

Hyperoside protects human kidney-2 cells against oxidative damage induced by oxalic acid

YONGLIANG CHEN, LIHONG YE, WANGJIAN LI, DONGZHANG LI and FENG LI

Department of Urology, Shaoxing Central Hospital, Shaoxing, Zhejiang 312030, P.R. China

Received January 12, 2017; Accepted August 8, 2017

DOI: 10.3892/mmr.2018.8948

Abstract. The majority of renal calculi (kidney stones) are calcium stones. Oxidative damage to renal tubular epithelial cells induced by reactive oxygen species (ROS) is the predominant cause of calcium oxalate stone formation. Hyperoside (Hyp) is a flavonol glycoside extracted from medicinal plants and appears to exhibit potent antioxidant activity in various cells. The aim of the present study was to investigate the protective effect of Hyp on renal cells exposed to oxidative stress simulated by oxalic acid (OA), and to determine whether the underlying mechanism involves the nuclear factor E2-related factor2 (Nrf2)-antioxidative response element signaling pathway. The study determined the indicators of high oxidative stress, including ROS and hydrogen peroxide (H_2O_2) in human kidney-2 cells and the results demonstrated that the levels of ROS, as evaluated by flow cytometry, and H_2O_2 were significantly increased following treatment with OA (5 mmol/l) for 24 h (OA group), compared with those in the untreated control group. The increased activity of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in these cells explained this observation, as it is a major source of ROS. The results demonstrated that, in the OA group, the adhesion of calcium oxalate crystals and lactate dehydrogenase (LDH) were significantly increased, and MTT assay demonstrated that cell viability was inhibited, compared with the control, which indicated that severe injury of cells was induced by OA. However, when the cells were pre-treated with Hyp prior to treatment with OA (drug group), the levels of ROS and H_2O_2 , and the activities of NADPH oxidase and LD were increased, and the adhesion of calcium oxalate crystals to cells was reduced, compared with the OA group. Western blot analysis and reverse transcription-quantitative polymerase chain reaction demonstrated that the protein and mRNA expression

levels of Nrf2, heme oxygenase-1 (HO-1) and NAD(P)H: quinineoxidoreductase 1 (NQO1) in the Hyp groups were significantly increased, compared with those in the OA group, with the exception of Nrf2 mRNA. These results suggested that Hyp had a marked protective effect on renal cells against the oxidative damage and cytotoxicity simulated by OA. This is the first report, to the best of our knowledge, demonstrating that the ability of Hyp to enhance the endogenous functions of antioxidation and detoxification in cells may involve the Nrf2/HO-1/NQO1 pathway.

Introduction

Next to prostate disease and urinary tract infections, kidney stones are the third most common urinary tract problem (1,2). The severe colic pain experienced by individuals with kidney stones cannot be relieved with conventional painkillers (3). Kidney stones are affected by several factors, including lifestyle, dietary, ethnicity and geographic location (4). The majority of kidney stones (60-80%) are calcium stones, containing calcium oxalate, calcium phosphate, cystine and uric acid (5). It has been suggested that the formation of calcium oxalate stones is closely associated with injury to kidney epithelial cells, which may be the initiating event in the development of calcium oxalate stones. Several investigations have confirmed that renal tubular epithelial cells are severely injured in the presence of high concentrations of oxalic acid (OA) and urinary calcium via lipid peroxidation (6,7). Following oxidative damage, there are changes to the structure and physiological characteristics in the cytomembrane of kidney epithelial cells supplying the effective adhesion site, which promotes the growth of calcium oxalate stones (2,8).

Previous studies have suggested that oxidative damage to renal tubular epithelial cells primarily involves reactive oxygen species (ROS), which are substances with high oxidative activity, including O_2^- , hydrogen peroxide (H_2O_2) and active hydroxyl (OH) (8,9). ROS exist as the normal metabolites in cells and tissues, and their levels are balanced by the antioxidative effects of substances, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), heme oxygenase-1 (HO-1) and nicotinamide adenine dinucleotide phosphate (NADPH) quinone oxidoreductase 1 (NQO1) (10,11). These antioxidants are important in metabolism, defense, antioxidation and detoxification in the body by protecting cells against injury simulated by ROS (12). However, cells

Correspondence to: Dr Lihong Ye, Department of Urology, Shaoxing Central Hospital, 1 Huayu Road, Keqiao, Shaoxing, Zhejiang 312030, P.R. China
E-mail: ebr965411@163.com

Key words: renal calculi, kidney stones, hyperoside, oxalic acid, reactive oxygen species, nuclear factor E2-related factor 2, heme oxygenase-1, NAD(P)H: quinoneoxidoreductase 1

are damaged when the production of ROS exceeds the free radical-scavenging activity of antioxidants (13). There are two sources producing ROS: Mitochondria and NADPH (14). The ROS derived from mitochondria are an important source in the majority of cells and tissues (14,15). The dysfunction of mitochondria has been shown to simulate the formation of kidney calculi, which indicates that ROS is induced by mitochondria (13,16). Human and animal experiments have demonstrated that another major source of ROS is NADPH oxidase, which is observed in chronic kidney diseases, including interstitial nephritis, hypertensive renal injury and diabetic renal diseases (17). Therefore, the simulation of antioxidants and inhibition of ROS are beneficial for patients with chronic kidney diseases.

In conventional treatment, the recurrence rate of calcium oxalate stones is high, despite advanced minimally invasive techniques, including extracorporeal shockwave lithotripsy, percutaneous nephrolithotomy and ureteroscopic lithotrips, being applied in clinical treatment; this leads to a heavy burden on patients with the disease (2,18). At present, there is a focus on medicinal plants in investigations, which are characterized by being a reliable source, and a cost-effective, safe and acceptable source of active compounds for pharmaceutical use. Hyperoside (Hyp) is a flavonol glycoside extracted from medicinal plants, and appears to show wide pharmacological activities, including anti-inflammatory, antidepressant, antioxidative, antibacterial and antiviral effects, and has been shown to protect cells from oxidative injury (19,20). Hyp exhibits potent antioxidative activity, and can upregulate the expression levels of protective proteins and reduce ROS levels (21). It has been shown that nuclear factor E2-related factor2 (Nrf2) is involved in the protective effect of Hyp on hepatocytes (22,23). Studies have shown that Nrf2 and its cytoplasmic protein, kelch-like ECH-associated protein 1 (Keap1), are central regulators of cell antioxidant responses (24,25). Nrf2 regulates the expression of antioxidant proteins and phase II detoxification enzymes by interacting with a cis-acting transcriptional regulatory element, designated as antioxidant response element or electrophile response element (EpRE). When the cells are exposed to a high level of oxidative stress, Nrf2 is transferred to the nucleus and forms a heterodimer with small Maf proteins, which upregulates the expression of genes containing an EpRE (AU-rich element (ARE)), including SOD, HO-1 and NQO1 (26).

Numerous investigations have revealed that the antioxidative function of Nrf2 involves the Keap1/Nrf2/NQO1/HO-1 pathway (27). However, to the best of our knowledge, there are no reports on the protective effect of Hyp against oxidative injury in renal cells caused by a high concentration of OA. The present study investigated the antioxidative effect of Hyp on renal cells *in vitro* and examined whether the underlying mechanism of the effects involve the Keap1/NRF2/NQO1/HO-1 pathway.

Materials and methods

Materials. Human kidney-2 (HK2) cells were obtained from America Type Culture Collection (Manassas, VA, USA). Hyp was purchased from TargetMol (Boston, MA, USA). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

(MTT) assay kit was a product of Beyotime Institute of Biotechnology (Haimen, China). DCFH-DA and the Lactate Dehydrogenase Activity Assay kit were obtained from Sigma; Merck Millipore (Darmstadt, Germany). The NADPH oxidase commercial kit was the product of GenMed Scientifics, Inc. (Arlington, MA, USA). The BCA Protein Assay kit was obtained from Pierce; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Monoclonal antibodies (Keap1; cat. no. ab139729; 1:1,000), Nrf2, (cat. no. ab31163; 1:1,000), HO-1 (cat. no. ab52947; 1:1,000) and NQO1; cat. no. ab10239; 1:100) were purchased from Abcam (Cambridge, UK). The RNeasy mini-kit was the product of Qiagen, Inc. (Valencia, CA, USA). TaqMan Gene master mix and the ABI PRISM 7700 sequence detection system were obtained from Applied Biosystems; Thermo Fisher Scientific, Inc. All chemical reagents, unless otherwise specified, were purchased from commercial sources.

Cell culture. The HK2 cells were cultured in Dulbecco's modified Eagle's medium Mixture F-12 Ham (DMEM/F12) containing 5% PBS and antibiotics, at 37°C under a humidified atmosphere of 5% CO₂ and 95% air, as previously described (28). The cells were grown to ~70-80% confluence and used for the following experiments.

Cell treatment. The cells were seeded in 96-well culture plates at a concentration of 1.5×10^5 cells/ml and incubated for 24 h with 10% fetal bovine serum (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) in DMEM. Cell viability was determined using an MTT assay following treatment with different concentrations of OA and Hyp. The cells were divided into three groups: Control group, OA group and drug groups. The cells in the control group and OA group were incubated with 1 ml of medium containing PBS at 37°C for 4 h. As described previously (29,30), the cells in the drug groups were pre-treated with 1 ml of medium containing PBS and different concentrations of Hyp (50, 100 and 200 μ M) 37°C for 4 h. Subsequently, the cells in the control group were incubated in 1 ml of normal medium for 24 h; the cells in the OA group and drug groups were then treated with 1 ml of medium containing OA (5 mmol/l) for 24 h. Calcium oxalate (0.75 mmol/l) was added to the culture medium of each group. After 15 min, the adhesion of cells to the crystals was observed under a microscope (EVOS FL; Life Technologies; Thermo Fisher Scientific, Inc.).

Evaluation of cell viability using an MTT assay. Cell viability was determined using an MTT assay. The cell suspension was transferred into 96-well plates at a density of 1×10^5 cells/per well, and treated as described above. Subsequently, MTT solution was added to each well at a concentration of 1 mg/ml per well and the cells were incubated at 37°C for 4 h. The absorbance at 540 nm was measured and a reference wavelength at 650 nm was read using a Multiskan FC microplate reader (Thermo Fisher Scientific, Inc.). Cell viability was calculated as a percentage of the average optical density value of the control.

Determination of ROS. The cells collected from the control group, OA group and drug groups were seeded into 96-well plates at a density of 1×10^5 cells/per well, and then washed

with PBS and resuspended in 1 ml Hank's solution. The cells were then incubated in a 20- μ M solution of DCFH-DA (Sigma; Merck Millipore) at 37°C for 1 h. Subsequently; the cells were washed with PBS three times. The production of ROS was measured by the changes in fluorescence (excitation, 488 nm; emission, 510 nm) using flow cytometry.

Determination of H_2O_2 . The production of H_2O_2 was determined as previously described (31) with minor modifications. The concentrations of H_2O_2 in the cells from the control group, OA group and drug groups were measured using the Amplex Red assay kit (Molecular Probes; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The absorbance at 560 nm was read using an Emax precision microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA). The concentration of H_2O_2 in cells was calculated by referring to a standard curve.

Determination of lactate dehydrogenase (LDH) activity. The cells from the control group, OA group and drug groups were resuspended in PBS and crushed using an ultrasonic cell crusher (Ningbo Scientz Biotechnology Co., Ltd., Ningbo, China). The activity of LDH in the cells was detected using the Lactate Dehydrogenase Activity Assay kit (Sigma-Aldrich; Merck Millipore) according to the manufacturer's protocol.

Determination of NADPH activity. The activity of NADPH oxidase in the cells from the control group, OA group and drug groups were detected with the cell NADPH oxidase commercial kit (GenMed Scientifics, Inc.) as described previously (32) with minor modification. Briefly, following treatment, the cells were washed with PBS twice and centrifuged at 12,000 \times g at 4°C for 3 min, following which they were resuspended at 5×10^4 in PBS. Following incubation in 150 μ M NADPH at 30°C (Boehringer Mannheim Biochemicals; Roche Diagnostics, Basel, Switzerland), the consumption of NADPH was measured by the change of absorbance at 340 nm, recorded on a SpectraMax 190 microplate reader (Molecular Devices, LLC). The activity of NADPH oxidase was determined as pmol/l substrate per min/mg protein.

Western blot analysis. The cells were washed with ice-cold PBS and solubilized in cold homogenization buffer (100 mM Tris, 150 mM NaCl and 1% triton X-100) with a cocktail of protease inhibitors. The protein expression levels were determined using the BCA Protein Assay kit according to the manufacturer's protocol. Briefly, the proteins in the cells were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 8-12% gels; Pharmacia; GE Healthcare Life Sciences; Piscataway, NJ, USA) and then transferred onto polyvinylidene difluoride membranes (EMD Millipore Bedford, MA, USA). Following blotting in 5% nonfat dry milk, the membranes were incubated overnight at 4°C with appropriate monoclonal antibodies as follows: Keap1 (cat. no. ab139729; 1:1,000), Nrf2 (cat. no. ab31163; 1:1,000), HO-1 (cat. no. ab52947; 1:1,000), NQO1 (cat. no. ab10239; 1:1,000) and GAPDH (cat. no. ab37168; 1:1,000) from Abcam. The blots were then washed with PBS and incubated with a 1:2,000 dilution of peroxidase-conjugated secondary antibodies (P448; Dako; Agilent Technologies GmbH, Waldbronn,

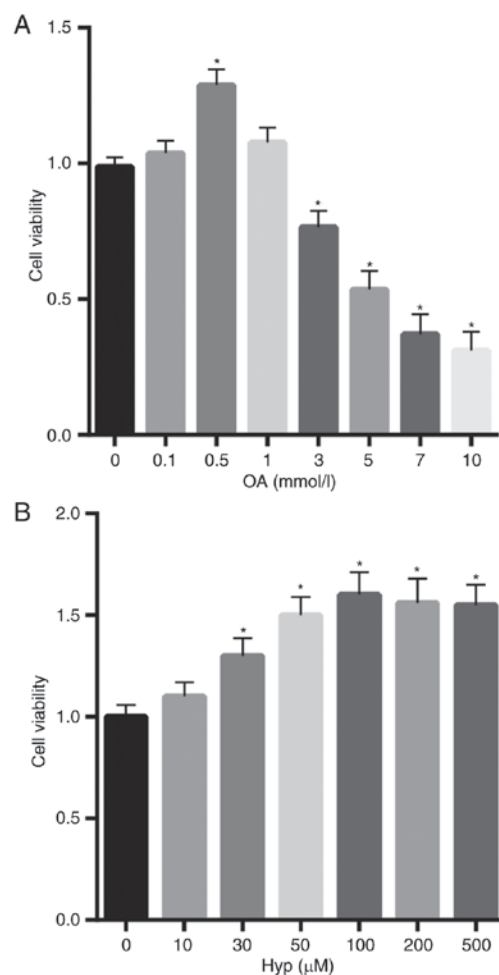


Figure 1. OA and Hyp affect the viability of HK2 cells. (A) Cell viability was decreased in cells exposed to increased levels of OA. (B) Cell viability was increased in cells treated with Hyp, compared with the control, and was marginally decrease data concentration >100 μ M. Data are expressed as the mean \pm standard deviation (n=3). *P<0.05, vs. control (without treatment). OA, oxalic acid; Hyp, hyperoside.

Germany) for 1 h at room temperature. The bands were visualized using an enhanced chemiluminescence (ECL) western blot detection system (ECL or ECL Plus; GE Healthcare Life Sciences). The intensities of the bands were measured using ImageJ software version 1.40 g (National Institutes of Health, Bethesda, MD, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA (1 μ g) was isolated from cells using the RNeasy mini kit (Qiagen, Inc.) according to manufacturer's protocol. Total RNA (1 μ g) was converted to cDNA using the TaqMan RT kit (Thermo Fisher Scientific, Inc.). The reaction conditions were: 4 μ l Oligo d T18 primer (25 pmol/ μ l), 1 μ l Forward Primer (10 μ M), 1 μ l ReversePrimer (10 μ M) and 1 μ l cDNA, 60°C for 42 min and 95°C for 5 min). RT-qPCR analysis was performed using TaqMan Gene master mix (Thermo Fisher Scientific, Inc.). The reaction conditions were: 5 μ l 10X Buffer, 5 μ l M-MLV RT Buffer (5X); denaturation at 95°C for 5 min followed by thermocycling: 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min (30 cycles) finally followed by 72°C for 10 min. The results were analyzed on an ABI PRISM 7700 sequence

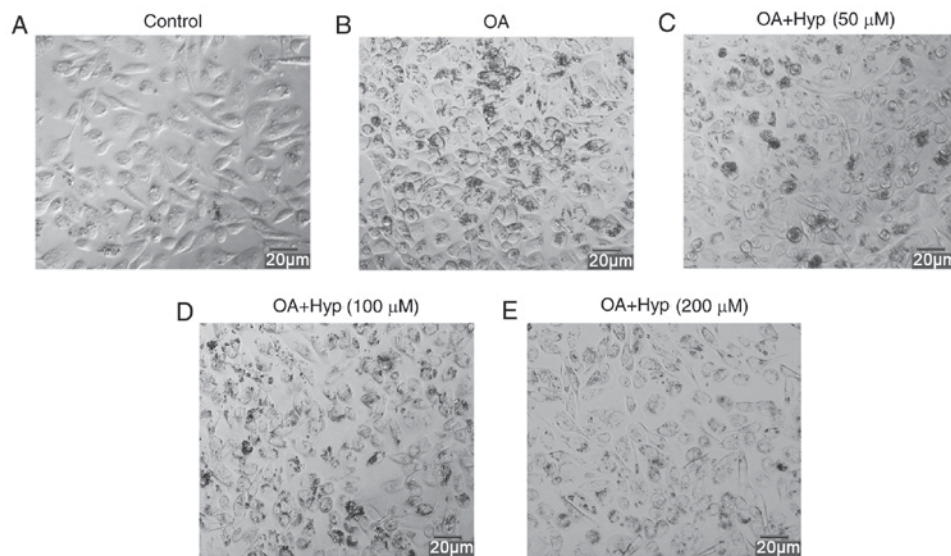


Figure 2. Inhibitory effect of Hyp on the formation of calcium oxalate crystals induced by OA (5 mmol/l; magnification, x400). (A) Control cells remained untreated. (B) Apparent adhesion of calcium oxalate crystals to cells was observed in cells treated with OA. Compared with the OA group, the adhesion of calcium oxalate crystals was increasingly inhibited following pre-treatment with Hyp at (C) 50, (D) 100 and (E) 200 μ M for 4 h prior to OA treatment. OA, oxalic acid; Hyp, hyperoside.

detection system (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The PCR products were verified by melting curve analysis and agarose gel electrophoresis. Data were calculated using the $2^{-\Delta\Delta C_q}$ method (33) and normalized to the levels of GAPDH. The reactions for each sample were performed in duplicate. The sequences of primers were as follows: Keap1, forward 5'-CAACTT CGCGGAGCAGATCG-3' and reverse 5'-AGCTGGCAG TGTGACAGGTT-3'; Nrf2, forward 5'-GGTTGGCCCTTT CCTGCTTT-3' and reverse 5'-ACAGCTCCAACCTGTCCC TT-3'; HO-1, forward 5'-CAGGAGCTGCTGACCCATGA-3' and reverse 5'-AAGGACCCATCGGAGAAGCG-3'; NQO1, forward 5'-TGTTACCCAGGCTGGAGTGC-3' and reverse 5'-CGCCTGTCATCCCAGCTACT-3'; GAPDH, forward 5'-CGGGAACTGTGGCGTGTAG-3' and reverse 5'-ATG ACCTTGCCACAGCCTT-3'.

Statistical analysis. For all assays, data are expressed as the mean \pm standard deviation. All tests were two-tailed and one-way analysis of variance or Student's t-test were used for the statistical comparison of multiple groups. $P < 0.05$ was considered to indicate a statistically significant difference. Statistical comparisons for all samples were calculated using SPSS software, version 18 (SPSS, Inc., Chicago, IL, USA).

Results

Effects of OA and Hyp on cell viability. To examine the effect of OA and Hyp on HK2 cells, the viability of cells was determined following cell treatment with different concentrations of OA and Hyp. The results, as shown in Fig. 1A and B, revealed that cell viability was decreased in the cells treated with increasing levels of OA, but was increased in the cells treated with Hyp, compared with that in the control. Of note, cell viability was marginally decreased following treatment at Hyp concentrations $>100 \mu$ M.

Adhesion of calcium oxalate crystals to cells is increased by OA treatment. Following treatment with OA (5 mmol/l) for 24 h, the cells in the model group (OA treatment only) exhibited apparent calcium oxalate crystals on the surface of cells, compared with the control cells (without treatment), as shown in Fig. 2A and B. However, there was a clear reduction in calcium oxalate crystals on the surface of cells in the drug group (pre-treated with Hyp prior treatment) and the inhibitory effects of Hyp on the adhesion of calcium oxalate crystals to the cells was dose-dependent (Fig. 2C-E). As shown in Fig. 2E, compared with the control, the adhesion of calcium oxalate crystals to cells induced by OA was completely inhibited when the cells were pre-treated with 200 μ M Hyp.

Hyp reduces injury to cells induced by OA through decreasing the levels of ROS. To further evaluate the oxidative damage of OA to cells, the levels of ROS in the cells were determined. The results demonstrated that the level of ROS in the OA group (treated with OA) was 13.9-fold higher than that of the control. In the drug group (pre-treated with Hyp), the relative level of ROS was significantly lower, compared with that in the OA group and decreased in a dose-dependent manner (Fig. 3A-F). The level of ROS in the cells treated with 200 μ M of Hyp demonstrated the maximum reduction of ROS, which was 2.6-fold lower than that of the control (Fig. 3A and F).

Hyp protects cells from cytotoxicity induced by OA treatment. The cell viability in each group was determined using an MTT assay, and the results demonstrated that cell viability in the OA group was reduced to 58%, compared with that in the control. In the drug group, the cell viabilities in the 50, 100 and 200 μ M Hyp pre-treated cells were increased to 69, 83 and 94% of that in the control (Fig. 4A).

LDH, an important enzyme involved in energy metabolism in cells, is simulated by oxidative damage to cells and is considered an indicator of cell damage. As shown in Fig. 4B,

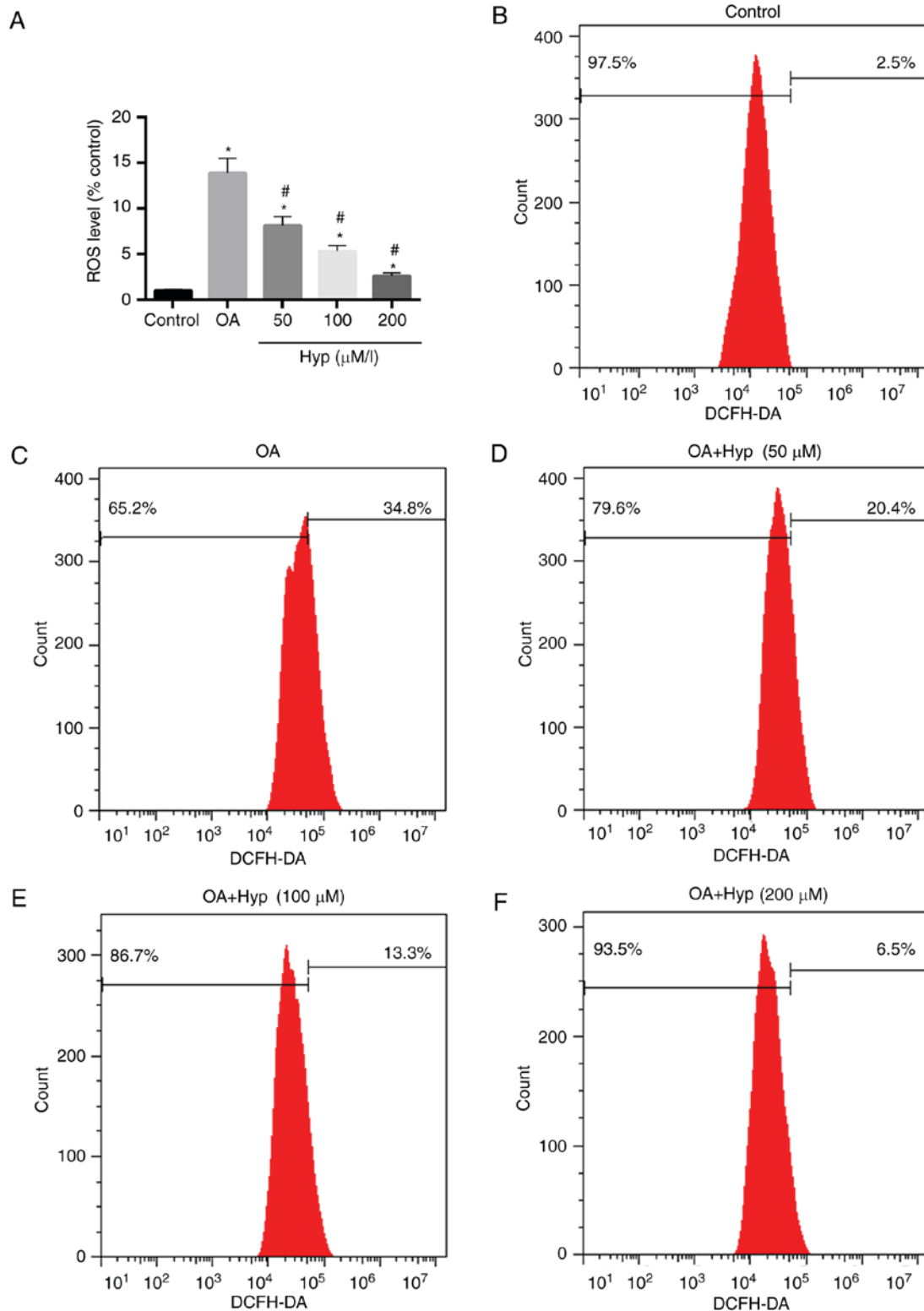


Figure 3. Free radical scavenging abilities of Hyp are effective and dose-dependent in cells treated with OA. (A-F) Flow cytometry demonstrated that ROS levels were significantly elevated following treatment with OA, compared with those in the control, and this effect was reversed in cells pre-treated with Hyp, compared with the OA group. Data are expressed as the mean \pm standard deviation (n=3) *P<0.05, vs. control; #P<0.05, vs. OA alone. OA, oxalic acid; Hyp, hyperoside; ROS, reactive oxygen species.

the activity of LDH in the cells of the OA group was almost 2-fold higher than that of the control group. The effect of OA on the activity of LDH in cells from the drug groups were significantly reduced when the cells were pre-treated with Hyp (50, 100, 200 and μM) for 4 h. These changes in the activity of

LDH in the cells suggested that OA treatment increased injury to the cells, and that Hyp inhibited the injury induced by OA.

In the OA group (Fig. 4C), the concentration of H_2O_2 in cells was significantly elevated following treatment with OA, compared with that in the control group. Hyp demonstrated

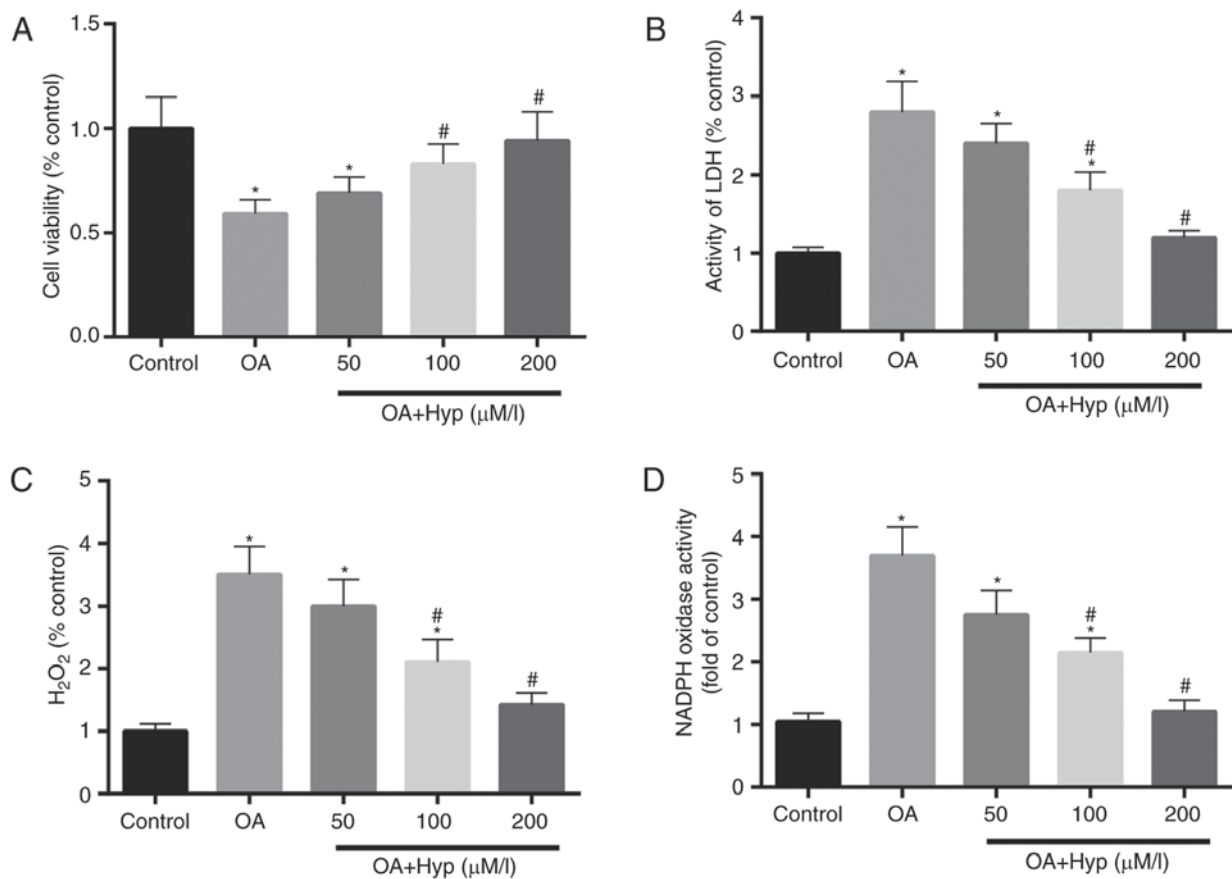


Figure 4. Hyp exhibits a protective effect on cells exposed treatment. (A) Cell viability was reduced by 58% following treatment with OA, and recovered by 69, 83 and 94% when pre-treated with Hyp at 50, 100 and 200 μM , respectively, prior to OA treatment, compared with that in the control. (B) LDH activity, an indicator of cell damage, was decreased to almost 60% in cells pre-treated with 200 μM Hyp, compared with that in the OA. (C) Levels of H_2O_2 were reduced in the Hyp-treated group with respect to the OA group. (D) Activity of NADPH oxidase, considered a major source of ROS, was reduced in a dose-dependent manner in the Hyp pre-treatment group, compared with that in the OA group. Data are expressed as the mean \pm standard deviation ($n=3$). *P<0.05, vs. control; #P<0.05, vs. OA group. OA, oxalic acid; Hyp, hyperoside; ROS, reactive oxygen species; LDH, lactate dehydrogenase; H_2O_2 , hydrogen peroxide; NADPH, nicotinamide adenine dinucleotide phosphate.

a significant reduction on the high level of H_2O_2 simulated by OA. Compared with the control, the level of H_2O_2 in cells recovered to almost a normal level when the cells were pre-treated with 200 μM Hyp.

It is well known that NADPH oxidase is one of the major sources of ROS and an important indicator for metabolic activity. Therefore, the present study measured the activity of NADPH oxidase in cells. The activity of NADPH oxidase appeared to show the same changes as observed for the LDH activity in cells of the control, OA and drug groups. Compared with the OA and drug groups, the increased activity of NADPH oxidase in cells induced by OA was decreased when the cells were pre-treated with Hyp (50, 100 and 200 μM) for 4 h (Fig. 4D).

Expression levels of Nrf2, HO-1 and NQO1 are reduced following treatment with OA and increased by Hyp. In the OA group, the cells were treated with OA for 24 h and the expression levels of Keap1, Nrf2, HO-1 and NQO1 were examined. The results demonstrated that the protein expression levels of Nrf2, HO-1 and NQO1 in the cells were reduced by 74.9, 68.9 and 80.1%, compared with those in the control group, respectively (Fig. 5A and B). In the drug group, the effect of OA on the reduced protein expression of Nrf2 was attenuated by pre-treatment of Hyp; this was dose-dependent and

demonstrated that the expression level of Nrf2 was recovered with pre-treatment with increasing concentrations of Hyp. Similarly, in the drug group, the expression levels of HO-1 and NQO1 were enhanced, compared with those in the OA group, and recovered to 75.7 and 85.4% of the control when pre-treated with 200 μM Hyp. The mRNA expression of HO-1 and NQO1 confirmed the effect of OA and Hyp on the expression levels of HO-1 and NQO1 (Fig. 5C). Similar to the protein expression, the mRNA levels of HO-1 and NQO1 in the OA group were significantly increased following treatment with OA, which was reversed when the cells were pre-treated with Hyp. The mRNA level of Nrf2 was reduced in the OA group, compared with that in the control, however, without significant difference (Fig. 5D and E). Compared with the control, the protein and mRNA levels of Keap1 were not affected by either OA or Hyp treatment. However, the mRNA levels of Nrf2 in the cells of the drug groups were significantly increased, compared with those in the OA and control groups (Fig. 5F and G).

Discussion

Several studies have revealed that hyperoside protects cells from reperfusion-, hydrogen peroxide-, carbon tetrachloride-, and neurotoxicity-induced injury through reducing oxidative

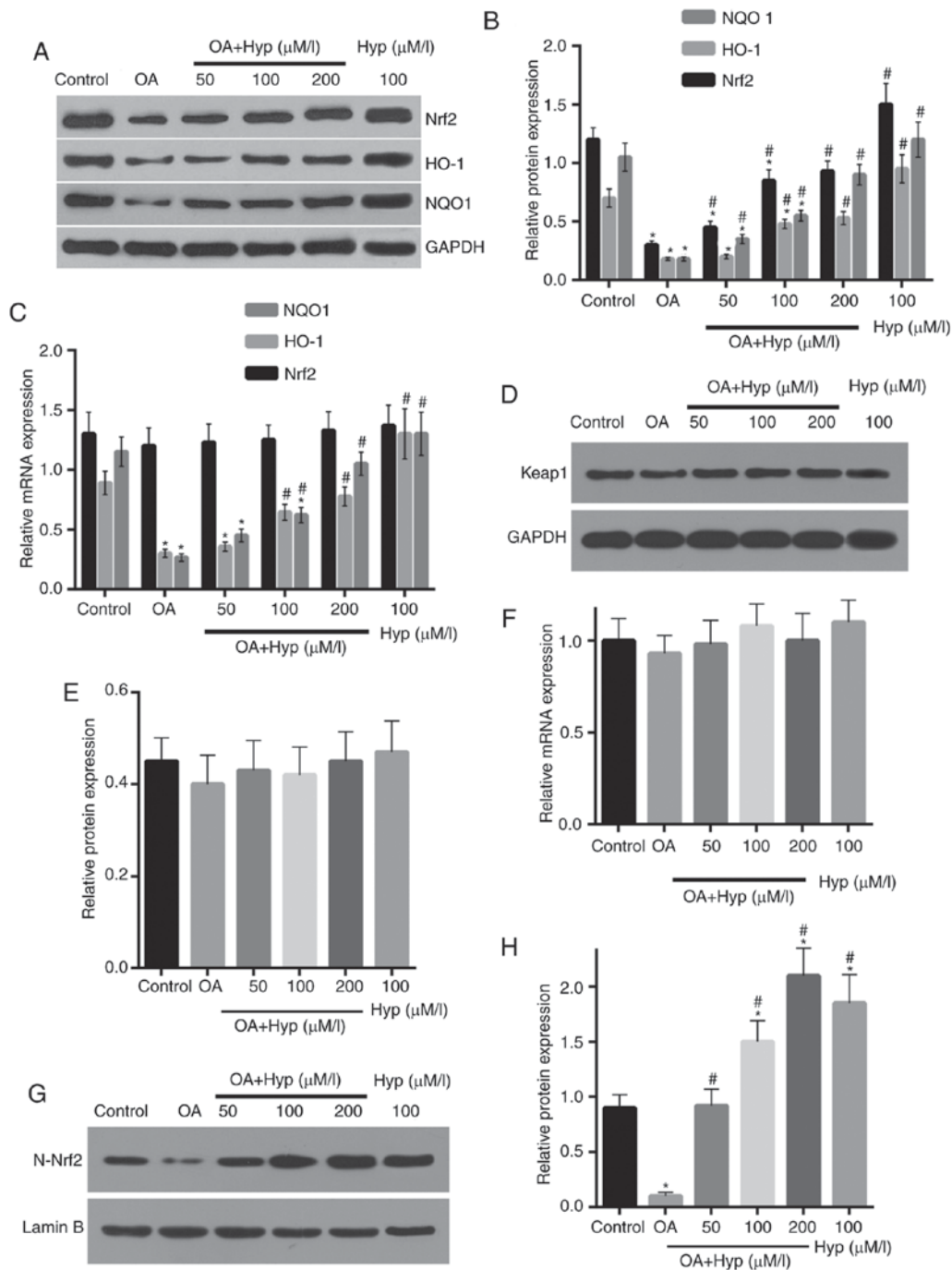


Figure 5. Expression levels of Nrf2, HO-1 and NQO-1 are reduced by OA treatment and enhanced by Hyp treatment. (A) Western blot assays and (B) quantification demonstrated that the protein expression levels of Nrf2, HO-1 and NQO-1 were decreased following OA treatment, and recovered by Hyp pre-treatment in a dose-dependent manner, compared with those in the control. (C) Reverse transcription-quantitative polymerase chain reaction assays demonstrated that the mRNA levels of HO-1 and NQO-1 were significantly reduced following OA treatment, and recovered in the Hyp pre-treated groups, compared with those in the control. No significant changes were observed in the mRNA levels of Nrf2 among groups. (D) Blots of the protein expression of Keap1 and (E) quantification, and the (F) mRNA expression of Keap1 demonstrated no significant changes. (G) Protein expression of nuclear Nrf2 was reduced following OA treatment and recovered in cells pre-treated with Hyp, as shown following (H) quantification. Data are expressed as the mean \pm standard deviation (n=3). *P<0.05, vs. control; #P<0.05, vs. OA group. OA, oxalic acid; Hyp, hyperoside; ROS, reactive oxygen species; Nrf2, nuclear factor E2-related factor 2; HO-1, heme oxygenase-1; NQO1, nicotinamide adenine dinucleotide phosphate quinoneoxidoreductase 1; Keap1; kelch-like ECH-associated protein 1.

stress by increasing the activity of antioxidant enzymes (19-21). Therefore, it is necessary to elucidate the effects of Hyp on kidney cells. The results from previous *in vitro* and animal experiments, and observations from clinical studies have suggested that oxidative damage within renal cells is a major event in the development or recurrence of kidney calculi (1,2). The results of the present study revealed that OA exhibited

cytotoxicity towards kidney cells when its concentration was >1 mmol/l (Fig. 1). In the present study, following treatment with OA (5 mmol/l) for 24 h, calcium oxalate crystals on the surface of cells were observed, compared with the cells in the control group (Fig. 2). The results also demonstrated that significantly higher levels of H₂O₂ and ROS were observed when the cells were treated with OA (5 mmol/l) for 24 h,

compared with those of the control group (Fig. 3). These observations were also supported by the observation that the levels of LDH (Fig. 4), which is an indicator of cell damage, increased following OA treatment and were reduced when pre-treated with Hyp. The levels of H₂O₂ and ROS in the cells pre-treated with Hyp were increased, compared with those in the OA group. These results indicated that Hyp had a protective effect against the oxidative injury simulated by OA. Similar to this result, several studies have shown that the abnormally increased levels of ROS caused by different stimuli in various cells were reduced following Hyp treatment (34-36).

Previous investigations have suggested that NADPH is the major source of ROS (37). NADPH is found in endothelial cells, myocardial cells and renal tubular cells, and are at low levels in normal state. However, levels are significantly upregulated in various diseases, including ischemia-reperfusion injury, diabetes, inflammation, cancer and atherosclerosis (38). The levels of ROS are also increased in cells or tissues (39). The results of the present study demonstrated that the activity of NADPH in cells was increased following OA treatment and decreased following additional pre-treatment with Hyp (Fig. 4). Similarly, a previous study demonstrated that the activities of NADPH in hepatocytes were decreased following Hyp treatment (40). Taken together, these results suggested that the elevated activity of NADPH caused by OA is one of the major sources of ROS in renal cells.

Studies (22,23) have shown that the protective effects of Hyp on oxidative injury in hepatocellular cells induced by H₂O₂ and hypoxia involve the Keap1/Nrf2/ARE pathway, and its downstream genes, including SOD, catalase (CAT), glutathione peroxidase (GPx), HO-1 and NQO1 (41). In the present study, the mRNA and protein expression levels of Keap1, Nrf2, HO-1 and NQO1 were determined following treatment with OA+/-pre-treatment with Hyp. There are two types of antioxidants in aerobic organisms: Enzymatic antioxidants and non-enzymatic antioxidants. HO-1 and NQO1 act as important antioxidants in enzymatic reactions in cells, involving antioxidation and detoxification in cells (42). The results of the present study demonstrated that the proteins expression levels of HO-1 and NQO1 were upregulated in cells treated with OA, compared with those in the control (Fig. 5). These levels were increased when the cells were pre-treated with Hyp prior to OA treatment, and remained lower than those in the control. Similarly, the mRNA levels of HO-1 and NQO1 were also increased in the OA group, compared with those in the control, and this was reversed when cells were pre-treated with Hyp. In addition, the investigations demonstrated that the levels of H₂O₂ and ROS were decreased in cells pre-treated with Hyp, compared with those in the OA group. Therefore, it was hypothesized that HO-1 and NQO1 are important endogenous anti-oxidation proteins in renal cells and are upregulated by Hyp.

ARE is a *cis*-acting regulatory element of genes encoding phase II detoxification enzymes and antioxidant proteins, including SOD, CAT, GPx, HO-1 and NQO1 (43). In addition, Nrf2 is an important transcription factor, which acts on ARE and mediates the expression of detoxification enzymes and antioxidant proteins (44). In the present study, the results demonstrated that the protein level of Nrf2 was reduced

following OA treatment and recovered when pre-treated with Hyp prior to treatment, compared with the control. The mRNA level of Nrf2 was marginally reduced and increased in the OA group and drug group, respectively, compared with that in the control, with no significant changes among the groups (Fig. 4). In addition, Keap1, a negative regulator of Nrf2, was suggested to be involved in the activation of Nrf2 according to a similar study (45), however, no significant changes in the expression of Keap1 were observed in the current study. In the present study, as reported in other investigations (43,44), the upregulated expression of Nrf2 explained the increasing expression levels of HO-1 and NQO1.

In conclusion, the findings of the present study suggested that Hyp is a potential candidate drug for the treatment of kidney calculi and diseases associated with ROS due to its ability to enhance endogenous antioxidation and detoxification functions in cells, the mechanism of which involves the Nrf2/HO-1/NQO1 pathway.

Acknowledgements

This study was supported by the Application Plan of Health Appropriate Technology in Zhejiang Province (grant no. 2014ZHB011).

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