# Matrine-Type Alkaloids Inhibit Advanced Glycation End Products Induced Reactive Oxygen Species-Mediated Apoptosis of Aortic Endothelial Cells In Vivo and In Vitro by Targeting MKK3 and p38MAPK Signaling

Zhongwei Liu, MD, PhD;* Ying Lv, MD;* Yong Zhang, MD; Fuqiang Liu, MD, PhD; Ling Zhu, MD, PhD; Shuo Pan, MD, PhD; Chuan Qiu, PhD; Yan Guo, PhD; Tielin Yang, PhD; Junkui Wang, MD, PhD

***Background***—The matrine-type alkaloids are bioactive components extracted from *Sophora flavescens*, which is used in treatment of diabetes mellitus in traditional Chinese medicine. Advanced glycation end products mediate diabetic vascular complications. This study was aimed to investigate the protective effects and molecular mechanisms of matrine-type alkaloids on advanced glycation end products–induced reactive oxygen species–mediated endothelial apoptosis.

***Methods and Results***—Rats aorta and cultured rat aortic endothelial cells were exposed to advanced glycation end products. Matrine-type alkaloids, p38 mitogen-activated protein kinase (MAPK) inhibitor, and small interference RNAs against p38 MAPK kinases MAPK kinase kinase (MKK)3 and MKK6 were administrated. Intracellular reactive oxygen species production, cell apoptosis, phosphorylation of MKKs/p38 MAPK, and expression levels of heme oxygenase/NADPH quinone oxidoreductase were assessed. The nuclear factor erythroid 2-related factor 2 nuclear translocation and the binding activity of nuclear factor erythroid 2-related factor 2 with antioxidant response element were also evaluated. Matrine-type alkaloids suppressed intracellular reactive oxygen species production and inhibited endothelial cell apoptosis in vivo *and* in vitro by recovering phosphorylation of MKK3/6 and p38 MAPK, nuclear factor erythroid 2-related factor 2 nuclear translocation, and antioxidant response element binding activity, as well as the expression levels of heme oxygenase/NADPH quinone oxidoreductase. p38 MAPK inhibitor treatment impaired the effects of matrine-type alkaloids in vivo and in vitro. MKK3/6 silencing impaired the effects of matrine-type alkaloids in vitro.

***Conclusions***—Matrine-type alkaloids exert endothelial protective effects against advanced glycation end products induced reactive oxygen species–mediated apoptosis by targeting MKK3/6 and enhancing their phosphorylation. (***J Am Heart Assoc***. 2017;6:e007441. DOI: 10.1161/JAHA.117.007441.)

**Key Words:** apoptosis • diabetes mellitus • endothelial cell • matrine-type alkaloids • reactive oxygen species

Atherosclerosis is believed to be one of the fundamental pathological manifestations of diabetic vascular complications.[1] Evidence from previous clinical trials indicated the role of hyperglycemia in the pathogenesis of diabetic vascular complications.[2] Under circumstances of sustained hyperglycemia, glucose participates in nonenzymatic glycation reactions with lipids, nucleic acids, and amino groups of proteins, generating Schiff bases and Amadori products. As a result, high-molecular-weight protein and fluorescent entities are produced. These products are referred to as advanced glycation end products (AGEs).[3] Studies pointed out that AGEs were closely correlated with the occurrence, progression, and development of diabetic vascular complications.[4]

Investigations have suggested that AGEs were responsible for induction and maintenance of oxidative stress.[5] After interacting with their receptors, AGEs trigger activation of multiple signaling pathways to induce generation of reactive oxygen species (ROS), including hydroxy radical (OH˙), superoxide anion ($O_2^-$), and peroxyl radical (RO˙). ROS participates in many intracellular pathological changes.[6,7] In accord with our and other previous studies, ROS triggers programmed cell death through the mitochondrial pathway, death receptor pathway, and endoplasmic reticulum stress-

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## Clinical Perspective

### What Is New?

- The intracellular antioxidant system in the arterial endothelium is impaired by advanced glycation end products exposure.
- The deactivation of mitogen-activated protein kinase kinases/p38 mitogen-activated protein kinase/erythroid 2-related factor 2 signaling pathway is involved in imbalance of antioxidant/oxidant leverage.
- Matrine-type alkaloids exert endothelial protective effects against advanced glycation end products–induced oxidative stress-mediated apoptosis by targeting mitogen-activated protein kinase kinase 3/6 and enhancing their phosphorylation.

### What Are the Clinical Implications

- Matrine-type alkaloids and related compounds have the potential clinical application value in treating diabetic vascular complications.
- Restoring the phosphorylation of mitogen-activated protein kinase kinases could be a novel strategy for enhancing intracellular antioxidant capacity, which protects against oxidative stress–associated cell injury.

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mediated pathway.[8–10] Previous studies indicated that AGEs induced endothelial cell apoptosis by exacerbating ROS–mediated apoptosis.[11]

Activation of antioxidant response element (ARE) is believed to be a modulator of the intracellular antioxidant defense system, which protects against ROS-mediated cell death. The nuclear factor erythroid 2–related factor 2 (Nrf2) signaling pathway triggers the activation of ARE. By initiating transcriptions of a set of antioxidant enzymes genes, Nrf2 exerts its crucial role in enhancing cellular antioxidant defense system. Kelch-like ECH-associated protein 1 (Keap1) binds to Nrf2 to maintain static status of Nrf2.[12] When encountering harmful stimuli, Nrf2 dissociates from Keap1 and further translocates to the nucleus to trigger the transcriptions of antioxidant enzymes genes by binding with ARE, which locates in the promoter region of these genes.[13] The activation of Nrf2/ARE signaling is regulated by the upstream kinases. Theses kinases are referred to as mitogen-activated protein kinases (MAPKs).[14] As a member of the MAPKs family, p38 MAPK is a key signaling transducer in response to harmful stimuli, including ROS.[15] The activation of p38MAPK is regulated by its upstream kinases, MAPK kinase kinase (MKK)3 and MKK6.[16]

Matrine, oxymatrine, and sophocarpine are typical matrine–type alkaloids, which are believed to be the main bioactive components extracted from Chinese medical herb *Sophora flavescens*,[17] which was used in treatment of diabetes mellitus from ancient times. These alkaloids have

been found to show a variety of pharmacological activities, including anti-inflammatory, -viral, -fibrotic, and –cancer properties. According to our and others' previous studies, the antioxidative and cardioprotective activities of matrine were featured.[18–20] Several previous studies indicated that matrine regulated some protein kinases, such as AMP-activated protein kinase and Janus-activated kinase.[21,22] The pharmacological activities of matrine–type alkaloids may be similar because they share several of the same key molecular structures.[23] Thus, it is reasonable for us to speculate that the matrine–type alkaloids may suppress AGEs-induced oxidative stress by affecting MKKs, which further regulates p38 MAPK/Nrf2 signaling. In the present study, both in vivo and in vitro investigations were implemented. The involvement of MKKs/p38 MAPK/Nrf2 signaling in matrine–type alkaloids' effects of attenuating AGEs induced oxidative stress–mediated endothelial cell apoptosis was investigated.

## Materials and Methods

The data, analytical methods, and study materials will not be made available to other researchers for purposes of reproducing the results or replicating the procedure.

## Preparation of AGEs-BSA

The preparation procedure was carried out in accord with previous studies.[24] Briefly, under sterile conditions, BSA (Hyclone Laboratories Inc, South Logan, UT) was incubated with glyceraldehyde (0.1 mmol/L; Sigma-Aldrich, St. Louis, MO) in $NaPO_4$ buffer (0.2 mmol/L; pH=7.4) at 37°C for 7 days. Then, by using chromatography with PD-10 desalting columns (GE Healthcare, Little Chalfont, UK) and dialysis against PBS, the unicorporated sugars were removed. Meanwhile, control nonglycated BSA was prepared by the same procedure without glyceraldehyde.

## Animals, Grouping, and Treatment

Seven-week-old male Sprague–Dawley rats (weighting 180–220 g) were provided by the Experimental Center of Xi'an Jiaotong University (Xi'an, China). The experimental procedures concerning animal studies were carried out according to the Recommended Guideline for the Care and Use of Laboratory Animals issued by Chinese Council on Animal Research. The experimental protocol in this study was approved by the Ethics Committee of Xi'an Jiaotong University.

For AGEs exposure, rats received an intraperitoneal injection of 1 mg of prepared AGEs-BSA daily for 10 consecutive days. Meanwhile, rats were treated with

intraperitoneal injections of matrine-type alkaloids (matrine, oxymatrine, and sophocarpine), respectively. Dosage for matrine (Sigma-Aldrich) was 200 mg/kg/day,[18] for oxymatrine (Sigma-Aldrich) 120 mg/kg/day,[25] and for sophocarpine (Invitrogen, Carlsbad, CA) 40 mg/kg/day for 10 consecutive days.[26] Several rats also received treatment of specific inhibitor of p38 MAPK SB203580 (1 mg/kg/day; Selleck Chemicals, Houston, TX) simultaneously.[27] For control animals, they received treatment of physiological saline with equal volume to agents. According to different treatment, an equal amount of rats (8 rats for each group) were assigned to control (control animals), AGEs (animals exposed to AGEs), AGEs+Mat (AGEs-exposed animals treated with matrine), AGEs+Oxy (AGEs-exposed animals treated with oxymatrine), AGEs+Soh (AGEs-exposed animals treated with sophocarpine), AGEs+Mat+SB (AGEs-exposed animals cotreated with matrine and SB203580), AGEs+Oxy+SB (AGEs-exposed animals cotreated with oxymatrine and SB203580), and AGEs+Soh+SB (AGEs-exposed animals cotreated with sophocarpine and SB203580). Animals were euthanitized by carbon dioxide asphyxia before samples were harvested.

## Cell Culture, Grouping, and Treatment

Rat aortic endothelial cells (RAECs) were isolated and cultured in accord with the method described previously.[28] RAECs were extracted from aorta segments. Isolated RAECs were cultured in endothelial-basal medium (EBM-2; Lonza, Basel, Switzerland) supplemented with FBS (5%; Hyclone) and an antibiotics mix (Invitrogen) in an incubator providing a humidified environment, a constant temperature at 37°C, and an atmosphere with 5%$CO_2$/95% fresh air. AGEs, SB203580 (final concentration, 10 μmol/L), and matrine-type alkaloids were used to incubate the cells, respectively. Several cells were also transfected with small interfering RNA (siRNAs). Specifically, the concentrations of matrine were 0.25, 0.5, 1.0, 2.0, and 2.5 mmol/L[18]; the concentrations of oxymatrine were 0.0625, 0.125, 0.25, 0.5, 1.0, and 2.0 mmol/L[29]; the concentrations of sophocarpine were 0.0625, 0.125, 0.25, 0.5, 1.0, and 2.0 mmol/L.[30]

## siRNA Transfection

In this study, MKK3 and MKK6 were silenced in RAECs by using an siRNA transfection technique. SignalSilence MKK3 siRNA (Cat.6294S; Cell Signaling Technology, Danvers, MA) was used to knock down mkk3. The specific sequence of siRNA against mkk6 was: 3′-CUACAGUAGUGAAGAGAUUTT-3′, which was designed and synthesized by GenePharma Co, Ltd (Shanghai, China). SignalSilence Control siRNA (Cat.6568S; Cell Signaling Technology) was used as control. Cultured RAECs (at 70% confluence) were transfected with the above

siRNAs with Mirus TransIT-TKO reagent (Mirus Bio LLC, Madison, WI) for 48 hours per the manufacturer's instructions.

## Cell Viability Assessment

The noncytotoxic concentrations of matrine-type alkaloids were evaluated by MTT assay. Cultured RAECs were seeded in a 96-well cell-culture plate and then cultured with matrine, oxymatrine, and sophocarpine at serial diluted concentrations for 48 hours. MTT (5 mg/mL; Sigma-Aldrich) was added into the wells to incubate RAECs for 4 hours. Then, DMSO was added to dissolve the formazan crystals. A plate reader was used to determine the absorbance at 490 nm (A490). Cell viability was calculated as (A490 of alkaloids-treated cell/A490 of DMSO-treated control cells)×100%.

## ARE-Luciferase Reporter Construct and Transfection

The ARE-luciferase reporter was constructed according to the description of previous studies[13] and accomplished by GenePharma. The pTl-ARE-luciferase contains a single copy of ARE of 41-base-pair length (5′-TAGCTTGGAAATGACATTGC-TAATGGTGACAAAGCAACTTT-3′) and a minimal TATA-lnr promoter. Plasmid was transfected into RAECs with HiPerFect Transfection Reagent (Qiagen, Hilden, Germany) per the manufacturer's instructions. After treatment of transfection reagent, cells were cultured in replaced fresh medium before further experiments.

## ARE-Luciferase Activity Assay

The ARE-luciferase activity assay was performed in accord with descriptions in previous studies.[13] Briefly, after being washed by PBS, RAECs were lysed by reporter lysis buffer (Promega, Fitchburg, WI). The resulting supernatant was used for ludiferase activity assay with a GloMax luminometer (Promega) after centrifugation at 3000g for 10 minutes. Luciferase activity was expressed as folds over control cells.

## In Situ ROS Detection

In situ ROS generation detection was performed by using DHE (Beyotime Biotech, Nantong, China) staining. Fresh thoracic aorta was harvested and immersed in optimal cutting temperature compound (Tissue-Tek) and frozen on dry ice. Tissue was cut into 10-μm-thick sections and placed on polylysine-coated slides. Slides were then incubated with DHE (10 μmol/L) in a humidified dark chamber at 37°C for 45 minutes according to the recommendations provided by the manufacturer. For cultured RAECs, intracellular ROS

production was detected by using DCFH-DA (Molecular Probes, Eugene, OR) staining. After PBS washing, RAECs were incubated with DCFH-DA at 37°C for 30 minutes in a humidified dark chamber according to the instruction provided by the manufacturer. Fluorescent images were captured by an inverted fluorescence microscope. Fluorescence intensities were analyzed by the software Image-Pro Plus (ver.5.0).

## Total Antioxidant Capacity Assessments

Aorta samples and cultured RAECs were collected and the homogenates were prepared. After centrifugation at 4°C at 12 000*g* for 5 minutes, the supernatant was used for total antioxidant capacity (TAC) detection. The TAC was measured by using a T-AOC Assay Kit (Beyotime) according to the manual provided by the manufacturer. A plate reader was used to determine the absorbance of $ABTS^+$ at 734 nm.

## Apoptosis Determination

In situ apoptosis was determined by terminal transferase UTP nick end labeling assay. Paraffin-embedded thoracic aorta tissue was cut into 5-μm-thick sections. After deparaffinization, sections were treated with proteinase K (20 μmol/L; Sigma-Aldrich). Then, terminal transferase UTP nick end labeling assay was carried out using a terminal transferase UTP nick end labeling assay kit (Roche, Indianapolis, IN) per the manufacturer's instructions. Fluorescent images were captured by an inverted fluorescence microscope. Fluorescence intensities were analyzed by the software Image-Pro Plus (ver.5.0). For cultured RAECs, apoptosis was evaluated by Annexin V/FITC and propidium iodide double staining with flow cytometry. Briefly, cells were resuspended in PBS. A 195-μL cell suspension (5000/mL) was mixed with 5 μL of Annexin V/FITC (BD Biosciences, San Jose, CA) at room temperature for 10 minutes. Then, cells were washed by PBS and resuspended in 190 μL of deliquated binding buffer. Five microliters of propidium iodide (BD Biosciences, San Jose, CA) was used to incubate RAECs in binding buffer for 15 minutes in a dark chamber. Then, a flow cytometer (FACS Calibur; BD Biosciences, San Jose, CA) was used to analyze apoptosis using CellQuest software.

## Nrf2 Nuclear Translocation Evaluation

Nuclear translocation of Nrf2 was assessed by western blotting in vivo and by both western blotting/immunofluorescent staining in vitro. By using a cell lysis buffer system (Santa Cruz Biotechnology), the whole-cell extract from cultured RAECs and harvested thoracic aorta tissue was prepared. Cytoplasmic protein and nuclear protein were extracted with Cytoplasmic Extraction Reagents (Pierce Biotechnology Inc,

Rockford, IL) and Nuclear Extraction Reagents (Pierce), respectively. Protein concentrations were determined by a BCA protein assay kit (Pierce). The protocol of western blotting is described in the below paragraph. Cultured RAECs were fixed and incubated with primary antibody against Nrf2 (1:250; Cell Signaling Technology) at 4°C for 8 hours. Then, the secondary antibody conjugated with Alexa 488 (Invitrogen) and DAPI was used to incubate the cells. Quenching of fluorescence was alleviated with SlowFade Lignt Antifade kit (Molecular Probes). Samples were exited at 519 nm and observed at 442 and 495 nm with an inverse fluorescence microscope (Axio Imager 2; Carl Zeiss, Jena, Germany). Fluorescent intensity was determined by using Zeiss Physiology software (Ver.3.2; Carl Zeiss).

## Western Blotting

A 20-μg protein sample was loaded and separated by vertical SDS-PAGE. Then, proteins were transferred to PVDF membranes electrically. Specific antibodies against Nrf2 (1:500Cell Signaling Technology), MKK3 (1:500; Sigma-Aldrich), phospho-MKK3 (1:500; Sigma-Aldrich), MKK6 (1:500; Sigma-Aldrich), phospho-MKK6 (1:500; Sigma-Aldrich), p38 (1:250; Cell Signaling Technology), phospho-p38 (1:250; Cell Signaling Technology), heme oxygenase (HO1; 1:1000; Cell Signaling Technology), NADPH quinone oxidoreductase (NQO1; 1:500; Cell Signaling Technology), histone H3 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), and GAPDH (1:1000; Invitrogen) were used to incubate membranes at 4°C for 10 hours. Then, membranes were washed by Tris-buffered saline-Tween 20 (0.02%). Corresponding second antibodies conjugated to HRP (Santa Cruz Biotechnology) were used to incubate membranes at room temperature for 2 hours. Membranes were developed with Super Signal West Pico chemiluminescence reagent and visualized. Intensities of immunoblots were quantified and analyzed by the software Image-Pro Plus (ver.5.0).

## Statistical Analysis

Data acquired in this study are presented in a mean±SD manner. One-way ANOVA and the Student *t* test were used for analyze the differences between values. The Bonferroni adjustments were performed as post hoc tests. The data analysis was performed by using SPSS software (ver. 16.0; SPSS, Inc, Chicago, IL). $P<0.05$ was considered statistically significant.

## Results

Matrine, oxymatrine, and sophocarpine administration alleviated AGEs-induced ROS generation in rat aorta.

The ROS produced in rat aorta was tagged by DHE fluorescent staining. The captured images are demonstrated in Figure 1A. ROS production was significantly upregulated in the AGEs group compared with the control group. However, after being treated with matrine (200 mg/kg/day), oxymatrine (120 mg/kg/ day), and sophocarpine (40 mg/kg/day), ROS production in rat aorta was dramatically reduced. Moreover, the p38 MAPK inhibitor administration significantly impaired matrine, oxymatrine, and sophocarpine's inhibition of ROS production in AGEs-exposed rat aorta. As shown in Figure 1B, the aortic TAC decreased significantly in rats exposed to AGEs, which was dramatically attenuated by matrine, oxymatrine, and sophocarpine administrations. However, the attenuation was significantly impaired by coadministration of p38 MAPK inhibitor SB203580.

Matrine, oxymatrine, and sophocarpine administration alleviated AGEs-induced cell apoptosis in rat aorta, which was reversed by SB203580 treatment.

In vivo cell apoptosis was detected by terminal transferase UTP nick end labeling assay. The captured images are shown in Figure 1C. Compared with control, apoptosis in aorta increased significantly in AGEs-exposed rats. However, the matrine (200 mg/kg/day), oxymatrine (120 mg/kg/day), and sophocarpine (40 mg/kg/day) administration significantly inhibited aortic cell apoptosis. The inhibitory effect of matrine-type alkaloids on apoptosis was dramatically impaired by coadministration with p38 MAPK inhibitor SB203580.

Matrine, oxymatrine, and sophocarpine administration recovered phosphorylation of MKK3/6, which facilitated p38 MAPK/Nrf2 antioxidative signaling transduction in AGEs-treated rat aorta, which was reversed by SB203580 treatment.

Immunoblots and their quantifications are demonstrated in Figure 2. The phosphorylation of MKK3, MKK6, and p38 MAPK was found to be significantly inhibited in AGEs-exposed aortas compared with control. The administration of matrine (200 mg/kg/day), oxymatrine (120 mg/kg/day), and sophocarpine (40 mg/kg/day) dramatically recovered the phosphorylation of MKK3, MKK6, and p38. The p38 MAPK inhibitor, SB203580, did not affect the phosphorylation levels of MKK3 and MKK6, but suppressed the phosphorylation of p38 MAPK. The nuclear expression of Nrf2 decreased significantly in AGEs-exposed aorta, indicating that the Nrf2 nuclear translocation was inhibited by AGEs. After matrine-type alkaloids treatment, however, the nuclear expression of Nrf2 was dramatically increased, indicating that the nuclear translocation of Nrf2 was recovered by matrine-type alkaloids administration. The expression levels of antioxidant proteins, namely HO1 and NQO1, were increased by administration of matrine-type alkaloids in aorta exposed to AGEs. The coadministration of SB203508 dramatically impaired the increasing expression of HO1 and NQO1 in AGEs-exposed aorta treated with matrine-type alkaloids.

## Selection of the Noncytotoxic Concentrations of Matrine, Oxymatrine, and Sophocarpine

The noncytotoxic concentrations of matrine, oxymatrine, and sophocarpin were selected by MTT assay. Matrine began to inhibit RAECs at a concentration of 2.0 mmol/L; both oxymatrine and sophocarpine began to show cytotoxic effects at a concentration of 0.25 mmol/L. Thus, matrine at a concentration of 1.5 mmol/L and oxymatrine and sophocarpin at concentrations of 0.125 mmol/L were selected for subsequent in vitro experiments. These results are demonstrated in Figure 3A.

Matrine, oxymatrine, and sophocarpine incubation suppressed AGEs-induced ROS production while strengthening TAC in cultured RAECs, which were impaired by SB203580 treatment and *mkk3/6* silencing.

ROS generation was determined by DCFH-DA staining in cultured RAECs. The captured fluorescent images are demonstrated in Figure 3B. AGEs exposure boosted the generation of ROS in cultured RAECs. The treatment of matrine (1.5 mmol/L), oxymatrine (0.125 mmol/L), and sophocarpine (0.125 mmol/L) significantly suppressed the production of ROS in AGEs-exposed RAECs. The administration of p38 MAPK inhibitor impaired the antioxidant effects of the matrine-type alkaloids on AGEs-exposed RAECs. MKK3 and MKK6 were silenced by specific siRNAs in RAECs, respectively. The results showed that both *mkk3* and *mkk6* silencing impaired the antioxidant effects of the matrine-type alkaloids on AGEs-exposed RAECs. Correspondingly, as shown in Figure 3C, the TAC of AGEs-exposed RAECs was reduced significantly, which was restored by matrine, oxymatrine, and sophocarpine incubations. However, coadministration with p38 MAPK inhibitor or *mkk3/6* silencing decreased TAC in AGEs-exposed RAECs incubated with matrine, oxymatrine, and sophocarpine.

Matrine, oxymatrine, and sophocarpine incubation suppressed AGEs-induced cell apoptosis of cultured RAECs, which were impaired by SB203580 treatment and *mkk3/6* silencing.

The apoptosis of RAECs was detected by flow cytometry in this study. The charts of flow cytometry are demonstrated in Figure 4. Compared with control, AGEs exposure significantly increased apoptosis of cultured RAECs. The treatment of matrine (1.5 mmol/L), oxymatrine (0.125 mmol/L), and sophocarpine (0.125 mmol/L) dramatically decreased apoptotic percentage of AGEs-exposed RAECs. Both coadministration of p38 MAPK inhibitor SB203508 and *mkk3/6* silencing impaired the matrine-type alkaloids' antiapoptotic effects on AGEs-exposed RAECs.
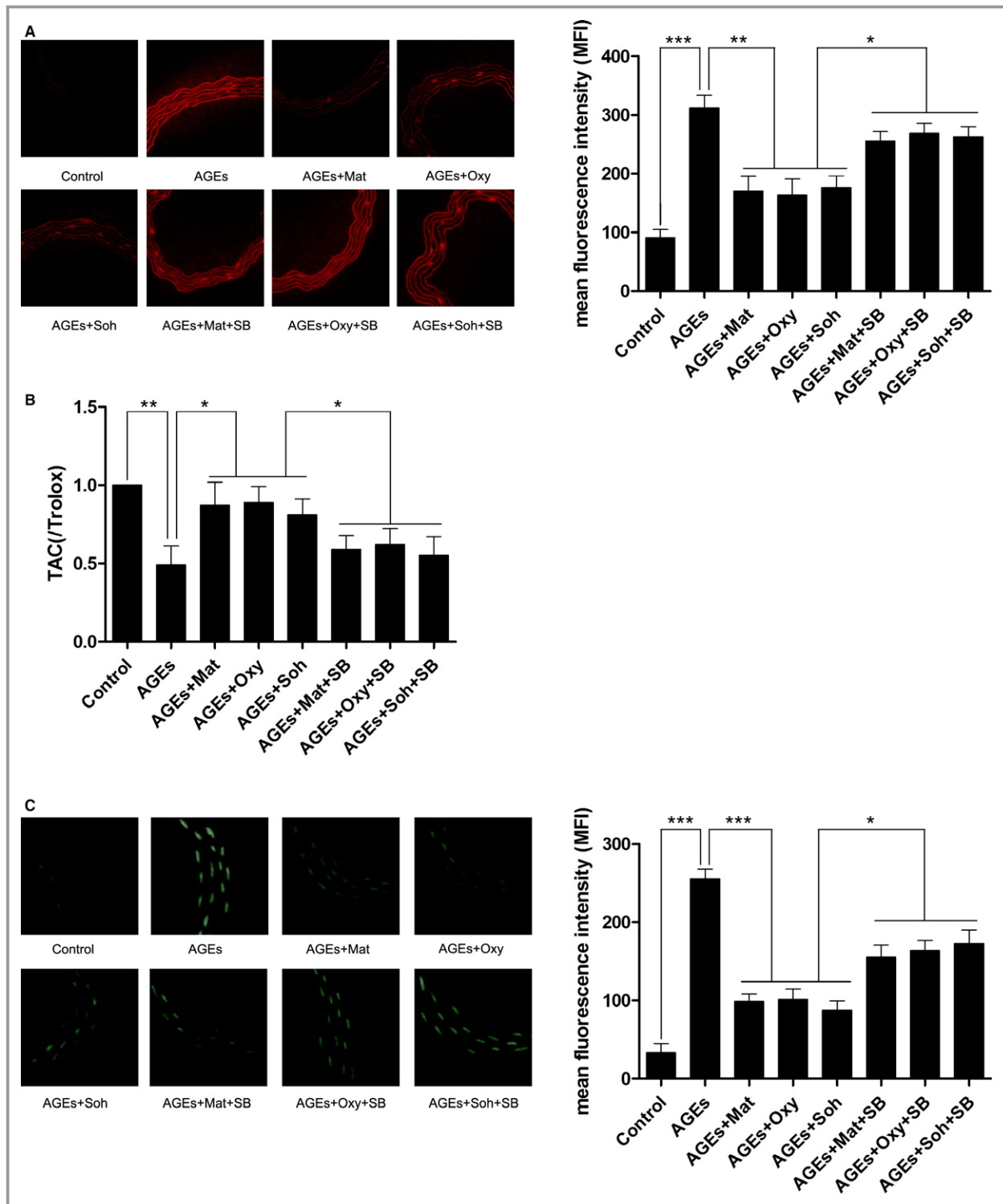
**Figure 1.** A, The left panel demonstrates the captured fluorescent images of aorta stained with DHE. ROS (reactive oxygen species) was positively stained. Columns on the right panel indicated the mean fluorescence intensity (MFI) of DHE stain in Control, AGEs (advanced glycation end products), AGEs+Mat, AGEs+Oxy, AGEs+Soh, AGEs+Mat+SB, AGEs+Oxy+SB, and AGEs+Soh+SB, respectively. B, Columns indicate the detected total antioxidant capacity (TAC) of aorta harvested from experimental animals. C, The left panel demonstrates the captured fluorescent images of TUNEL (terminal transferase UTP nick end labeling) assay of aorta. Apoptotic cells were positively stained. Columns on the right panel indicate the MFI of TUNEL staining in Control, AGEs, AGEs+Mat, AGEs+Oxy, AGEs+Soh, AGEs+Mat+SB, AGEs+Oxy+SB, and AGEs+Soh+SB, respectively (3 independent experiments were performed; *$P<0.05$; **$P<0.01$; ***$P<0.001$).
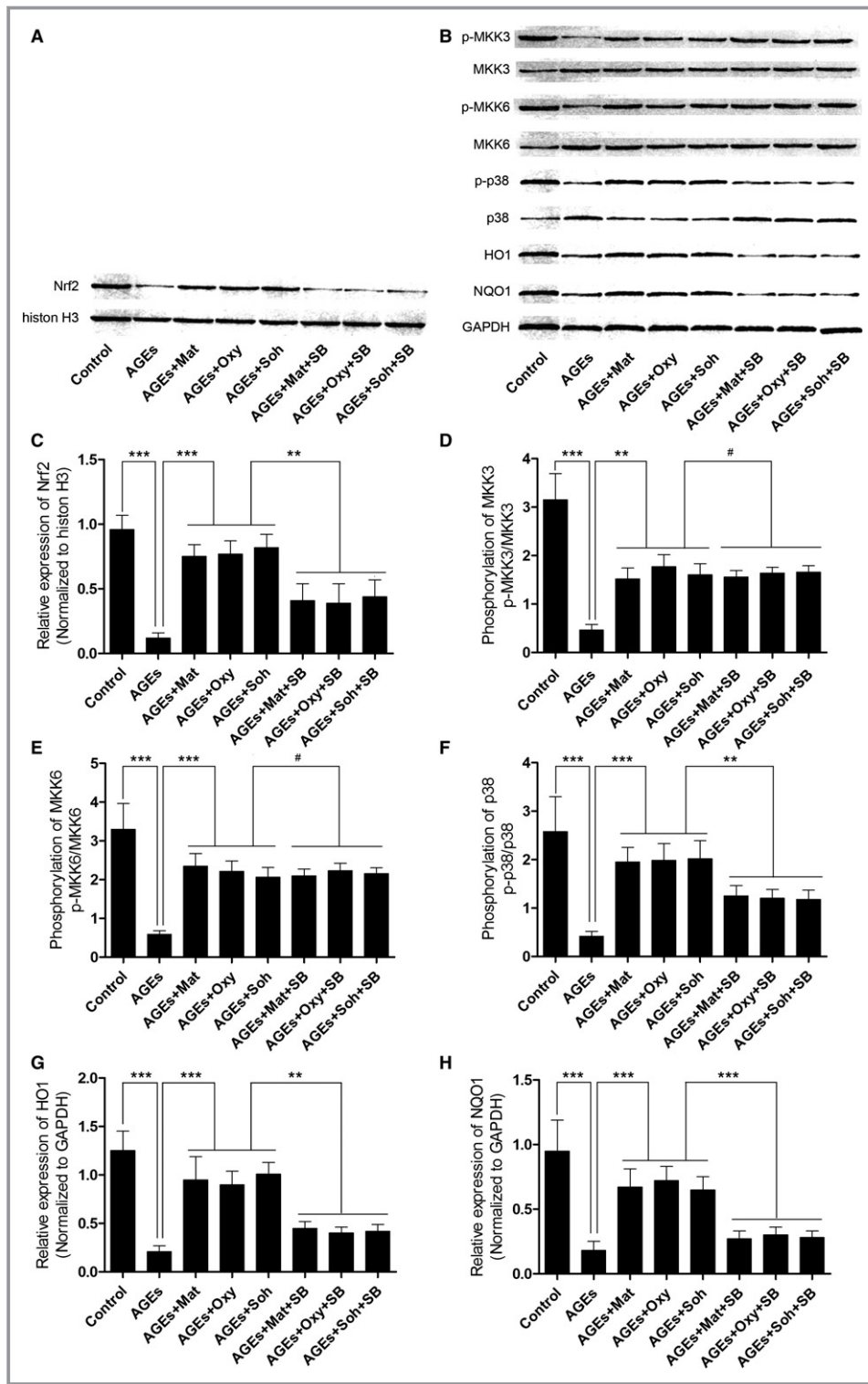
**Figure 2.** A, The immunoblots of Nrf2 in nuclear protein when histone H3 was used as the internal reference. B, The immunoblots of phosphorylated MKK3 (p-MKK3), MKK3, p-MKK6, MKK6, p-p38, p38, HO1 (heme oxygenase 1), and NQO1 (NADPH quinone oxidoreductase 1) in total protein when GAPDH was introduced as the internal reference. C through H, Columns in these panels indicate the relative expression level of Nrf2 (Nrf2/histon H3), phosphorylation level of MKK3 (p-MKK3/MKK3), phosporylation level of MKK6 (p-MKK6/MKK6), phosphorylation level of p38 MAPK, the relative expression levels of HO1 (HO1/GAPDH), and the relative expression level of NQO1 (NQO1/GAPDH), respectively (3 independent experiments were performed; *$P<0.05$; **$P<0.01$; ***$P<0.001$; #$P>0.05$).
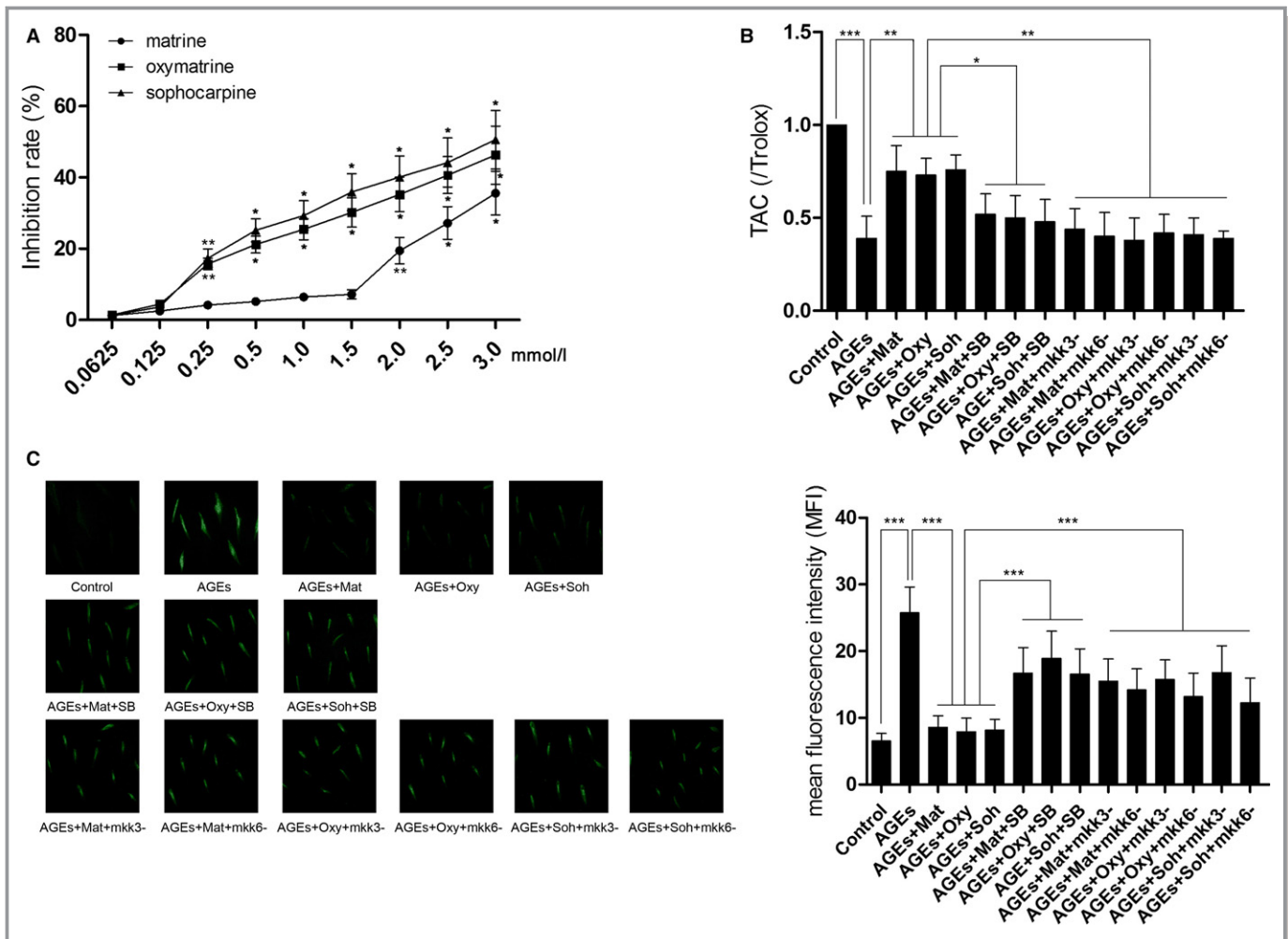
**Figure 3.** A, Results of MTT assay are indicated by the line chart in this figure. Cultured RAECs (rat aortic endothelial cells) were incubated with matrine, oxymatrine, and sophocarpine at serial diluted concentrations. B, Columns indicate the TAC (total antioxidant capacity) levels in cultured RAECs in Control, AGEs, AGEs+Mat, AGEs+Oxy, AGEs+Soh, AGEs+Mat+SB, AGEs+Oxy+SB, AGEs+Soh+SB, AGEs+Mat+mkk3−, AGEs+Mat+mkk6−, AGEs+Oxy+mkk3−, AGEs+Oxy+mkk6−, AGEs+Soh+mkk3−, and AGEs+Soh+mkk6−, respectively. C, Left panel demonstrates the captured fluorescent images of cultured RAECs stained with DCFH-DA. ROS (reactive oxygen species) was positively stained. Columns on the right panel indicate the MFI of DCFH-DA stain in Control, AGEs, AGEs+Mat, AGEs+Oxy, AGEs+Soh, AGEs+Mat+SB, AGEs+Oxy+SB, AGEs+Soh+SB, AGEs+Mat+mkk3−, AGEs+Mat+mkk6−, AGEs+Oxy+mkk3−, AGEs+Oxy+mkk6−, AGEs+Soh+mkk3−, and AGEs+Soh+mkk6−, respectively (3 independent experiments were performed; *$P<0.05$; **$P<0.01$; ***$P<0.001$).

Matrine, oxymatrine, and sophocarpine incubation facilitated Nrf2 nuclear translocation and ARE-binding, which were impaired by SB203580 treatment and *mkk3/6* silencing.

Results of Nrf2 nuclear translocation and ARE binding activity are demonstrated in Figure 5. After AGEs exposure, both the Nrf2 nuclear translocation and ARE binding activity were inhibited. Matrine (1.5 mmol/L), oxymatrine (0.125 mmol/L), and sophocarpine (0.125 mmol/L) incubation dramatically restored Nrf2 nuclear translocation and ARE binding activity. However, both coadministration of p38 MAPK inhibitor SB203508 and *mkk3/6* silencing impaired the above restoring effects in AGEs-exposed RAECs.

Matrine, oxymatrine, and sophocarpine incubation recovered activation of MKK3/6-p38MAPK/Nrf2 antioxidative signaling in AGEs-treated RAECs, which were impaired by SB203580 treatment and *mkk3/6* silencing.

As shown in Figure 6, the phosphorylation of MKK3, MKK6, and p38 MAPK was found to be significantly inhibited in AGEs-treated RAECs. Moreover, the expression of Nrf2 in nuclei and expression levels of HO1 and NQO1 were suppressed in AGEs-treated RAECs. After incubation with matrine (1.5 mmol/L), oxymatrine (0.125 mmol/L), and sophocarpine (0.125 mmol/L), the phosphorylation of MKK3, MKK6, and p38 MAPK were restored. The expression levels of HO1 and NQO1 and nuclear expression of Nrf2
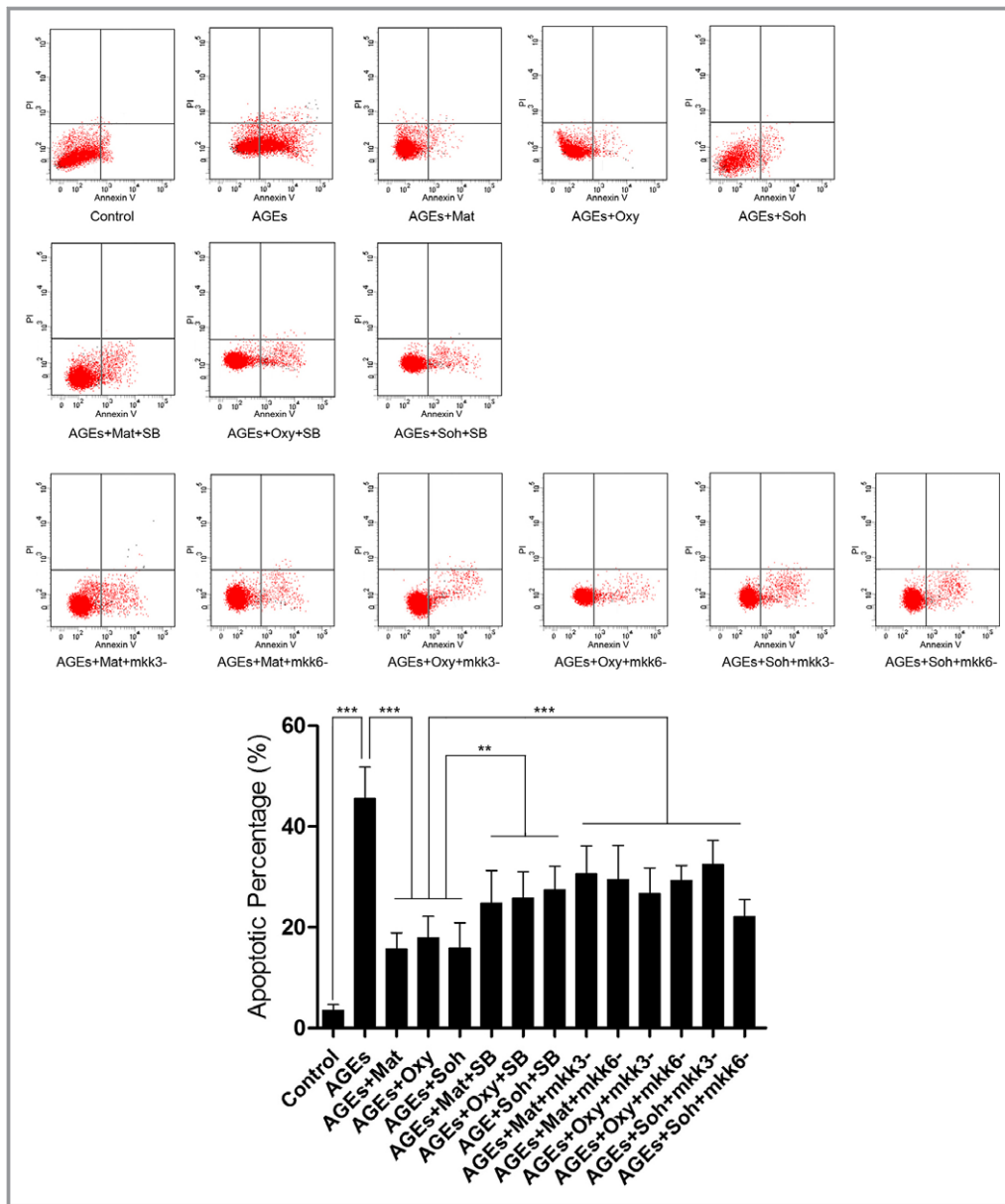
**Figure 4.** Charts on the upper panel of this figure demonstrate PI/Annexin V double staining detected by a flow cytometer. Columns on the lower panel indicate the apoptotic percentage in Control, AGEs, AGEs+Mat, AGEs+Oxy, AGEs+Soh, AGEs+Mat+SB, AGEs+Oxy+SB, AGEs+Soh+SB, AGEs+Mat+mkk3−, AGEs+Mat+mkk6−, AGEs+Oxy+mkk3−, AGEs+Oxy+mkk6−, AGEs+Soh+mkk3−, and AGEs+Soh+mkk6−, respectively (3 independent experiments were performed; $**P<0.01$; $***P<0.001$).

were also recovered. However, the administration of p38 MAPK inhibitor inhibited the restoring effects of matrine-type alkaloids on p38 MAPK phosphorylation without affecting MKK3/6 phosphorylation. As a result, coadministration with SB203508 decreased nuclear expression of Nrf2 and HO1/NQO1 expressions compared with matrine-type alkaloids-incubated AGEs-exposed RAECs. Treatment of *mkk3/6* silencing significantly reduced phosphorylation of MKK3/6, respectively. The *mkk3/6* silencing also decreased nuclear expression of Nrf2 and HO1/NQO1 expressions

compared with matrine-type alkaloids-incubated AGEs-exposed RAECs.

## Discussion

AGEs are fostered and further raise oxidative stress to cause cell damages in type 2 diabetes mellitus.[31] There is a strong association between apoptosis of endothelial cells and diabetic cardiovascular complications.[32] AGEs-induced oxidative stress plays important roles in the pathogenesis of diabetic
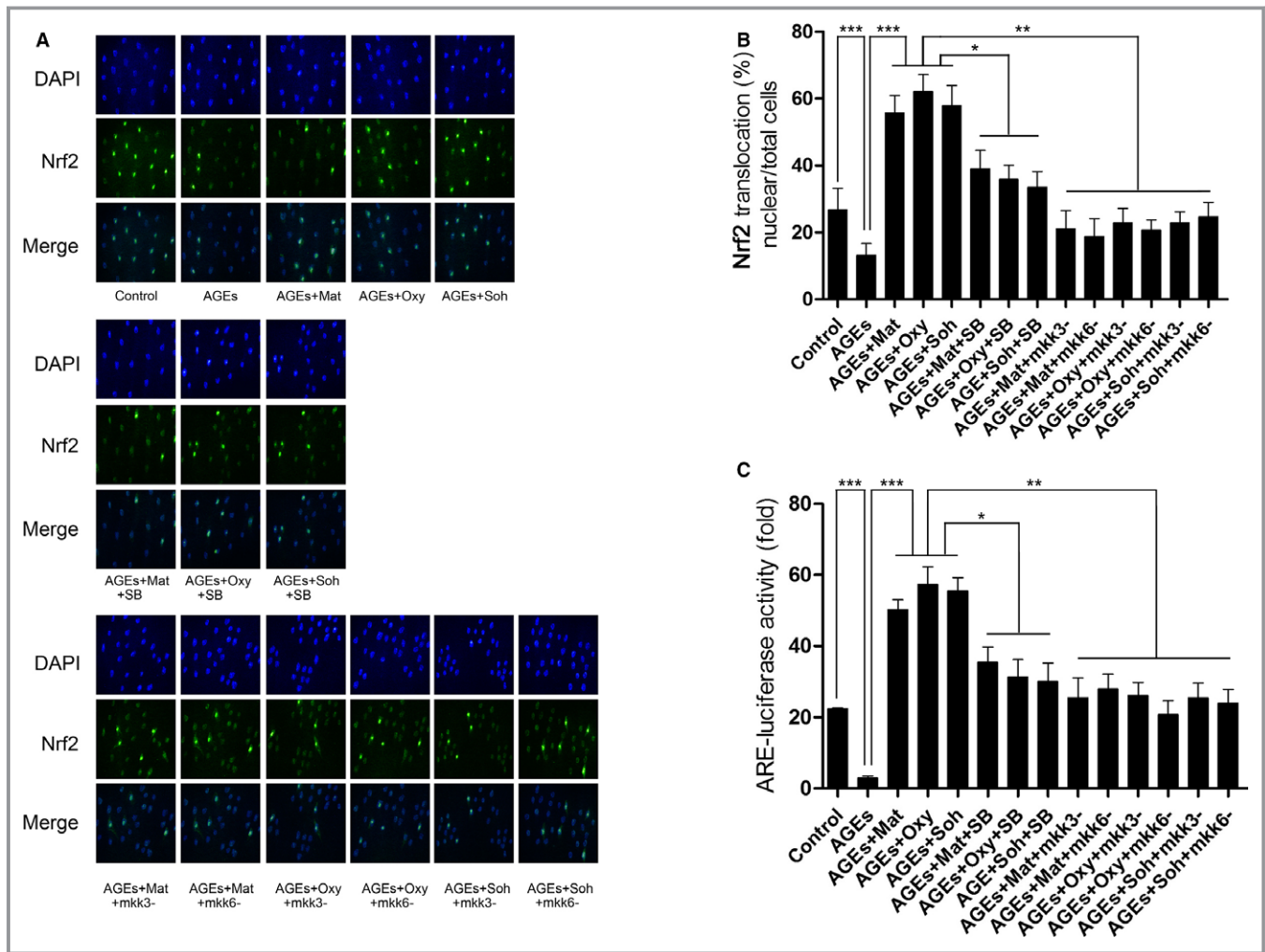
**Figure 5.** A, This panel demonstrates the captured images of Nrf2 and DAPI fluorescent immunochemistry staining and their merged images in cultured RAECs (rat aortic endothelial cells). B, Columns indicate the ratio of Nrf2 nuclear translocated cell count against total cell count in RAECs in Control, AGEs, AGEs+Mat, AGEs+Oxy, AGEs+Soh, AGEs+Mat+SB, AGEs+Oxy+SB, AGEs+Soh+SB, AGEs+Mat+mkk3−, AGEs+Mat+mkk6−, AGEs+Oxy+mkk3−, AGEs+Oxy+mkk6−, AGEs+Soh+mkk3−, and AGEs+Soh+mkk6−, respectively. C, Columns indicate the ARE-luciferase activities in RAECs in Control, AGEs, AGEs+Mat, AGEs+Oxy, AGEs+Soh, AGEs+Mat+SB, AGEs+Oxy+SB, AGEs+Soh+SB, AGEs+Mat+mkk3−, AGEs+Mat+mkk6−, AGEs+Oxy+mkk3−, AGEs+Oxy+mkk6−, AGEs+Soh+mkk3−, and AGEs+Soh+mkk6−, respectively (3 independent experiments were performed; *$P<0.05$; **$P<0.01$; ***$P<0.001$).

cardiovascular complications. It was reported that by coupling with its downstream receptor, receptor for advanced glycation end products, AGEs stimulated ROS production by activating oxidases.[11] The AGEs-induced ROS-mediated cell apoptosis was linked with AGEs/receptor for advanced glycation end products interaction.[33] In this study, rats and cultured RAECs were exposed to AGEs. As a result, the ROS-mediated apoptosis of RAECs was found to be increased significantly both in vivo and in vitro. Therefore, inhibition of ROS-mediated RAECs apoptosis would ameliorate vascular injuries caused by AGEs.

A Chinese medical herb named Shan-Dou-Gen (*S. flavescens*) has been used for treatment of diabetes mellitus from ancient times. The effective components were mainly quinolizidine alkaloids, principally matrine-type alkaloids. Matrine, oxymatrine, and sophocarpine are typical matrine-type alkaloids extracted from *S. flavescens* exhibiting similar bioactive effects attributed to their comparable molecular structures. Matrine, oxymatrine, and sophocarpine were known as cardiovascular protective agents and showed potent antioxidant effects.[20,26,34]

The antioxidant system exists and maintains intracellular antioxidant-oxidant balance. Enhancing the antioxidant system counterweights the cell injuries brought about by excessively produced ROS under certain pathological conditions. As a member of the "cap 'n' collar" family of basic leucine zipper transcription factors, Nrf2 induces the transcriptions of antioxidant enzymes by binding with ARE.[35]
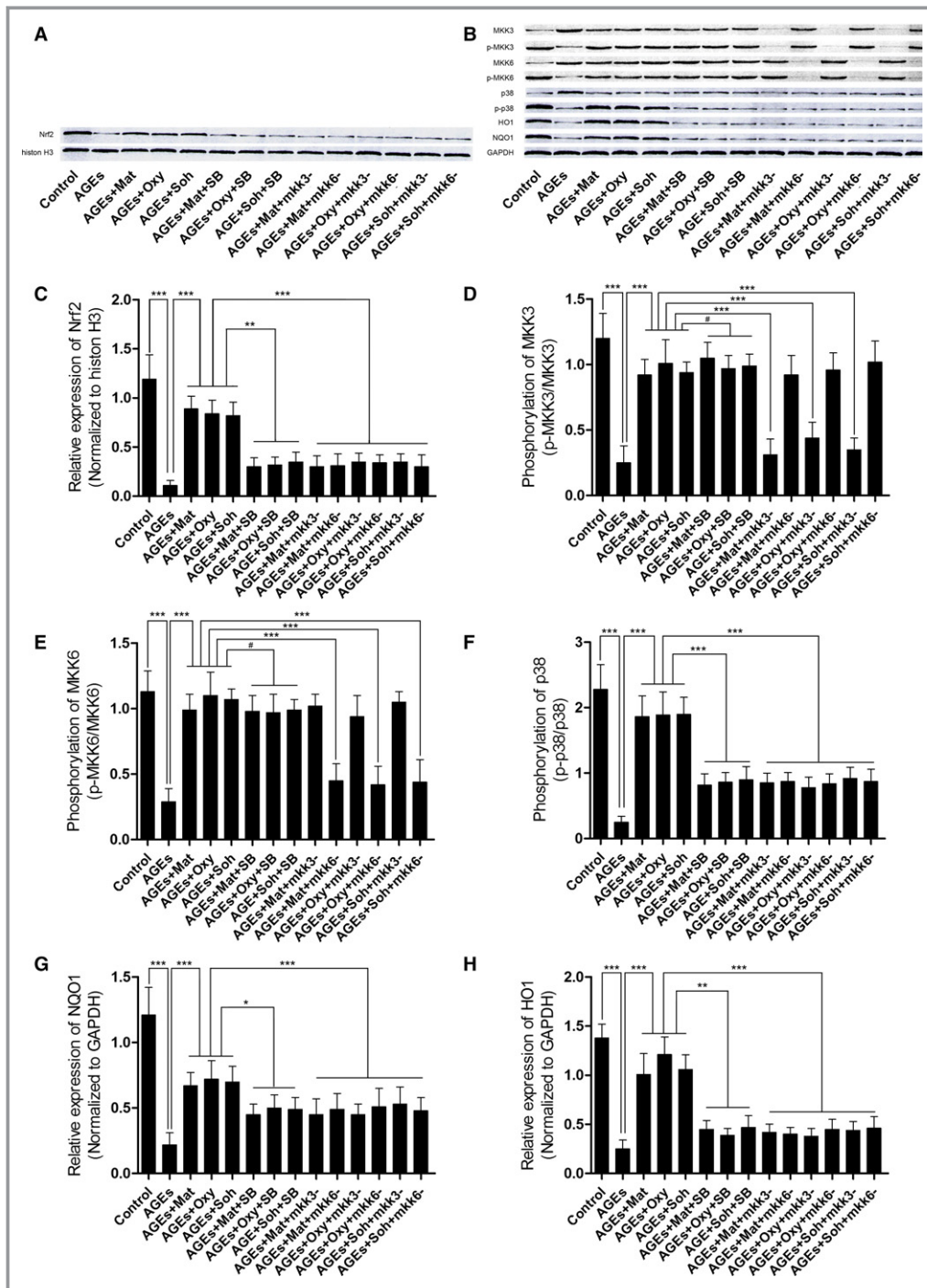
**Figure 6.** A, The immunoblots of Nrf2 in nuclear protein when histone H3 was used as the internal reference. B, The immunoblots of phosphorylated MKK3 (p-MKK3), MKK3, p-MKK6, MKK6, p-p38, p38, HO1 (heme oxygenase 1), and NQO1 (NADPH quinone oxidoreductase 1) in total protein when GAPDH was introduced as the internal reference. C through H, Columns in these panels indicate the relative expression level of Nrf2 (Nrf2/histone H3), phosphorylation level of MKK3 (p-MKK3/MKK3), phosporylation level of MKK6 (p-MKK6/MKK6), phosphorylation level of p38 MAPK, the relative expression levels of HO1 (HO1/GAPDH), and the relative expression level of NQO1 (NQO1/GAPDH), respectively (3 independent experiments were performed; *$P<0.05$; **$P<0.01$; ***$P<0.001$; #$P>0.05$).

Several genes of antioxidant enzymes were the downstream targets of Nrf2/ARE signaling. HO1, which has the ability of conversing heme into biliverdin, is one of the key phase II enzymes maintaining intracellular redox homeostasis by neutralizing oxidative stress.[36] As a detoxifiying enzyme, NQO1 catalyzes the 2-electron reductive metabolism and
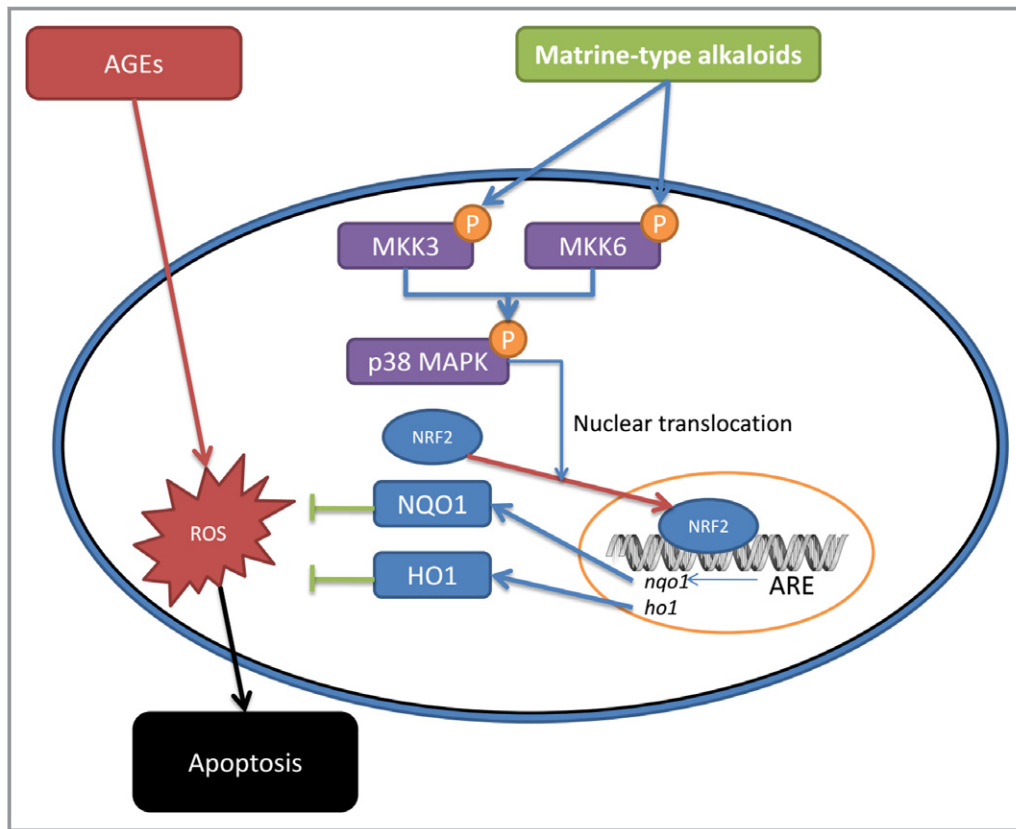
**Figure 7.** The schematic diagram of the antioxidant molecular mechanisms of matrine-type alkaloids. AGEs (advanced glycation end products) trigger intracellular ROS (reactive oxygen species) production, which induces apoptosis of aortic endothelial cells. The matrine-type alkaloids administration promotes the phosphorylation of MKK3 and MKK6. As the kinases of p38 MAPK, phosphorylated MKK3 and MKK6 further phosphorylate p38 MAPK, which facilitate the nuclear translocation of Nrf2. Binding with ARE (antioxidant response element), the nuclear transcription factor Nrf2 initiates the transcriptions of antioxidant proteins such as NQO1 (NADPH quinone oxidoreductase 1) and HO1 (heme oxygenase 1). Thus, the intracellular ROS accumulation is alleviated and apoptosis is suppressed.

detoxification of quinies, protecting cells from oxidative stress–induced injury.[37]

Indeed, in the current study, the Nrf2 nuclear translocation was found to be inhibited in AGEs-exposed aorta. After treatment of matrine-type alkaloids, the nuclear translocation of Nrf2 increased dramatically. Moreover, Nrf2/ARE binding activities were found to be increased by matrine-type alkaloids treatment. As a result, the expression levels of HO1 and NQO1, as well as TAC, were elevated dramatically in thoracic aorta. These results indicated that matrine-type alkaloids increased antioxidant capability of aortic endothelium by activating Nrf2/ARE signaling.

The Nrf2/ARE signaling activation was regulated by their upstream kinase, p38 MAPK. In physiological conditions, p38 MAPK is at a static status by binding with its inhibitor, Keap1.[14] Various pathological stimuli and intracellular responses activate p38 MAPK by phosphorylation, which facilitates disassociation of Nrf2/Keap1 complex and nuclear translocation of Nrf2.[38] In both in vivo and in vitro studies of

this investigation, p38 MAPK phosphorylation was found to be impaired in RAECs exposed to AGEs. In the in vitro study, specifically, in RAECs exposed to AGEs, evidenced by ARE-binding activity assay, the suppression of Nrf2/ARE signaling transduction was found. The matrine-type alkaloids administration, however, significantly increased the p38 MAPK phosphorylation and Nrf2 nuclear translocation. As a result, the expression levels of antioxidant enzymes HO1 and NQO1, as well as TAC, were upregulated. ROS production and its mediated apoptosis were suppressed in RAECs exposed to AGEs. We also found that the p38 MAPK inhibitor, SB203580, dramatically impaired the protective effects of matrine-type alkaloids. The treatment of SB203580 blocked p38 MAPK phosphorylation, which further inhibited Nrf2 nuclear translocation and its binding with ARE, as well as the HO1 and NQO1 expressions in RAECs exposed to AGEs, leading to excessive ROS accumulation and ROS-mediated apoptosis. Results from both in vivo and in vitro studies suggested that matrine-type alkaloids could increase antioxidant ability of RAECs to inhibit

ROS-mediated apoptosis by regulating the p38 MAPK/Nrf2/ARE pathway.

MKKs are kinase enzymes phosphorylating and activating MAPKs, including extracellular signal-regulated kinases, c-Jun N-terminal kinases, and p38.[39,40] Among them, MKK3 and MKK6 were reported to be specifically involved in the activation of p38 MAPK.[16] In this study, the small RNA interfering technique was used to silence MKK3 and MKK6 in AGEs-exposed RAECs. The results showed that both MKK3 and MKK6 silencing impaired matrine-type alkaloids' antioxidant ability in RAECs exposed to AGEs. The results suggested that matrine-type alkaloids exerted protective effects against oxidative stress–induced cell death by affecting p38 MAPK antioxidant signaling. Their targets were supposed to be the upstream kinases, MKK3 and MKK6, particularly.

In summary, results from the current study indicated that AGEs exposure would trigger ROS overproduction, inducing cell apoptosis in aortic endothelium in vivo and in vitro. The antioxidant signaling transduction by p38 MAPK/Nrf2/ARE was inhibited. Administrations of matrine-type alkaloids, namely matrine, oxymatrine, and sophocarpine, were found to recover the activation of p38 MAPK/Nrf2/ARE both in vivo and in vitro. p38 MAPK kinases MKK3/6 were believed to be the molecular targets of these matrine-type alkaloids. The schematic diagram of the molecular mechanisms is shown in Figure 7. We believe results from this study would not only enrich our knowledge of molecular pharmacological mechanisms of matrine-type alkaloids, but also provide the clues for potential clinical application of matrine-type alkaloids and related compounds in treatment of diabetic vascular complications.

## Limitations

There were several limitations of this study. (1) Although male experiment animals were more physically stabilized, female animals should also be included because sex is an important biological variant. (2) The concentration gradients of matrine-type alkaloids were not included in the experiment design of the in vivo study because it would bring a heavy workload. Our pre-experiments confirmed their effective dosages, which were used in the subsequent experiments. It would be better if in vivo toxicity assays were implemented to prove the biosafety of these alkaloids. Interestingly, we also noticed that the effective dosages of these matrine-type alkaloids were not consistent in the in vivo and in vitro studies. We think that maybe the effective concentrations of the alkaloids were various, though they share several similar molecular structures; the in vivo metabolisms of these agents may be different. Perhaps studies on the pharmacokinetics of these agents in the future would be helpful to elucidate it. (3) Administration of p38 MAPK inhibitor impaired the effects of matrine-type alkaloids partially. This result indicated that

there might be other signaling pathways modulating matrine-type alkaloids' protective effects on RAECs. There are other MKKs regulating MAPKs. For instance, MKK4/7 could regulate the phosphorylation of another MAPK subgroup, the c-Jun N-terminal kinase. Moreover, it was reported that c-Jun N-terminal kinase was also involved in ROS-mediated apoptosis of several types of cells.[41] Thus, further investigations of other possibly involved pathways would also be meaningful. (4) Results from this study just answered whether the MKK3/6 were phosphorylated by matrine-type alkaloids, but did not answer how. Maybe some bioinformatic software could be helpful in anticipating the phosphorylation sites of MKK3/6 by these agents, which could be further confirmed by corresponding antibodies. (5) Keap1 binds to Nrf2 to maintain the static status of Nrf2 under normal conditions. When we studied the initiation of nuclear translocation of Nrf2, it would have been better for us to investigate the expression of Keap1 at the same time. This would make the evidence more solid.

## Author Contributions

Zhongwei Liu wrote the manuscript and implemented the experiments; Lv implemented the experiments; Zhang researched data; Fuqiang Liu accomplished the figures; Zhu analyzed the data; Pan revised the manuscript; Qiu analyzed the data; Guo participated in the experiments; Yang participated in the experiments; Wang designed the study and revised the manuscript. Prof Wang is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data.

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## Disclosures

None.

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