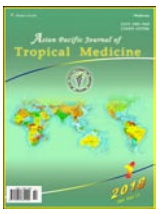

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Autophagy plays a protective role in advanced glycation end products-induced apoptosis of chondrocytes via regulation of tumor necrosis factor-α, nuclear factor-κB and reactive oxygen species

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ABSTRACT

Objective: To study the adverse effects of advanced glycation end products (AGEs) on chondrocytes and the role of autophagy in this process. **Methods:** Chondrocytes were harvested from the human articular cartilage tissues in surgery. AGEs were administered during chondrocytes culture. The rapamycin was used to induce autophagy. The cell viability was determined by 3-[4,5-dimethylthiazol2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. The expression of tumor necrosis factor-α (*TNF-α*) and nuclear factor-κB (*NF-κB*) was detected by quantitative real-time polymerase chain reaction. The reactive oxygen species (ROS) production and apoptosis of the chondrocytes were determined by fluorescent probe and flow cytometer, respectively. **Results:** The chondrocytes viability was significantly reduced after 12 h incubation with AGEs (P<0.01)). In contrast, rapamycin pretreatment increased the chondrocytes viability through autophagy. AGEs increased *TNF-α* and *NF-κB* mRNA expression of chondrocytes and autophagy receded or proceeded the change. AGEs increased intracellular ROS accumulation and autophagy reversed the change. AGEs accelerated chondrocytes apoptosis and autophagy suspended apoptosis. **Conclusions:** Accumulation of AGEs may have an adverse role for chondrocytes by increasing *TNF-α* and *NF-κB* expression, ROS accumulation and apoptosis; meanwhile, autophagy ameliorates the AGEs-induced adverse effects.

1. Introduction

Advanced glycation end products (AGEs) are formed *in vivo* by metal-catalyzed glucose auto-oxidation and lipid peroxidation. A reducing sugar reacts with a protein (free amino groups in lysine, arginine or hydroxylysine residues) to form a labile, subsequently stabilized product, which produces an irreversible, non-enzymatic post-translational modification[1,2]. Once these are formed, AGEs are only degraded in the case of protein degradation that is prone to AGE formation[3]. The accumulation of AGEs affects the extracellular and intracellular structure and function in many different tissues and cell types. The most massive accumulation of AGEs will produce in tissues with low turnover, for instance, crystalline in the lens and collagen in the extracellular matrix of connective tissues, e.g., amyloid plaques[4,5], skin[6], tendon[7], bone and cartilage[8,9].

AGEs accumulation affects the function of tissues, contributing to the pathogenesis of diseases. Recent studies found that AGEs play

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a significant role in the development of age-related diseases, such as osteoarthritis[10,11]. Articular cartilages are prone to accumulate AGEs contributing to their low turnover. The accumulated AGEs increase the brittleness and stiffness of articular cartilage by reducing the synthesis of proteoglycan and collagen in the chondrocytes[11]. As such, AGEs accumulation adversely affects the mechanical properties of the matrix driving the development of osteoarthritis.

Autophagy refers to the cellular degradative pathway that involves on turnover of cell constituents and serves as a short-lived survival mechanism under starvation environment by clearing away unfolded protein. Autophagy occurs at the low level in virtually all cells to perform the homeostatic function. However, it is rapidly up-regulated when cells need to generate intracellular nutrients and energy under certain stress conditions, such as starvation, infection, oxidative stress, protein aggregate accumulation and another irritation[12]. It is reported that AGEs also can induce autophagy in vascular smooth muscle cells and endothelial cells[13,14]. Generally, autophagy can promote cell survival by blocking apoptosis. However, autophagy also can result in cell death under certain conditions[15].

This study examined the effect of AGEs on cell viability, *TNF-* α and *NF-* κ *B* expression, ROS accumulation and apoptosis in human chondrocytes. Meanwhile, the role of autophagy on AGEs-induced adverse effect was investigated.

2. Materials and methods

2.1. Samples collection and cell culture

Cartilages were harvested from osteoarthritis patients with surgical procedures of total knee replacement (n=5, three males and two females, age range: 49–56 years old). The harvested cartilage was stored with amicrobic physiological saline. The cartilage was cut into 5–10 mm^3 slices after removing the subchondral lamina. Consequently, the complete treatment procedures were followed. Trypsin solution was used to soak the cartilage slices at room temperature for 30 min and then washed with phosphate buffer saline (PBS), followed by type II collagenase solution treatment at 37 ℃ for 10 h. The isolated cells were maintained at 37 ℃ with 5% CO_2 in DMEM/F12 supplemented with 10% fetal bovine serum (HyClone, Logan, Utah, USA). The above procedures were performed under sterile conditions. Phase contrast images of the live chondrocytes were obtained using Olympus microscope.

2.2. AGEs treatment

AGE-BSA full-length protein was purchased from Abcam Company (ab51995). When the confluence of chondrocytes reached 80%, 100 μg/mL AGE-BSA was administered in the medium during the culture.

2.3. Cell viability assay

After AGEs treatment for 12, 24 and 48 h, the viability of chondrocyte populations in culture was quantified by 3-[4,5-dimethylthiazol2-yl]-2,5-diphenyl tetrazolium bromide (MTT). Cells were seeded at 3×10^3 cells/well in a 96-well microplate in culture media with 10% FBS. MTT solution (0.5 mg/mL in PBS) was put into each well and then incubated at 37 ℃ for 4 h. Dimethyl sulfoxide (DMSO) was added after removing the supernatant. The absorbance was quantified using a microplate reader at 490 nm.

2.4. Rapamycin treatment induced autophagy

Autophagy inductor rapamycin (1 mmol/L) (Sigma, Co., St. Louis, MO, USA) was pretreated 2 h before AGEs incubation. MTT was used to measure cell viability at 12, 24, and 48 h after AGEs treatment. The time points for the experiments of *TNF-* α and *NF-* κ *B* expression, intracellular ROS level and apoptosis were decided based on the result of MTT assay.

2.5. Western blot analysis

The chondrocytes were lysed using radio immunoprecipitation lysis buffer to extract protein. The protein in cell lysates was quantified using a spectrophotometer. The western blot analyses were performed using the rabbit monoclonal microtubule-associated protein 1 light chain 3 antibody (1:1 000 dilution; Cell Signaling Technology, Boston, MA, USA) and β-actin antibody (1: 3 000 dilution; Wuhan Sanying, Hubei, China), then labeled by horseradish peroxidase-conjugated antibody. The binds were visualized using enhanced chemiluminescent. Immunoblot signals were quantified using Gel-Pro Analyzer 4.0.

2.6. Expression of cellular TNF-α and NF-κB using quantitative real-time polymerase chain reaction (qRT-PCR)

The expression of *TNF-* α and *NF-* κ *B* were evaluated using qRT-PCR. Total RNA of chondrocytes was extracted using Trizol regent (Sigma, Co., St.Louis, MO, USA) and reverse-transcribed into cDNA according to instruction manual of an iScriptTMcDNA Synthesis Kit. The mRNA expression was quantified by two step SYBR Green RT-PCR. Relative fold change of gene was calculated using the comparative C_t equation. The relative amount of transcript was normalized against glyceraldehyde phosphate dehydrogenase (*GAPDH*) transcript. The amplification was in the presence of the following specific primer sets: 5′-CCT CAT CTA CTC CCA GGT-3′ and 5′-TAG ATG GGC TCA TAC CAG-3′ for *TNF-* α, 5′-TGG TGG AGG ATT TGC TGA GG-3′ and 5′-CCG TTG GGG TGG TCA AGA AG-3′ for *NF-* κ *B*, 5′-CGG AGT CAA CGG ATT TGG TCG TAT-3′ and 5′-AGC CTT CTC CAT GGT GGT GAA GAC-3′ for *GAPDH*.

2.7. Measurement of intracellular ROS

Intracellular ROS level was measured using Carboxy-2',5'-dichlorofluorescein diacetate (Carboxy-H_2DCFDA) molecular probe (Invitrogen, Spartak Calder, CF, USA). After treatment, cells were incubated in medium containing 1 μmol/L DCFDA for 30 min at 37 ℃ in the dark. Cells were washed with PBS twice and dissociated enzymatically with trypsin added EDTA. Then cells were harvested by centrifugation and re-suspended in 500 μL PBS. Fluorescence absorbance was detected using a microplate reader (FCM, Thermo

Fisher Scientific, Boston, MA, USA) with 488 nm/535 nm (excitation/emission wavelength).

2.8. Apoptosis analysis

The apoptosis rate was measured by flow cytometer (FCM, Thermo Fisher Scientific, Boston, MA, USA) using the Annexin V-FITC/ propidium iodide (PI) apoptosis detection kit (Nanjing Jiancheng, Jiangsu, China) according to the manufacturer's instruction. Briefly, the chondrocytes were harvested by enzymolysis and centrifugation, re-suspended in binding buffer and added into FCM tube. Annexin V-FITC and PI were added in cells-suspension and incubated in the dark for 15 min before FCM measurement.

2.9. Statistical analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS, Inc.). Statistically significant differences of means between three groups were determined by one-way ANOVA followed by the Post-Hoc test. The results are showed as mean ± standard deviation (Mean ± SD). $P<0.05$ was considered significant.

3. Results

3.1. Effects of AGEs and autophagy amelioration on cell viability

MTT assay showed that chondrocytes viability was remarkably decreased to 91.91% after 12 h incubation with AGEs ($P<0.001$), to 85.69% after 24 h incubation ($P<0.001$), and to 80.06% after 48 h incubation ($P<0.001$). At the same time point, the chondrocytes viabilities were 94.44%, 86.98% and 79.94% when the rapamycin was pretreated before AGEs incubation; It was significant only at 12 h ($P=0.010$) which suggested the response time to rapamycin. The chondrocytes viability under AGEs treatment or AGEs-added rapamycin treatment is shown Figure 1. The following results were that of experiments for chondrocytes treated with AGEs for 12 h.

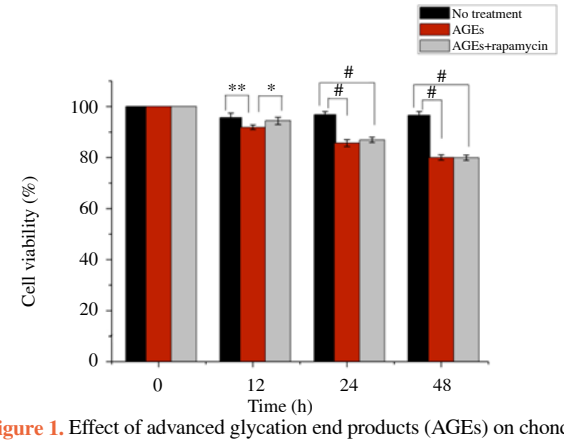

Figure 1. Effect of advanced glycation end products (AGEs) on chondrocytes viability.

This data were compared between no treatment group, AGEs group and AGEs+raspamycin group. $^{*}P<0.05$, $^{**}P<0.01$ and $^{\#}P<0.001$.

3.2. LC3 expression after rapamycin indicating autophagy

Rapamycin was administered to chondrocytes to induce autophagy. LC3 involved in formation of autophagosomal vacuoles, especially LC3-II is regarded as the biomarker of autophagy. Hence, the expression of cellular LC3 protein was measured using western blot to present the level of autophagy. Compared to that with no treatment, the total LC3 (including LC3-I and LC3-II) expression of chondrocytes was significantly increased both with AGEs treatment ($P=0.015$) and with AGEs-added rapamycin treatment ($P<0.001$) (Figure 2). The densitometry of LC3-II/ β -actin in chondrocytes treated with AGEs added rapamycin was much higher than that in chondrocytes without treatment and AGEs treatment (both $P<0.001$). There was no significance between chondrocytes with AGEs treatment and chondrocytes without treatment (Figure 2).

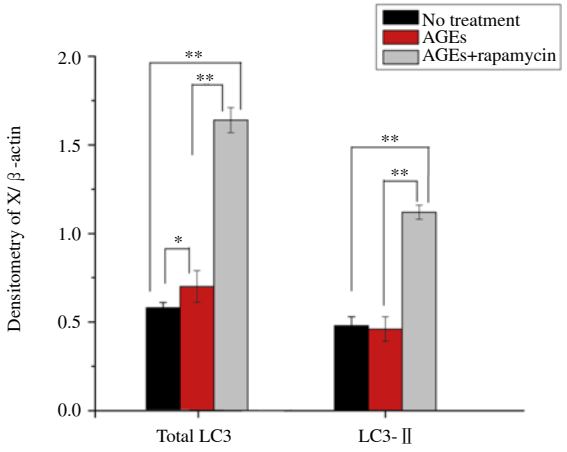

Figure 2. Densitometric analysis of cellular light chain (LC3) protein.

In the bar, $n = 5$ in each group. $^{*}P<0.05$, $^{**}P<0.001$.

3.3. AGEs increased the expression of TNF- α and NF- κ B of chondrocytes and autophagy receded the change

The effects of AGEs and autophagy on the expression of *TNF-* α and *NF-* κ *B* in chondrocytes were measured by qRT-PCR. Compared to that without treatment, the RNA expression levels of *TNF-* α and *NF-* κ *B* in chondrocytes with AGEs incubation were significantly higher (2.46-fold and 2.16-fold, both $P<0.001$). In the case of AGEs-added rapamycin treatment, the RNA expression levels of *TNF-* α and *NF-* κ *B* in chondrocytes were reduced compared to that with single AGEs incubation. However, they were also higher than those without treatment (1.75-fold and 1.62-fold, $P=0.004$ and $P<0.001$, respectively).

3.4. AGEs increased intracellular ROS accumulation and autophagy reversed the change

To test the effect of AGEs on the cellular ROS levels, cells were stained with DCFDA and detected by FCM. The levels of cellular ROS increased when chondrocytes were with AGEs incubation for 12 h ($P<0.001$). The fluorescence absorbance of cellular ROS production was 284.4–334.4. When the chondrocytes were pretreated with rapamycin before AGEs incubation, the average fluorescence

absorbance of cellular ROS production was 308.4, which was lower than that with AGEs incubation (P=0.012) but higher than that with no treatment (P=0.007).

3.5. AGEs accelerated chondrocytes apoptosis and autophagy suspended apoptosis

To evaluate the apoptosis of chondrocytes under different conditions, the chondrocytes were stained by Annexin V-FITC/PI and detected by FCM (Figure 3). AGEs accelerated chondrocytes apoptosis, and the rates of apoptosis were from 6.41% (95% *CI*: 4.32%–8.50%) to 24.31% (95% *CI*: 18.00%–30.63%). With the rapamycin pretreatment, the rate of apoptosis returned to 15.18% (95% *CI*: 13.48%–16.88%) under the autophagy protection.

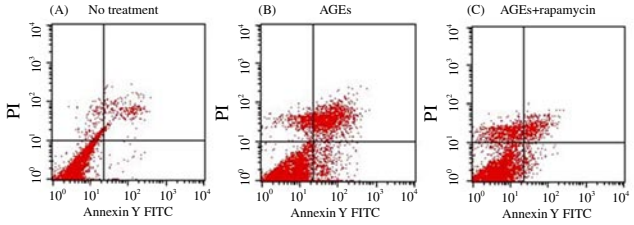

Figure 3. Chondrocytes apoptosis after culture with advanced glycation end products (AGEs) treatment and AGEs-added rapamycin treatment.
Dot-plots depict live cells (lower left quadrant), early apoptotic (lower right quadrant), late apoptotic (upper right), and necrotic (upper left).

4. Discussion

AGEs are lipids or proteins which turn glycated after sugars exposure. AGEs accumulation plays a pivotal role in osteoarthritis which is predisposed by aging[10,16,17]. Accumulation of AGEs decreases collagen synthesis and turnover, increases matrix metalloproteinase (MMP)-1, MMP-3, and MMP-13 in human osteoarthritic chondrocytes[18], and alters the tensile properties of articular cartilage[19]. Our results indicate that AGEs decreased cell viability, increased TNF-α and NF-κB expression, up-regulated ROS production and induced apoptosis in human articular chondrocytes; however, autophagy significantly reversed AGEs-induced above damages.

AGEs-bound AGEs receptor stimulates signaling pathways linked to pro-inflammatory, activating various inflammatory genes and many signaling cascades[20,21]. Some researchers have showed that TNF-α is in the upstream in the signaling cascades. In human umbilical vein endothelial cells, the evoked sequences by TNF-α include the following events: NADPH oxidase stimulation to ROS generation to mitochondrial respiratory chain activation to NF-κB activity stimulation to RAGE expression induction[22]. But some researches showed that TNF-α is in the downstream of AGEs-bound AGEs receptor and NF-κB[23]. In this study, although we cannot identify the relationship of sequenced activation between TNF-α and NF-κB, we verified AGEs increased TNF-α and NF-κB expression in human chondrocytes.

AGEs play a crucial role in the oxidative stress injury and the apoptosis[24]. In the endothelium, AGEs block nitric oxide activity and induce mitochondrial dysfunction thus cause the ROS production, which facilitates the production of mitochondrial superoxide afterward[25,26]. The mitochondrial dysfunction and high levels of ROS cause apoptosis through caspase activation. The results of this study also showed AGEs increased the levels of ROS accumulation and the apoptosis in chondrocytes. AGEs-induced ROS generation is partly through NF-κB activation in human aortic endothelial cells[27]. Additionally, as mentioned above, ROS generation also can induce NF-κB activity. TNF-α can activate both apoptotic and survival signals mediating apoptosis, proliferation, differentiation, and survival of cells[28]. Tumor necrosis factor (TNF) activates apoptosis and anti-apoptosis pathways simultaneously. TNF binding to TNF receptor 1 sequentially recruits TNF receptor-associated death domain, Fas-associated death domain, Fas-associated death domain-like interleukin-1 β converting enzyme, and caspase-3, leading to apoptosis[29]. However, both effects of TNF are mediated through the production of reactive oxygen intermediates. There may be a cross talking among TNF-α, NF-κB, ROS generation and apoptosis under AGEs provoking.

Autophagy is a protection mechanism that involves in the homeostasis maintenance of cells in reaction to many forms of stress such as oxygen, nutrient, chemotherapeutics and growth factor deprivation. The relationship between ROS and autophagy is mutual. ROS accumulation induces autophagy, in turn, autophagy serves to lower ROS level[30,31]. In general, autophagy is valuable to cell survival in a diverged condition; however, excessive autophagy can cause cell death. In this study, we showed that AGEs exerted their adverse effects on the cell viability, TNF-α and NF-κB expression, ROS accumulation and apoptosis. Meanwhile, when the chondrocytes were pretreated with the autophagy inductor rapamycin, all the adverse effects were improved. This indicated autophagy was a defense mechanism to protect cartilage damage when it is adversely attacked by AGEs against damaged organelles and harmful metabolites, delaying the ROS accumulation and reducing apoptosis.

In conclusion, this study confirms autophagy plays a protective role in AGEs-induced apoptosis of chondrocytes possibly via regulation of TNF-α, NF-κB and ROS. However, the potential molecular mechanisms are still elusive to a large extent. The future studies should focus on the important question. The results also suggest that the novel pharmacological actions against AGEs-stimulated oxidative stress and apoptosis of chondrocytes are a potential measure for treating osteoarthritis.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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