Western Blot Analysis

A Western and IP Cell Lysis Kit (Beyotime, China) was used to extract total protein. Protein concentrations were measured by a BCA Protein Assay Kit (Beyotime, China). Equal aliquots of protein from each group were separated by 12% SDS-PAGE and subsequently transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Burlington, MA), which were blocked with 5% nonfat milk in buffer solution contained Tris-Hcl, NaCl, tween20 buffer before being incubated with primary antibodies against GAPDH (1:5000, Abcam, Cambridge, United Kingdom), cytochrome c (1:1000, Novus, Colorado), Bax (1:1000, Bioss, China), Bcl-2 (1:1000, Bioss, China), caspase-9 (1:500, Proteintech, China), and caspase-3 (1:500, Abcam, Cambridge, United Kingdom) overnight at 4 °C. The membranes were then incubated with the indicated secondary antibodies (goat anti-rabbit or goat anti-mouse, 1:10,000) for 1 hour at room temperature. The bands were visualized in a darkroom and were analyzed by AlphaEaseFC (Alpha Innotech, California).

Real-time PCR

After the cells in each group were treated, total RNA was extracted from them by Trizol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. The isolated RNA was subsequently reverse-transcribed into complementary DNA with reverse transcriptase (ToYobo, Japan). The mRNA sequences for GAPDH, pro-apoptotic Bax, and anti-apoptotic Bcl-2 were as follows:

GAPDH: forward: 5′-CGCCTGGAGAAAGCTGCTA-3′
reverse: 5′-ACGACCTGGTCCTCGGTGTA-3′;
Bax: forward: 5′-CAAGAAGCTGAGCGAGTGTCTC-3′
reverse: 5′-TGCACAGGGCCTTGAGTACC-3′;
Bcl-2: forward: 5′-GGCCTTCTTTGAGTTCGGTG-3′
reverse: 5′-GAGGGTGATGCAAGCTCCTATC-3′. The expression levels of the above genes in each group and those of GAPDH were detected by StepOne Real-Time PCR (Life Technologies, Carlsbad, CA), and the data were analyzed using the $2^{-\triangle\triangle CT}$ method. GAPDH was used as an internal control.

DCFH-DA

Intracellular reactive oxygen species (ROS) levels were determined by DCFH-DA (Sigma-Aldrich, St. Louis, MO). AF cells from each treatment group were harvested and washed once with PBS before being incubated with 10 mM DCFH-DA in the dark for 20 minutes at 37 °C. The cells were subsequently washed twice with DMEM/F12, and the mean fluorescence intensity was measured by flow cytometry (Becton Dickinson, Franklin Lakes, NJ). Intracellular ROS levels in each group were also determined by a fluorescence microscope (Olympus, Japan).

JC-1 Staining

JC-1 staining (Beyotime, China) was used to assess mitochondrial membrane potential (MMP) by flow cytometry. AF cells from each treatment group were harvested and washed once with PBS before being resuspended in 1 mL of culture medium containing 0.5 mL of JC-1 staining fluid. The cells were subsequently incubated in the dark for 20 minutes at 37 °C with 5% CO_2. The cells were washed twice with cold staining buffer and resuspended in 0.5 mL of staining buffer. The MMP of each group was evaluated by flow cytometry (Becton Dickinson, Franklin Lakes, NJ) and was expressed as the ratio of red fluorescence intensity to green fluorescence intensity.

Statistical Analysis

All data are presented as the mean ± standard deviation (SD) from at least three independent experiments. Statistical analysis was performed by Student *t* test or one-way analysis of variance (ANOVA) using GraphPad Prism (GraphPad Software Inc; La Jolla, CA). Bonferroni post hoc test was used to determine the source of the observed differences. $P < 0.05$ was considered statistically significant.

RESULTS

AGEs Had Inhibitory Effects on AF Cell Viability and Cell Proliferation

CCK-8 assay was used to detect cellular dehydrogenase activity to evaluate AF cell viability. As shown in Figure 1A, AGEs

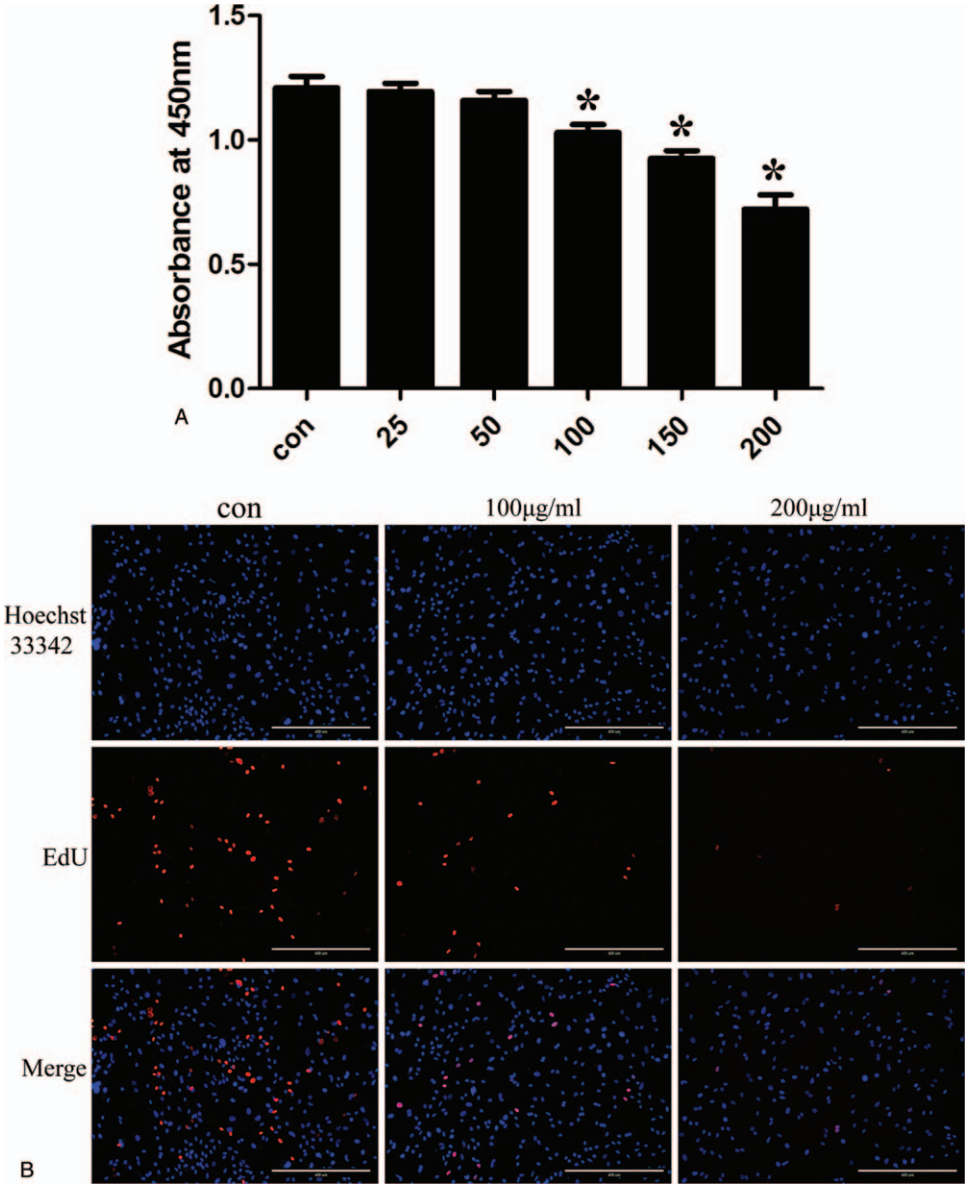

Figure 1. Effects of AGEs on AF cell viability and cell proliferation. (**A**) CCK-8 assay was used to evaluate the cell viability after treatment with AGEs at a concentration of con, 25, 50, 100, 150, and 200 μg/mL on AF cells. Con served as a control group. Data are presented as the mean ± SD of three independent experiments. $*P < 0.05$ *versus* control group. (B) Representative micrographs of EdU staining, as demonstrated by fluorescence microscopy, after treatment with AGEs at a concentration of con, 100, and 200 μg/mL. The red fluorescence indicates EdU-positive cells. AF indicates annulus fibrosus; AGEs, advanced glycation end-products; CCK-8, Cell Counting Kit-8; EdU, 5-ethynyl-2′-deoxyuridine.

significantly decreased AF cell viability in a dose-dependent manner in all treated groups except the 25 and 50 μg/mL-treated groups compared with the control group ($P < 0.05$).

To investigate the inhibitory effects of AGEs on AF cell proliferation, we performed EdU incorporation assay evaluate cell proliferation after the cells were treated with AGEs at different doses (100 and 200 μg/mL). The number of EdU-positive cells (red fluorescence) was decreased in the AGE-treated groups compared with the control group, as shown in Figure 1B. These data indicated that AGEs inhibit AF cell proliferation.

AGEs Had Cytotoxic Effects on AF Cells

To determine whether AGEs had cytotoxic effect on AF cells, we quantified the changes in the numbers of live/dead cells under a fluorescence microscope. Live and dead cells were indicated by green and red fluorescence, respectively. As shown in Figure 2, the number of red cells were increased with increasing AGE concentrations, a change that was accompanied by a dose-dependent decrease in green fluorescence. These data implied that AGEs have cytotoxic effects on AF cells.

AGEs Induced AF Cell Apoptosis

To investigate whether AGEs induce AF cell apoptosis, we performed Annexin V/PI double-staining to examine cell apoptosis. As shown in Figure 3A, AGEs significantly induced AF cell apoptosis in treated cells compared with control cells. AGE treatment elicited a dose-dependent increase in the rate of cell apoptosis in AF cells. In addition, we noted a significant difference in the rate of cell apoptosis between the control and treatment groups ($P < 0.05$).

To examine AGE-induced AF cell apoptosis further, we used Hoechst 33342 staining to detect apoptosis-related morphological changes under a fluorescence microscope.

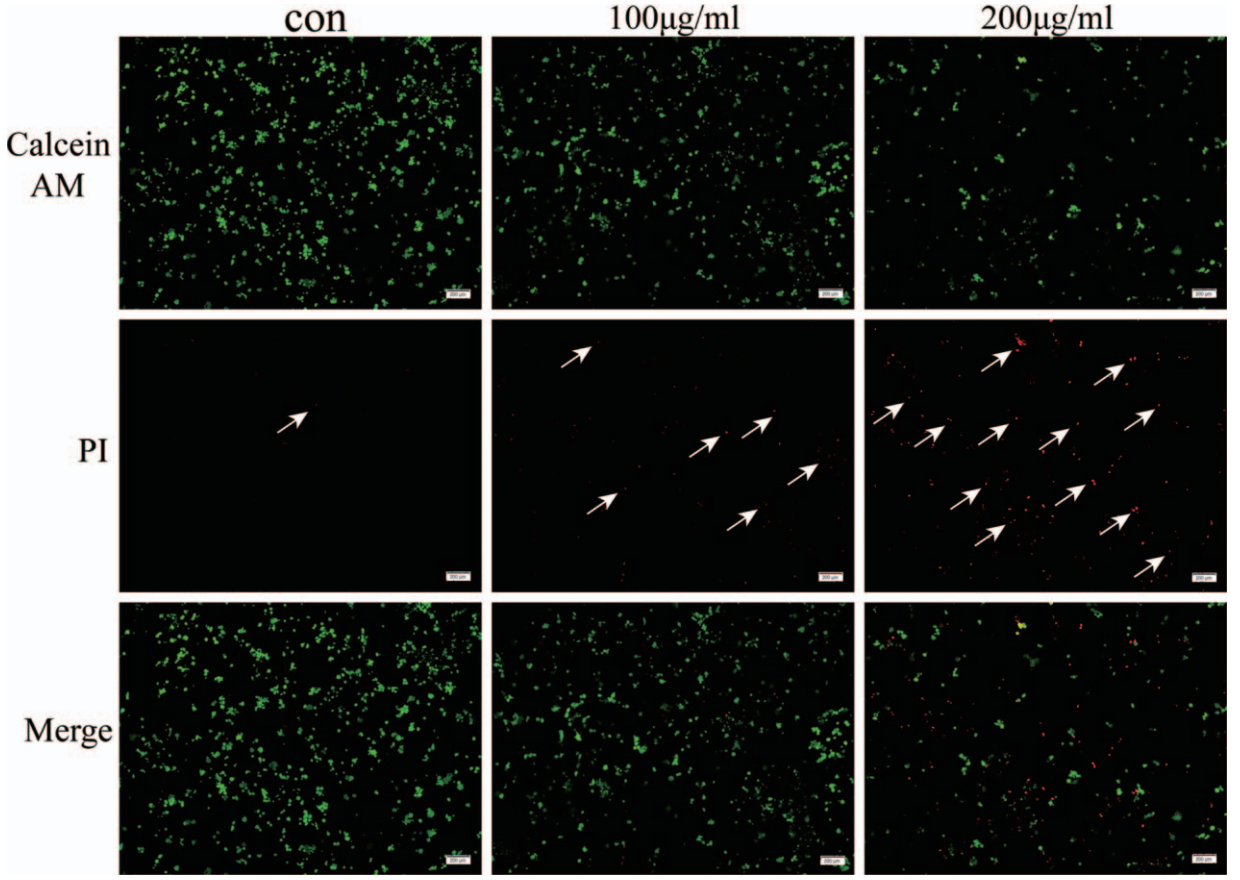

Figure 2. Cytotoxic effects of AGEs on AF cells. Calcein-AM/PI dye was used to evaluate AF cell damage under a fluorescence microscope. The green fluorescence indicates live cells, and the red fluorescence indicates dead cells. AF indicates annulus fibrosus; AGEs, advanced glycation end-products.

The apoptotic nuclei showed condensed DNA, which stained brightly with Hoechst 33342. As shown in Figure 3B, the number of apoptotic nuclei in the AGE (100 and 200 μg/mL)-treated group was increased compared with that in the control group.

AGE Induced Changes in the Expression of Mitochondrial Apoptosis Pathway-Related Proteins

To determine the role of the mitochondrial apoptosis pathway in AGE-induced cell apoptosis, we detected the expression of apoptosis-related proteins by western blotting. As shown in Figure 4A, after treatment with AGEs, the expression of the pro-apoptotic protein Bax increased, while that of the anti-apoptotic protein Bcl-2 decreased in a dose-dependent manner in the AGE-treated groups compared with the control group. In addition, we found that AGEs increase the release of cytochrome c into the cytosol and induce caspase-9 and 3 activation.

To further assess the mechanism by which AGE-induced cell apoptosis occurs, we measured the mRNA expression levels of the pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 by RT-PCR. As shown in Figure 4B, treatment with AGEs up-regulated Bax mRNA expression levels, a change accompanied by the suppression of Bcl-2 expression. Bax and Bcl-2 expression levels were significantly different between the AGE-treated groups and the control group ($P < 0.05$). These data indicated that the mitochondrial apoptosis pathway is likely involved in AGE-mediated AF cell apoptosis.

AGEs Increased Intracellular ROS Production in AF Cells

Excessive ROS can hamper mitochondrial function and affect cell viability. To determine whether ROS generation is related to AGE-induced cell apoptosis, we used DCFH-DA fluorescent probes to evaluate intracellular ROS levels in AF cells. As shown in Figure 5A, after AGE treatment, intracellular ROS levels increased in a dose-dependent manner in the AGE-treated group compared with the control group. ROS production peaked at a level that was of 2.12-fold greater in the 200 μg/mL AGE-treated group than in the control group. There was a statistically significant difference in ROS levels between the control and treatment groups (100 and 200 μg/mL). As shown in Figure 5B, after treatment with AGEs, ROS-related green fluorescence was significantly increased in treated AF cells compared with control AF cells. These data demonstrated that AGEs increase intracellular ROS production in AF cells.

AGEs Induced the Loss of MMP in AF Cells

The loss of MMP is considered an important step in the mitochondrial apoptotic pathway. To further illustrate the

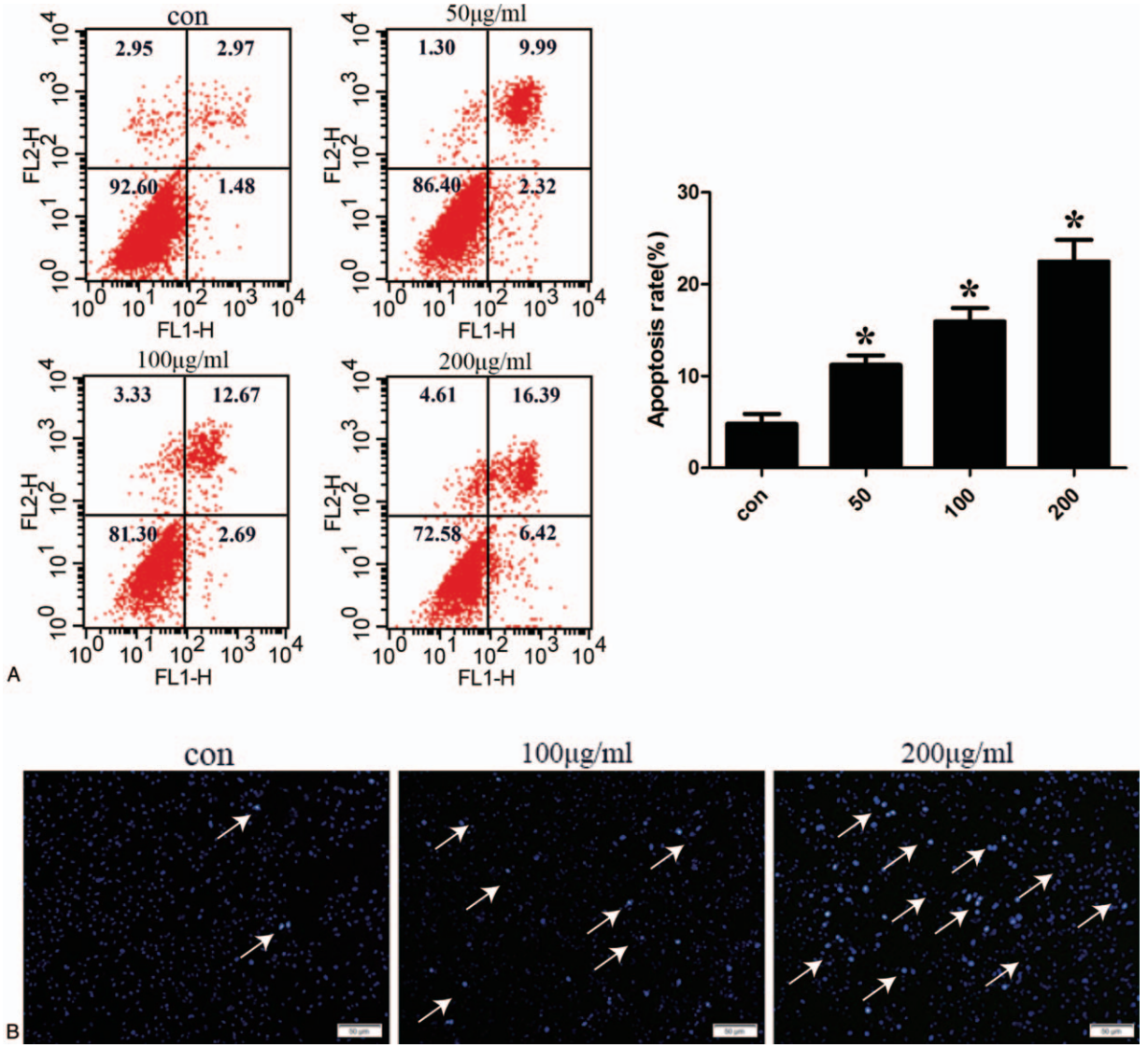

Figure 3. Effects of AGEs on AF cell apoptosis. (**A**) Flow cytometric analysis was used to detect cell apoptosis. The con served as a control group. Data are presented as the mean ± SD of three independent experiments. $^{*}P < 0.05$ *versus* control group. (**B**) AGE-induced apoptosis-related morphological changes in AF cells. Hoechst 33342 staining was used to detect apoptotic cells according to their morphology under a fluorescence microscope. The apoptotic nuclei showed condensed DNA, which stained brightly with Hoechst 33342. AF indicates annulus fibrosus; AGEs, advanced glycation end-products.

role of the loss of MMP in AGE-induced cell apoptosis, we performed JC-1 staining to measure MMP in AF cells by flow cytometric analysis. The results shown in Figure 6 indicate that treatment with AGEs induces the loss of MMP in a dose-dependent manner, a change that presents as a decrease in the red (JC-1 aggregates)/green (JC-1 monomers) ratio. AGEs (50, 100, and 200 μg/mL) significantly reduced the red/green ratio from 7.82 to 5.19 and 3.56, respectively ($P < 0.05$), in the treatment groups compared with the control group.

NAC Protects Against Cell Apoptosis Induced by AGEs

N-acetyl cysteine (NAC), one of the major intracellular antioxidant precursors of glutathione, exerts anti-apoptotic effects by inhibiting oxidative stress-induced cell apoptosis. To explore whether ROS inhibition protects against AGEs induced apoptosis, we pretreated AF cells with 5 mmol/L NAC for 1 hour and then incubated them with AGEs. As shown in Figure 7A–C, pretreatment with NAC significantly inhibited AGE-induced ROS production and alleviated AGE-induced MMP loss. Pretreatment with NAC also protected against AGE-induced cell apoptosis. These results showed that an antioxidant (NAC) decreased AGE-induced ROS production, which plays an important role in the AGE-induced apoptotic pathway.

DISCUSSION

The present study is the first to investigate AGE-induced rabbit AF cell apoptosis and the possible molecular

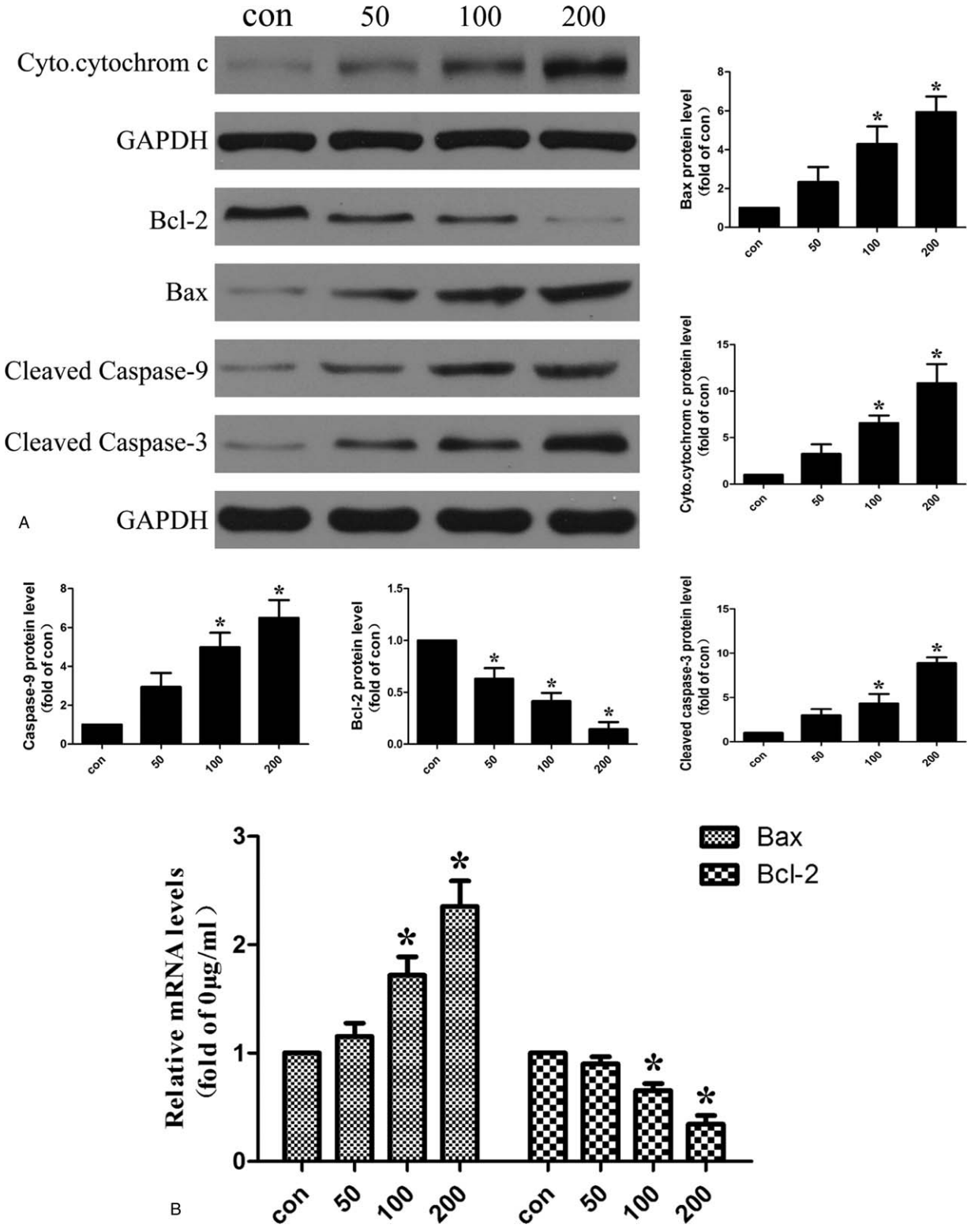

Figure 4. Effect of AGEs on mitochondrial apoptosis-related markers in AF cells. (**A**) Representative western blotting results for Bax, cytochrome c, cleaved caspase-9, Bcl-2, and cleaved caspase-3 expression after treatment with AGEs. Quantitative analysis of Bax, cytochrome c, cleaved caspase-9, Bcl-2, and cleaved caspase-3 expression in AF cells. (**B**) Pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 mRNA expression levels were detected by RT-PCR. The con group served as a control group. Data are presented as the mean ± SD of three independent experiments. $^{*}P < 0.05$ *versus* control group. AF indicates annulus fibrosus; AGEs, advanced glycation end-products.

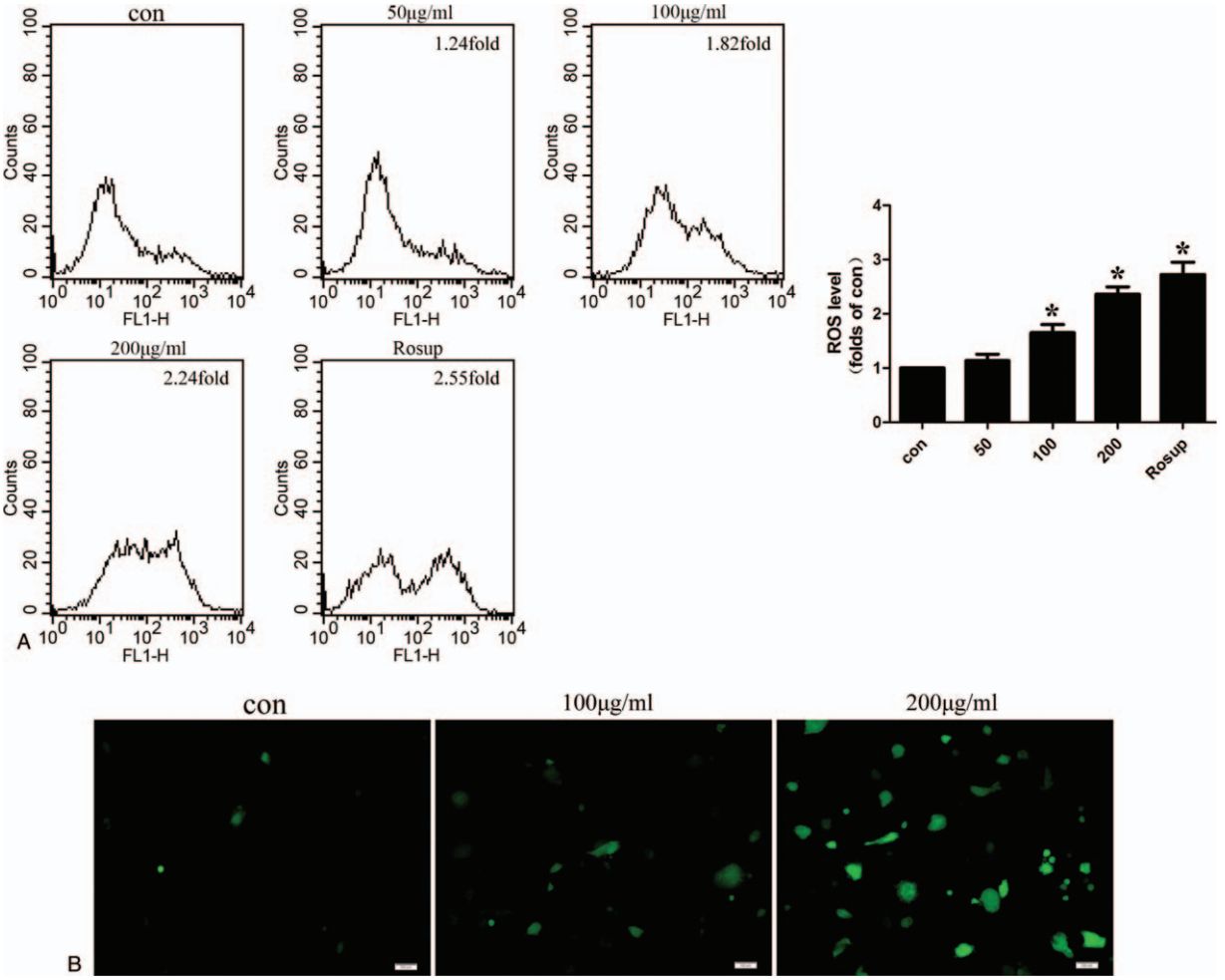

Figure 5. Effect of AGEs on intracellular ROS production in AF cells. (**A**) Flow cytometric analysis was performed to detect intracellular ROS production in AF cells. Quantitative analysis of intracellular ROS production in AF cells by flow cytometry. The con group served as a control group. Data are presented as the mean ± SD of three independent experiments. $^{*}P < 0.05$ *versus* control group. (**B**) Intracellular ROS levels were observed by in situ ROS staining under a fluorescence microscope. AF indicates annulus fibrosus; AGEs, advanced glycation end-products; ROS, reactive oxygen species.

mechanisms underlying AGE-induced AF cell apoptosis. In the current study, we demonstrated that AGEs inhibit AF cell proliferation and have dose-dependent cytotoxic effects on AF cells. AGEs also induce AF cell apoptosis. Moreover, the mitochondrial pathway is involved in AGE-mediated cell apoptosis. Furthermore, we found that AGEs induce the loss of MMP and increase intracellular ROS production. In addition, NAC-mediated inhibition of ROS production protects against AGE-induced cell apoptosis.

IVD degeneration is considered the main factor associated with LBP, which is a global health problem affecting quality of life that has imposed an enormous economic burden on society.[1–3] DM is believed to an important inducer of IVD degeneration. Previous studies have reported that the incidence of lumbar disk disease in diabetic patients was significantly higher than that in patients with other diseases.[8] Agius *et al*[30] also showed that the disc height of patients with diabetes is significantly lower than that of non-diabetic patients. Previous studies have reported that diabetes significantly induces degenerative changes, including undermining disc glycosaminoglycan, adversely affected biomechanical behavior, and reduced water content in a rat model of type 2 diabetes, changes that contributed to IVD degeneration.[31]

Many studies have recently shown that IVD degeneration in DM is related to AGE accumulation.[9,11] Many researchers thought that AGEs have toxic effects on cells and decrease cell viability. Researchers showed that cardiomyocyte viability decreased in a dose-dependent manner in diabetic cardiomyopathy after treatment with AGEs.[32] In addition, Liu *et al*[28] reported that AGEs significantly decreased chondrocyte viability. Similarly, studies by other researchers have shown that decreases in chondrocyte viability occurred after treatment with AGEs.[33] Consistent with these results, our CCK-8 assay results showed that AGEs have an inhibitory effect on AF cell viability. Similar to the CCK-8 assay, the EdU incorporation assay also showed that AGEs inhibit cell proliferation. In addition, the live/dead staining results demonstrated that AGEs had cytotoxic effects on AF cells.

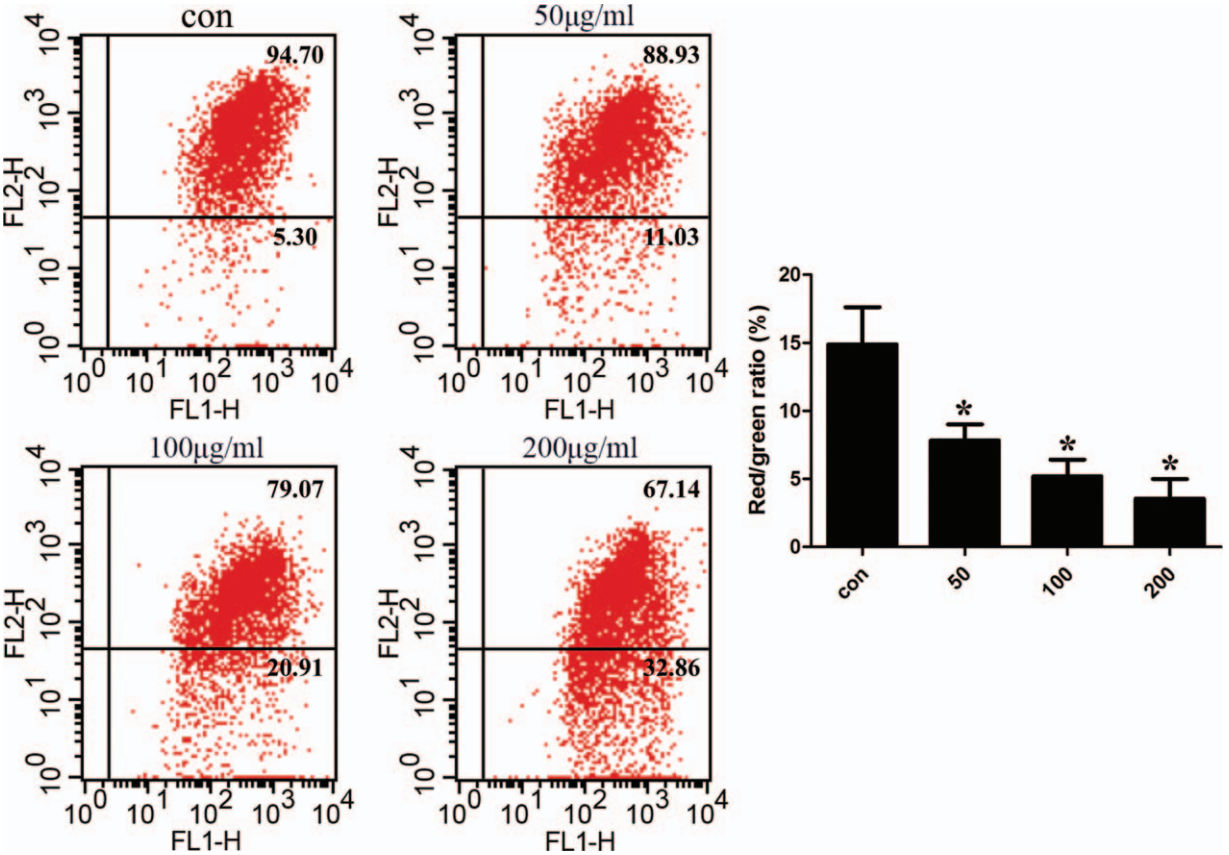

Figure 6. Effect of AGEs on MMP in AF cells. Flow cytometric analysis was used to detect MMP in AF cells. Quantitative analysis of MMP in AF cells by flow cytometry. The con group served as a control group. Data are presented as the mean ± SD of three independent experiments. $^{*}P < 0.05$ *versus* control group. AF indicates annulus fibrosus; AGEs, advanced glycation end-products; MMP, mitochondrial membrane potential; SD, standard deviation.

However, the potential mechanism by which AGE decreases cell viability remains unclear. To date, several studies have shown that the cytotoxic effects of AGEs on other cells are associated with cell apoptosis. Previous studies suggested that AGEs induced apoptosis in mesangial cells by promoting ROS generation.[21] The study by Xie *et al*[34] showed that AGEs increase the apoptotic rate in endothelial cells, while other researchers also demonstrated that the rate of AGE-induced apoptosis was increased in a dose-dependent manner in chondrocytes.[25] Yang *et al*[24] also found that AGEs significantly decrease chondrocyte cell viability and induce chondrocyte apoptosis. Given that IVD cell apoptosis is an important factor in IVD degeneration, we performed flow cytometry to investigate the effect AGE on AF cell apoptosis and found that AGEs significantly induced apoptosis in a dose-dependent manner in AF cells. Furthermore, Hoechst 33342 staining revealed that after treatment with AGEs, the number of apoptotic nuclei was increased in the AGE-treated groups compared with the control group.

The mitochondrial apoptosis pathway is initiated by apoptotic signals, which cause an increase in intracellular ROS levels, the loss of MMP and the release of cytochrome c into the cytosol. The release of cytochrome c is suppressed by the anti-apoptotic members of the Bcl-2 family and stimulated by the pro-apoptotic members of the family, such as Bax. Cytochrome c then recruits caspase-9 to form a complex known as the apoptosome, which indirectly induces caspase-3 activation and triggers the caspase cascade.[7,16,26,27,35] Our results indicated that AGEs promote the release of cytochrome c into the cytosol, a change accompanied by the activation of caspase-9 and caspase-3. Furthermore, we found that the expression of Bax was significantly increased, while that of Bcl-2 was decreased in AF cells after treatment with AGEs. In addition, we also assessed mitochondrial function after treatment with AGEs and found that AGEs induce mitochondrial membrane depolarization. These data indicate that AGEs induce AF cells apoptosis by activating the mitochondrial apoptosis pathway.

ROS are widely considered a potent proapoptotic factor in NP cells *in vitro*.[36] Intracellular ROS are produced mainly in the mitochondria, whose function is directly damaged when ROS production is enhanced.[37] Many studies strongly support the idea that ROS are associated with apoptosis induction. One study showed that ROS overproduction induced increased NP cell apoptosis through the mitochondrial apoptosis pathway,[4,38] and another previous study

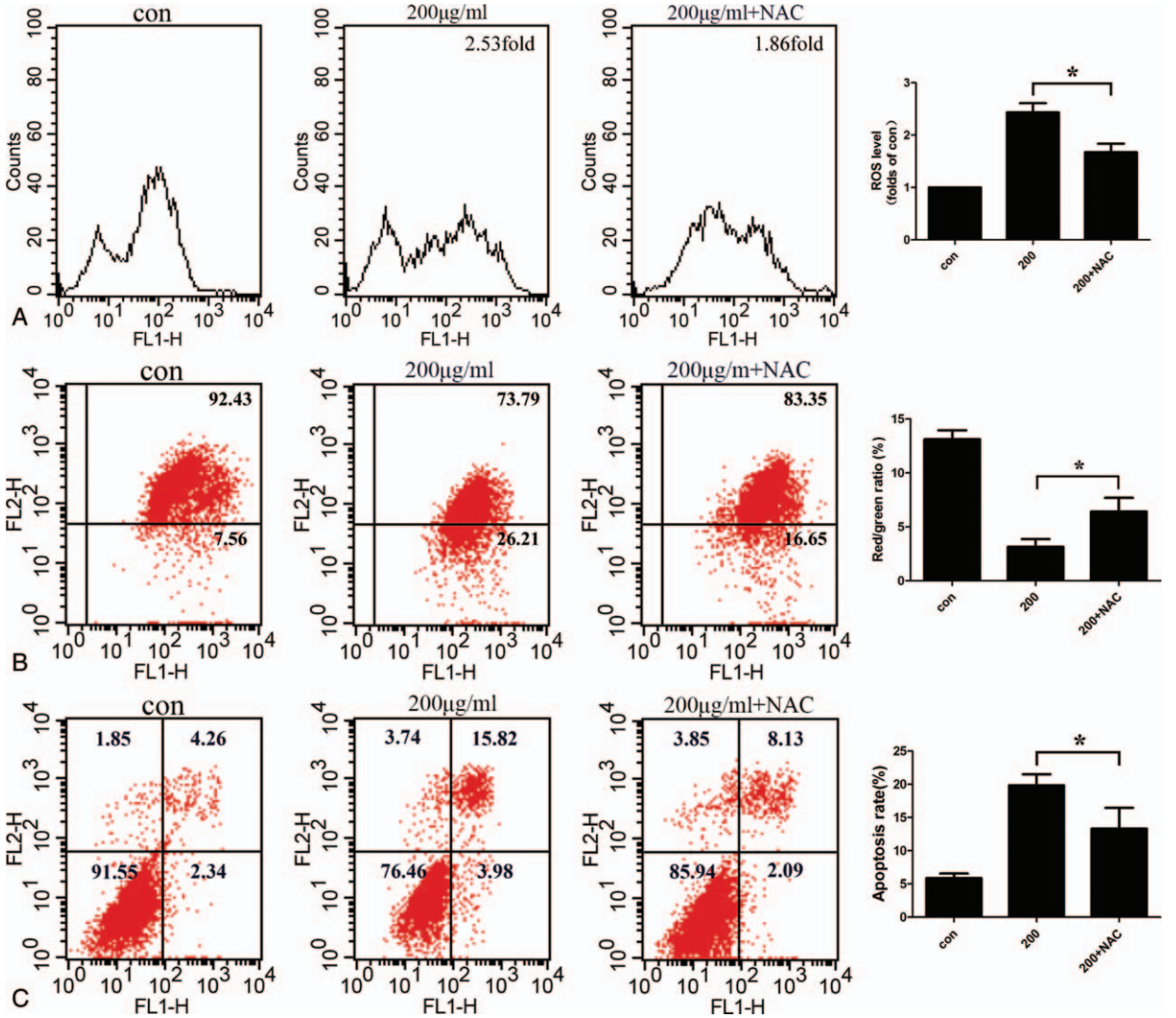

Figure 7. Effect of the antioxidant NAC on AGE-induced AF cell apoptosis. (**A**) Effects of NAC on intracellular ROS production in AF cells, as determined by flow cytometry. Quantitative analysis of ROS levels in AF cells. (**B**) Effects of NAC on MMP in AF cells, as determined by flow cytometry. Quantitative analysis of MMP in AF cells. (**C**) The effects of NAC on AF cell apoptosis were assessed by flow cytometric analysis. Quantitative analysis of AF cell apoptosis. The group treated without NAC served as a control group. Data are presented as the mean ± SD of three independent experiments. $^{*}P < 0.05$ *versus* treatment group (200 μg/mL). AF indicates annulus fibrosus; AGEs, advanced glycation end-products; MMP, mitochondrial membrane potential; NAC, N-acetyl cysteine; SD, standard deviation.

showed that ROS, particularly mitochondrial-derived ROS, cause cellular and molecular damage.[4,27,39] A study regarding osteoarthritis suggested that the mechanism underlying AGE-mediated chondrocyte apoptosis mainly involved ROS production and the mitochondrial apoptosis pathway.[24] In this study, AGEs increased intracellular ROS levels in a dose-dependent manner. In addition, the antioxidant NAC effectively prevented increases in ROS levels and the loss of MMP, thereby protecting against AGEs-induced cell apoptosis. Those results indicated that ROS play an important role in AGE-induced AF cell apoptosis.

However, there were some limitations to this study. First, this study was conducted *in vitro*, and its conclusions may not necessarily be indicative of that which occurs *in vivo*. Second, it is difficult to obtain healthy human IVD tissue. Thus, we used rabbit AF cells to determine whether AGEs induce AF cell apoptosis. If possible, it will be necessary to investigate the effects of AGEs on human IVD cells in the future. Besides, we will use 3D-culture systems culture and a hypoxic condition to simulate microenvironment of the body in further study.

In conclusion, our study demonstrated that treatment with AGEs at different doses significantly induces rabbit AF cell apoptosis *in vitro*. The underlying molecular mechanism of AGEs-induced cell apoptosis involves the mitochondrial pathway. Taken together, these findings may provide a theoretical basis for diabetic IVD degeneration.

➢ Key Points

- ❑ AGEs have cytotoxic effects on AF cells and induce rabbit AF cell apoptosis in a dose-dependent manner.
- ❑ The mitochondrial pathway is involved in AGE-mediated AF cell apoptosis.
- ❑ NAC can significantly reverse AGE-induced MMP decrease and AF cell apoptosis.
- ❑ ROS is an important mediator in AGE-mediated AF cell apoptosis.

References

1. Risbud MV, Shapiro IM. Role of cytokines in intervertebral disc degeneration: pain and disc content. *Nat Rev Rheumatol* 2014;10:44–56.
2. Liu C, Yang H, Gao F, et al. Resistin promotes intervertebral disc degeneration by upregulation of ADAMTS-5 through p38 MAPK signaling pathway. *Spine (Phila Pa 1976)* 2016;41:1414–20.
3. Suzuki S, Fujita N, Fujii T, et al. Potential involvement of the IL-6/JAK/STAT3 pathway in the pathogenesis of intervertebral disc degeneration. *Spine (Phila Pa 1976)* 2017;42:E817–24.
4. Ding F, Shao ZW, Yang SH, et al. Role of mitochondrial pathway in compression-induced apoptosis of nucleus pulposus cells. *Apoptosis* 2012;17:579–90.
5. Anekstein Y, Smorgick Y, Lotan R, et al. Diabetes mellitus as a risk factor for the development of lumbar spinal stenosis. *Isr Med Assoc J* 2010;12:16–20.
6. Chen S, Liao MM, Li JP, et al. The correlation between microvessel pathological changes of the endplate and degeneration of the intervertebral disc in diabetic rats. *Exp Ther Med* 2013;5:711–7.
7. Jiang LB, Zhang XL, Zheng XH, et al. Apoptosis, senescence, and autophagy in rat nucleus pulposus cells: implications for diabetic intervertebral disc degeneration. *J Orthop Res* 2013;31:692–702.
8. Sakellaridis N. The influence of diabetes mellitus on lumbar intervertebral disk herniation. *Surg Neurol* 2006;66:152–4.
9. Goh SY, Cooper ME. Clinical review: the role of advanced glycation end products in progression and complications of diabetes. *J Clin Endocrinol Metab* 2008;93:1143–52.
10. Vlassara H, Striker GE. AGE restriction in diabetes mellitus: a paradigm shift. *Nat Rev Endocrinol* 2011;7:526–39.
11. Tsai TT, Ho NY, Lin YT, et al. Advanced glycation end products in degenerative nucleus pulposus with diabetes. *J Orthop Res* 2014;32:238–44.
12. Peppa M, Uribarri J, Vlassara H, et al. advanced glycation end products, and diabetes complications: what is new and what works. *Clin Diabetes* 2003;21:186–7.
13. Wagner DR, Reiser KM, Lotz JC. Glycation increases human annulus fibrosus stiffness in both experimental measurements and theoretical predictions. *J Biomech* 2006;39:1021–9.
14. Tang SY, Sharan AD, Vashishth D. Effects of collagen crosslinking on tissue fragility. *Clin Biomech (Bristol, Avon)* 2008;23:122–3.
15. Zhang WL, Chen YF, Meng HZ, et al. Role of miR-155 in the regulation of MMP-16 expression in intervertebral disc degeneration. *J Orthop Res* 2017;35:1323–34.
16. Ding F, Shao ZW, Xiong LM. Cell death in intervertebral disc degeneration. *Apoptosis* 2013;18:777–85.
17. Yokosuka K, Park JS, Jimbo K, et al. Advanced glycation end-products downregulating intervertebral disc cell production of proteoglycans in vitro. *J Neurosurg Spine* 2006;5:324–9.
18. Wako M, Ohba T, Ando T, et al. Mechanism of signal transduction in tumor necrosis factor-like weak inducer of apoptosis-induced matrix degradation by MMP-3 upregulation in disc tissues. *Spine (Phila Pa 1976)* 2008;33:2489–94.
19. Zhao CQ, Jiang LS, Dai LY. Programmed cell death in intervertebral disc degeneration. *Apoptosis* 2006;11:2079–88.
20. Zhang F, Zhao XL, Shen HX, et al. Molecular mechanisms of cell death in intervertebral disc degeneration (Review). *Int J Mol Med* 2017;37:1439–48.
21. Xu L, Fan Q, Wang X, et al. Inhibition of autophagy increased AGE/ROS-mediated apoptosis in mesangial cells. *Cell Death Dis* 2016;7:e2445.
22. Kong X, Wang GD, Ma MZ, et al. Sesamin ameliorates advanced glycation end products-induced pancreatic **β**-cell dysfunction and apoptosis. *Nutrients* 2015;7:4689–704.
23. Yang YC, Tsai CY, Chen CL, et al. Pkc **δ** activation is involved in ROS-mediated mitochondrial dysfunction and apoptosis in cardiomyocytes exposed to advanced glycation end products (Ages). *Aging Dis* 2018;9:647–63.
24. Yang QS, Guo SF, Wang S, et al. Advanced glycation end products-induced chondrocyte apoptosis through mitochondrial dysfunction in cultured rabbit chondrocyte. *Fundam Clin Pharmacol* 2015;29:54–61.
25. Zhang HB, Zhang Y, Chen C, et al. Pioglitazone inhibits advanced glycation end product-induced matrix metalloproteinases and apoptosis by suppressing the activation of MAPK and NF-kB. *Apoptosis* 2016;21:1082–93.
26. Green DR, Reed JC. Mitochondria apoptosis. *Science* 1998;281:1309–12.
27. Cai XY, Xia Y, Yang SH, et al. Ropivacaine- and bupivacaine -induced death of rabbit annulus fibrosus cells in vitro: involvement of the mitochondrial apoptotic pathway. *Osteoarthritis Cartilage* 2015;23:1763–75.
28. Liu YL, Feng XB, Zhang YK, et al. Establishment and characterization of a novel osteosarcoma cell line: CHOS. *J Orthop Res* 2016;34:2116–25.
29. Cai XY, Liu YL, Yang W, et al. Long noncoding RNA MALAT1 as a potential therapeutic target in osteosarcoma. *J Orthop Res* 2016;34:932–41.
30. Agius R, Galea R, Fava S. Bone mineral density and intervertebral disc height in type 2 diabetes. *J Diabetes Complications* 2016;30:644–50.
31. Fields AJ, Berg JB, Metz LN, et al. Alterations in intervertebral disc composition, matrix homeostasis and biomechanical behavior in the UCD-T2DM rat model of type 2 diabetes. *J Orthop Res* 2015;33:738–46.
32. Zhang B, Shen Q, Chen YP, et al. Myricitrin alleviates oxidative stress-induced inflammation and apoptosis and protects mice against diabetic cardiomyopathy. *Sci Rep* 2017;7:44239.
33. Huang WZ, Ao P, Li J, et al. Autophagy protects advanced glycation end product-induced apoptosis and expression of MMP-3 and MMP-13 in rat chondrocytes. *Biomed Res Int* 2017;2017:6341919.
34. Xie YP, Yu D, Wu JH, et al. Protective effects of physiological testosterone on advanced glycation end product-induced injury in human endothelial cells. *Mol Med Rep* 2017;15:1165–71.
35. Liu Q, Jin L, Mahon BH, et al. Novel treatment of neuroinflammation against low back pain by soluble fullerol nanoparticles. *Spine (Phila Pa 1976)* 2013;38:1443–51.
36. Ishibashi H, Tonomura H, Ikeda T, et al. Hepatocyte growth factor/c-met promotes proliferation, suppresses apoptosis, and improves matrix metabolism in rabbit nucleus pulposus cells in vitro. *J Orthop Res* 2016;34:709–16.
37. Ott M, Gogvadze V, Orrenius S, et al. Mitochondria, oxidative stress and cell death. *Apoptosis* 2007;12:913–22.
38. Chen JW, Ni BB, Li B, et al. The responses of autophagy and apoptosis to oxidative stress in nucleus pulposus cells: implications for disc degeneration. *Cell Physiol Biochem* 2014;34:1175–89.
39. Nasto LA, Robinson AR, Ngo K, et al. Mitochondrial-derived reactive oxygen species (ROS) play a causal role in aging-related intervertebral disc degeneration. *J Orthop Res* 2013;31:1150–7.