Curcumin activates autophagy and attenuates high glucose-induced apoptosis in HUVECs through the ROS/NF-κB signaling pathway

QI-HUI JIN[1], XU-JUN HU[2] and HAI-YAN ZHAO[3]

[1]Department of Geriatric Medicine, The Second Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou, Zhejiang 310009; [2]College of Medical Technology, Zhejiang Chinese Medical University, Hangzhou, Zhejiang 310053; [3]Department of Internal Medicine, Shangcheng District Hospital of Traditional Chinese Medicine, Hangzhou, Zhejiang 310000, P.R. China

Received April 25, 2022; Accepted July 4, 2022

DOI: 10.3892/etm.2022.11533

Abstract. Curcumin (CUR) is well known for its anti-inflammatory and antioxidant effects. However, the endothelial protective effect of CUR in diabetes and the underlying signaling pathway remains unclear. The goal of the current study was to provide evidence regarding the protective mechanism of CUR against the high glucose (HG)-induced damage to human umbilical vein endothelial cells (HUVECs). HG-induced HUVECs injury model was used to evaluate the protective effect and the underlying mechanism of CUR against endothelial injury. The cell viability was determined by the MTT method. The cell reactive oxygen species (ROS) were determined by using flow cytometry. The protein expression levels of Bcl-2, Bax, LC3-II/I, Beclin-1, p62, cleaved caspase-3, IκBα and NF-κB were measured by the western blotting. Results showed that CUR significantly decreased the cell apoptosis, the ROS generation and the inflammatory cytokine NF-κB activity in the HG-induced HUVECs versus the control, P<0.05. In addition, CUR significantly increased the expressions of LC3-II/I, Beclin-1, IκBα and Bax/Bcl-2 in the HG-induced HUVECs versus the control, P<0.05. Furthermore, the addition of autophagy inhibitor 3-MA impaired the autophagy, exacerbated the apoptotic death and increased the ROS and NF-κB levels in HUVECs under the high glucose condition, P<0.05. In brief, autophagy served a protective role in the HG-induced apoptosis in HUVECs and CUR alleviated apoptosis by promoting autophagy and inhibiting the ROS/NF-κB signaling pathway.

Correspondence to: Dr Xu-Jun Hu, College of Medical Technology, Zhejiang Chinese Medical University, 548 Binwen Road, Hangzhou, Zhejiang 310053, P.R. China
E-mail: huxujun10@hotmail.com

Key words: curcumin, autophagy, high glucose, apoptosis, NF-κB, reactive oxygen species

Introduction

Diabetes mellitus (DM) is a chronic metabolic disease, characterized by hyperglycemia. Diabetic angiopathy, one of the most common and serious complications, is also the main cause of mortality in type 2 diabetes mellitus (T2DM) (1,2). Long-term exposure of endothelial cells (ECs) to high glucose (HG) contents leads to inflammation and oxidative stress in diabetics, resulting in vascular endothelial cell injury and eventually vascular endothelial dysfunction (3).

The mechanisms by which hyperglycemia influences endothelial function are multiple. The vascular dysfunction in the setting of diabetes is associated with increased vascular oxidative stress and low-grade inflammation (4). Under hyperglycemic conditions, the excessive production of reactive oxygen species (ROS) is one of the key factors in the development of pathological changes in the endothelium, causing endothelial dysfunction or cell apoptosis (5). The endothelial pathology, accompanied by excessive production of factors, is related to the inflammatory process, such as TNF-α and IL-6 (6,7). Hyperglycemia induces the production of proinflammatory cytokines and growth factors by activating the key signal pathways that are associated with the MAPK and NF-κB (8). Oxidative stress and inflammation are the major mechanisms underlying the pathogenesis of DM complications. The overproduction of ROS, which results in oxidative damage, including lipid peroxidation, protein oxidation and DNA damage, can lead to cell death. Furthermore, ROS can act as the second messengers to activate transcription factors such as NF-κB and the activation of NF-κB is involved in the multiple aspects of diabetic pathology, including cellular differentiation, survival, autophagy and apoptosis (9).

Autophagy serves a vital role in cellular homeostasis by acting as a housekeeper to eliminate the damaged organelles. The impairment of autophagy, which is observed in the endothelial injury, is implicated especially in the DM-induced endothelial dysfunction. In vascular pathogenesis, autophagy can act as a survival pathway, protecting endothelial cells from oxidative stress (10,11). Vascular endothelial dysfunction is recognized as an initial step of diabetic vascular complications,

which might be caused by hyperglycemia-induced apoptosis (12). A complex interplay is engaged between autophagy and apoptosis, which occurs simultaneously within the same cell under various stresses. The interaction between autophagy and ROS is frequently correlated with cell survival/death in various diseases, including DM-related endothelial oxidative damage (13,14). The regulation of autophagy, therefore, can attenuate the endothelial oxidative damage, eliminate the endothelial dysfunction and promote tissue repair, especially the therapeutic angiogenesis (15). The drug 3-Methyladenine (3-MA), which is widely used as an autophagy inhibitor in animal experiments, has significant neurological impacts on cerebral infarction (16,17).

Curcumin [(CUR); 1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is a polyphenol extracted from the *Curcuma longa* plant, commonly known as turmeric. Fig. 1A shows the chemical structure of CUR. Accumulating evidence suggests that CUR can act as an agent with anti-inflammatory, antioxidant, anticarcinogenic and antilipidemic effects (18,19). Not only has the natural CUR been investigated extensively for clinical application, but also some synthetic curcumin analogs (20,21). CUR can improve gastric emptying in rats by blocking the production of oxidative stress and abolishing the NF-κB signal transduction with diabetic gastroparesis (22). In addition, CUR also induces autophagy to protect the vascular endothelial cells and reduces the cell apoptosis from the oxidative stress damage (23-25), suggesting a potential mechanism underlying the anti-apoptosis effects of CUR (26,27).

Overall, previous studies have confirmed that HG contents can promote oxidative stress and apoptosis and CUR has a strong antioxidant and antiapoptosis effect, but the exact mechanisms are uncertain. To understand the pharmacological mechanisms of CUR, the current study investigated the antiapoptosis effects of CUR in human umbilical vein endothelial cells (HUVECs). HUVECs were pretreated with various concentrations of CUR before the HG stimulation. Alterations in the expression of autophagy, inflammation and apoptosis-related proteins, cell viability and activation of the ROS were observed. The goal was to provide evidence regarding the protective mechanism of CUR against the HG-induced damage to HUVECs.

Materials and methods

Experimental design. The whole experiment was conducted in several parts. To explore the dose-dependent effects of CUR on the viability of HUVECs, the cells were pretreated for 4 h at 37˚C with different concentrations of CUR (5, 10, 20 and 40 μM) and then incubated with HG concentration for 24 h. Exposure of vascular ECs to a glucose level greater than 10 mmol/l (*in vitro* or *in vivo*, as in diabetes mellitus) is considered an HG condition. As the cell viability is relatively high under normal glucose exposure with no obvious glucose toxicity and excessive oxidative stress, the effects of CUR on cell viability were examined only with CUR added to an HG concentration. The cell viability was determined with the use of an MTT assay. To further clarify the effect of CUR on apoptosis and autophagy, HUVECs that were pretreated with 20 μM CUR and then incubated in 33.3 mmol/l glucose (HG) were used. The apoptosis was evaluated using the TUNEL assay and the expression of the autophagy proteins was measured by western blotting. To gain an insight into the apoptosis signaling pathway, the expression of caspase proteins was examined by immunoblotting. To confirm whether ROS and NF-κB would participate in the anti-apoptosis effect of CUR, HUVECs were incubated for 4 h at 37˚C with 20 μM CUR and then for 24 h with or without the autophagy inhibitor 3-methyladenine (3-MA, 10 mM) in the HG condition. The ROS production was detected with a flow cytometer while the NF-κB-related protein was detected with the western blotting.

Cell culture. Immortalized HUVECs (Lonza Group Ltd.) were cultured in Dulbecco's modified Eagle's medium (DMEM; MilliporeSigma), supplemented with 10% FBS and 1% penicillin-streptomycin in a humidified 5% CO_2, 37˚C incubator (Thermo Fisher Scientific, Inc.). All parts of the experiment on HUVECs were conducted at the ~3-7 passages. Those HUVECs that were cultured in a medium of 33.3 mmol/l glucose for 24 h served as the HG group (8), while those HUVECs that were cultured in a medium of 5.5 mmol/l glucose for an equivalent time served as the control (Con) group.

MTT cell viability assay. The colorimetric MTT assay was used to detect the cell viability in the 96-well plates. The MTT substrate was prepared in a physiologically balanced solution, added to HUVECs in culture and incubated for 4 h at 37˚C. Viable cells with active metabolism converted MTT into purple formazan. The optical density of formazan (directly proportional to the number of viable cells) after dissolution in DMSO was measured at 450 nm with a microplate reader (Molecular Devices, LLC.). The mean optical density of 6 wells was used to calculate the cell viability percentage relative to the cell viability of control wells.

Caspase-3 activity. The caspase-3 activity was determined with a Caspase-3 Assay kit (Colorimetric) ab39401 (Abcam) according to the manufacturer's protocol. Briefly, the cells were lysed with the cell lysis buffer, incubated on ice for 10 min at 37˚C and centrifuged at 10,000 g for 1 min. The supernatant was collected to measure the protein concentration with the bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Inc.). To measure the caspase-3 activity, the sample was mixed with an equal volume of 2X reaction buffer (containing 10 mM DTT) and 200 μM of DEVD-p-NA substrate. The mixture was incubated at 37˚C for 2 h. The optical density was measured at 400 nm with a microplate reader (BioTek Instruments, Inc.).

Assay of intracellular ROS. The cells were seeded in a 6-well plate at a density of $5x10^4$ cells/well. The levels of intracellular ROS were measured with the fluorescent probe dichloro-dihydro-fluorescein diacetate (DCFH-DA; Beijing Baiaosentai Biotechnology). Following treatment with stimuli, the cells were incubated with DCFH-DA (10 μM) at 37˚C for 30 min. The cells and probe were mixed thoroughly for 30 min by inverting the flask once every 3-5 min. The cell suspension was centrifuged at 12,000 x g for 5 min at 4˚C, and the supernatant was discarded. The cells were resuspended and washed 3 times with 1 ml serum-free medium to remove the DCFH-DA that did not enter the cells. The cells were

then centrifuged again at 12,000 x g for 5 min at 4˚C, and the supernatant was discarded, followed by the addition of 500 ml phosphate-buffered saline (PBS) to resuspend the cells. After 30 min, the cells were subjected to flow cytometry (using the parameters set for FITC) at an excitation wavelength of 535 nm and an emission wavelength of 610 nm to detect the fluorescence intensity before and after stimulation. The fluorescence images were obtained with fluorescence microscopy (BX41F, Olympus Corporation).

Western blotting analysis. HUVECs were homogenized in RIPA lysis (Wuhan Servicebio Technology Co., Ltd.) buffer to obtain total proteins. According to the previous studies (28,29), the protein concentrations were measured by a BCA Protein Assay kit (Thermo Fisher Scientific, Inc.) and denatured at 72˚C for 10 min. Protein (~50 μg) was separated by 10% SDS-PAGE and then transferred onto a PVDF membranes (MilliporeSigma). After blocking for 1 h at room temperature with 5 % skimmed milk in TBS buffer (10 mM Tris, 150 mM NaCl), the membranes were probed with primary antibodies, including rabbit polyclonal antibodies (Cell Signaling Technology, Inc.): Anti-LC3 (1:500; cat. no. 4108), anti-Beclin1 (1:500; cat. no. 3738), anti-cleaved-caspase3 (1:500; cat. no. 9661), anti-Bcl-2 (1:1,000; cat. no. 3498), anti-Bax (1:1,000; cat. no. 2774) and a mouse monoclonal antibody against β-actin (1:1,000; cat. no. 8457) at 4˚C overnight. Next, the membranes were washed four times, 15 min each, with TBST buffer (10 mM Tris, 150 mM NaCl, and 0.1% Tween-20) and incubated for 30 min at 37˚C with appropriate HRP-conjugated secondary antibodies (anti-rabbit IgG, HRP-linked antibody cat. no. 7074; Cell Signaling Technology, Inc.). The protein bands were visualized with chemiluminescent reagents following the manufacturer's instructions and exposed to Hyperfilm-ECL (Amersham; Cytiva). Densitometry analysis of band intensity was performed using ImageJ software v 1.8.0 (National Institutes of Health).

Apoptosis assay. Cell apoptosis was determined with an Annexin V-FITC Apoptosis kit (cat. no. C1062s; Beyotime Institute of Biotechnology) according to the manufacturer's protocol. In total, 1x10^6 cells were collected and the cells from each sample were suspended in 195 μl of 1X Annexin V-FITC binding buffer and 5 μl Annexin V-FITC. The cells were incubated at room temperature for 10 min. Then, each sample was centrifuged at 12,000 x g for 5 min, suspended again in 190 μl of binding buffer, to which was added 10 μl of propidium iodide (PI) working solution. The suspension was mixed and incubated in the dark at room temperature for 15 min. Finally, cell apoptotic rates (early + late) were determined using a flow cytometer (FACSCanto II; BD Biosciences). FlowJo version 7.6.1 software (FlowJo LLC) was used to analyze the data.

Statistical analysis. Data analysis was conducted with the GraphPad Prism 8 software. All results were presented as mean ± standard deviation (SD). To compare measurements obtained in different test conditions, a one-way analysis of variances (ANOVA) was used to examine the effect of test condition on the dependent variable. The post-hoc analysis was conducted with Tukey's test for pairwise comparisons. P<0.05 was considered to indicate a statistically significant difference.

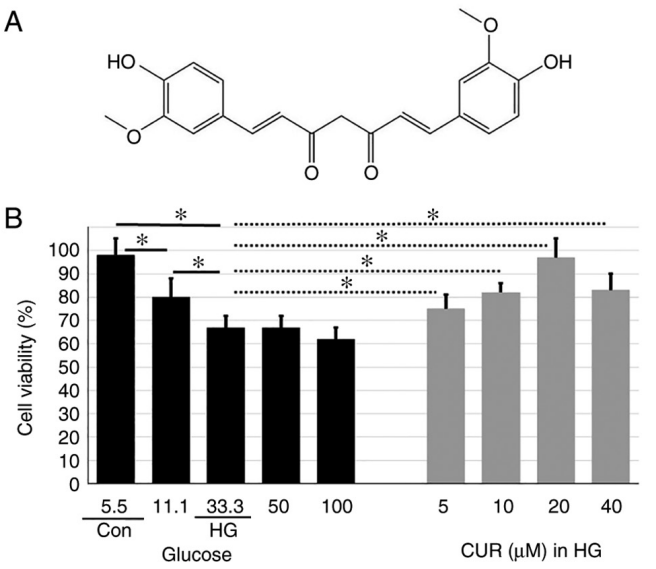

Figure 1. CUR increases the cell viability in HG. (A) The chemical structure of CUR. (B) The cell viability was measured for cells treated with glucose in different concentrations (5.5-100 mmol/l) and for cells treated with curcumin in different concentrations (5-40 μM) under HG conditions. Each error bar is the standard deviation of the mean (n=6). Significance level between two test conditions: *P<0.05. Con, control group treated with 5.5 mmol/l glucose; HG, high glucose group treated with 33 mmol/l glucose; CUR, curcumin.

Results

CUR enhances HUVEC viability in HG. The first part of the experiment was to measure the cell viability in different cell groups with the purpose to show the protective ability of CUR against the damage induced by HG conditions (Fig. 1B). The cell variability for cells that were treated only with different glucose concentrations (5.5-100 mmol/l) for 24 h was also included in Fig. 1B. The cell viability decreased in a dose-dependent manner from the Con group (5.5 mmol/l glucose) to the HG group (33.3 mmol/l glucose). In addition, the cell viability for the HG group was similar to the cell viability for cells treated with higher glucose concentrations (50 and 100 mmol/l). The cell viability for the HG group was significantly lower than the cell viability for the Con group, P<0.01 (Fig. 1B), indicating HG conditions reduced the number of healthy cells in the sample. Meanwhile, for those cells that were pretreated with 5, 10, 20 and40 μM CUR for 4 h and then incubated in 33.3 mmol/l HG for 24 h, the cell activity increased in a dose-dependent manner that was peaked at 20 μM CUR. Compared to the cell viability (67%) for the HG group, the cell viability (96%) for cells treated with 20 μM CUR in the HG condition was significantly higher, P<0.05 (Fig. 1B), suggesting the 20 μM CUR treatment could significantly enhance the cell viability in HG conditions. Thus, the 20 μM CUR concentration provided the optimal cell viability enhancement in HG conditions and the cells treated with 20 μM CUR in HG were labeled as the HG+CUR group for the subsequent parts of the experiment.

CUR reduces ROS generation in HG. The second part of the experiment was conducted to measure the ROS content for the

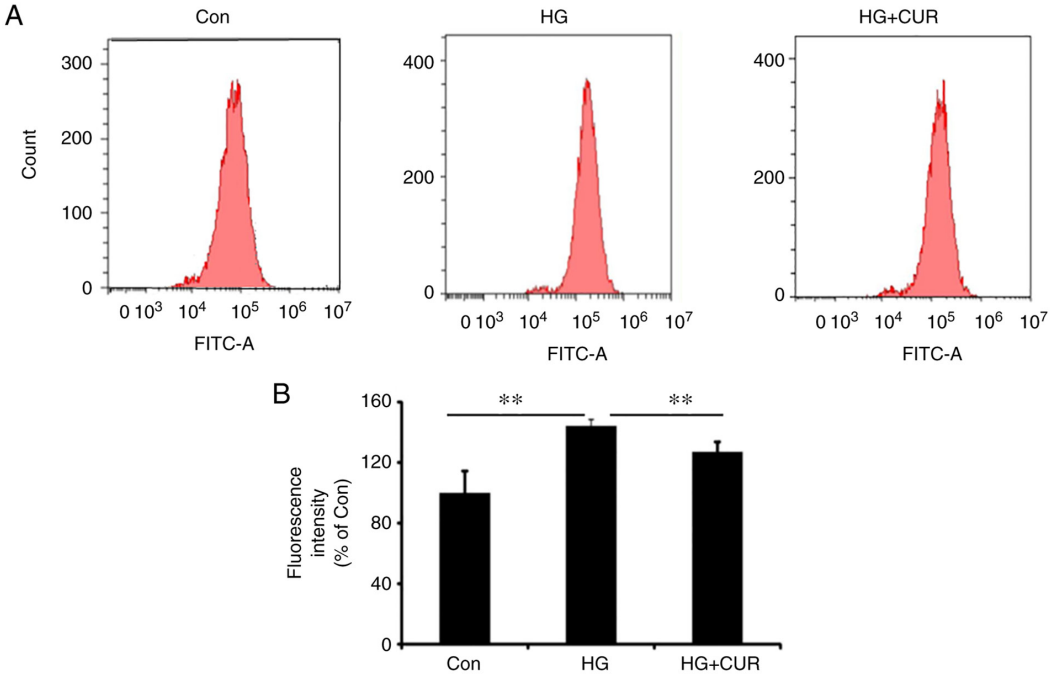

Figure 2. CUR reduces the high glucose-induced secretion of ROS. (A) The histogram of counting ROS was measured with the flow cytometry (FITC-A) for the Con, HG and HG+CUR groups. (B) The fluorescence intensity was measured for the three groups. Each error bar is the standard deviation of the mean (n=6). Significance level between two test conditions: **P<0.01. CUR, curcumin; ROS, reactive oxygen species; Con, control group treated with 5.5 mmol/l glucose; HG, high glucose group treated with 33 mmol/l glucose.

three groups (Con, HG and HG+CUR) to show the effect of CUR on ROS generation in a HG condition. Fig. 2A shows the histogram of counting the ROS content using a flow cytometer for the three groups. The fluorescence intensity of the ROS content (%) among the three groups is presented in Fig. 2B. The ROS content in the HG group was significantly higher than the ROS content in the Con group, P<0.01; and the ROS content in the HG+CUR group was significantly lower than the ROS content in the HG group, P<0.01 (Fig. 2B). Therefore, HG conditions could induce ROS production in HUVECs, while the CUR treatment significantly decreased the intracellular ROS generation in the HG condition.

CUR reduces HG-induced HUVEC apoptosis. The third part of the experiment determined whether CUR could reduce cell apoptosis in the HG-induced injury model. Fig. 3A shows the detection of apoptosis in two-dimension using FITC Annexin V and PI for the three groups: Con, HG and HG+CUR. Fig. 3B shows the apoptosis rate detected with the TUNEL assay. The apoptosis rate in the HG group was significantly higher than the apoptosis rate in the Con group, P<0.01 (Fig. 3B), suggesting the HG treatment for 24 h could sufficiently induce HUVEC apoptosis. In addition, the cell apoptosis rate in the HG+CUR group was reduced significantly when compared with the apoptosis rate in the HG group, P<0.01 (Fig. 3B), demonstrating that the CUR treatment could reduce the apoptosis of HUVECs in the HG condition. These findings suggested that CUR might have a protective effect on the endothelial cell apoptosis that was induced by HG conditions.

CUR enhances autophagy in HG-cultured HUVECs. The fourth part of the experiment was to determine whether the CUR treatment could enhance the cell autophagy of endothelial cells in HG stress. The autophagy-related protein expressions of p62, Beclin1 and LC3-II/I were detected with the western blotting (Fig. 4A). In Fig. 4B, when compared with the Con group, the stress in the HG group could significantly increase the expression of p62 (P<0.05) while decreasing the expression of Beclin1 and LC3-I/II (P<0.01). However, when compared with the protein expressions in the HG group, the expression of p62 in the HG+CUR group was significantly lower (P<0.01) and the expressions of Beclin1 and LC3-II/I were significantly higher (P<0.05). These results indicated that CUR enhanced autophagy for HUVECs that were incubated in the HG condition.

Autophagy inhibition attenuates the antioxidant effect of CUR. The fifth part of the experiment was to investigate the interaction between autophagy and ROS in the HG+CUR group (Fig. 5). The amount of ROS was measured with the flow cytometer (fluorescence intensity). In Fig. 5B, by comparing the results in the Con group, the intracellular ROS in the HG group were significantly increased, P<0.01. However, compared with the ROS in the HG group, the CUR treatment in the HG+CUR group significantly decreased the overproduced ROS that was triggered by HG, P<0.01. Furthermore, the inhibition of the HG-induced ROS in the HG+CUR group was significantly attenuated by the autophagy inhibitor 3-MA treatment as observed in the HG+CUR+3MA group, P<0.05. Therefore, the CUR treatment could decrease ROS production in the HG condition, whereas the 3-MA treatment significantly reversed this effect. These findings suggested that the CUR supplementation might have increased the autophagy effect by inhibiting the HG-induced ROS.

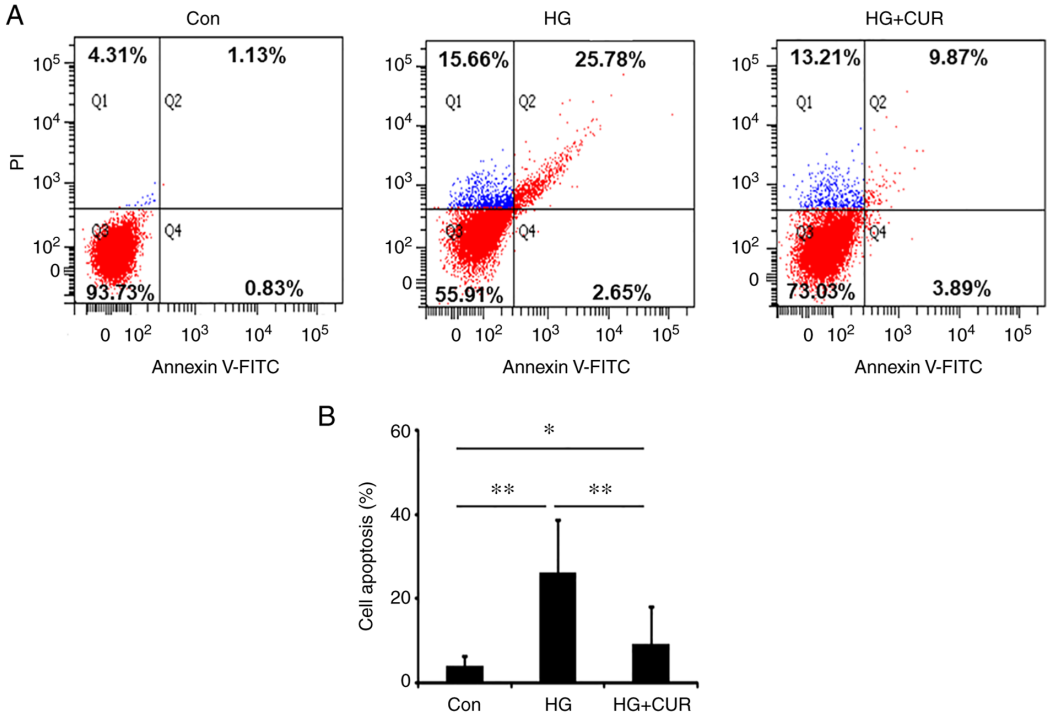

Figure 3. CUR reduces the high glucose-induced cell apoptosis. (A) Detection of apoptosis was shown in a two-dimension graph using FITC Annexin V and PI for the Con, HG and HG+CUR groups. (B) The cell apoptosis rate was measured with the TUNEL method for the three groups. Each error bar is the standard deviation of the mean (n=6). Significance level between two test conditions: *P<0.05, **P<0.01. CUR, curcumin; FITC, fluorescein isothiocyanate; PI, propidium iodide; Con, control group treated with 5.5 mmol/l glucose; HG, high glucose group treated with 33 mmol/l glucose.

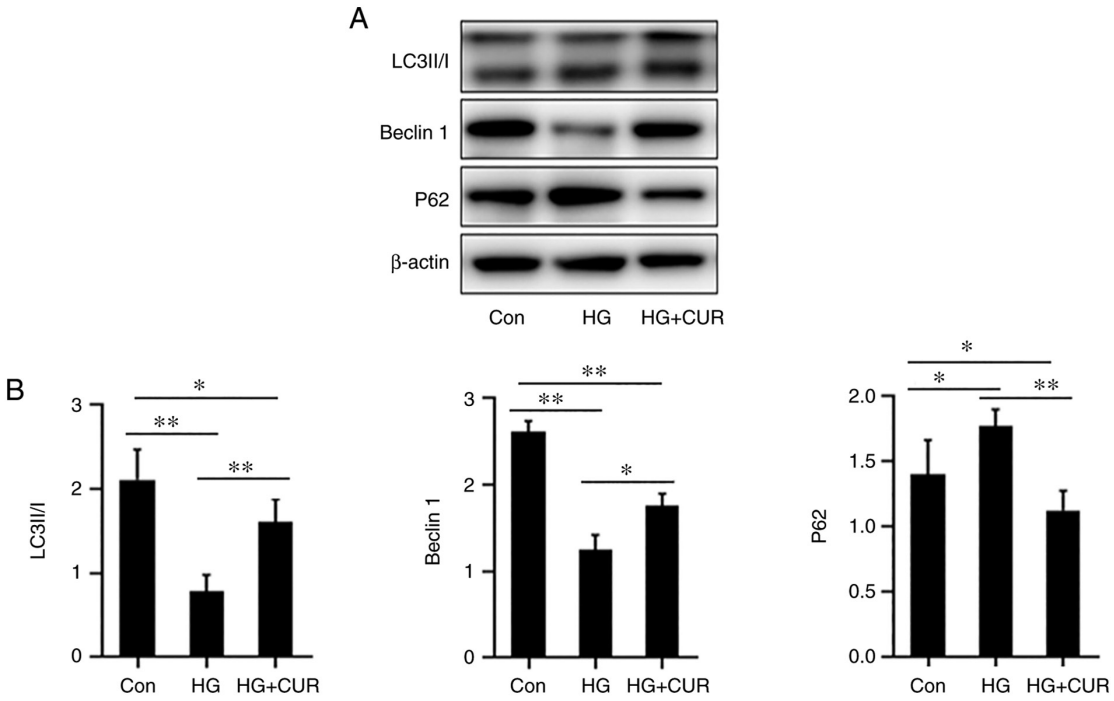

Figure 4. CUR enhances autophagy for HUVECs incubated in high glucose conditions. (A) The autophagy-related protein expressions were measured with western blotting for the Con, HG and HG+CUR groups. (B) The expressions of LC3II/I, Beclin1 and p62 (after normalization to β-actin) for the three groups. Each error bar is the standard deviation of the mean. Significance level between two test conditions: *P<0.05, **P<0.01. CUR, curcumin; HUVECs, human umbilical vein endothelial cells; Con, control group treated with 5.5 mmol/l glucose; HG, high glucose group treated with 33 mmol/l glucose.

Autophagy inhibition attenuates the anti-inflammatory effect of CUR. To investigate the interaction between cell autophagy and inflammation in the HG+CUR group, the sixth part of the experiment measured the expressions of NF-κB (mediator of inflammatory responses) and IκBα (inhibitor of NF-κB) in different cell groups (Fig. 6). By comparing the results in the

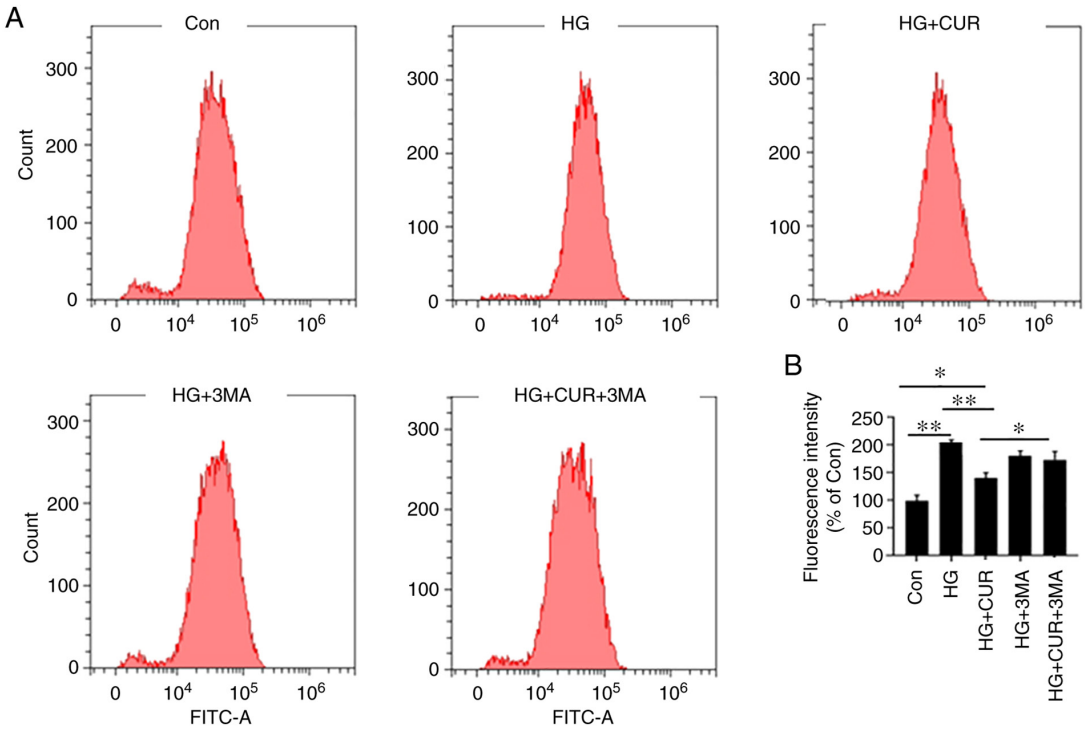

Figure 5. 3-MA treatment attenuates the antioxidant effect of CUR. (A) Histogram of ROS was measured with a flow cytometer for the Con, HG and HG+CUR, HG+3MA and HG+CUR+3MA groups. (B) The fluorescence intensity was measured for the five groups. Each error bar is the standard deviation of the mean. Significance level between two test conditions: *P<0.05, **P<0.01. CUR, curcumin; ROS, reactive oxygen species; Con, control group treated with 5.5 mmol/l glucose; HG, high glucose group treated with 33 mmol/l glucose.

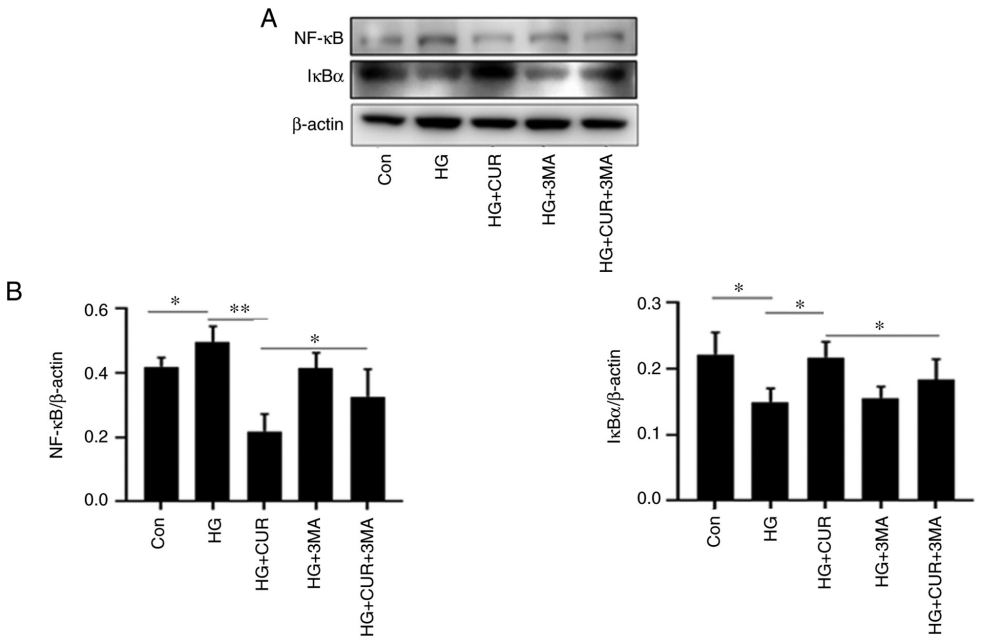

Figure 6. 3-MA treatment attenuates the anti-inflammatory effect of CUR. (A) Different expressions were measured with western blotting for the Con, HG and HG+CUR, HG+3MA and HG+CUR+3MA groups. (B) The expressions of NF-κB/β-actin and IκBα/β-actin were measured for the five groups. Each error bar is the standard deviation of the mean. Significance level between two test conditions: *P<0.05, **P<0.01. CUR, curcumin; Con, control group treated with 5.5 mmol/l glucose; HG, high glucose group treated with 33 mmol/l glucose.

Con group (Fig. 6B), the NF-κB level in the HG group was significantly increased and the IκBα level was downregulated when HUVECs were exposed to HG, P<0.05. The CUR treatment, however, significantly decreased the overproduction of HG-triggered NF-κB (P<0.01) and increased the IκBα level (P<0.05). Furthermore, this anti-inflammatory effect of CUR was significantly attenuated by the 3-MA treatment as observed in the HG+CUR+3MA group, P<0.05. Thus, the CUR treatment decreased inflammation in the HG condition, whereas the 3-MA treatment significantly reversed the effect.

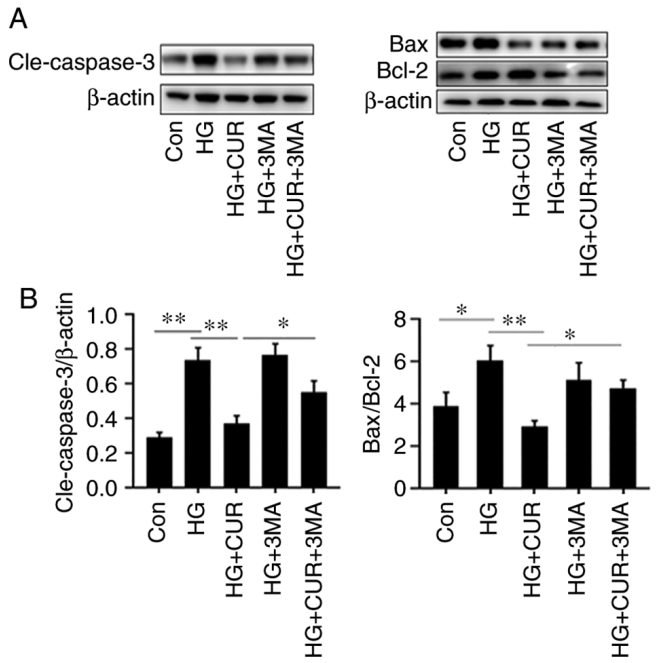

Figure 7. 3-MA treatment attenuates the anti-apoptosis effect of CUR. (A) Different expressions were measured with western blotting for the Con, HG and HG+CUR, HG+3MA and HG+CUR+3MA groups. (B) The levels of Cle-caspase-3/β-actin and Bax/Bcl-2 were measured for the five groups. Each error bar is the standard deviation of the mean. Significance level between two test conditions: *P<0.05, **P<0.01. CUR, curcumin; Con, control group treated with 5.5 mmol/l glucose; HG, high glucose group treated with 33 mmol/l glucose; Cle, cleaved.

These findings suggested that the CUR supplementation might have increased the autophagy effect as protection by inhibiting the HG-induced inflammation.

Autophagy inhibition attenuates the anti-apoptosis effect of CUR. The last part of the experiment investigated the interaction between apoptosis and autophagy by measuring the levels of apoptosis-related cleaved (Cle-) caspase-3, Bcl-2 and Bax in different cell groups (Fig. 7). Compared with the results in the HG group (Fig. 7B), the CUR treatment in the HG+CUR group could significantly lower the apoptotic levels of Cle-caspase-3/β-actin and Bax/Bcl-2 that were induced by HG, P<0.01. By contrast, compared with the results in the HG+CUR group, the Cle-caspase-3/β-actin and Bax/Bcl-2 expressions in the CUR+HG+3MA group were significantly increased after the 3-MA treatment, P<0.05. Taken together, these findings suggested that CUR could protect HUVECs from HG-induced apoptosis through autophagy.

Discussion

The current study investigated the effects of CUR on apoptosis and autography in HUVECs under HG conditions. It also explored the molecular mechanism of CUR and the ROS/NF-κB pathway. The current results have shown that CUR can promote autophagy and decrease apoptosis in HUVECs by inhibiting ROS and NF-κB. The present study further supported the putative role of CUR on autophagy induction, revealing the underlying mechanisms that may account for the beneficial effects on endothelial cell apoptosis.

CUR, a hydrophobic polyphenol compound extracted from the spice turmeric, has different pharmacological effects on both *in vitro* and *in vivo* models. A number of studies have reported that CUR is capable of triggering autophagy in several types of cells. For example, CUR is known for its potential anti-inflammatory and antioxidant properties (30). Increasing evidence suggests that CUR may take a protective effect against diabetic complications (31). CUR, therefore, protects against diabetic cardiomyopathy by modulating the crosstalk between autophagic and apoptotic machinery. The modulation of autophagy may be an effective strategy for the treatment of cardiovascular diseases associated with diabetes (32-35). Focusing on how CUR can target autophagy in different cellular settings may extend our knowledge of the new pharmacological agents in overcoming the relevant diseases.

In T2DM, elevated oxidative stress can cause injury to the vascular endothelial cells during the process of diabetic vascular complications (36). Evidence has revealed that HG conditions induce the production of ROS, causing endothelial cell apoptosis (37). Constant oxidative stress eventually leads to apoptosis or cell death. The current findings demonstrated that the HG group promoted more ROS generation. However, the CUR treatment markedly restored the HG-reduced ROS protein levels, while the HG-reduced Bax/Bcl-2 ratio was significantly reverted and the Cle-caspase-3 under HG stimulation was also downregulated.

Autophagy is usually an adaptive mechanism that regulates the cell response to stress and enhances the resistance to apoptosis, and defective autophagy has been linked to increased apoptosis (38). Abundant evidence indicates that HG decreases autophagy in different cell types (39). The current study demonstrated that HG decreased both the protein expressions of LC3II/I and Beclin-1 and increased the level of p62. Thus, insufficient autophagy has been observed in HUVECs treated with HG (40). In addition, compared to the HG group, the CUR treatment markedly upregulated the expressions of LC3II/I and Beclin-1 and downregulated p62 in HUVECs. The present study also has found that the Cle-caspase-3 and Bax/Bcl-2 levels were downregulated when autophagy was activated and that treatment with autophagy inhibitor 3-MA abolished the autophagy upregulation triggered by CUR in the HG condition with aggravated apoptosis. In summary, autophagy had a protective effect on the HG-induced apoptosis in HUVECs. Previous studies have confirmed the current findings and have shown that CUR protects against diabetic cardiomyopathy and nephropathy by promoting autophagy and alleviating apoptosis (16,32).

Inflammation is probably another key factor for the onset and progression of endothelial dysfunction. Inflammatory processes can enhance vascular ROS generation and endothelial cell apoptosis. The present study found that the CUR treatment inhibited the HG-induced inflammatory response, as evidenced by a decrease in IκBα protein level and an induced NF-κB activity. The enhanced generation of ROS and NF-κB was markedly suppressed by CUR in cells subjected to the HG-induced injury. Furthermore, although the current study investigated ROS and NF-κB separately after autophagy inhibition, the ROS/NF-κB pathway is speculative and generally accepted. Based on these findings, it is hypothesized that

ROS could be an important cellular mediator that triggers the NF-κB-dependent pathway after the administration of CUR in HUVECs. It appears that ROS/NF-κB activation is involved in the pathogenesis of the HG-induced cell dysfunction and apoptosis, suggesting that CUR, which can block the ROS/NF-κB signaling, may be effective in protecting the cell activity. A previous study shows that the stimulation of autophagy can reduce oxidative status and rapamycin can protect HUVECs from the damages caused by HG (41). The present study found that HG could increase ROS generation and activate the downstream NF-κB pathway in endothelial cells, which indicated that HG induced an oxidative stress reaction in endothelial cells. This result is consistent with previous studies that demonstrated that oxidative stress may be a potential mechanism for HG-related cell degenerative changes (42,43).

Overall, the present study indicated that CUR could promote autography and decrease apoptosis in HUVECs under HG conditions and that CUR treatment markedly restored the HG-reduced ROS protein levels and NF-kB expression. The ROS/NF-κB signaling pathway was, therefore, considered as the potential mechanism involved in the autophagy activation by curcumin. Previous reports have shown that there are a number of different potential intracellular sources of ROS which are capable of influencing or being influenced by NF-κB (44,45). ROS can activate NF-κB through IκB kinase (IKK)-dependent pathway (46). In other words, ROS can modulate an NF-κB response and NF-κB target genes can decrease ROS to promote survival in a number of ways. Depending on the setting, ROS can both act as promoters or inhibitors of NF-κB signaling (47). For example, NF-κB activity is able to influence ROS levels via increased expression of antioxidant proteins and NF-κB would also induce inflammatory enzymes that could promote the production of ROS (46). Therefore, the ROS/NF-κB signaling pathway in autophagy activated by CUR may serve a role in the potential anti-inflammatory and antioxidant properties of CUR.

To confirm that the enhanced autophagy due to CUR has a potential role in reducing the generation of NF-κB, caspase-3 and Bax/Bcl-2 in the HG-treated HUVECs, the present study adopted the autophagy inhibitor 3-MA. The results showed that 3-MA induced an additional increase in the Cle-caspase-3, Bax/Bcl-2 and NF-κB levels when compared to the levels in HG treated with CUR. This finding suggested that autophagy served an essential role in reducing caspase-3, Bax/Bcl-2 and NF-κB generation in HG and that CUR could promote autophagy and reduce apoptosis and inflammation. Therefore, the effect of inhibition on autophagy not only exacerbates the HG-induced apoptosis but also diminishes the suppression effects of CUR in NF-κB, providing a scientific basis for further research and clinical application of CUR.

The present study had several limitations. Since it was an *in vitro* simulation of the diabetic endothelial cells exposed to HG, it may not be equivalent to a T2DM model. It is also agreed that the mechanisms by which CUR activates autophagy should be the focus of research. The current study may be considered a pilot study in understanding some fundamental effects of CUR on cells exposed to HG, the protective mechanisms of CUR need to be examine further in another study.

The activation of autophagy is to protect the HG-induced HUVECs. Meanwhile, curcumin protects the HG-induced HUVECs by restoring autophagy, an effect attributed to the inhibition of the ROS/NF-B pathway. Therefore, it was hypothesized that enhancing autophagy by inhibiting ROS/NF-B expression may be a potential therapeutic strategy for treating vascular complications in diabetes. Furthermore, the regulation of endothelial cell autophagy may be another key point of control in regulating vascular function under disease conditions associated with oxidative stress.

Acknowledgements

The authors would like to thank Dr Chi C Lau, an independent scholar, for his invaluable advice in preparing the manuscript.

Funding

The present study was supported by a grant from the Administration of Traditional Chinese Medicine of Zhejiang Province (grant no. 2017ZA087).

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

The study was designed by QHJ, data were collected by HYZ, data analysis and interpretation were performed by XJH and QHJ, and the manuscript was written by QHJ and XJH. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Zheng Y, Ley SH and Hu FB: Global aetiology and epidemiology of type 2 diabetes mellitus and its complications. Nat Rev Endocrinol 14: 88-98, 2018.
2. Fowler MJ: Microvascular and macrovascular complications of diabetes. Clin Diabetes 26: 77-82, 2008.
3. Popov D: Endothelial cell dysfunction in hyperglycemia: Phenotypic change, intracellular signaling modification, ultrastructural alteration, and potential clinical outcomes. Int J Diabetes Mellitus 2: 189-195, 2010.
4. Giacco F and Brownlee M: Oxidative stress and diabetic complications. Circ Res 107: 1058-1070, 2010.
5. Zhang X, He N, Xing Y and Lu Y: Knockdown of GCN2 inhibits high glucose-induced oxidative stress and apoptosis in retinal pigment epithelial cells. Clin Exp Pharmacol Physiol 47: 591-598, 2020.

6. Wang J, Li G, Wang Z, Zhang X, Yao L, Wang F, Liu S, Yin J, Ling EA, Wang L and Hao A: High glucose-induced expression of inflammatory cytokines and reactive oxygen species in cultured astrocytes. Neuroscience 202: 58-68, 2012.
7. Liu T, Gong J, Chen Y and Jiang S: Periodic vs constant high glucose in inducing pro-inflammatory cytokine expression in human coronary artery endothelial cells. Inflamm Res 62: 697-701, 2013.
8. Sweet IR, Gilbert M, Maloney E, Hockenbery DM, Schwartz MW and Kim F: Endothelial inflammation induced by excess glucose is associated with cytosolic glucose 6-phosphate but not increased mitochondrial respiration. Diabetologia 52: 921-931, 2009.
9. Verma N and Manna SK: Advanced glycation end products (AGE) potently induce autophagy through activation of RAF protein kinase and nuclear factor κB (NF-κB). J Biol Chem 291: 1481-1491, 2016.
10. Xie Y, You SJ, Zhang YL, Han Q, Cao YJ, Xu XS, Yang YP, Li J and Liu CF: Protective role of autophagy in AGE-induced early injury of human vascular endothelial cells. Mol Med Rep 4: 459-464, 2011.
11. Ge D, Jing Q, Meng N, Su L, Zhang Y, Zhang S, Miao J and Zhao J: Regulation of apoptosis and autophagy by sphingosylphosphorylcholine in vascular endothelial cells. J Cell Physiol 226: 2827-2833, 2011.
12. Allen DA, Yaqoob MM and Harwood SM: Mechanisms of high glucose-induced apoptosis and its relationship to diabetic complications. J Nutr Biochem 16: 705-713, 2005.
13. Ao H, Li H, Zhao X, Liu B and Lu L: TXNIP positively regulates the autophagy and apoptosis in the rat müller cell of diabetic retinopathy. Life Sci 267: 118988, 2021.
14. Li Q, Yin Y, Zheng Y, Chen F and Jin P: Inhibition of autophagy promoted high glucose/ROS-mediated apoptosis in ADSCs. Stem Cell Res Ther 9: 289, 2018.
15. Liu H, Yu S, Zhang H and Xu J: Angiogenesis impairment in diabetes: Role of methylglyoxal-induced receptor for advanced glycation endproducts, autophagy and vascular endothelial growth factor receptor 2. PLoS One 7: e46720, 2012.
16. Tu Q, Li Y, Jin J, Jiang X, Ren Y and He Q: Curcumin alleviates diabetic nephropathy via inhibiting podocyte mesenchymal transdifferentiation and inducing autophagy in rats and MPC5 cells. Pharm Biol 57: 778-786, 2019.
17. Wang L, Xiong X, Zhang X, Ye Y, Jian Z, Gao W and Gu L: Sodium Tanshinone IIA sulfonate protects against cerebral ischemia-reperfusion injury by inhibiting autophagy and inflammation. Neuroscience 441: 46-57, 2020.
18. Kelany ME, Hakami TM and Omar AH: Curcumin improves the metabolic syndrome in high-fructose-diet-fed rats: Role of TNF-α, NF-κB, and oxidative stress. Can J Physiol Pharmacol 95: 140-150, 2017.
19. Zhang Z and Li K: Curcumin attenuates high glucose-induced inflammatory injury through the reactive oxygen species-phosphoinositide 3-kinase/protein kinase B-nuclear factor-κB signaling pathway in rat thoracic aorta endothelial cells. J Diabetes Investig 9: 731-740, 2018.
20. Huang J, Fu J, Liu B, Wang R and You T: A synthetic curcuminoid analog, (2E,6E)-2,6-bis(2-(trifluoromethyl)benzylidene) cyclohexanone, ameliorates impaired wound healing in streptozotocin-induced diabetic mice by increasing miR-146a. Molecules 25: 920, 2020.
21. Shimizu K, Sunagawa Y, Funamoto M, Wakabayashi H, Genpei M, Miyazaki Y, Katanasaka Y, Sari N, Shimizu S, Katayama A, *et al*: The synthetic curcumin analogue GO-Y030 effectively suppresses the development of pressure overload-induced heart failure in mice. Sci Rep 10: 7172, 2020.
22. Jin QH, Shen HX, Wang H, Shou QY and Liu Q: Curcumin improves expression of SCF/c-kit through attenuating oxidative stress and NF-κB activation in gastric tissues of diabetic gastroparesis rats. Diabetol Metab Syndr 5:12, 2013.
23. Han J, Pan XY, Xu Y, Xiao Y, An Y, Tie L, Pan Y and Li XJ: Curcumin induces autophagy to protect vascular endothelial cell survival from oxidative stress damage. Autophagy 8: 812-825, 2012.
24. Yu W, Zha W, Ke Z, Min Q, Li C, Sun H and Liu C: Curcumin protects neonatal rat cardiomyocytes against high glucose-induced apoptosis via PI3K/Akt signaling pathway. J Diabetes Res 2016: 4158591, 2016.
25. Tu Y, Guo C, Song F, Huo Y, Geng Y, Guo M, Bao H, Wu X and Fan W: Mild hypothermia alleviates diabetes aggravated cerebral ischemic injury via activating autophagy and inhibiting pyroptosis. Brain Res Bull 150: 1-12, 2019.
26. Wei Y, Gao J, Qin L, Xu Y, Shi H, Qu L, Liu Y, Xu T and Liu T: Curcumin suppresses AGEs induced apoptosis in tubular epithelial cells via protective autophagy. Exp Ther Med 14: 6052-6058, 2017.
27. Guo S, Long M, Li X, Zhu S, Zhang M and Yang Z: Curcumin activates autophagy and attenuates oxidative damage in EA.hy926 cells via the Akt/mTOR pathway. Mol Med Rep 13: 2187-2193, 2016.
28. Wang Y, Gao A, Xu X, Dang B, You W, Li H, Yu Z and Chen G: The neuroprotection of lysosomotropic agents in experimental subarachnoid hemorrhage probably involving the apoptosis pathway triggering by cathepsins via chelating intralysosomal iron. Mol Neurobiol 52: 64-77, 2015.
29. Yan H, Ma Y, Li Y, Zheng X, Lv P, Zhang Y, Li J, Ma M, Zhang L, Li C, *et al*: Insulin inhibits inflammation and promotes atherosclerotic plaque stability via PI3K-Akt pathway activation. Immunol Lett 170: 7-14, 2016.
30. Arshad L, Haque MA, Abbas Bukhari SN and Jantan I: An overview of structure-activity relationship studies of curcumin analogs as antioxidant and anti-inflammatory agents. Future Med Chem 9: 605-626, 2017.
31. Parsamanesh N, Moossavi M, Bahrami A, Butler AF and Sahebkar A: Therapeutic potential of Curcumin in diabetic complications. Pharmacol Res 136: 181-193, 2018.
32. Yao Q, Ke ZQ, Guo S, Yang XS, Zhang FX, Liu XF, Chen X, Chen HG, Ke HY and Liu C: Curcumin protects against diabetic cardiomyopathy by promoting autophagy and alleviating apoptosis. J Mol Cell Cardiol 124: 26-34, 2018.
33. Zhang X, Li ZL, Crane JA, Jordan KL, Pawar AS, Textor SC, Lerman A and Lerman LO: Valsartan regulates myocardial autophagy and mitochondrial turnover in experimental hypertension. Hypertension 64: 87-93, 2014.
34. Wu S, Lu Q, Ding Y, Wu Y, Qiu Y, Wang P, Mao X, Huang K, Xie Z and Zou MH: Hyperglycemia-driven inhibition of AMP-activated protein kinase α2 induces diabetic cardiomyopathy by promoting mitochondria-associated endoplasmic reticulum membranes in vivo. Circulation 139: 1913-1936, 2019.
35. Yu W, Gao B, Li N, Wang J, Qiu C, Zhang G, Liu M, Zhang R, Li C, Ji G and Zhang Y: Sirt3 deficiency exacerbates diabetic cardiac dysfunction: Role of Foxo3A-Parkin-mediated mitophagy. Biochim Biophys Acta Mol Basis Dis 1863: 1973-1983, 2017.
36. Ihnat MA, Thorpe JE, Kamat CD, Szabó C, Green DE, Warnke LA, Lacza Z, Cselenyák A, Ross K, Shakir S, *et al*: Reactive oxygen species mediate a cellular 'memory' of high glucose stress signalling. Diabetologia 50: 1523-1531, 2007.
37. Quagliaro L, Piconi L, Assaloni R, Martinelli L, Motz E and Ceriello A: Intermittent high glucose enhances apoptosis related to oxidative stress in human umbilical vein endothelial cells: The role of protein kinase C and NAD(P)H-oxidase activation. Diabetes 52: 2795-2804, 2003.
38. Ortiz-Cordero C, Bincoletto C, Dhoke NR, Selvaraj S, Magli A, Zhou H, Kim DH, Bang AG and Perlingeiro RC: Defective autophagy and increased apoptosis contribute toward the pathogenesis of FKRP-associated muscular dystrophies. Stem Cell Reports 16: 2752-2767, 2021.
39. Xu J, Kitada M, Ogura Y, Liu H and Koya D: Dapagliflozin restores impaired autophagy and suppresses inflammation in high glucose-treated HK-2 cells. Cells 10: 1457, 2021.
40. Zhang Z, Zhang S, Wang Y, Yang M, Zhang N, Jin Z, Ding L, Jiang W, Yang J, Sun Z, *et al*: Autophagy inhibits high glucose induced cardiac microvascular endothelial cells apoptosis by mTOR signal pathway. Apoptosis 22: 1510-1523, 2017.
41. Rezabakhsh A, Ahmadi M, Khaksar M, Montaseri A, Malekinejad H, Rahbarghazi R and Garjani A: Rapamycin inhibits oxidative/nitrosative stress and enhances angiogenesis in high glucose-treated human umbilical vein endothelial cells: Role of autophagy. Biomed Pharmacother 93: 885-894, 2017.
42. Hou G, Zhao H, Teng H, Li P, Xu W, Zhang J, Lv L, Guo Z, Wei L, Yao H and Xu Y: N-Cadherin attenuates high glucose-induced nucleus pulposus cell senescence through regulation of the ROS/NF-κB pathway. Cell Physio Biochem 47: 257-265, 2018.

43. Cao G, Fan J, Yu H and Chen Z: Resveratrol attenuates high glucose-induced cardiomyocytes injury via interfering ROS-MAPK-NF-κB signaling pathway. Int J Clin Exp Pathol 11: 48-57, 2018.
44. Teng JF, Mei QB, Zhou XG, Tang Y, Xiong R, Qiu WQ, Pan R, Law BY, Wong VK, Yu CL, *et al*: Polyphyllin VI induces Caspase-1-mediated pyroptosis via the Induction of ROS/NF-κB/NLRP3/GSDMD signal axis in non-small cell lung cancer. Cancers (Basel) 12: 193, 2020.
45. Ma B, Wang X, Zhang R, Niu S, Rong Z, Ni L, Di X, Han Q and Liu C: Cigarette smoke extract stimulates PCSK9 production in HepG2 cells via ROS/NF-κB signaling. Mol Med Rep 23: 331, 2021.
46. Morgan MJ and Liu Z: Crosstalk of reactive oxygen species and NF-κB signaling. Cell Res 21: 103-115, 2010.
47. Xu X, Huang X, Zhang L, Huang X, Qin Z and Hua F: Adiponectin protects obesity-related glomerulopathy by inhibiting ROS/NF-κB/NLRP3 inflammation pathway. BMC Nephrol 22: 218, 2021.