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Original Research Article

Glechoma longituba (Lamiaceae) alleviates apoptosis in calcium oxalate-induced oxidative stress in kidney proximal tubule epithelial cell line, HK-2

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Abstract

Purpose: *To investigate the effects of Glechoma longituba on calcium oxalate (CaOx)-induced stress in HK-2 cells as a possible treatment strategy for nephrolithiasis (kidney stones).*
Methods: *Human kidney HK-2 cells were treated with CaOx and Glechoma longituba at different concentrations. The levels of reactive oxygen species (ROS), lactate dehydrogenase (LDH), and malondialdehyde (MDA) were measured. Cell apoptosis and viability were assessed by flow cytometry and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, respectively, while apoptosis-related proteins were determined using western blotting. The levels of the nuclear factor-erythroid 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1), and NADPH-quinone-oxidoreductase 1 (NQO-1) genes were evaluated using quantitative real-time polymerase chain reaction (qRT-PCR). Using lentivirus, Nrf2 was knocked down in HK-2 cells, and this was confirmed by both qRT-PCR and western blotting. Scramble and si-Nrf2 transfected HK-2 cells were treated with CaOx and Glechoma longituba, and ROS levels and apoptosis were also assessed.*
Results: *CaOx significantly increased the levels of ROS, LDH and MDA, while Glechoma longituba pre-treatment attenuated these elevations in a dose-dependent manner. CaOx treatment increased cell apoptosis and decreased cell viability ($p < 0.05$), while Glechoma longituba pre-treatment abolished these effects in a dose-dependent manner. Glechoma longituba pre-treatment significantly upregulated the expressions of Nrf2, HO-1 and NQO-1 ($p < 0.05$). In HK-2 cells, Si-Nrf-2 attenuated the effects of Glechoma longituba pre-treatment on cell oxidative stress and apoptosis induced by CaOx.*
Conclusion: *Glechoma longituba pre-treatment attenuates cell apoptosis and oxidative stress induced by CaOx via Nrf2/HO-1 signalling pathway. Thus, the plant is a potential source of agents for the treatment of nephrolithiasis.*

Keywords: *Glechoma longituba, Nephrolithiasis, Nuclear factor-erythroid 2-related factor 2, Oxidative stress, Apoptosis*

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INTRODUCTION

Nephrolithiasis is a common kidney disease and a major cause of morbidity worldwide [1]. Over the past two decades, the prevalence of nephrolithiasis has increased by 70% among

adults [2]. In addition, the incidence of nephrolithiasis among adolescents has also increased by 6–10% annually [3,4]. The increase of nephrolithiasis is a challenge to human health, and an economic burden for families and society [5]. Currently, surgery and medical treatments are the main therapies for nephrolithiasis, but the outcome remains unsatisfactory. The recurrence of nephrolithiasis is approximately 50% within 10 years of diagnosis [6]. Thus, it is important to determine the mechanism of nephrolithiasis to provide new insight for improved treatment of nephrolithiasis.

Calcium oxalate (CaOx) stones are considered to constitute the majority of calculi in kidney stones, which are characterized by hypercalciuria, attached CaOx stones, and interstitial calcium phosphate deposits [7]. CaOx not only obstructs ureters, but also induces reactive oxygen species (ROS), which results in renal cellular injury and inflammation [8,9]. Liang *et al.* have reported that androgen receptor degradation enhancer, ASC-J9, inhibits the formation of CaOx crystals by regulating oxalate biosynthesis and oxidative stress both *in vivo* and *in vitro* [10]. In addition, erythrocyte oxidative damage contributes to the tubular damage and stone formation [11]. Recently, several herbal remedies have been used as treatments for nephrolithiasis in clinics, owing to their regulation of CaOx formation [12,13]. However, their underlying mechanisms remain unclear.

In the current study, a traditional Chinese medicinal plant, *Glechoma longituba,* was explored to identify its effects on oxidative damage of renal epithelial cell induced by CaOx, to provide new insights into the treatment of nephrolithiasis.

EXPERIMENTAL

Cell culture and treatment

Human kidney epithelial cells, HK-2, and a human embryonic kidney cell line, 293T, were used in this study. The HK-2 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM)/F12 medium (Gibco, Grand Island, NJ, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) at 37°C in a humidified incubator with 5% CO_2. In addition, 293T cells were maintained in DMEM medium supplemented with 10% FBS at 37°C in a humidity incubator with 5% CO_2 . When grown to 70–80% confluency, as monolayers, the HK-2 cells were seeded in a 6-well plate and treated with or without 1.0, 2.0, or 4.0 mg/mL of *Glechoma longituba* solution. One hour later, the cells were treated with or without 67 μg/cm^2 CaOx.

Preparation of CaOx crystals

To obtain CaOx crystals, equal volumes of 10 mM NaOx and $CaCl_2$ were mixed at room temperature. After crystals formed, the suspension was equilibrated at 4°C for 3 days. Then, the crystals were washed with double-distilled water (DDW) twice, and dried at 60°C. Subsequently, Fourier transform infrared (FT-IR) spectroscopy analysis was used to confirm the crystals of the formed CaOx. After confirmation, 5 mg/mL CaOx solution was prepared in sterile phosphate-buffer saline (PBS).

Preparation of *Glechoma longituba* solution

Glechoma longituba were purchased from Beijing Tongrentang (Beijing, China). One thousand grams of raw material was immersed in 5.0 L of DDW for 60 min and further extracted for 25 min. The mixed materials were filtered, and the supernatant was collected. The residue was mixed with 3.0 L of DDW, boiled, and filtered. Then, the first and second aqueous extracts were mixed, re-filtered, concentrated, lyophilized, and stored at -80°C. In the current study, *Glechoma longituba* powder was prepared at three concentrations: 1.0, 2.0, and 4.0 mg/mL, for subsequent investigations.

Assessment of reactive oxygen species (ROS), lactate dehydrogenase (LDH), and malondialdehyde (MDA)

ROS levels in HK-2 cells in 6-well plates were evaluated using the ROS-specific fluorescent dye, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (Invitrogen, Carlsbad, CA, USA), according the manufacture's protocol. The LDH cytotoxicity assay detection kit (Beyotime, Haimen, China) was utilized to determine LDH levels in the cell medium according to the manufacturer's protocol. A commercial TBARS Assay Kit (Cayman Chemical Co., Ann Arbor, MI, USA) was used to detect MDA, which is a thiobarbituric acid reactive substance (TBARS) and an indicator of cell lipid peroxidation, according to the manufacturer's protocol.

Assessment of apoptosis

After treatment with *Glechoma longituba*, the apoptosis levels of HK-2 cells in a 6-well plate were determined using the V-FITC/PI apoptosis kit (MaiBio, Hong Kong, China) according to the manufactures protocol. The fluorescence of fluorescein isothiocyanate (FITC) and propidium

iodide (PI) were measured using a Beckman flow cytometer and estimated using Expo32 software (Beckman Coulter, Sacramento, CA, USA).

Assessments of cell viability

Cell viability of HK-2 cells was estimated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HK-2 cells were seeded onto a 96-well plate at a density of 5.0×10^3 cells/well, and treated with *Glechoma longituba* and CaOx as previously mentioned. After treatment for 48 h, cells were incubated with 2.0 mg/mL MTT solution (Sigma-Aldrich, St. Louis, MO, USA) for each well at 37°C for 4 h. Following this, formazan crystals in cells were dissolved with dimethylsulfoxide and the optical density of the product was measured at 540 nm using a Varioskan® Flash Top Spectral Scanning Multimode Reader (Thermo Fisher Scientific, Waltham, MA, USA). Each sample had five replicates and each experiment was performed in triplicate.

Western blotting

After treatment, the medium was discarded and cells were lysed using RIPA lysis buffer containing a protease inhibitor cocktail (Roche, Indianapolis, IN, USA). The suspension was then ultrasonicated and centrifuged at 4°C, at 1,000 g, for 10 min. The supernatant was collected, quantified using the Bradford assay, and boiled with an equal volume of loading buffer. Then, 10 µg of total protein was loaded for SDS-PAGE followed by transfer of the proteins onto a polyvinylidene difluoride (PVDF) membrane. Blots were blocked with 5% non-fat milk at room temperature for 1 h, and then incubated with rabbit anti-human Nrf2 (1:1000, Sigma Aldrich), anti-pro-caspase-3 (1:3000; Cell Signalling Technology, Danvers, MA, USA), anti-pro-caspase-9 (1:1000; Cell Signalling Technology), anti-cleaved-caspase-3 (1:3000; Cell Signalling Technology), anti-cleaved-caspase-9 (1:1000; Cell Signalling Technology), anti-Bcl-2 (1:1000; Sigma-Aldrich), anti-Bax (1:2000; Sigma-Aldrich), or anti-β-actin (1:5000; Sigma-Aldrich) at 4°C overnight. Blots were incubated with secondary antibody at room temperature for 1 h and visualized using the Enhanced Chemiluminescence Detection Kit (SuperSignal West Femto, Thermo Scientific).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA from HK-2 cells was extracted using TRIzol reagent (Thermo Scientific, Shanghai, China) following the manufacture's protocol. Then, 2–3 µg of total RNA was used to synthesize cDNA using a cDNA synthesis kit (TakaRa, Tokyo, Japan). Subsequently, qRT-PCR was performed using SYBR Green premix (TakaRa) on an ABI Prism® 7500 instrument (ABI, Foster city, CA, USA). The amplification conditions were as follows: 94°C for 20 s, 40 cycles at 94°C for 3 s, and 55°C for 30 s. The primer sequences were as follows: Nrf2, forward: 5'-TCTTGCCTCCAAAGTATGTCAA-3' and reverse: 5'-CACGGTCCACAGCTCATC-3'; HO-1, forward: 5′-TGAAGGAGGCCACCAAGGAGG-3′ and reverse: 5′-AGAGGTCACCCAGGTA GCGGG-3′; NQO-1, forward, 5′-GCAGTGC TTTCCATCACCAC-3′ and reverse: 5′-TGGAG-TGTGCCCAATGCTAT-3′; and β-actin: forward: 5'-GGGGACCTGACTGACTA-3' and reverse: 5'-TGAAGGTAGTTTCGTGGATGC-3'. Gene expression levels were assessed using the $2^{-\Delta\Delta Ct}$ method [14] and β-actin was used as the internal control.

Statistical analysis

GraphPad Prism (version 6.0; GraphPad Software, La Jolla, CA, USA) was utilized to perform statistical analyses. Continuous variables are presented as the mean ± standard deviation (SD). Comparisons between groups were assessed using Student's *t*-test, and a significant difference was considered when $p < 0.05$.

RESULTS

Glechoma longituba inhibited the oxidative stress induced by CaOx.

ROS, LDH, and MDA levels in HK- 2 cells were significantly decreased in CaOx-treated cells compared with the control group ($p < 0.01$). However, *Glechoma longituba* significantly abolished these elevations in a dose-dependent manner. (Figure 1).

Effect of *Glechoma longituba* on HK-2 cell proliferation and apoptosis

CaOx treatment significantly decreased cell viability of HK-2 cells, but pre-treatment with *Glechoma longituba* significantly attenuated the effect of CaOx on HK-2 cells in a dose-dependent manner (Figure 2 A). Moreover, *Glechoma longituba* pre-treatment reversed the elevated apoptosis induced by CaOx in HK-2 in a dose-dependent manner (Figure 2 B). The western blotting results showed that CaOx induced the expression of Bax, cleaved-caspase-3 and cleaved-caspase-9, and reduced the expression levels of Bcl-2, pro-caspase-3 and

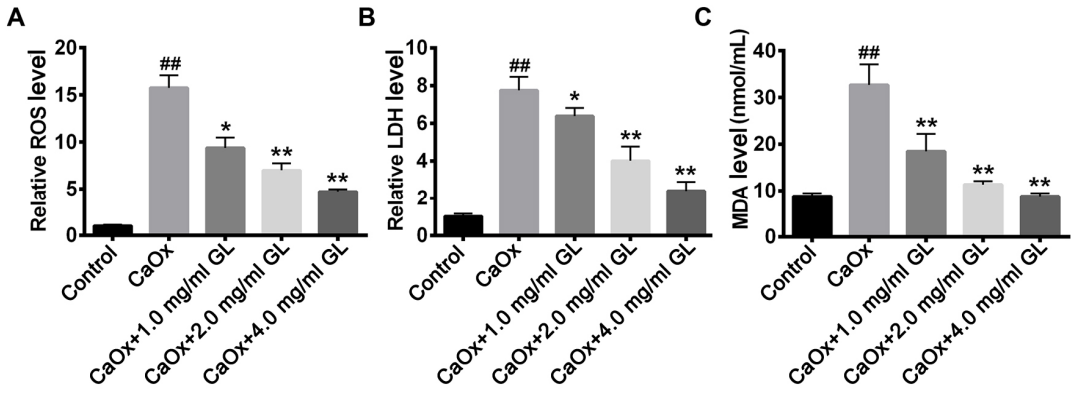

Figure 1: Expression levels of ROS, LDH and MDA in HK-2 cells treated with CaOx and *Glechoma longituba*. A, Relative ROS levels in HK-2 cells; B, relative LDH levels in HK-2 cells; and C, relative MDA levels in HK-2 cells. ROS, reactive oxygen species; LDH, lactate dehydrogenase; MDA, malondialdehyde; GL, *Glechoma longituba*. CaOx, calcium oxalate. $^{\#\#}p < 0.01$, compared with the control group; $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the CaOx group

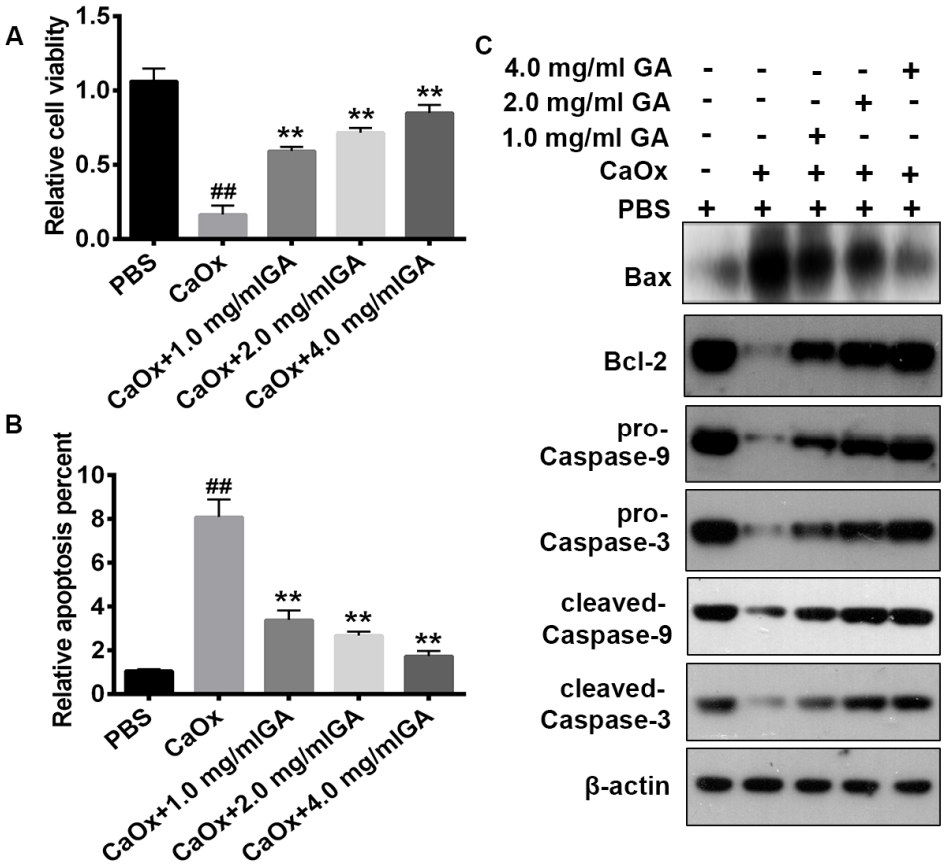

Figure 2: *Glechoma longituba* (GA) attenuated the effects of CaOx on the cell viability and inhibited apoptosis of HK-2. A, Cell viability of HK-2 cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay; B, apoptosis of HK-2 cells was determined using flow cytometry; C, the expression of apoptosis-associated proteins in HK-2 cells was determined using western blotting. $^{\#\#}p < 0.01$ compared with the control group; $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the CaOx group. CaOx, calcium oxalate; PBS, phosphate-buffered saline

pro-caspase-9 in HK-2 cells. However, *Glechoma longituba* pre-treatment reversed these effects in a dose-dependent manner (Figure 2 C).

Effect of *Glechoma longituba* on the expression of Nrf2, HO-1 and NQO1

The expression levels of *Nrf2*, *HO-1* and *NQO1* in HK-2 cells were determined using qRT-PCR. The analytical results showed that the expression

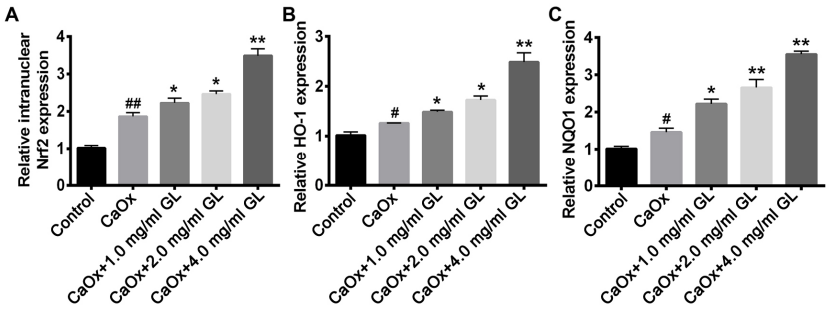

Figure 3: Relative expressions of the *Nrf2*, *HO-1* and *NQO1* genes using quantitative real-time PCR. A, Relative expression of *Nrf2*; B, relative expression of *HO-1*; and C, relative expression of *NQO1*. $^{\#\#}p < 0.01$ compared with the control group; $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the CaOx group. CaOx, calcium oxalate; GL, *Glechoma* longituba

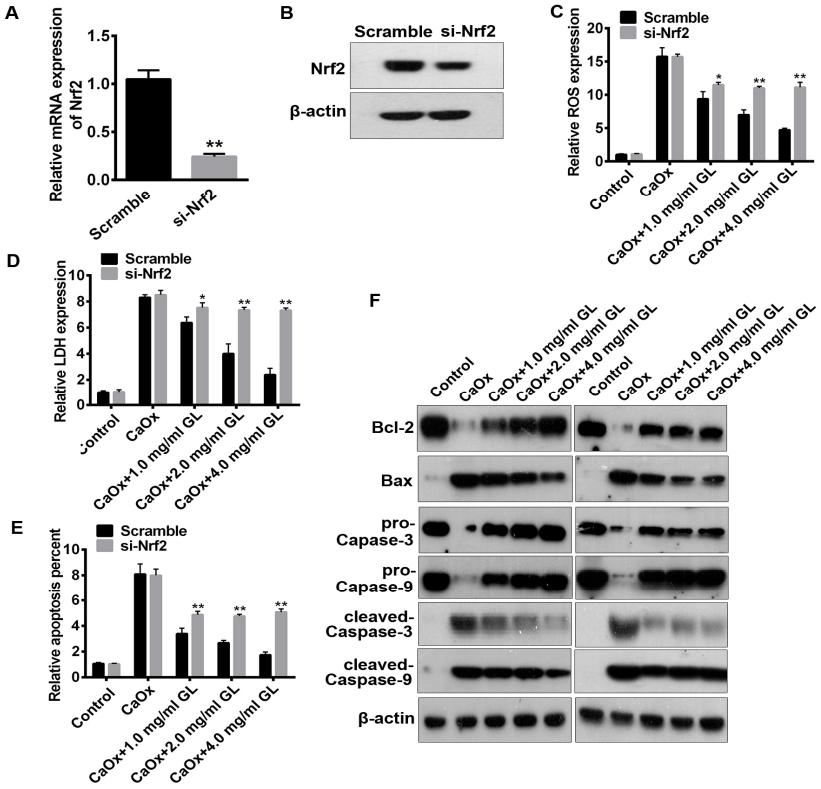

Figure 4: GL effects on si-Nrf2 in HK-2 cells attenuated the effects of GL against CaOx. **A,** Expression of the *Nrf2* gene using quantitative real-time PCR in HK-2 cells; B, relative expression of Nrf2 protein as measured by western blotting in HK-2 cells; C, relative expression of reactive oxygen species (ROS) levels determined in HK-2 cells; D, relative expression of lactate dehydrogenase (LDH) determined using a commercial kit in HK-2 cells; E, relative expression of apoptosis determined using flow cytometry in HK-2 cells; and F, expression levels of apoptosis-associated proteins using western blotting in HK-2 cells. ROS, reactive oxygen species; GL, *Glechoma longituba;* CaOx, calcium oxalate. $^{*}p < 0.05$, $^{**}p < 0.01$, compared with the scramble group

levels of *Nrf2*, *HO-1* and *NQO1* were significantly upregulated following treatment with CaOx, but *Glechoma longituba* pre-treatment markedly improved these effects in a dose-dependent manner (Figure 3).

Knock-down Nrf2 abolished the effects of *Glechoma longituba* on cell apoptosis

To further determine the underlying mechanism of *Glechoma longituba*, Nrf2 was knocked down in HK-2 cells, and the levels of ROS, LDH, and apoptosis were estimated after treating with CaOx. The knockdown efficacy of Nrf2 was confirmed using both qRT-PCR (Figure 4 A) and western blotting (Figure 4 B). Compared with the control group, CaOx significantly increased the levels of ROS, LDH, and apoptosis in all groups, while *Glechoma longituba* pre-treatment abolished these elevations in all groups, but this effect was attenuated in the si-Nrf2 transfected group as compared with the scramble

transfected group (Figure 4 C–E). In addition, the western blotting also showed that *Glechoma longituba* pre-treatment did not reverse the increased expression of Bax-2, cleaved-caspase-3 and cleaved-caspase-9, and the decreased expression of Bcl-2, pro-caspase-3 and caspase-9 induced by CaOx in the si-Nrf2 HK-2 cells (Figure 4 F).

DISCUSSION

Oxidative damage is considered an important promoter of renal epithelial damage and CaOx formation. In the current study, *Glechoma longituba* pre-treatment significantly abolished the elevations of ROS and apoptosis in HK-2 cells induced by CaOx via the Nrf2/HO-1 signalling pathway. *Glechoma longituba* pre-treatment also increased the viability of HK-2 cells after treatment with CaOx. These findings indicated that *Glechoma longituba* had a promising effect on the alleviation of renal epithelial damage caused by CaOx.

Glechoma longituba is a common plant distributed in Asia, America, and Europe. It has been widely used in disease treatment for centuries in China, including for dropsy, urolithiasis, asthma, and cholelithiasis [15]. The chemical constituents of this plant include phenolic acids, triterpenoids, essential oils, and flavonoids [16]. Among them, phenolic acids, such as caffeic, rosmarinic, chlorogenic acids, and flavonoids possess promising antioxidant properties [17-19]. Liang *et al* have shown that *Glechoma longituba* had anti-urolithic affects via inhibiting urinary stone formation and urolithiasis-related protein expression levels, and elevating antioxidant levels [20].

Wang *et al* reported that *Glechoma longituba* decreased the oxidative, inflammatory and fibrotic levels via downregulating NF-κB, AP-1, and TGF-β/Smad signalling pathway components in a bile duct ligation-induced liver injury model [21]. In the present study, *Glechoma longituba* pre-treatment significantly down-regulated ROS, LDH and MDA levels induced by CaOx. It also decreased the elevation of apoptosis, but increased the reduction of cell viability induced by CaOx in a dose-dependent manner. Thus, it was shown that CaOx treatment increased cell apoptosis via upregulating oxidative damage in renal cells, while *Glechoma longituba* possessed a relatively high antioxidant ability, which significantly reversed the elevated oxidative stress to suppress cell apoptosis and promote cell viability. These findings indicated that *Glechoma longituba* may provide a promising therapeutic method for nephrolithiasis treatment via decreasing cell oxidative stress.

Nrf2 is an oxidative stress responding transcriptional factor that regulates the expression of several antioxidant genes via binding to their antioxidant response elements [22]. Specifically, HO-1 and NQO1 are two important downstream targets of Nrf2 that respond to oxidative stimuli [23, 24]. Liang *et al* have demonstrated that dihydroquercetin decreased oxidative stress by inducing the expression of HO-1 and NQO-1 via a Nrf2-dependent antioxidant pathway [25]. Kang *et al* reported that Schisandrae semen essential oil attenuated oxidative damage via upregulating the Nrf2/HO-1 signalling pathway in C2C12 murine skeletal muscle cells [26].

Sahu *et al* found that *Lagerstroemia speciosa* L. decreased apoptosis in isoproterenol-induced cardio-toxic mice via downregulating oxidative stress dependent on Nrf2/HO-1 [27]. CaOx is a common component of calculi in stone formers, and induces ROS to increase the oxidative damage in cells [8,9]. In the current study, we observed that CaOx treatment increased the expression of Nrf2, HO-1 and NQO-1, indicating that CaOx upregulated oxidative stress in HK-2 cells, while HK-2 cells had the ability to respond to this stimulation via the Nrf-2 signalling pathway. A similar result was also reported by Lin *et al* in H_2O_2-treated HK-2 cells [28]. However, *Glechoma longituba* pre-treatment promoted increased levels of Nrf2, HO-1 and NQO-1 in HK-2 cells, to prevent oxidative damage induced by CaOx.

To further confirm this signal transduction mechanism, Nrf2 was knocked down in HK-2 cells and treated with *Glechoma longituba* pre-treatment and/or CaOx. The results showed that si-Nrf2 prevented the inhibitory effects of *Glechoma longituba* on the increased cell oxidative stress and apoptosis induced by CaOx. These findings suggest that *Glechoma longituba* pre-treatment significantly attenuated cell oxidative damage and apoptosis induced by CaOx via Nrf2/HO-1 signalling pathway.

CONCLUSION

CaOx exerts oxidative stress on HK-2 cells, which significantly inhibits cell viability and increases cell apoptosis. *Glechoma longituba* has antioxidant effects that attenuate the cell oxidative damage and apoptosis induced by CaOx via the Nrf2/HO-1 signalling pathway, in a dose-dependent manner. These findings indicate that *Glechoma longituba* is a promising

therapeutic agent for the treatment of nephrolithiasis.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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