Chlorogenic acid attenuates hydrogen peroxide-induced oxidative stress in lens epithelial cells

JIKE SONG[1], DADONG GUO[2] and HONGSHENG BI[2]

[1]Medical College of Ophthalmology and Optometry, Shandong University of Traditional Chinese Medicine; [2]Shandong Provincial Key Laboratory of Integrated Traditional Chinese and Western Medicine for Prevention and Therapy of Ocular Diseases; Eye Institute of Shandong University of Traditional Chinese Medicine, Jinan, Shandong 250002, P.R. China

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Abstract. Oxidative stress has an important role in the degradation, oxidation, cross-linking and aggregation of lens proteins, and can trigger lens epithelial cell apoptosis. To investigate the protective effect of chlorogenic acid (CGA) against hydrogen peroxide (H_2O_2)-induced oxidative stress, human lens epithelial cells (hLECs) were exposed to various concentrations of H_2O_2 in the presence and absence of CGA. Using MTT assay, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and ELISA techniques, cell viability, and protein/mRNA levels of BCL2 apoptosis regulator (Bcl-2) and BCL2 associated X apoptosis regulator (Bax) were investigated. Additionally, the levels of intracellular reactive oxygen species (ROS) and apoptosis within cells were measured using flow cytometry to determine the protective effect of CGA on H_2O_2-induced oxidative stress. Furthermore, the protective effect of CGA on H_2O_2-induced apoptosis was also examined using rabbit lenses *ex vivo*. The results indicated that CGA reduced H_2O_2-induced cytotoxicity in a dose-dependent manner. Flow cytometry analysis demonstrated that simultaneous exposure of hLECs to H_2O_2 and CGA significantly decreased apoptosis and the levels of ROS. RT-qPCR analysis revealed a decrease in Bcl-2 and an increase in Bax in hLECs following exposure to H_2O_2 for 24 h, regardless of CGA presence. Furthermore, ELISA results indicate that CGA increased Bcl-2 expression and decreased Bax expression following treatment with H_2O_2 for 24 h and the Bax/Bcl-2 ratio was significantly decreased by CGA treatment. Lens organ culture experiments indicated a dose-dependent decrease in H_2O_2-induced lens opacity following CGA treatment. These results suggest that CGA suppresses hLECs apoptosis and prevents lens opacity induced by H_2O_2 via Bax/Bcl-2 signaling pathway. CGA may provide effective defenses against oxidative stress and, thus, haσ potential as treatment for a variety of diseases in clinical practice.

Correspondence to: Professor Hongsheng Bi, Shandong Provincial Key Laboratory of Integrated Traditional Chinese and Western Medicine for Prevention and Therapy of Ocular Diseases Eye Institute of Shandong University of Traditional Chinese Medicine, 48 Yingxiongshan Road, Jinan, Shandong 250002, P.R. China
E-mail: azuresky1999@163.com

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Introduction

Human lens epithelium is the most metabolically active cell layer of the lens, and is the initial cell layer exposed to environmental and oxidative insult (1). Oxidative stress has an important role in the degradation, oxidation, cross-linking and aggregation of lens proteins, and also triggers lens epithelial cell apoptosis. The apoptosis of lens epithelial cells has been proposed as a common basis for the initiation of noncongenital cataract formation, with oxidative stress as a major contributor to cataract formation (2-4). Exposure to oxidative stress results in lens opacification in experimental animal models (5,6) and cultured lens systems (7-9).

Hydrogen peroxide (H_2O_2) is one of the most physiologically relevant oxidants of the lens and aqueous humor, and has been reported to deplete glutathione and damage ion pump activity in lens epithelial cells (LECs) (10,11). Elevated levels of H_2O_2 are reported in the aqueous humor of patients with cataract and can lead to opacification of the lens *in vitro* (12,13). Overall, previous studies have demonstrated that H_2O_2-induced apoptosis in human lens epithelium cells is a useful model of cataractogenesis (14-16).

It has been confirmed that loss of transparency during human cataract formation is involved in a variety of complex metabolic and physiologic mechanisms, and an increase in antioxidant levels in the lens may prevent or ameliorate oxidative damage, and reduce cataract risk (7). Therefore, it is important to develop protective strategies against apoptosis in human lens epithelial cells to prevent cataractogenesis.

Chlorogenic acid (CGA) is one of the most abundant polyphenol compounds in coffee, strawberries, pineapple, apple, sunflower and blueberries (17). The molecular structure of CGA is presented in Fig. 1. It can exert various biological properties and modulatory effects on lipid and glucose metabolism under metabolic dysregulation conditions, such as antioxidant, antiangiogenic, anticarcinogenic and antiglycation (18-21). Kim *et al* (22) reported that CGA may provide a potential therapeutic approach for prevention of diabetic complications, such as cataracts. Akila *et al* (23) reported that CGA is an effective protective agent to maintain the activities of enzymic antioxidants, including superoxide dismutase, catalase, glutathione peroxidase and glutathione-S-transferase. Similarly,

Ye *et al* (24) demonstrated that CGA efficiently protected kidney function against oxidative stress in a rat model of diabetic nephropathy.

Based on these observations, we hypothesize that CGA may protect human lens epithelial cells (hLECs) against oxidative stress-induced apoptosis, and may offer benefits in the treatment of cataract associated with oxidative stress. In the present study, H_2O_2-treated hLECs and rabbit lenses were used as models to examine the protective effect of CGA on LECs exposed to H_2O_2-mediated oxidative stress.

Materials and methods

Materials. Human HLE-B3 lens epithelial cell line was obtained from the American Type Culture Collection (Manassas, VA, USA), and chlorogenic acid (purity, 98.7%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products of China (Beijing, China). MTT and H_2O_2 (30%) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). A 500 μM H_2O_2 solution was prepared in phosphate-buffered saline (PBS) immediately prior to application. The Annexin V/propidium iodide (PI) apoptotic detection kit was purchased from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China). All other chemicals used were purchased from Sigma-Aldrich (Merck KGaA) unless otherwise stated.

Cell culture and treatment. HLE-B3 cells were cultured in RPMI-1640 medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) containing 1 g/l glucose, 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences), 100 U/ml penicillin and 100 μg/ml streptomycin (HyClone; GE Healthcare Life Sciences) under a humidified atmosphere with 5% CO_2 at 37˚C. The cells were seeded into a 60 mm culture dish (Corning Incorporated, Corning, NY, USA). When at 75-80% confluence, the cells were treated with H_2O_2 (10-500 μM) for 24 h or pretreated with CGA for 2 h prior to H_2O_2-treatment. At the indicated time-points, the cells were collected for different assays.

Cell viability assay. The concentrations of CGA and H_2O_2 were optimized using MTT assay. Briefly, hLECs were cultured in 96-well plates and treated with a broad range of concentrations for each reagent (H_2O_2 or CGA) for 24 h. To examine the effect of CGA, hLECs were incubated with 100 μM H_2O_2 in the absence or presence of different concentrations of CGA for 24 h. Subsequently, 20 μl MTT solution (5 mg/ml) was added into each well. After another 4 h incubation at 37˚C, the medium was removed and the formazan crystals formed by oxidation of the MTT dye were dissolved with 150 μl DMSO. The absorbance was measured at 490 nm using the spectrophotometer [Unico (shanghai) Science Instruments Co., Ltd., Shanghai, China]and the cell survival ratio was expressed as a percentage of the blank.

Measurement of intracellular reactive oxygen species (ROS). To obtain further evidence for the protective effect of CGA against H_2O_2 induced oxidative stress, alterations of intracellular ROS levels were determined. The production of intracellular ROS was measured using 2',7'-dichlorofluorescin diacetate (DCFH-DA; Invitrogen, Carlsbad, CA, USA) by flow cytometry. Briefly, hLECs were incubated either with 100 μM H_2O_2 alone or treated with different concentrations (10, 30 and 50 μM) of CGA for 2 h prior to treatment with 100 μM H_2O_2 for 24 h. After harvest of hLECs, the cells were incubated with DCFH-DA solution (10 μM) in the dark at 37˚C for 30 min, washed with PBS (pH 7.4), and analyzed within 30 min using a flow cytometer (Accuri C6; Accuri Cytometers Inc., Ann Arbor, MI, USA). The specific fluorescence signals that correspond to DCFH-DA were collected with a 525 nm band pass filter. For each determination, $2.0x10^4$ cells were counted.

Table I. Primer sequences for Bcl-2, Bax and GAPDH.

Target gene	Primer sequence
Bcl-2	F: 5'-GAGTGGATGACCGTCTACCTG-3'
	R: 5'-CCTGAGACCTTCTGCTTTCG-3'
Bax	F: 5'-TTTTGCTTCAGGGTTTCATC-3'
	R: 5'-GACACTCGCTCAGCTTCTTG-3'
GAPDH	F: 5'-CCATGTTCGTCATGGGTGTGAACCA-3'
	R: 5-GCCAGTAGAGGCAGGGATGATGTTC-3'

Bcl-2, BCL2, apoptosis regulator; Bax, BCL2 associated X, apoptosis regulator; GAPDH, Glyceraldehyde-phosphate dehydrogenase.

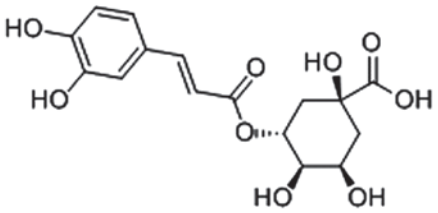

Figure 1. Molecular structure of chlorogenic acid.

Apoptosis assay. For quantification of the apoptosis rate in hLECs, cells were cultured on a 6-well plate at $5.0x10^5$ cells/well and treated with 100 μM H_2O_2 with or without CGA (0, 10, 30 and 50 μM) for 24 h. Subsequently, hLECs were collected and stained using Annexin V/PI kit and assessed by a flow cytometer (Accuri C6; Accuri Cytometers), following the instructions of the manufacturer.

Revere transcription-quantitative polymerase chain reaction (RT-qPCR). The effect of CGA on the gene expression of BCL2 associated X, apoptosis regulator (Bax) and BCL2, apoptosis regulator (Bcl-2) mRNA using RT-qPCR in the presence and absence of H_2O_2. The cells ($6x10^5$) were incubated either with 100 μM H_2O_2 for 24 h or with CGA for 2 h prior to treatment with 100 μM H_2O_2. Total RNA was extracted with TRIzol reagent (Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and cDNA was generated using a Superscript cDNA kit (Thermo Fisher Scientific, Inc.) based on the manufacturer's protocol. Following quantification with a micro-spectrophotometer (Beijing Kaiao Technology Development Co., Ltd., Beijing, China), cDNA was synthesized using total RNA. qPCR was performed with SYBR-Green Master Mix (Takara Biotechnology Co., Ltd., Dalian, China) in a Stratagene Mx3000P sequence detection system (Agilent Technologies, Inc., Santa Clara, CA, USA). GAPDH was

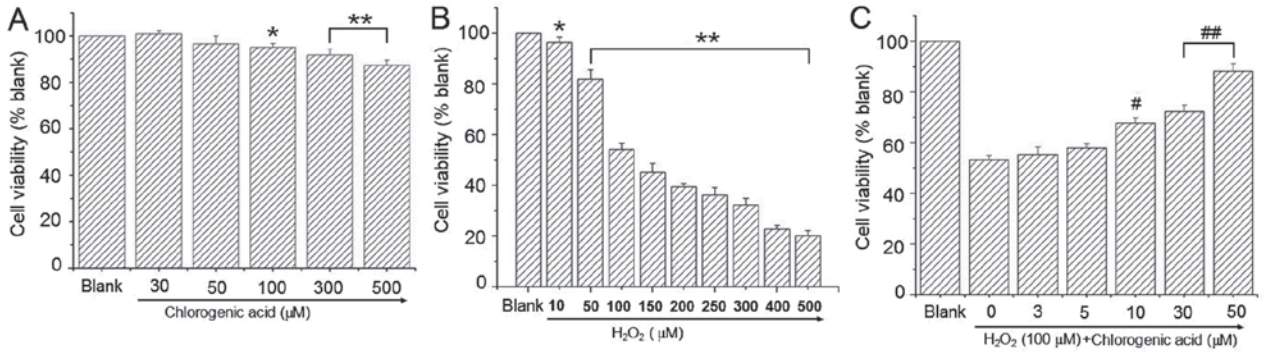

Figure 2. Cell viability was assessed by MTT assay. (A) Cells were treated with chlorogenic acid (0-500 μM) for 24 h. (B) Cells were treated with H_2O_2 (0-500 μM) for 24 h. (C) Effect of chlorogenic acid on HLE-B3 cell viability in co-treatment with H_2O_2 (100 μM). Blank cells were cultured in media alone, while control cells were treated with H_2O_2 (100 μM) alone. Data are presented as the mean + standard deviation. (*P<0.05 and **P<0.01 vs. blank; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. control; one-way analysis of variance and followed by Tukey's honest significant difference post hoc test).

used as a positive control, and a negative control without template RNA was also included. The primer sequences are presented in Table I. The reactions were performed in a total volume of 20 μl using the SensiMix One-Step kit (http://www.quantace.com; Quantace, Finchley, UK). The conditions of PCR amplification for Bcl-2 and Bax were as follows: 95˚C for 10 min, followed by 40 cycles of a 95˚C denaturation for 15 sec, annealing at 55˚C for 30 sec, and 72˚C extension for 50 sec. Each experiment was carried out four times and the ΔΔCq values were calculated by normalizing the gene expression levels to the expression of GAPDH (25). The relative expression level of each gene was expressed as a fold change.

ELISA. The levels of Bcl-2/Bax (anti-/pro-apoptotic) proteins were determined using commercially available ELISA kits [BCL-2 kit (JYM0302Hu), BAX kit (JYM0265Hu); Colorfulgene Biological Technology, Co., Ltd., Wuhan, China]. The hLECs ($6x10^5$) were incubated with 100 μM H_2O_2 for 24 h alone or treated with CGA for 2 h prior to treatment with 100 μM H_2O_2 for 24 h. The cells were then collected and cell concentration was diluted to 10^6/ml with PBS (pH 7.2-7.4). Following three freeze-thaw cycles, damaged cells were centrifuged at 5,000 x g at 4˚C for 20 min. The supernatant was carefully collected and stored at -80˚C prior to use. The protein in the cell lysate was determined using the ELISA kit according to the manufacturer's instructions. Subsequently, the plates were read at 450 nm using a microplate reader (BioTek ELX800; BioTek Instruments, Inc., Winooski, VT, USA). The protein level of each sample was determined by comparison to a standard curve.

Lens organ culture and treatment. All animal procedures were in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research (26), and the animal experiments were approved by Shandong University of Traditional Chinese Medicine Animal Care and Ethics Committee (Jinan, China). In the present study, New Zealand White rabbits (n=30; weighing 1.8-2.0 kg) aged 10-12 weeks were euthanized with an overdose of sodium pentobarbital injection through the marginal ear vein. The eyes were removed and the lenses were carefully dissected by a posterior approach. Lenses were immediately transferred into a 24-well culture plate containing 2 ml Dulbecco's modified Eagle's medium (HyClone, Beijing, China), 1 g/l glucose, 100 U/ml penicillin and 100 μg/ml streptomycin (HyClone; GE Healthcare Life Sciences). Approximately, 24 h after the preparation of organ cultures, transparent lenses were selected for further experimentation. During the experiment, H_2O_2 and CGA were maintained at indicated concentrations for a period of 12 h, and the medium was changed. Conditioned medium was stored for further culture. Lenses were cultured in a 5% CO_2 incubator at 37˚C for 48 h, and were images using a stereomicroscope under a cross background (1.0x1.0 cm). Each sample contained three lenses and lens opacity was analyzed using ImageJ-1.46 software (National Institutes of Health, Bethesda, MD, USA). This experiment was repeated three times independently.

Flow cytometry with Annexin V/PI staining. The apoptosis rate of lens epithelial cells was assessed by flow cytometry with Annexin V/PI staining. The lenses exposed to 500 μM H_2O_2 were cultured with various concentrations (i.e., 0, 10, 30 and 50 μM) of CGA for 48 h. At the indicated time-points (0, 12, 24 and 48 h, respectively), lens epithelial explants were carefully detached from rabbit lens under an operation microscope (YZ20P5; 66 Vision Tech Co., Ltd., Suzhou, China). Lens epithelial explants were then sheared, digested with trypsin, and RPMI-1640 medium was added to terminate the digestion (27). The cell suspension was then passed through a cell strainer and centrifuged (300 x g) at 4˚C for 5 min. The cells were resuspended in RPMI-1640 medium and collected by centrifugation at 300 x g at 4˚C for 10 min. The collected cells were washed with cold PBS and stained with Annexin V/PI apoptotic detection kit. Finally, the cells were resuspended in 500 μl PBS for further analysis using a flow cytometer (Accuri C6; Accuri Cytometers, Inc.).

Statistical analysis. All experiments were repeated three times and data are expressed as the mean ± standard deviation, and were analyzed by one-way analysis of variance, followed by Tukey's honest significant difference post hoc test, using SPSS software (version 17.0; SPSS Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Cell viability assessed by MTT assay. To determine the effect of CGA on hLECs, cells were treated with a broad range of CGA concentrations for 24 h. Cell viability was presented as a

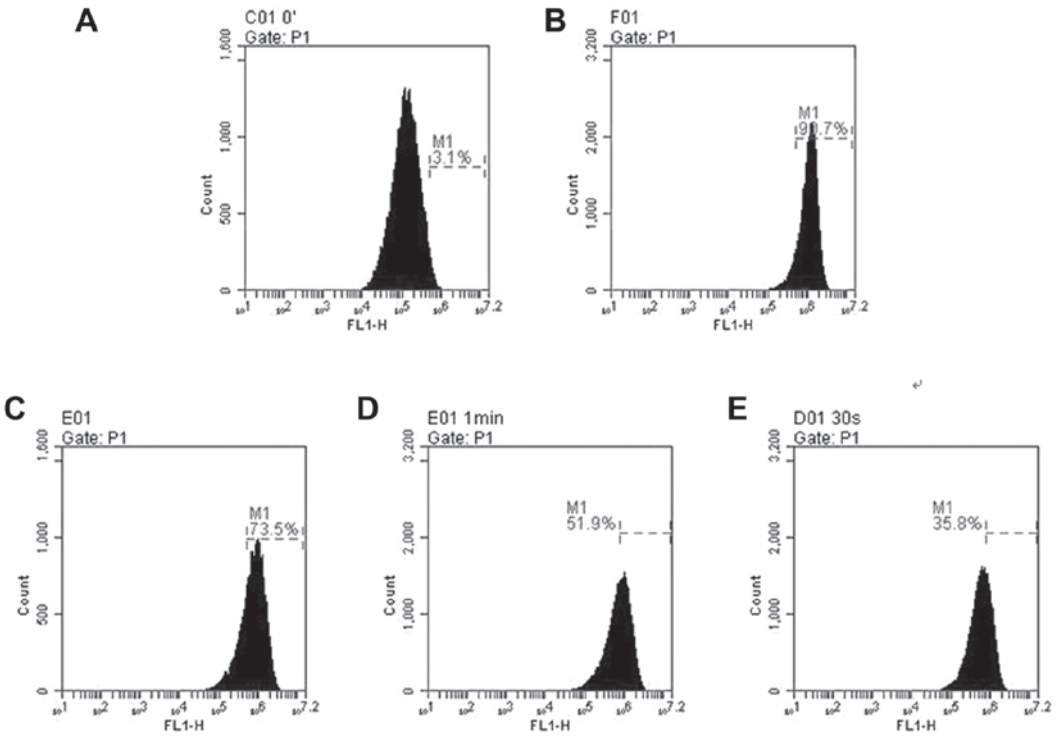

Figure 3. The alterations of ROS levels in human lens epithelial cells determined by flow cytometry. Cells were cultured following exposure to 100 μM H_2O_2 with or without CGA (0, 10, 30 and 50 μM) for 24 h. (A) Untreated cells; (B) cells exposed to 100 μM H_2O_2; (C) cells exposed to 100 μM H_2O_2 + 10 μM CGA; (D) cells exposed to 100 μM H_2O_2 + 30 μM CGA; (E) cells exposed to 100 μM H_2O_2 + 50 μM CGA. Data re the percentage of cells producing ROS and are the mean value from three independent experiments performed by flow cytometry. ROS, reactive oxygen species; CGA, chlorogenic acid.

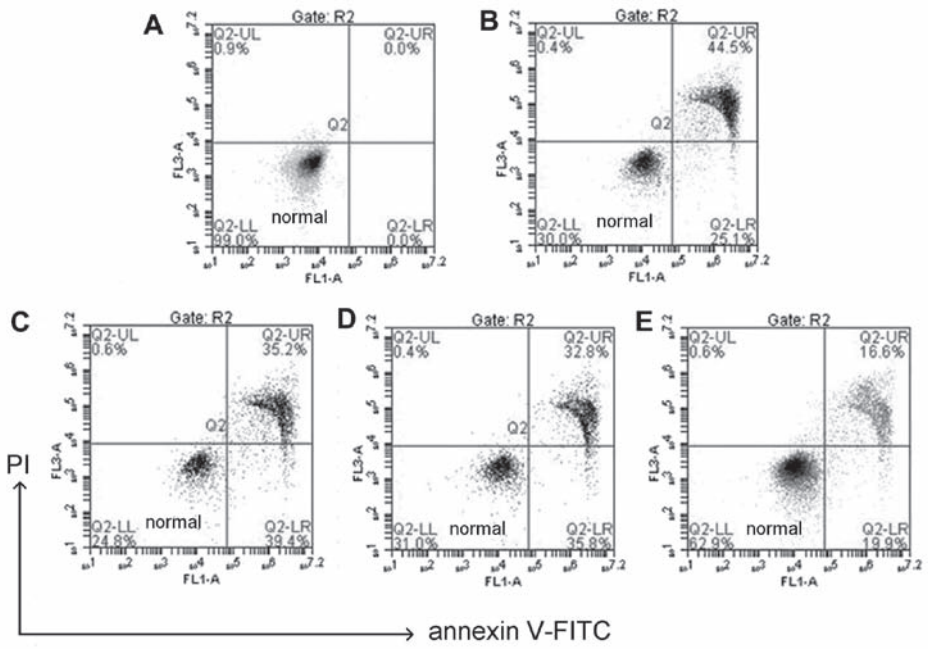

Figure 4. Apoptosis analysis of human lens epithelial cells. (A) Apoptosis in blank cells without any treatment, or pretreated with different concentration of chlorogenic acid: (B) 0 μM, (C) 10 μM, (D) 30 μM, (E) 50 μM, followed by 100 μM H_2O_2 for 24 h. Apoptosis was analyzed using flow cytometry with Annexin V/PI staining. Control cells were treated with H_2O_2 (100 μM) alone. P<0.01 control group vs. all other groups. PI, propidium iodide; FITC, fluorescein isothiocyanate.

percentage of the blank value. The results indicated that CGA had no cytotoxicity in hLEC cells when the CGA concentration was <100 μM (Fig. 2A). H_2O_2-treated cells exhibited lower cell viability at H_2O_2 concentrations ≥50 μM (Fig. 2B). Co-treatment with CGA resulted in a dose-dependent reduction in cytotoxicity induced by H_2O_2 (100 μM; Fig. 2C).

Effect of CGA on intracellular ROS. hLECs treatment with 100 μM H_2O_2 alone for 24 h resulted in the production of ROS with a ~3-fold increase compared with 50 μM CGA-treated cells (Fig. 3). Pretreatment with CGA prior to H_2O_2 exposure markedly reduced the ROS levels. When hLECs were treated with CGA (0, 10, 30 and 50 μM) for 2 h prior to treatment

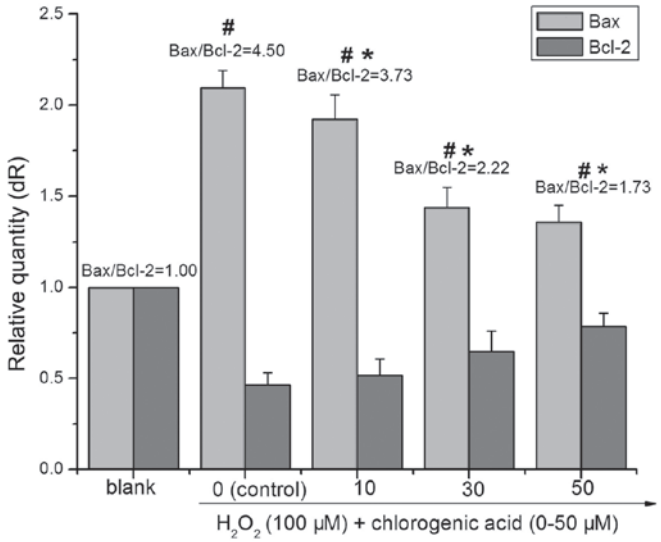

Figure 5. Bax and Bcl-2 mRNA were examined using reverse transcription-quantitative polymerase chain reaction. Changes in mRNA levels of either Bax or Bcl-2 after treatment with 100 μM H_2O_2 plus chlorogenic acid (0, 10, 30 and 50 μM, respectively). Blank cells were cultured in media alone, while control cells were treated with H_2O_2 (100 μM) alone. Data are presented as the mean + standard deviation. ($^{\#}$P<0.01 vs. blank; *P<0.01 vs. control, one-way analysis of variance and followed by Tukey's honest significant difference post hoc test). Bax, BCL2 associated X, apoptosis regulator; Bcl-2, BCL2, apoptosis regulator.

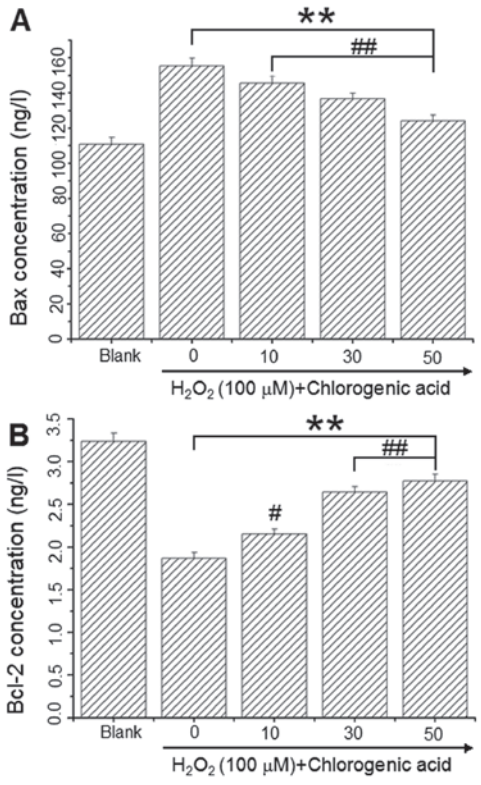

Figure 6. Determination of Bcl-2 and Bax protein expression using ELISA. (A) Protein level of Bax following treatment with 100 μM H_2O_2 plus chlorogenic acid (0, 10, 30 and 50 μM) for 24 h. (B) Protein level of Bcl-2 following treatment 100 μM H_2O_2 plus chlorogenic acid (i.e., 0, 10, 30 and 50 μM) for 24 h. Blank cells were cultured in medium alone. Control cells were treated with H_2O_2 (100 μM) alone. Data are expressed as the mean ± standard deviation. (*P<0.05 and **P<0.01 vs. blank; $^{\#\#}$P<0.01 and $^{\#}$P<0.05 vs. control, one-way analysis of variance and followed by Tukey's honest significant difference post hoc test). Bax, BCL2 associated X, apoptosis regulator; Bcl-2, BCL2 apoptosis regulator.

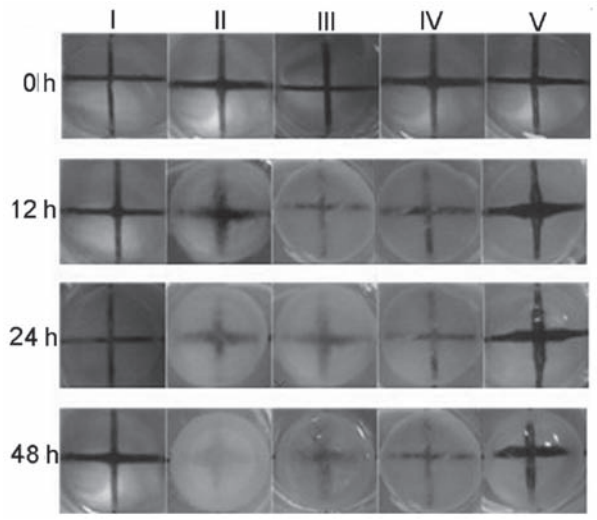

Figure 7. Effect of H_2O_2 and/or chlorogenic acid on the transparency of rabbit lens. Rabbit lenses were treated untreated (I) or treated with 500 μM H_2O_2 plus different concentrations of chlorogenic acid [0 μM (II), 10 μM (III), 30 μM (IV), 50 μM (V)] and were images with a cross background (1.0x1.0 cm) at indicated time-points (12, 24 and 48 h, respectively).

with 100 μM H_2O_2 for 24 h, the ROS generation reduced from 90.7±7.75 to 73.5±5.98%, 51.9±4.74 and 35.8±3.53% (Fig. 3B-E), respectively. These findings demonstrated that with the increase of concentrations of CGA, the intracellular ROS level induced by H_2O_2 was reduced and the ROS reduction was in a concentration-dependent manner. In addition, there was a significant difference in the level of H_2O_2-induced ROS compared with that of untreated cells (Fig. 3).

CGA prevents H_2O_2-induced apoptotic changes in hLECs. The use of Annexin V/PI double staining allows distinction between live cell populations, cells entering early apoptosis and those in late-stage apoptosis/necrosis. Q2-LL quadrant indicates healthy cells, Q2-UR quadrant indicates necrosis cells (Fig. 4). Any late-stage apoptosis cells were considered as necrosis as this technique is not sensitive enough to differentiate between the two. The data in Fig. 4 demonstrated that following treatment with 100 μM H_2O_2 and co-treatment with CGA (10, 30 and 50 μM) for 24 h, the late apoptotic rate of HLE-B3 cells decreased from 44.5 to 35.2, 32.8 and 16.6%, respectively (Fig. 4).

CGA modulates the expression of Bcl-2/Bax. The members of the Bcl-2 protein family are pivotal role in the regulation of the mitochondrial apoptotic pathway (28). Both pro-apoptotic and anti-apoptotic Bcl-2 family members can affect the execution of apoptosis. As presented in Fig. 5, treatment with 100 μM H_2O_2 for 24 h decreased the expression of Bcl-2 (0.47-fold higher compared with blank) and increased the expression of Bax (2.09-fold higher compared with blank), whereas pretreatment with CGA inhibited the downregulation of Bcl-2 and upregulation of Bax. The ratio of Bax/Bcl-2 in the 100 μM H_2O_2-treated group was significantly higher than that in the blank group (P<0.01). With elevated CGA concentrations, the ratio of Bax/Bcl-2 in the CGA-treated (i.e., 10, 30 and 50 μM) groups decreased significantly compared with the H_2O_2-treated group (P<0.01). Following treatment with 100 μM H_2O_2 and co-treatment with different concentrations of CGA (10, 30

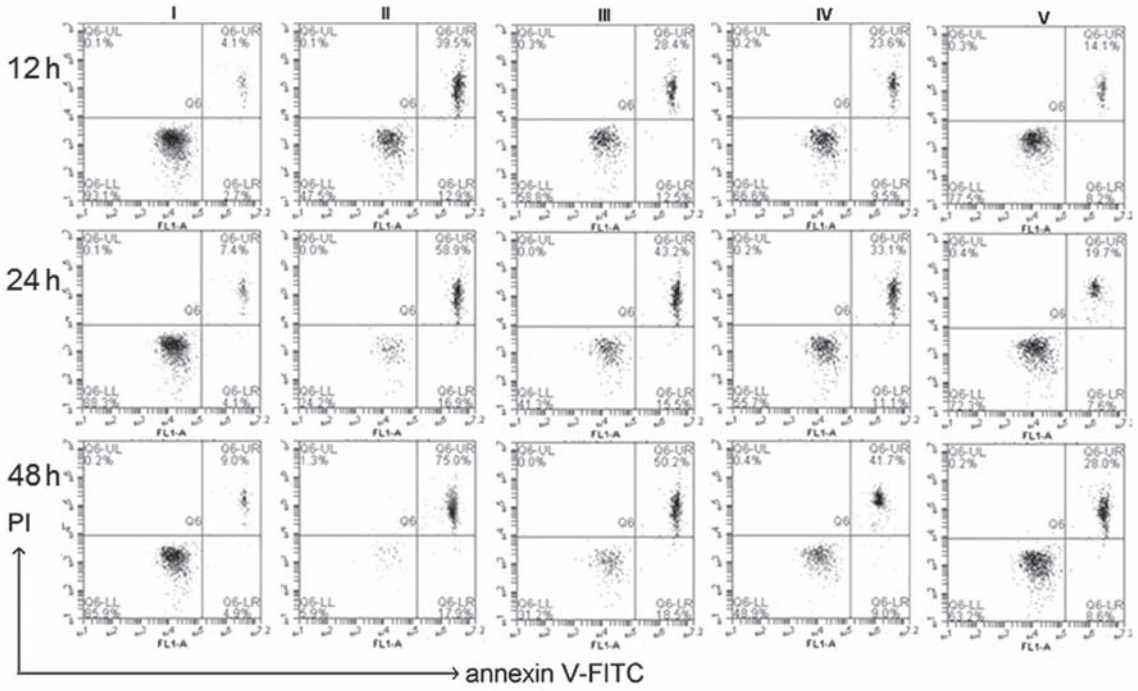

Figure 8. Apoptotic analysis of isolated lens epithelial cells by flow cytometry after staining with Annexin V/PI. Cells were treated untreated (blank; I) or treated with 500 μM H_2O_2 (control) plus different concentrations of chlorogenic acid [0 μM (II), 10 μM (III), 30 μM (IV), 50 μM (V)]. At the indicated time-points (i.e., 12, 24 and 48 h), the apoptosis rates were (II): 51.8±3.81, 77.2±4.12 and 94.57±4.77%; (III): 42.3±3.76, 58.3±3.67 and 65.9± 4.12%; (IV): 33.7±2.95, 42.3±3.29 and 50.43±4.13%; (V): 22.4±1.09, 28.5±2.54 and 39.2±3.71% of control value. Data are expressed as the mean ± standard deviation. P<0.01 blank vs. II, III, IV, V; P<0.01 control vs. I, III, IV, V; one-way analysis of variance and followed by Tukey's honest significant difference post hoc test). PI, propidium iodide; FITC, fluorescein isothiocyanate.

and 50 μM) for 24 h the ratios of Bax/Bcl-2 were 4.50±0.61, 3.73±0.46, 2.22±0.47 and 1.73±0.33, respectively.

CGA downregulates Bcl-2 expression and upregulates Bax expression. Bcl-2 and Bax levels were assessed using commercially available ELISA kits to determine whether these key regulators of apoptosis were involved in the H_2O_2-induced apoptosis mechanism. As presented in Fig. 5, Bax protein levels were 110.91±3.97, 155.49±4.30, 145.59±4.17, 136.78±3.07 and 124.19±3.11 ng/l (Fig. 6A) and Bcl-2 levels were 3.24±0.10, 1.87±0.07, 2.15±0.06, 2.64±0.09 and 2.77±0.10 ng/l (Fig. 6B) in the blank, 100 μM H_2O_2 and H_2O_2 + CGA 10, 30 and 50 μM groups, respectively. These results demonstrated that CGA increased Bcl-2 protein expression and decreased Bax protein expression in hLECs following incubation with 100 μM H_2O_2 for 24 h.

CGA prevents H_2O_2-induced lens opacity. The protective effects of CGA against cataractogenesis of rabbit lenses induced with H_2O_2 (500 μM) were investigated further. As presented in Fig. 7, loss of transparency in lenses exposed to 500 μM H_2O_2 was first noted in the equatorial region, spreading throughout the superficial cortex by 24 h and into deeper regions by 48 h. By contrast, there was little change in transparency of untreated lenses during the entire exposure period. The opacities of rabbit lenses incubated in H_2O_2 (500 μM) media containing various concentrations of CGA were measured every 12 h and compared with the control and blank samples. The opacities of the lenses began to increase after 12 h treatment with H_2O_2, and were gradually improved by CGA treatment in a dose-dependent manner.

At the indicated time-point, lens epithelial explants were detached. LECs were harvested and stained with Annexin V/PI for further analysis using a flow cytometer. Lenses treated with 500 μM H_2O_2 for 12, 24 and 48 h, caused a time-dependent increase of apoptosis rates (51.8±3.81, 77.2±4.12 and 94.57±4.77% of control value, respectively) whereas co-treatment with different concentrations of CGA [10 μM (III), 30 μM (IV), 50 μM (V)] the decreased the apoptosis rates (Fig. 8).

Discussion

As the most anterior part of the lens, LECs are the primary site of external insult that ultimately leads to cataracts (29). LECs are also the most metabolically-active part of the lens and are responsible for maintaining homeostasis and transparency. Li *et al* (3) have reported that apoptosis in lens epithelium may be a common cellular basis for noncongenital cataract formation, and that blocking apoptosis may prevent cataract formation. To investigate the protective effects of CGA on H_2O_2-induced apoptosis in hLECs, we performed a dose-response experiment using a range of concentrations (10, 30 and 50 μM). H_2O_2 was used as the oxidant model of classical oxidative stress. The results demonstrated that all concentrations of CGA exerted a protective effect against oxidative stress and an inhibitory effect against apoptosis, as measured by the cell morphology and cell viability studies. In addition, CGA inhibited cytotoxicity in hLECs caused by 100 μM H_2O_2 compared with H_2O_2 treatment alone. Apoptosis is an intracellular suicide mechanism that cause morphologic

changes and biochemical responses. In the presence of CGA, the proportions of apoptotic cells were significantly decreased. Thus, it is suggested that CGA may have a potentially beneficial role in the prevention of cataract formation.

It is well established that the proto-oncogene Bcl-2 can prevent apoptosis induced by a variety of factors. Regarding the mechanism by which Bcl-2 prevents cell death, one theory suggests that it acts by protecting cells from oxidative stress. Mao *et al* (30) reported that through downregulation of the αB-crystallin gene, Bcl-2 reduces the tolerance of rabbit lens epithelial cells against H_2O_2-induced apoptosis. Bcl-2 and Bax proteins are widely regarded as the most important apoptotic regulators, and their relative levels determine the fate of cells. Bcl-2 protein expression in the mitochondrial outer membrane inhibits cytochrome translocation into the cytosol, which is a critical step in the apoptotic process. By contrast, Bax is a pro-apoptotic antagonist of Bcl-2, and has been characterized as a Bcl-2 binding protein that shares significant sequence homology with Bcl-2 (31). An altered ratio of anti-apoptotic to pro-apoptotic Bcl-2 family genes is critical in determining whether apoptosis is performed. In the current study, RT-qPCR analysis revealed that the Bax/Bcl-2 ratio was significantly increased by the treatment with 100 μM H_2O_2, and this increase was inhibited by pretreatment with CGA (10, 30 and 50 μM). This result indicates that the Bcl-2 family may have a critical role in regulating hLEC death induced by H_2O_2, and that CGA is able to protect against H_2O_2-stimulated apoptosis through modulation of Bax/Bcl-2 expression.

Rabbit lenses were cultured *ex vivo* in an attempt to mimic the potential *in vivo* pathological environment present in patients with cataracts (12). Superoxide damages the lens, leading to loss of transparency and the formation of a cataract *in vivo* and *ex vivo*. Charakidas *et al* (32) suggested that the accumulation of small-scale epithelial losses during a lifetime may induce alterations in lens fiber formation and homeostasis, resulting in loss of lens transparency. Therefore, it is important to develop protective strategies for apoptosis of hLECs. As demonstrated in the current study, damage to lenses exposed to H_2O_2 *ex vivo* was initially observed in the equatorial region, spreading throughout the superficial cortex by 24 h and into the deeper regions by 48 h. The H_2O_2-induced opacity of lenses was improved following treatment with CGA. Evidence from these *ex vivo* experiments indicated that the observed lens opacity can be induced by oxidative stress, and can be ameliorated by CGA.

In conclusion, the present study demonstrates that H_2O_2 can induce human lens epithelial cell apoptosis and lens opacification, whereas CGA can effectively attenuate human lens epithelial cell apoptosis and lens opacity under oxidative stress mediated by H_2O_2. CGA, a potent antioxidant, can effectively protect HLE-B3 cells against H_2O_2-induced oxidative stress and apoptosis via Bcl-2/Bax signaling pathway, suggesting that CGA may be applied clinically as a potential protective treatment for cataract formation.

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