Int J Clin Exp Pathol 2019;12(1):64-76
www.ijcep.com /ISSN:1936-2625/IJCEP0085501

Original Article

Hyperoside alleviated *N*-acetyl-para-amino-phenol-induced acute hepatic injury via Nrf2 activation

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Received September 14, 2018; Accepted November 7, 2018; Epub January 1, 2019; Published January 15, 2019

Abstract: *N*-acetyl-para-amino-phenol (APAP) acute hepatic injury is receiving increasing attention. In the present study, we examined the effects of Hyperoside (Hype) on APAP-induced acute hepatic injury. Oral administration of Hype dose-dependently attenuated the index of hepatic injury, including the production of AST, ALT, and ALP. Increased glutathione (GSH) and decreased ROS production induced by Hype demonstrated its potential antioxidant capacity. In addition, Nrf2 and its downstream genes were markedly activated by Hype. Furthermore, enhanced levels of SOD, GST, and GSH-Px were markedly suppressed by Hype in a dose-dependent manner. At the same time, decreased LPO was also detected in Hype-treated mice. The in vitro study verified a protective effect of Hype on APAP-induced injuries in LO2 cells. Moreover, the regulatory effect was found to be mostly dependent on Nrf2 which decreased LDH and ALT generation and increased cell viability. Nrf2-silenced LOS cells were sensitive to APAP-induced injury, while Hype did not exhibit any further effects on LO2 cells, which demonstrate the critical role of Nrf2 in this process. Taken together, our results demonstrated the ability of Hype to inhibit APAP-induced acute hepatic injury and its potential use in the treatment of Nrf2-associated diseases.

Keywords: Hyperoside, acute hepatic injury, Nrf2

Introduction

Liver, a main organ of detoxification, is the primary site of drug exposure in the body [1]. It was reported that drug-induced liver injury represents a major clinical problem, which causes public concern worldwide [2]. Previous study demonstrated that APAP is widely regarded as analgesic and antipyretic [3]. Even though APAP is safe with therapeutic dose, it can cause severe hepatotoxicity and even lead to death at an overdose [4]. APAP-induced hepatic injury not only can change its biotransformation, with affecting the isoform 2E1 of cytochrome P450 enzymes to form a reactive metabolite, N-acetyl-parabenzo-quinone imine (NAPQI), but also enhance excessive ROS production [5-7]. A high level of ROS led to a depletion of glutathione (GSH) and the inhibited antioxidant enzyme activation, resulting to oxidative stress [2, 8, 9]. Moreover, overproduction of ROS caused dysfunction of organelles, which induced apoptosis of hepatocytes and liver injury [10]. Furthermore, many studies have illustrated that oxidant stress activates a specific downstream signaling pathway, which contributes to APAP-induced hepatotoxicity.

Nuclear factor-erythroid 2 (NF-E2)-related factor 2 (Nrf2) is a redox-sensitive transcription factor, whose activation results in cellular antioxidant responses via modulating several stress-responsive proteins and phase II detoxifying [11, 12]. In the normal conditions, Nrf2 combines with cytoskeleton associated protein, named Kelch-like ECH-associated protein 1 (Keap1), which is a negative regulator of Nrf2. Upon the stimulation of oxidants and electrophiles, Nrf2 could accumulate in the nucleus and then binds to antioxidant response element (ARE), which improves the transcription of its target genes, including heme oxygenase-1 (HO-1), NAD(P)H: quinoneoxidoreductase (NQO1), glutathione Stransferase (GST), and

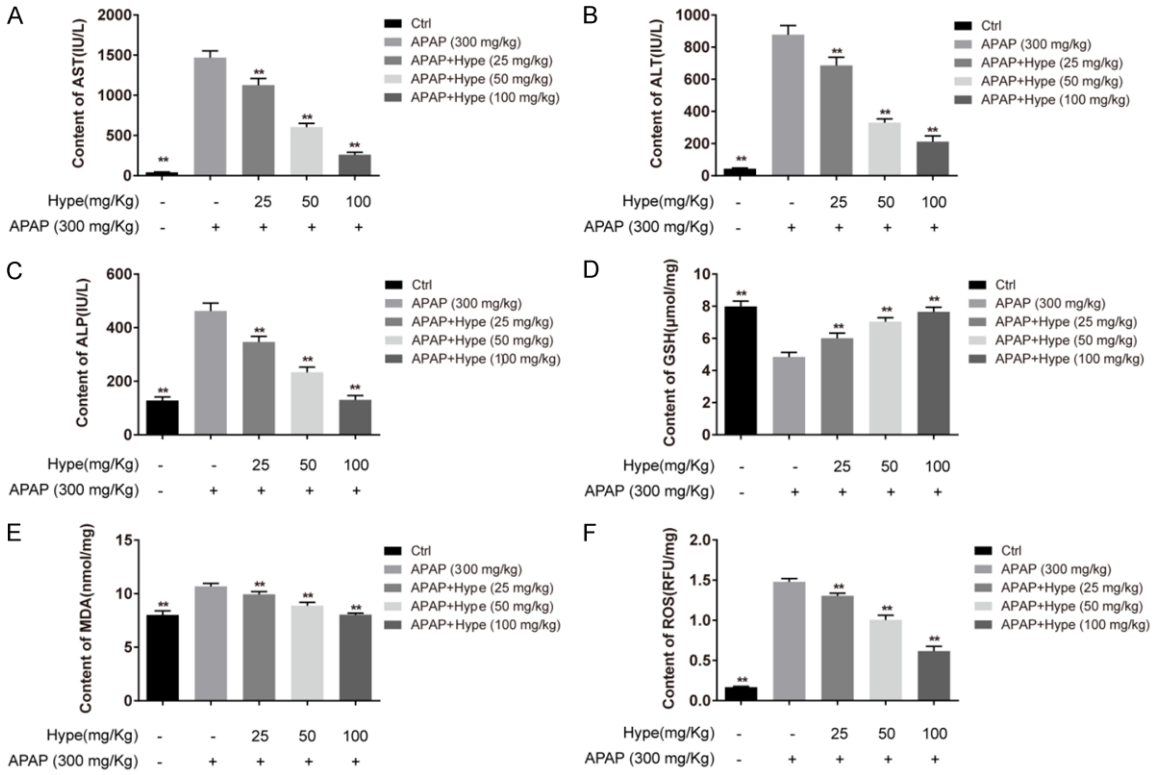


Figure 1. Effect of Hype on APAP-induced acute hepatic injury in vivo. C57BL/6 male mice were treated with Hype (0 mg/Kg), Hype (0 mg/Kg), Hype (25 mg/Kg), Hype (50 mg/Kg) and Hype (100 mg/Kg) at once a day for 7 d. After one hour of the last gavage, mice in each group were injected intraperitoneally with APAP (300 mg/kg), and the blank control group was injected intraperitoneally with equal volume of physiological saline. A-C. ALT, AST and ALP were measured by automatic biochemical analyzer in mice serum. D-F. GSH, MDA and ROS were measured by the GSH, MDA and ROS Kit in mice tissue. Results are mean ± SD for ten individual experiments which, for each condition, were performed in triplicate. Compared with the APAP group: *P < 0.05, **P < 0.01.

glutathione peroxidase (GPx) [13, 14]. The increased expressions of Nrf2 target genes lead to the protection against many types of plentiful inflammatory diseases and other injuries [15]. It was reported that Nrf2 inactivation was detected in an acute hepatic injury mice model. Consistent with this, many Nrf2 inducers could be used for the treatment of hepatic injury [16-18]. Moreover, the detailed mechanism of Nrf2 in APAP-induced hepatic injury should be put forward.

Hyperoside, a main pharmacologically active component from Hypericumperforatum and Prunella vulgaris L., exerts many kinds of biological activities, including antioxidant [19], anti-inflammatory [20], anticancer, and cardiovascular protective function [21]. However, the underlying molecular mechanisms are worth fully elucidating. The present study aimed to determine the protective effect of Hype on APAP-induced liver injuries and identify the potential mechanism involved. We demonstrated that Hype was regarded as an Nrf2 inducer, which decreased the damage to the liver induced by APAP though decreasing ROS production.

Result

The effect of hype on APAP-induced acute hepatic injury in vivo

To explore the regulatory activity of Hype, APAP-induced acute hepatic injury was adopted. As shown in **Figure 1A**, APAP significantly increased the contents of AST (**Figure 1A**) and ALT (**Figure 1B**), while Hype remarkably decreased their production in a dose dependent manner. Moreover, ALP, a marker of acute hepatic injury, was upregulated in APAP group. Different doses of hype administration suppressed this enhancement, and almost decreased the content of ALP to a normal level at 100 mg/kg

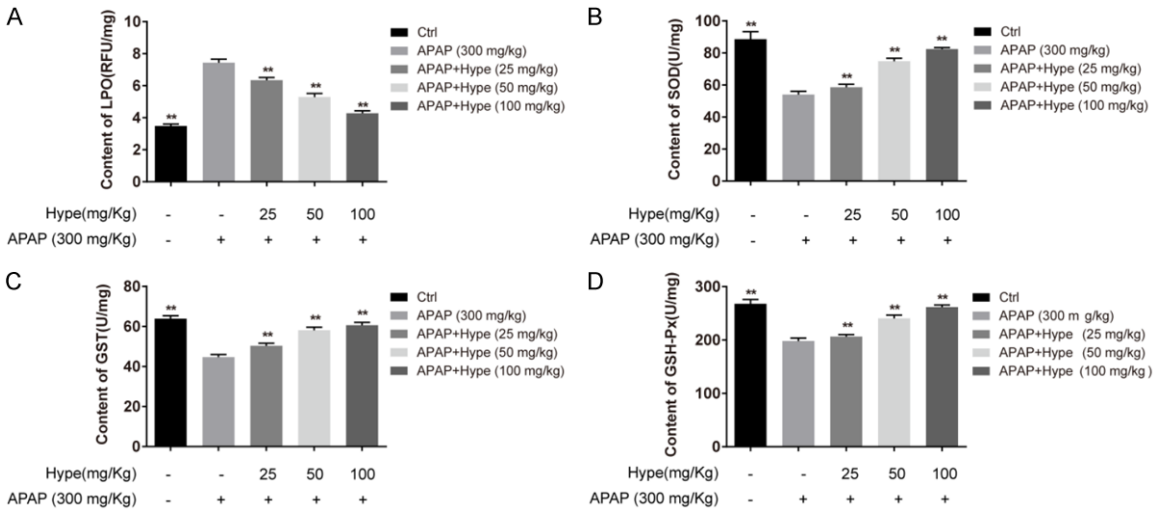


Figure 2. Effect of Hype on APAP-induced acute hepatic injury in vivo. C57BL/6 male mice were treated with Hype (0 mg/Kg), Hype (0 mg/Kg), Hype (25 mg/Kg), Hype (50 mg/Kg) and Hype (100 mg/Kg) at once a day for 7 d. After one hour of the last gavage, mice in each group were injected intraperitoneally with APAP (300 mg/kg), and the blank control group was injected intraperitoneally with equal volume of physiological saline. A-D. LPO, SOD, GST and GSH-Px were measured by the LPO, SOD, GST and GSH-Px Kit in mice tissue. Results are mean ± SD for ten individual experiments which, for each condition, were performed in triplicate. Compared with the APAP group: *P < 0.05, **P < 0.01.

(**Figure 1C**). Increased production of ROS was reported in acute hepatic injury. As expected, compared with control group, APAP obviously suppressed the production of GSH, but Hype rescued decreased GSH in a dose-dependent manner (**Figure 1D**). Higher level of MAD2 induced by APAP was inhibited by hype administration (**Figure 1E**). Furthermore, compared with control group, significant ROS production was detected in APAP group, while Hype suppressed this elevated level dose dependently (**Figure 1F**). Taken together, these results suggested that Hype attenuated the severity of APAP-induced acute hepatic injury, and these therapeutic effects were associated with oxidative stress.

Hype attenuated oxidative stress in APAP-induced acute hepatic injury

Based on the fact that Hype obviously inhibited the increased production of ROS induced by APAP, we further detected the regulation of Hype on other oxidative stress-associated markers. As shown in **Figure 2A**, LPO in liver from hype-treated mice were significantly attenuated more than that of the APAP group. In addition, the reduced SOD activity (**Figure 2B**), GST (**Figure 2C**) and GSH-Px (**Figure 2D**) levels in the liver after APAP treatment were significantly rescued by hype in a dose-dependent manner. These results indicated that Hype attenuated oxidative stress in APAP-induced acute hepatic injury dose-dependently.

Hype induced the Nrf2/ARE pathway in APAP-induced acute hepatic injury

A recent study reported that hype induced the Nrf2/ARE pathway through up-regulating Nrf2 protein level and promoting its nuclear translocation. As shown in **Figure 3B**, APAP treatment led to a decreased Nrf2 protein level, while treatment with Hype dose-dependently reversed it. In the meantime, we found that the mRNA and protein levels of Nrf2 target genes NAPDH-quinone oxidoreductase-1 (NQO1), heme oxygenase-1 (HO-1) and GCLC were down-regulated by APAP administration, which was also reversed by different doses of Hype (**Figure 3A**). Moreover, the suppressed nuclear translocation of Nrf2 (**Figure 3C**, **3D**) and the expressions of its target genes, including GCLC (**Figure 3E**), HO-1 (**Figure 3F**) and NQO1 (**Figure 3G**) were remarkably improved by hype dose-dependently. Immunochemical staining assay revealed that the decreased p-Nrf2, GCLC, HO-1 and NQO1 levels were reversed by Hype dose-dependently. Consistently, ROS production was improved by APAP, while Hype

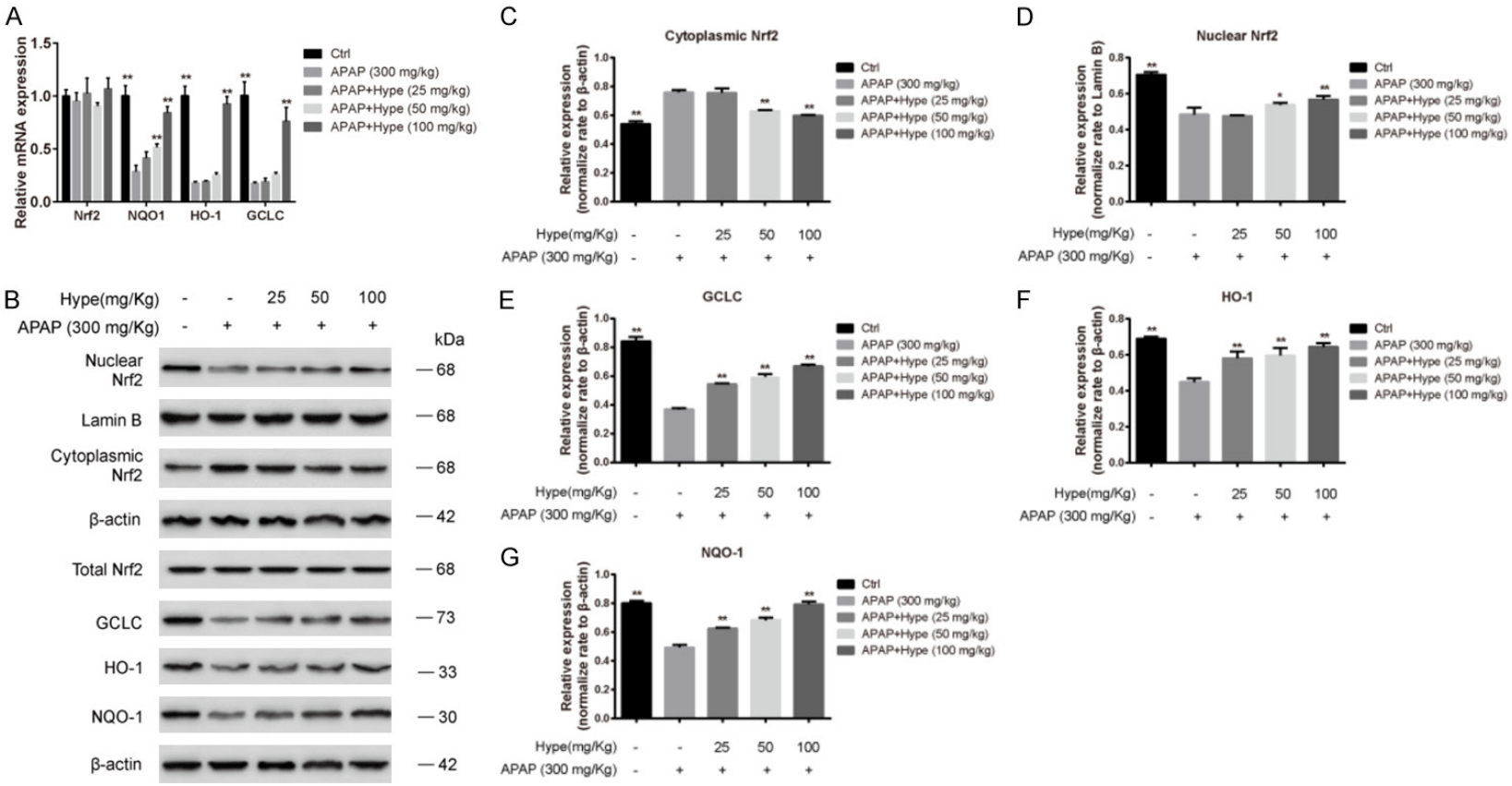
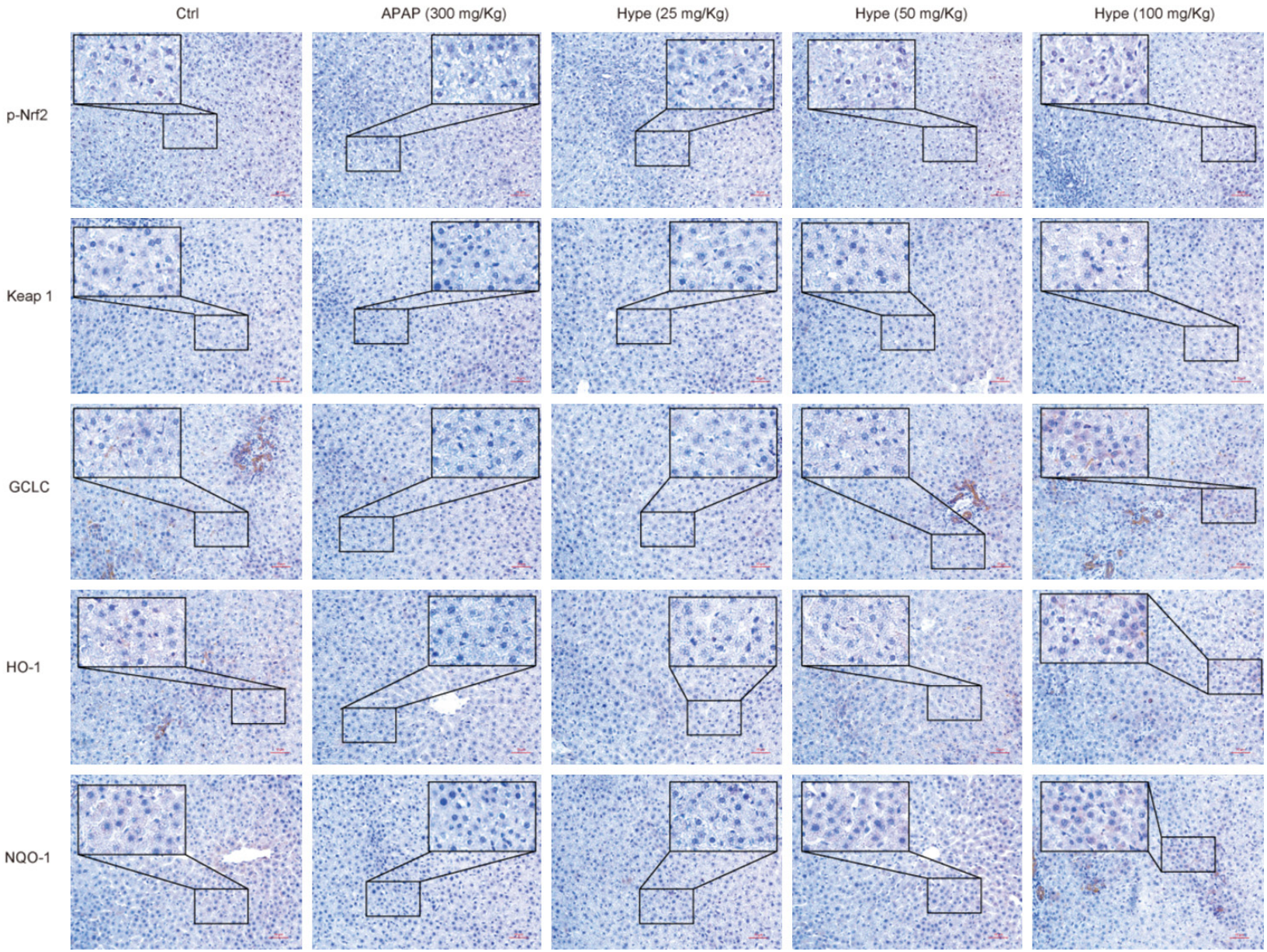


Figure 3. Effect of Hype on Nrf2/ARE antioxidant pathways in vivo. C57BL/6 male mice were treated with Hype (0 mg/Kg), Hype (0 mg/Kg), Hype (25 mg/Kg), Hype (50 mg/Kg) and Hype (100 mg/Kg) at once a day for 7 d. After one hour of the last gavage, mice in each group were injected intraperitoneally with APAP (300 mg/kg), and the blank control group was injected intraperitoneally with equal volume of physiological saline. A. mRNA levels of Nrf2, NQO1, HO-1 and GCLC were measured with Real-Time PCR. B-G. Expressions of Nrf2, NQO1, HO-1 GCLC and Nrf2 nuclear translocation were measured with western blot assay. Results are mean ± SD for three individual experiments which, for each condition, were performed in triplicate. Compared with the APAP group: *P < 0.05, **P < 0.01.


Ctrl
APAP (300 mg/Kg)
Hype (25 mg/Kg)
Hype (50 mg/Kg)
Hype (100 mg/Kg)
p-Nrf2
Keap 1
GCLC
HO-1
NQO-1

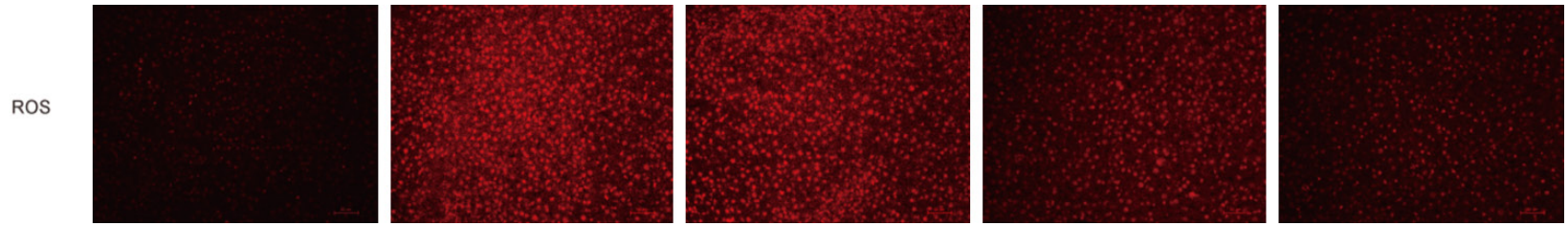


Figure 4. Hype did not increase the expressions of Nrf2/Keap1 pathway in C57BL/6 male mice. Nrf2 and Keap1 staining are shown and positive signal was revealed by yellow-brown staining (scale bar, 10 μm). GCLC and HO-1 staining are shown and positive signal was revealed by yellow-brown staining (scale bar, 10 μm). NQO-1 staining is shown and positive signal was revealed by yellow-brown staining (scale bar, 10 μm). ROS staining is shown and positive signal was revealed by red staining (scale bar, 50 μm).

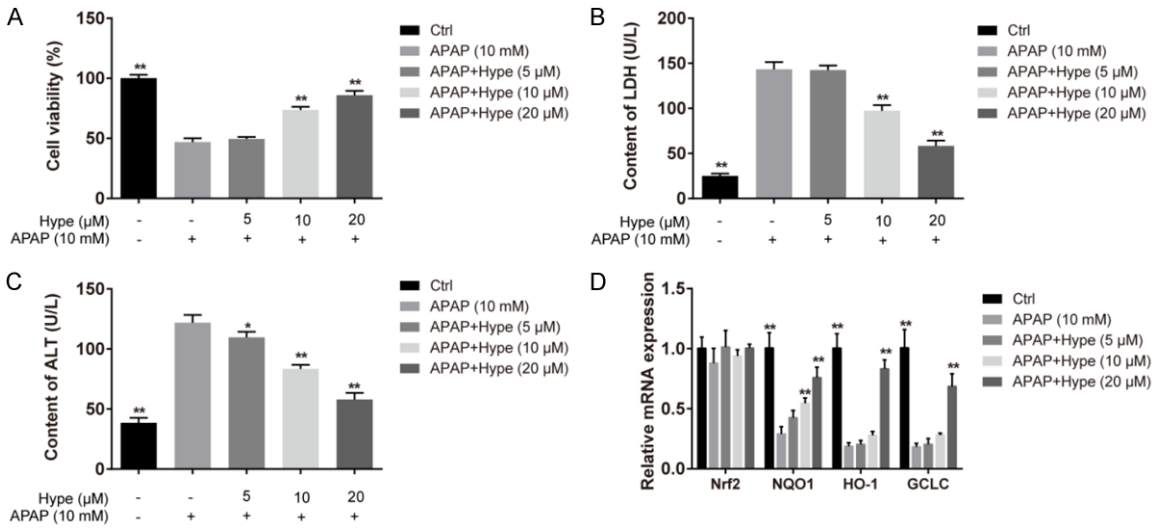


Figure 5. Effect of Hype on Nrf2/ARE Antioxidant pathways in vitro. A. MTT assay. B, C. LDH and ALT were measured by the LDH and ALT Kit in LO2 cells. D. mRNA levels of Nrf2, NQO1, HO-1 and GCLC were measured with Real-Time PCR. Results were mean ± SD for three individual experiments which, for each condition, were performed in triplicate. Compared with the APAP group: *P < 0.05, **P < 0.01.

significantly decreased the enhanced levels (**Figure 4**). These results suggested that hype activated Nrf2/ARE pathway in APAP-treated mice.

Effect of hype on Nrf2/ARE antioxidant pathways in vitro

Our in vivo study proved that Hype attenuated oxidative stress in APAP-induced acute hepatic injury. To investigate the underlying mechanism, we further explored if Hype could rescued oxidative stress in vitro. As shown in **Figure 5A**, APAP inhibited the viability of LO2 cells. Hype dose-dependently decreased increased LO2 cells viability. Consistent with this, increased LDH (**Figure 5B**) and ALT (**Figure 5C**) release caused by APAP treatment distinctly reduced when LO2 were exposed to different concentrations of Hype. Next, we investigated whether Hype affected Nrf2/ARE pathway in vivo. As seen in **Figure 5D**, Hype rescued the decreased mRNA levels of Nrf2, NQO1, HO-1, GCLC in a dose-dependent manner. Furthermore, Nrf2 protein accumulation occurred mostly within the nuclear fraction of these cells when stimulated by Hype (**Figure 6A-C**). Consistently, the expressions of its target genes, including NQO1 (**Figure 6F**), HO-1 (**Figure 6E**) and GCLC (**Figure 6D**) were upregulated by Hype dose-dependently. Taken together, our data showed that Hype induced-Nrf2 activation in LO2 cells.

The effects of hype on LO2 cells were dependent on Nrf2

To determine the role of Nrf2 in Hype-induced inhibition of LO2 proliferation, Nrf2 was silenced by small interfering RNA (siRNA) in LO2. Nrf2 knockdown significantly decreased viability of LO2, while treatment of Hype exhibited no effects on the inhibition of cell proliferation (**Figure 7A**). To further confirm this, the secretion of LDH and ALH were tested in Nrf2 silenced LO2. The results showed that the inhibition of Hype on LDH and ALH production were abolished almost completely when Nrf2 was absent (**Figure 7B**, **7C**). Taken together, our data showed that Nrf2 activation was required for the regulation of Hype on LO2 cells.

Discussion

Liver is an important organ of the body, which is impacted first by ingested xenobiotics. It is responsible for the majority of detoxification and biotransformation of ingested xenobiotics [22]. The index of toxicity to the liver represents overall toxicity to the system. Currently, APAP, an anti inflammatory drug, is known to induce liver injury. It can inactivate Nrf2 and then exhauste GSH [23]. Our present study revealed that APAP could induce acute hepatic injury, and the index of liver function was changed by APAP. Also, more production of ROS and obvi-

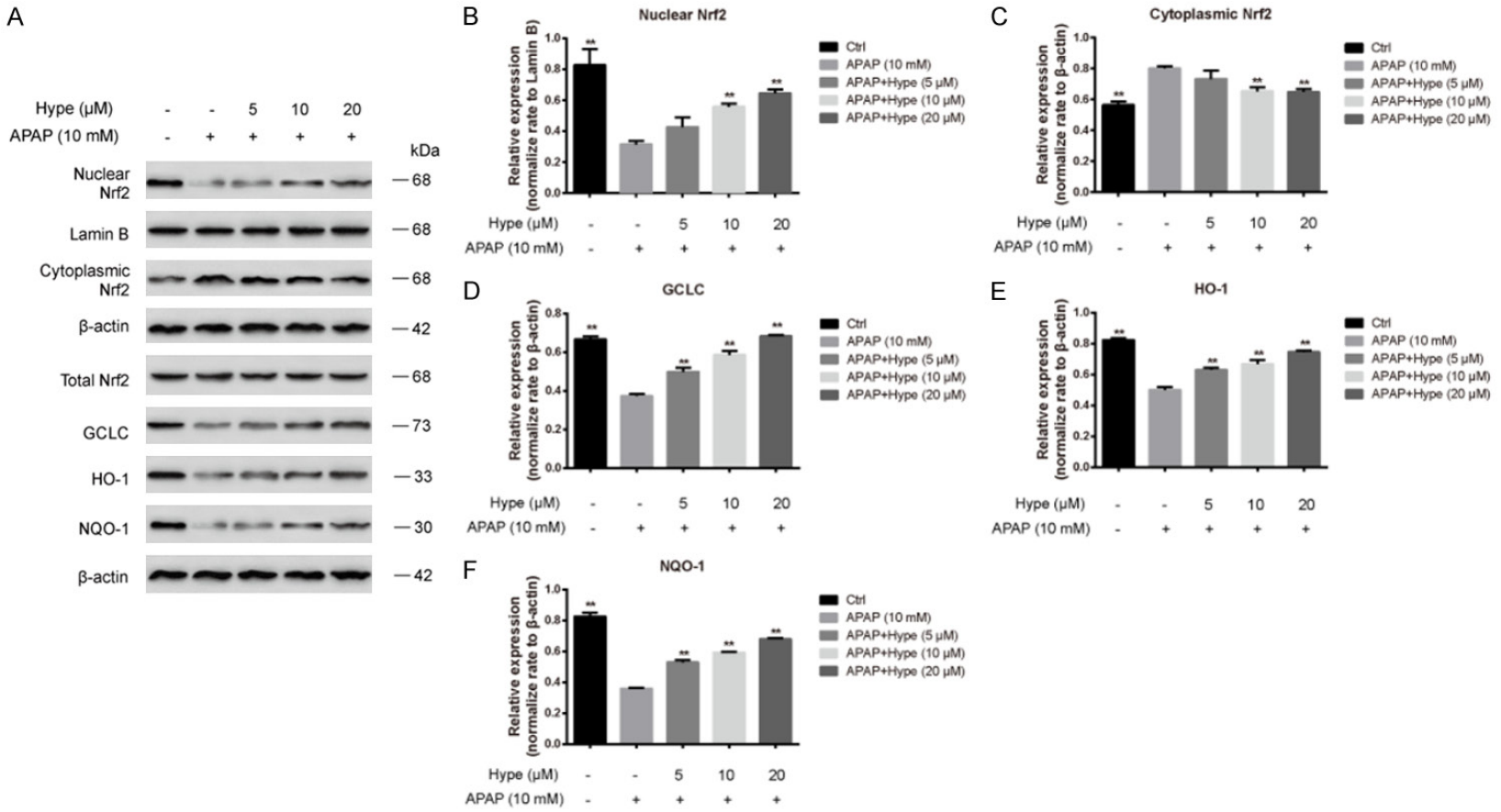


Figure 6. Effect of Hype on Nrf2/ARE antioxidant pathways in vitro. LO2 cells were treated with Hype 0 μM, APAP 10 mM, Hype 5 μM + APAP 10 mM, Hype 10 μM + APAP 10 mM, Hype 20 μM + APAP 10 mM for 24 h. A-F. Nrf2 nuclear translocation, and GCLC, HO-1 and NQO-1 expressions were measured with western blot assay. Results were mean ± SD for three individual experiments which, for each condition, were performed in triplicate. Compared with the APAP group: *P < 0.05, **P < 0.01. Compared with the siCtrl APAP + Hype group: ##P < 0.01.

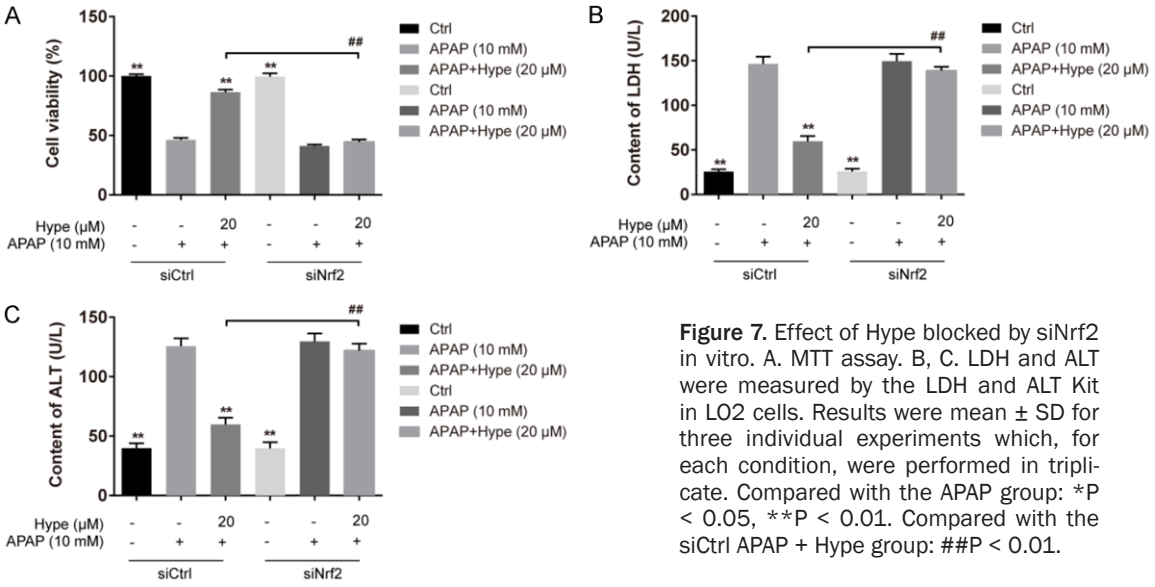


Figure 7. Effect of Hype blocked by siNrf2 in vitro. A. MTT assay. B, C. LDH and ALT were measured by the LDH and ALT Kit in LO2 cells. Results were mean ± SD for three individual experiments which, for each condition, were performed in triplicate. Compared with the APAP group: *P < 0.05, **P < 0.01. Compared with the siCtrl APAP + Hype group: ##P < 0.01.

ous oxidative damage in liver were detected in the APAP administered group.

Nrf2 is a redox sensitive transcription factor, and its activation protects against a variety of reactive toxicants, especially different sources of ROS [24]. As higher production of ROS was observed in APAP group. Scavenging cellular ROS could be a useful strategy to alleviate liver injuries induced by APAP [25-27]. Our present study revealed that Hype dose dependently activated Nrf2 and promoted its nuclear translocation. Moreover, Hype also suppressed ROS production and upregulated Nrf2-targeted genes expressions. Even though many compounds were demonstrated to protect liver from APAP-induced injuries, the specific mechanism is still worth exploring.

GSH is a downstream protein of endogenous antioxidants, which is involved in redox reactions and regulation of antioxidant enzymes, with scavenging free radicals and ROS [28]. Nrf2 inducers and dietary antioxidants can help to maintain the balance between the endogenous antioxidants and oxidative stress induced by xenobiotic transformation of drugs, including APAP [29]. It appears that Hype was able to decrease the generation of ROS, and reversed GSH depletion caused by APAP, thereby complementing the endogenous antioxidants. Increased GSH production led to higher activity of antioxidant enzyme [30, 31]. In our study, Nrf2 was identified as a potential target of Hype. Its activation contributed to GSH production, but whether GSH was a mediator of Nrf2-mediated protection should be explored.

We hypothesized that Nrf2 activation caused by Hype leads to inhibiting ROS production and increasing antioxidant enzymes. Indeed, SOD and other non-enzymes were improved by Hype in a dose-dependent manner in vivo and vitro. It was reported that Nrf2 played a critical role in controlling basal GSH levels by regulating the rate of GSH synthesis. GSH was greatly involved in protection against a soft and hard electrophile in liver injuries [32, 33], which indicated that increasing the production of GSH would be more important than the other enzymes induced by Nrf2 activation, including HO-1, and NQO1 in scavenging ROS generation [34, 35]. In this study, we detected the mRNA levels of Nrf2-targeted ARE genes, such as GCLC, HO-1, and NQO1, and found that all of them could be upregulated by Hype in a dose-dependent manner. In summary, we found that the co-expressions of these target genes help to decrease ROS production and protect LO2 cells from the damage by APAP. However, whether the effects of Hype on APAP-induced acute hepatic injury are dependent on Nrf2 should be validated. APAP-induced acute hepatic injury should be conducted in Nrf2-/- mice.

In conclusion, the potential of Hype in the treatment of acute hepatic injury were studied in a mice model in which liver injury was induced by

administration of APAP. Our data showed that oral administration of Hype dose-dependently attenuated index of hepatic injury, including the production of AST, ALT and ALP. Increased glutathione (GSH) and decreased ROS production induced by Hype demonstrated its potential antioxidant capacity. The regulation of Hype on acute hepatic injury was dependent on Nrf2/ARE pathway activation. Furthermore, our results provided a strategy to cure APAP-induce liver injury and suggested that Nrf2 could be a potential target for the therapeutic modulation of oxidative stress-associated diseases.

Materials and methods

Reagents

The hyperoside, was purchased from Dalian Meilun Biotechnology Co., Ltd. (Dalian, China) and were dissolved in DMSO. Primary antibodies for Nrf2, β-actin, IL-1β, horseradish peroxidase (HRP) conjugated second antibodies were from Bioworld Technology Inc. (Bioworld Technology Inc., CA). Lamin A, NQO1, HO-1 antibodies were purchased from Santa Cruz Biotechnology Inc. (SantaCruz Biotechnology Inc., CA). Nrf2 siRNA and control siRNA were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz Biotechnology Inc., CA).

Cell culture

LO2 cells were obtained from the Cell Bank of Shanghai, Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China) and maintained in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY). LO2 cells were cultured under a humidified 5% (v/v) CO_2 atmosphere at 37°C.

Mice

6- to 8-week-old female C57BL/6 mice were purchased from Model Animal Genetics Research Center of Nanjing University (Nanjing, China). Animal welfare and experimental procedures were carried out strictly in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, the United States) and the related ethical regulations of our university. All efforts were made to minimize animals' suffering and to reduce the number of animals used.

Western blot assay

Cells with different treatments were washed twice with PBS, then collected and lysed in lysis buffer (100 mM of Tris-Cl, pH 6.8, 4% (m/v) SDS, 20% (v/v) glycerol, 200 mM of β-mercaptoethanol, 1 mM of PMSF, 0.1 mM NaF and DTT) for 1 hon the ice. The lysates were then subjected to centrifugation (13,000 rpm) at 4°C for 20 min. Protein concentration in the supernatants was detected by BCA protein assay (Thermo, Waltham, MA). Then an equal amount of protein was separated with 12% SDS-PAGE and transferred to polyvinylidenedifluoride (PVDF) membranes (Millipore, Bedford, MA) using a semi-dry transfer system (Bio-Rad, Hercules, CA). Proteins were detected using specific antibodies of Nrf2, NQO1, HO-1, GCLC, β-actin and Lamin A overnight at 4°C followed by HRP-conjugated secondary antibodies for 1 h at 37°C. All of the antibodies were diluted in PBST containing 1% BSA. Enhanced chemilumine scentreagents (Beyotime, Jiangsu, China) were used to detect the HRP on the immunoblots. The signals were analyzed using the ECL chemiluminescence detection system (Tanon, Nanjing, China).

Nuclear and cytoplasmic extraction

Following treatments, cells were harvested by centrifugation and washed twice with PBS. Cells were then lysed on ice in 3 volumes of cytoplasmic extraction buffer. Nuclear and cytosol lysates were isolated using a Nuclear/Cytosol Fractionation Kit (BioVision, Mountain View, CA) according to the manufacturer's instruction. The protein concentration of the nuclear and cytoplasmic extracts was determined by a Bio-Rad protein assay dye using the Bradford method. Extracts were stored at 70°C until further experimentation.

Measurement of production of cellular ALT, AST, ALP and GSH

LO2 cells were exposed with different stimulations and the amount of ALT, AST, ALP and GSH were quantified by ELISA kit (Boster, Wuhan, China).

Immunohistochemistry

Immunohistochemical stains against Nrf2, HO-1, NQO1 and Keap1 were performed using immunohistochemistry kit (Key-GEN, Nanjing,

China). Briefly, paraffin-embedded slides were deparaffinized, rehydrated and washed in 1% PBS-Tween. Then they were treated with 3% hydrogen peroxide and blocked with 10% goat serum for 1 h at 37°C. Slides were incubated with primary antibodies in PBS containing 1% BSA (1:50) for 1 h at 37°C. Biotinylated secondary anti-rabbit antibodies were added and incubated at room temperature for 1 h. Streptavidin-HRP was added, and after 40 min the sections were stained with DAB substrate and counterstained with hematoxylin.

Real-time RT-PCR

Real-time RT-PCR was performed as follows: RNA samples were reverse transcribed to cDNA and subjected to quantitative PCR, which was performed with the LightCycler_ 96 Real-Time PCR System (Roche, Basel, Swiss) using AceQqPCR SYBR Green Master Mix (Vazyme, Nanjing, China). The program for amplification was 1 cycle of 95°C for 2 min followed by 40 cycles of 95°C for 10 s, 60°C for 30 s, and 95°C for 10 s.

Nrf2 siRNA transfection

The Nrf2 siRNA and control siRNA were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The interference was performed in 35-mm^2 dishes. Nrf2 siRNA and control siRNA were introduced into each well using LipofectamineTM2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

Statistics analysis

All results shown represent mean ± SD from triplicate experiments performed in a parallel manner unless otherwise indicated. Statistical analysis was performed using a one-way ANOVA. See details of each statistical analysis used in figures and figure legends.

Disclosure of conflict of interest

None.

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