Article

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Chlorogenic Acid Alleviates High Glucose-induced HK-2 Cell Oxidative Damage through Activation of KEAP1/NRF2/ARE Signaling Pathway

Tian Dai[1,*], Yunfang Xiao[1], Hong Zhang[1], Yingying Shi[1], Fangyuan Wu[1]

[1]Renal Rheumatology and Immunology Department, The People's Hospital of PingYang, 325400 Wenzhou, Zhejiang, China
*Correspondence: daitianm@163.com (Tian Dai)
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Objective: To investigate the alleviating effect of chlorogenic acid (CGA) on oxidative damage in high glucose (HG)-induced HK-2 cells and to explore its potential mechanisms.

Methods: We cultured the human proximal tubular cell line HK-2 and divided them into the control group and different concentrations of CGA groups (0, 5, 10, 25, 50, 100, 200 μM). The trypan blue dye test was used to detect CGA's potential cytotoxicity on HK-2 cells. Then, we treated HK-2 with HG and CGA; the Cell Counting Kit-8 (CCK-8) method was used to detect the cell viability of HK-2 cells in each group. Flow cytometry was employed to measure the apoptosis rate of cells. Western blot was performed to detect the expression of apoptosis proteins B-cell lymphoma-2 (BCL-2), BCL-2-associated X protein (BAX), cysteinyl aspartate specific proteinase (CASPASE)-9, and CASPASE-3. In addition, enzymatic activities, including superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), and lipid peroxide (LPO), were measured with the corresponding detection kits. 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) assay and flow cytometry were performed to detect reactive oxygen species (ROS) production. Western blot analysis and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) were conducted to evaluate protein and mRNA expressions of the Kelch-like ECH-associated protein-1 (KEAP1)/Nuclear factor erythroid 2-related factor 2 (NRF2)/Antioxidant Response Elements (ARE) signaling pathway.

Results: The outcomes showed that, in a dose-dependent way, CGA dramatically increased the vitality of HK-2 induced by HG. Furthermore, CGA significantly reduced the HG-stimulated HK-2 cell apoptosis, which may be linked to the promotion of BCL-2 and the suppression of BAX, cleaved-CASPASE-3, and cleaved-CASPASE-9 expression. In HK-2 cells, CGA reduced the formation of ROS generated by HG levels and markedly boosted the activity of the antioxidant enzymes SOD, GSH-Px, and CAT. Furthermore, compared with the HG group, CGA significantly raised NRF2 nuclear expression and downregulated NRF2 cytosolic expression and increased the mRNA expression of *NRF2* and its target genes, heme oxygenase-1 (*HO-1*), *KEAP1*, and NAD(P)H dehydrogenase quinone 1 (*NQO1*).

Conclusion: These results show that CGA might be useful in managing oxidative damage in HG-induced HK-2 cells.

Keywords: chlorogenic acid; high glucose; HK-2 cells; apoptosis; oxidative stress

Introduction

The most frequent consequence of diabetes is diabetic nephropathy (DN), which also poses a significant risk for cardiovascular disease and is a primary cause of end-stage renal disease [1]. According to statistics, DN affects at least 30% of diabetic patients, raising the risk of diabetes-related death [2]. Most DN lesions were glomerulosclerosis, although previous reports have also revealed that diabetic renal damage can happen in the renal tubules [3–5]. Due to an increase of the apoptosis rate in renal tubular epithelial cells, hyperglycemia aggravates renal tubular damage in DN [6]. Although various treatment approaches, including medications, dietary changes, and other complementary therapies, have been developed to slow the progression of DN and kidney damage, their efficacy has not been sufficiently demonstrated. Therefore, creating a unique and consistently successful treatment method against DN is imperative.

The study has observed oxidative damage and renal tubular cell death in the early stages of DN [7]. Additionally, reactive oxygen species (ROS) may cause renal tubular epithelial cells to undergo apoptosis, which might worsen kidney damage in DN [8]. Other reports have shown that inhibition of oxidative stress can improve the symptoms associated with streptozotocin-induced DN [9–11]. Furthermore, mitochondrial ROS can lessen tubular damage brought on by hyperglycemia [12]. As a result, DN treatment that inhibits oxidative stress seems to be a potential approach.

When the body undergoes oxidative stress, Nuclear factor erythroid 2-related factor 2 (NRF2) undergoes phos-

phorylation, leading to its detachment from Kelch-like ECH-associated protein-1 (KEAP1) [13]. It then translocates to the nucleus and attaches itself to the appropriate spot on the Antioxidant Response Elements (ARE). In response to oxidative stress, NRF2 decouples from KEAP1, phosphorylates, moves to the nucleus, and binds to the corresponding site on the ARE. As a result, ARE can trigger the expression of genes encoding antioxidant proteins and downstream phase II detoxification enzymes, thereby augmenting the capacity of cells to withstand oxidative stress [13]. Research has indicated that the KEAP1/NRF2/ARE signaling pathway is a prospective target for therapeutic intervention in illnesses driven by redox imbalance, including DN [14,15].

One type of phenylpropanoid produced by plant cells is chlorogenic acid (CGA). It is produced by condensing the quinic acid's hydroxyl group with the caffeic acid's carboxyl group. This process is known as the shikimic acid pathway. Sweet potatoes, eggplants, and green coffee beans are rich in it [16–18]. In addition to decreasing blood fat and sugar, CGA demonstrates several pharmacological benefits [19–21]. The anti-diabetic and antilipidemic properties of CGA have been demonstrated [22]. The mechanism of CGA's ability to reduce inflammation in renal tissue, prevent cell apoptosis, and preserve renal function in DN rats may involve controlling the production of proteins that cause cell death and blocking the Toll-like receptor 4 (TLR4)/Nuclear factor kappa-B (NF-κB) signaling pathway [23]. Nevertheless, little is known about the function and mechanisms of CGA reducing oxidative stress and HK-2 cell death brought on by high glucose (HG). Consequently, this study aims to examine the impact of CGA on high glucose-induced HK-2 cell death and oxidative stress. This may offer adequate proof of CGA's potential as a therapeutic agent for managing DN.

Materials and Methods

Cell Culture

The BeNa Culture Collection (BNCC339833, Suzhou, China) provided the human proximal tubular cell line HK-2. HK-2 cells were cultured at 37 °C with 5% CO_2 in Dulbecco's modified eagle medium (DMEM)/F12 (A4192002, Gibco, Grand Island, NY USA) containing 10% FBS (10099, Gibco, Grand Island, NY, USA) and 100 units/mL penicillin (V900929, Sigma-Aldrich, St. Louis, MO, USA).

HK-2 cells used underwent testing provided by the BeNa Culture Collection Suzhou, China). Mycoplasma contamination was assessed using the Polymerase Chain Reaction (PCR)-based assay provided by North Na Company to ensure the absence of contamination. Additionally, cytomorphological characterization was conducted using Short tandem repeat (STR) analysis to verify the authenticity and stability of the cell lines. The results indicated the absence of mycoplasma contamination in the tested HK-2 cells and provided authenticated cell characteristics.

Trypan Blue Staining

After being cultivated on 6-well plates for 24 hours, the cells were treated for another 24 hours with various dosages of CGA (5, 10, 25, 50, 100, or 200 µM) (327-97-9, Shanghai Yuanye Biological Technology Co., Ltd., Shanghai, China). Trypan blue solution (72-57-1, Solarbio, Beijing, China) was added to the cell suspension.

Cell Counting Kit-8 (CCK-8)

After being seeded into 96-well plates, HK-2 cells were treated to various dosages of CGA (5, 10, 25, 50, or 100 µM) and 30 mmol/L glucose. After treatment, each well received the addition of 10 µL of CCK-8 (CK04, Dojindo, Shanghai, China) and was allowed to sit at 37 °C for one hour. The microplate reader was utilized to record the absorbance at 450 nm.

Cell Apoptosis Assay

Annexin V-FITC apoptosis detection kit (C1062L, Beyotime Biotechnology, Shanghai, China) was used in flow cytometry to test for cell apoptosis. In 6-well plates, cells (2×10^5 per well) were seeded, adhered overnight, and given varying CGA concentrations for 24 h. TrypLE was used to separate the cells, and 1 mL of binding buffer containing anti-annexin V-APC and HEPS-buffered PBS with 2.5 mM calcium chloride was used to resuspend the separated cells. Following a 15-minute incubation of the mixture at room temperature in a dark environment, PI solution (20 g/mL) was added. Flow cytometry was used to quantify the apoptotic rate (Becton-Dickinson and Company; BD Biosciences, San Jose, CA, USA).

Western Blot

For 30 minutes, cells were lysed in 1 mL of RIPA solution containing Cocktail (K1015, Apexbio, Suzhou, China) and PMSF (P0100, Solarbio, Beijing, China). The Radio Immunoprecipitation Assay (RIPA) buffer, Phenylmethanesulfonyl fluoride (PMSF), and Cocktail have a volume ratio of 100:1:1. The resulting lysate was centrifuged for 15 minutes at 4 °C and 14,000 ×g. Proteins (30 µg/well) were loaded in equal proportions onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), separated at 120 V by electrophoresis, and then transferred at 220 mA onto PVDF membranes (GVWP02500, Millipore, Billerica, MA, USA). Subsequently, the membranes were sealed for four hours using 5% non-fatty milk. They were then incubated for an additional night at 4 °C in the diluted primary antibodies (cysteinyl aspartate specific proteinase (CASPASE)-3: 9662, CASPASE-9: 9508, B-cell lymphoma-2 (BCL-2): 2772, and BCL-2-associated X protein (BAX): 2774, Cell Sig-

Table 1. Designed primer.

Gene	Accession Number (NCBI)	5′- 3′
NRF2	NM_006164	F: CACATCCAGTCAGAAACCAGTGG
		R: GGAATGTCTGCGCCAAAAGCTG
HO-1	NM_002133	F: CCAGGCAGAGAATGCTGAGTTC
		R: AAGACTGGGCTCTCCTTGTTGC
KEAP1	NM_203500	F: CAACTTCGCTGAGCAGATTGGC
		R: TGATGAGGGTCACCAGTTGGCA
NQO1	BC000906	F: CCTGCCATTCTGAAAGGCTGGT
		R: GTGGTGATGGAAAGCACTGCCT
GAPDH	NM_002046	F: GTCTCCTCTGACTTCAACAGCG
		R: ACCACCCTGTTGCTGTAGCCAA

NRF2, Nuclear factor erythroid 2-related factor 2; *HO-1*, heme oxygenase-1; *KEAP1*, Kelch-like ECH-associated protein-1; *NQO1*, NAD(P)H dehydrogenase quinone 1; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

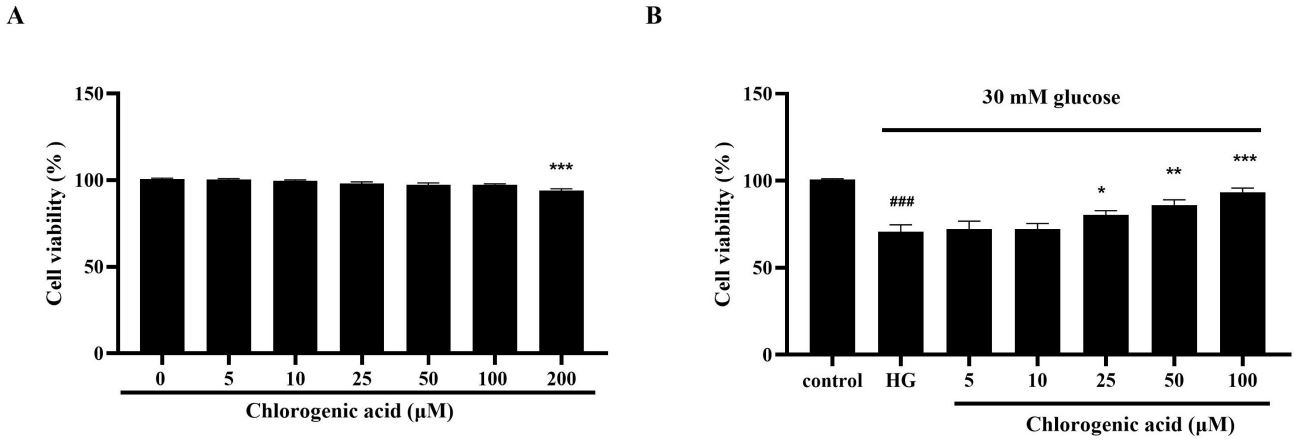

Fig. 1. Cell viability in HK-2 cells stimulated with high glucose (HG) is dramatically increased by chlorogenic acid (CGA). (A) The trypan blue dye test was used to measure the cytotoxicity of 5, 10, 25, 50, 100, and 200 μM CGA on cells (6-well plates, cells 2×10^5 per well). (B) HK-2 cells treated with 5, 10, 25, 50, and 100 μM CGA after being stimulated with HG were used to determine the vitality of the cells with Cell Counting Kit-8 (CCK-8) assay (96-well plates). $^{###}p < 0.001$ vs. control group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. HG group.

naling Technology, Danvers, MA, USA. 1:1000). Following a two-hour incubation period with the goat anti-mouse horseradish peroxidase-labeled secondary antibody (7076, Cell Signaling Technology, USA. 1:2000), membranes were then twice more rinsed with Tris Buffered Saline with Tween 20 (TBST) buffer, each time lasting 15 minutes. The signals were finally detected with an Enhanced chemiluminescence (ECL) detection kit (P0018S, Beyotime, Shanghai, China) with an enhanced chemiluminescence reagent system (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the loading control. The protein bands were analyzed using ImageJ software (version 1.46, U. S. National Institutes of Health, Bethesda, MD, USA).

Measurement of Antioxidant Enzyme Activities

In 12-well plates, cells (1×10^5 per well) were seeded, adhered overnight, and given varying CGA concentrations for 24 h. Cells were collected and washed 1–2 times with 4 °C PBS. The precipitates were homogenized with precooled PBS at 4 °C. The homogenate was then centrifuged at 4 °C and the supernatant was taken as the sample to be tested. The enzyme activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), and lipid peroxide (LPO) were assessed using specific detection kits (A001-1-2, A006-2-1, A007-1-1, A106-1-2, Nanjing Institute of Biological Engineering, Nanjing, China).

2′,7′-Dichlorodihydrofluorescein Diacetate (DCFH-DA) Assay

The DCFH-DA assay was used to measure the amount of ROS. In 24-well plates, 6×10^4 cells were cultured in each well. Cells were stained for one hour in the dark us-

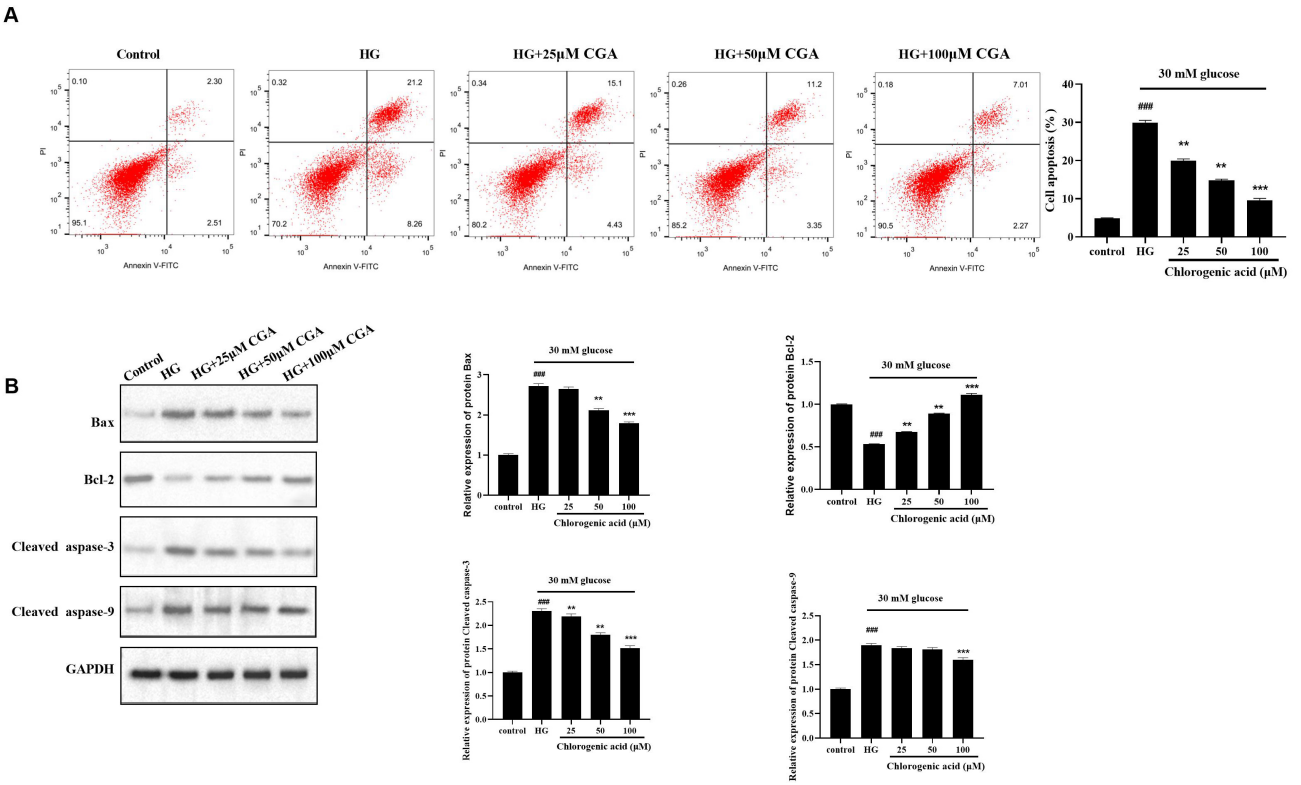

Fig. 2. CGA prevents HK-2 cells stimulated with HG from going through apoptosis. (A) As detected by flow cytometry, HK-2 cells were treated with HG and CGA underwent apoptosis (6-well plates, cells 2×10^5 per well). (B) Expression of the apoptosis-related proteins cysteinyl aspartate specific proteinase (CASPASE)-3, CASPASE-9, BCL-2-associated X protein (BAX), and B-cell lymphoma-2 (BCL-2) was assessed by Western blot analysis (6-well plates, cells 2×10^5 per well). $^{###}p < 0.001$ vs. control group. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. HG group.

ing 20 μM of DCFH-DA (Sigma-Aldrich; Merck KGaA) at room temperature. Next, fluorescence intensity was measured using fluorescence spectrophotometry (RF-5300, Hitachi, Tokyo, Japan) and excitation and emission wavelengths of 485 and 530 nm.

RT-qPCR

Trizol (AM9738, Thermo Fisher Scientific, Waltham, MA, USA) was used to separate total RNA from cells, and DNaseI (K2981, Thermo Fisher Scientific, Waltham, MA, USA) was then applied. The High-Capacity cDNA Reverse Transcription Kit (4368814, Thermo Fisher Scientific, Waltham, MA, USA) generated cDNA from 1 μg of total RNA. The amplification process was conducted using ABI PRISM 7700 (Thermo Fisher Scientific, Waltham, MA, USA). The $2^{-\Delta\Delta Ct}$ technique was utilized to standardize the relative mRNA levels of target genes to the expression of *GAPDH*. Table 1 lists the primer pairs used in this investigation.

Statistical Analysis

GraphPad Prism (version 5.0, GraphPad Software, Inc., San Diego, CA, USA) was used to conduct all statistical analysis. Every test set comprises three duplicates. The means of the two groups were compared using a *t*-test, and the results are shown as the means ± SEM. $p < 0.05$ was the threshold for statistical significance.

Results

Cytotoxic Effect of CGA on HK-2 Cells

We assessed the potential cytotoxicity of CGA on HK-2 cells. The results of the trypan blue dye test revealed that treatment with CGA after 24 hours, there was a significant change in HK-2 cell cytotoxicity at concentrations of 200 μM in contrast to the control group ($p < 0.001$), treatment with CGA for 24 h was not significantly changed at concentrations ranging from 5 to 100 μM compared with the control ($p > 0.05$) (Fig. 1A). Based on the trypan blue dye test results, 200 μM CGA was not selected as one of the experimental concentrations for the following investigations.

Impact of CGA on HK-2 Cell Survival after HG Stimulation

The effect of CGA on HG-induced HK-2 cell viability was examined using the CCK-8 test. Results of CCK-8 showed that HG significantly inhibited cell viability compared to the control group. Nevertheless, the administration

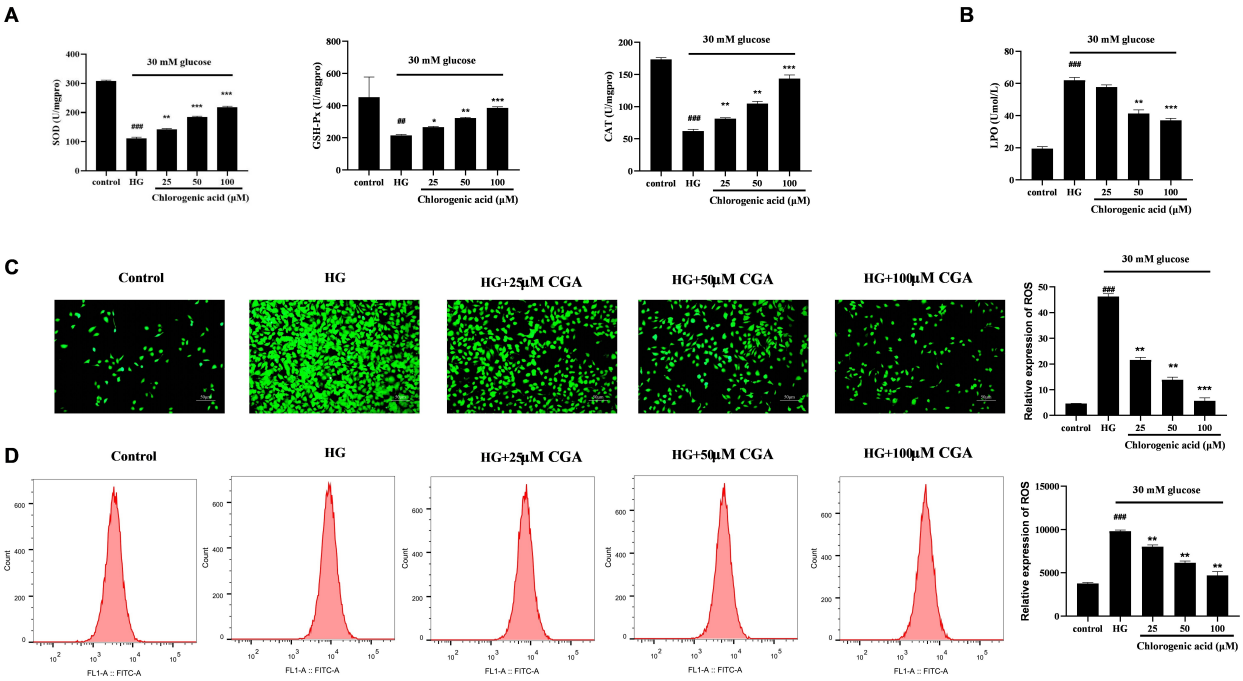

Fig. 3. CGA dramatically boosts the activities of antioxidant enzymes and reduces the generation of reactive oxygen species (ROS) in HK-2. (A) After being treated with HG and CGA, the activity of the antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) was assessed (12-well plates, 1×10^5 cells). (B) After receiving HG and CGA therapy, lipid peroxide (LPO) activity was noted (12-well plates, 1×10^5 cells). (C) HK-2 cell generation of ROS was analyzed using the 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) assay (24-well plates, 6×10^4 cells), ×200. (D) HK-2 cells stimulated with HG and CGA were tested for ROS generation using flow cytometry (6-well plates, cells 2×10^5 per well). $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. control group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. HG group.

of 25, ($p < 0.05$), 50, ($p < 0.01$), and 100, ($p < 0.001$) μM of CGA markedly improved cell viability when compared to the HG group, ($p < 0.001$) (Fig. 1B). Based on the results of the CCK-8 test, 25, 50, and 100 μM CGA were selected as the experimental concentrations for subsequent experiments.

Impact of CGA on HG-induced Apoptosis in HK-2 Cells

Flow cytometry was used to ascertain how CGA affected the HG-induced apoptosis of HK-2 cells. The proportion of apoptotic cells in the HG was significantly higher compared to the control group, ($p < 0.001$, Fig. 2A) of apoptotic cells. Treatment with CGA significantly decreased the amount of cell apoptosis caused by HG in HK-2 cells treated with 25, ($p < 0.01$), 50, ($p < 0.01$), and 100 μM of CGA, ($p < 0.001$) (Fig. 2A).

Western blotting results showed that HG significantly decreased BCL-2 expression while increasing BAX, cleaved CASPASE-3, and cleaved CASPASE-9 expressions ($p < 0.001$). 100 μM of CGA therapy significantly attenuated these effects ($p < 0.05$) (Fig. 2B).

Impact of CGA on Antioxidant Enzyme Activity and ROS Generation in HK-2 Cells Stimulated with HG

The antioxidant enzymes CAT, GSH-Px, and SOD were assessed for their activity. The findings showed that, in comparison to the control, exposure to HG dramatically reduced the activities of SOD, GSH-Px, and CAT ($p < 0.001$). However, when treated with CGA at concentrations of 25 μM, 50 μM and 100 μM, a significant increase in antioxidant activity was observed ($p < 0.05$, Fig. 3A). Additionally, it was shown that, in comparison to the control, HK-2 cells stimulated with HG had considerably greater LPO activity, an indicative of lipid peroxidation ($p < 0.001$). However, CGA reduced this activity dose-dependently (Fig. 3B).

The findings of the DCFH-DA assay showed that while CGA significantly reduced ROS generation, HG significantly increased ROS production ($p < 0.001$) (Fig. 3C). Moreover, the findings of flow cytometry demonstrated that CGA significantly and dose-dependently reduced ROS generation by HK-2 cells stimulated with HG ($p < 0.05$) (Fig. 3D).

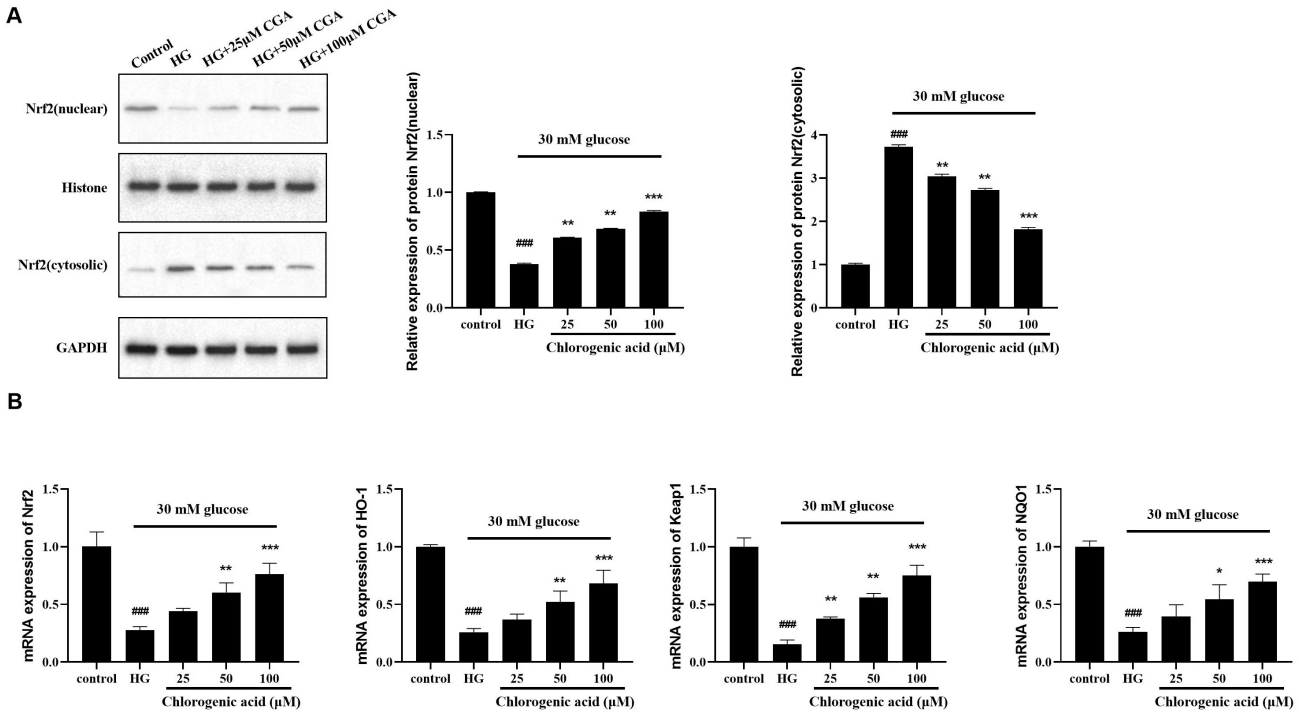

Fig. 4. CGA controls the NRF2/Antioxidant Response Elements (ARE) signaling pathway. (A) Nuclear and cytosolic expression of Nrf2 was examined using Western blot analysis (6-well plates, cells 2×10^5 per well). (B) Reverse Transcription-Polymerase Chain Reaction (RT-PCR) assessed NRF2's mRNA expression in relation to its target genes, *NQO1*, *HO-1*, and *KEAP1* (6-well plates, cells 2×10^5 per well). $^{###}p < 0.001$ vs. control group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. HG group.

Impact of CGA on the HK-2 KEAP1-NRF2-ARE Signaling Pathway in HK-2 Cells Stimulated with HG

The findings of Western blot analysis showed that HG significantly reduced NRF2 nuclear expression ($p < 0.001$), whereas CGA significantly raised it ($p < 0.05$) Furthermore, HG elevated the cytosolic expression of NRF2 ($p < 0.001$), which was dramatically downregulated in the CGA-treated group ($p < 0.05$) (Fig. 4A).

Compared with the control group, the results of Reverse Transcription-Polymerase Chain Reaction (RT-PCR) showed that HG substantially decreased the mRNA expression of *NRF2* and its target genes, heme oxygenase-1 (*HO-1*), *KEAP1*, and NAD(P)H dehydrogenase quinone 1 (*NQO1*) ($p < 0.001$). But in a dose-dependent way, CGA significantly reduced this impact ($p < 0.05$) (Fig. 4B).

Discussion

The investigation results showed that whereas CGA treatment considerably enhanced the viability of HK-2 cells induced by glucose, excessive glucose significantly decreased cell viability. These results imply that CGA might be a helpful tool in reducing the harmful effects of elevated hyperglycemia on HK-2 cells. Our findings concur with earlier research suggesting that CGA enhances the inhibition of HG on cell division [24].

The safeguarding influence of CGA against HG-induced cell death has been documented in earlier research [25,26]. This investigation showed that CGA significantly inhibited the expression of BAX, cleaved CASPASE-3, and cleaved CASPASE-9 and promoted the production of BCL-2, which reduced the amount of cell death caused by HG. These results add to the evidence supporting CGA's protective ability against elevated glucose.

Oxidative stress stands out as a pivotal factor in the progression of DN, as numerous investigations have demonstrated [27–30]. ROS generation is significantly increased by hyperglycemia, which is the leading cause of diabetic complications [31]. One of the first things that cause DN to develop is excessive glucose stimulation [32]. Moreover, oxidative stress and ROS are produced by HG. In DSS-induced colitis mice, a prior study discovered that CGA supplementation significantly raised T-AOC and the activities of GSH-Px, CAT, and SOD, among other antioxidant enzymes [33]. Similar antioxidant qualities were observed with CGA in our investigation; it also decreased ROS generation and increased the activity of SOD, GSH-Px, and CAT. This indicates that elevated glucose might cause oxidative damage while CGA can prevent it.

One of the most significant endogenous defense systems against oxidative stress is the KEAP1/NRF2/ARE pathway, which is now targeted by medications to treat disorders of the kidneys, lungs, digestive tract, central ner-

vous system, and cardiovascular system [34]. Numerous downstream defensive genes, including *HO-1* and *NQO1*, can be activated by the NRF2/ARE pathway [35]. The KEAP1/NRF2/HO-1 signaling pathway has been demonstrated to be modulated by CGA in thioacetamide-induced acute liver toxicity [36]. Through modulation of the NRF2/HO-1 and NF-κB pathways, CGA has also been demonstrated to protect DN by decreasing oxidative stress and inflammation [37]. Under typical circumstances, ubiquitination of Keapl and proteasome degradation in the cytoplasm are the primary mechanisms behind the downregulation of NRF2 expression in cells. To protect the cells from excessive ROS stimulation, Nrf2 enters the nucleus when activated, for example, by ROS, and joins with the ARE sequence to trigger the transcription of several antioxidant genes [38]. Our research shows that whereas CGA markedly boosted NRF2 nuclear expression, HG dramatically lowered it. This further confirms the conclusions of our study. Furthermore, in contrast to the control group, excessive glucose dramatically reduced the mRNA expression of *NRF2* and its target genes including *HO-1*, *KEAP1*, and *NQO1*. CGA, however, significantly lessened this impact. These findings suggest that CGA modulates the KEAP1/NRF2/ARE signaling pathway to reduce oxidative stress and HK-2 cell apoptosis caused by elevated glucose.

Finally, the study offered new proof that, in HG-induced HK-2 cells, CGA may both increase cell survival and suppress cell apoptosis and oxidative stress. By stimulating the synthesis of antioxidant enzymes and activating the KEAP1/NRF2/ARE PAThway, CGA improves a cell's capacity to eliminate ROS, preserve the redox equilibrium, and lessen oxidative damage. Taken together, these results showed that CGA could be helpful in DN management.

Conclusion

These results show that CGA might be useful in managing oxidative damage in HG-induced HK-2 cells by activating THE KEAP1/NRF2/ARE PATHway.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

Author Contributions

TD, YX and HZ designed the research study. YX and HZ performed the research. TD and YS provided help and advice on the experiments. YS and FW analyzed the data. All authors were involved in drafting and critical revision of the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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