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Trehalose prevents cadmium-induced hepatotoxicity by blocking Nrf2 pathway, restoring autophagy and inhibiting apoptosis

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ABSTRACT

Cadmium (Cd) is a ubiquitously distributed environmental pollutant that is highly toxic to liver. Trehalose (Tr), a novel autophagy activator, has been shown to exert cytoprotective effect in numerous pathological processes. However, it is yet to be established whether Tr affords protection against Cd-induced hepatotoxicity. Here, we aimed to investigate the protective effect of Tr on Cd-induced hepatic injury in rats. First, Cd-elevated serum hepatic enzymes and liver pathological changes were significantly ameliorated by Tr treatment. Also, Tr remarkably improved Cd-mediated oxidative stress and antioxidant status in serum, indicating its anti-oxidant action for the whole body. Cd-stimulated nuclear factor erythroid 2-related factor 2 (Nrf2) nuclear translocation and subsequent elevated expression of Nrf2-downstream targets in rat liver were significantly inhibited by Tr treatment. Simultaneously, Cd-elevated protein levels of hepatic antioxidant enzymes were markedly down-regulated by administration with Tr. Moreover, Cd-induced autophagy inhibition in liver tissues was noticeably restored by Tr, evidenced by immunohistochemical analysis and immunoblot assays. Additionally, Tr treatment significantly mitigated Cd-induced apoptosis in hepatic tissues via inhibiting caspase-dependent apoptotic pathway. In conclusion, these observations demonstrate that Tr treatment alleviates Cd-induced liver injury by blocking Nrf2 pathway, restoring autophagy and inhibiting apoptosis.

1. Introduction

Cadmium (Cd) is a widespread environmental contamination that seriously harms human health. Cadmium production, consumption and emissions to the atmospheric, terrestrial and aquatic environment have continuously increased due to its extensive application in various anthropogenic and industrial activities [1]. Unlike organic pollutants, Cd cannot be chemically biodegraded by microorganisms; instead, it can be accumulated by organisms. Routes of Cd exposure include inhalation of Cd-contaminated dust particles or aerosols or cigarette smoke and ingestion of Cd-contaminated food or water [1–3]. As a multi-organ toxicant, Cd exerts potent toxic effects on different tissues including the liver, kidney, testes, spleen and bone [4–8]. Liver is known to be the highest depot of Cd accumulation in soft tissues and the primary target organ of following acute or chronic Cd exposure [9].

There is general consensus that oxidative stress contributes to the development of Cd-induced hepatotoxicity [8,10,11]. Cd-induced oxidative stress is accompanied by the activation of redox-sensitive transcription factors [12,13]. Nuclear factor erythroid 2-related factor 2 (Nrf2) is an essential transcription factor that translocated into the nucleus and interacts with the antioxidant response element (ARE) to initiate the transcription of target genes to enhance detoxification and attenuate oxidative stress [14], and previous study has revealed that Nrf2 activation prevents Cd-induced oxidative stress and liver injury [10]. Thus, Nrf2 pathway is an important target for protection against Cd-induced liver damage. Additionally, oxidative stress is closely correlated with apoptosis and autophagy, two distinct cellular processes that regulate cell death and survival [15–17]. It has been demonstrated that oxidative stress-mediated apoptotic death played a key role in Cd-induced hepatotoxicity [5,8], and impaired autophagy due to excessive

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oxidative stress causes severe liver injury characterized by increased liver function enzymes in serum, hepatocyte hyperplasia and degeneration [18–20]. Given these obtained results, we speculated that the application of active ingredients with antioxidant activity, anti-apoptotic and autophagy enhancing effects might be useful in the treatment of Cd-induced liver damage.

Trehalose (Tr), a naturally occurring disaccharide that presents in a diverse range of organisms including insects, bacteria, yeast and fungi, is a novel autophagy activator in many cells through mammalian target of rapamycin (mTOR) independent pathway [21]. Several studies have reported that Tr acts as an antioxidant to effectively prevent lipid peroxidation [13,22,23]. Tr has also been demonstrated to prevent apoptosis in an autophagy-dependent manner [22,24,25]. These scientific findings give us a hint that Tr might prevent Cd-induced liver damage. Therefore, in this study, we investigated the protective effect of Tr on Cd-induced hepatotoxicity, and the roles of Nrf2 pathway, apoptosis and autophagy in this putative protective action.

2. Materials and methods

2.1. Chemicals and antibodies

All chemicals available were of highest grade purity. Anhydrous cadmium chloride ($CdCl_2$, 439,800) and D-(+)-Trehalose dihydrate (Tr, T0167) were purchased from Sigma-Aldrich (Carlsbad, CA, USA). Commercial kits for detection of six serum oxidative stress assays, i.e., malonaldehyde (MDA), total antioxidant capacity (T-AOC), superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), glutathione (GSH), and three serum hepatic enzyme levels, i.e., aspartate aminoteansferase (AST), alanine transaminase (ALT), lactic dehydrogenase (LDH), were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). Nuclear Protein Extraction Kit and in Situ cell death detection kit were obtained from Beyotime Institute of Biotechnology (Haimen, Jiangsu, China). Enhanced chemiluminescence (ECL) kit and bicinchonininc acid (BCA) protein assay kit were obtained from Pierce Biotechnology (Rockford, IL, USA). The following primary antibodies were used: Nrf2 (M200-3) antibody was obtained from MEDICAL & BIOLOGICAL LABORATORIES CO. LTD (Nagoya, Japan). Kelch-like epichlorohydrin- associated protein 1 (Keap1, ab66620), heme oxygenase 1 (HO-1, ab68477), NAD(P)H dehydrogenase [quinone] 1 (NQO1, ab80588), CAT (ab76110), GPx (ab108427), SOD1 (ab51254), SOD2 (ab68155) were purchased from Abcam (Cambridge Science Park, Cambridge, UK). Sequestosome 1(SQSTM1/p62) (P0067), microtubule-associated protein 1 light chain 3B (LC3B, L7543), α-tubulin (T6199) and β-actin (A5441) were purchased from Sigma (St. Louis, MO, USA). Histone H3 (4499), cleaved caspase-3 (9661), cleaved caspase-9 (9507) and cleaved poly (adenosine diphosphate-ribose) polymerase (cleaved PARP, 9545) were obtained from Cell Signaling Technology (Danvers, MA, USA). All secondary antibodies were obtained from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. (ZSGB, Beijing, China).

2.2. Experimental design

Thirty-two 6-week-old male Sprague-Dawley rats, with body weight ranging from 100 g to 110 g, were obtained from Jinan Pengyue Experimental Animal Breeding Co., Ltd. (Shandong, China). All animals were housed in an animal facility (24 ± 0.5 °C, 12 h alternating light-dark cycle) and were fed a standard pellet diet with free access to water. All procedures were approved by the Animal Care and Use Committee at Shandong Agricultural University.

After one-week acclimatization to the laboratory conditions, rats were allocated randomly to four experimental groups of eight animals each, maintained under the supervision of a licensed veterinarian in accordance with the principles set forth in the U.S. National Institute of Health Guide for the Care and Use of Laboratory Animals [26]. Cd-intoxicated model was established by drinking an aqueous solution of $CdCl_2$ (20 mg/L) as the only drinking fluid for eight weeks, and Tr-treated rats received a daily oral gavage administration of Tr at dose of 2 g/(kg body weight) for eight weeks. Cd plus Tr rats received both Cd and Tr at the doses, periods and routes of administration described above. Particularly, Cd and Tr solutions were prepared fresh in distilled water and the doses of them were selected on the basis of previous studies and our preliminary experiments [27,28]. Tr solution was prepared daily and its concentration was adjusted such that a rat would receive 0.3 mL per day. Cd solution was replaced daily to minimize the precipitation, which was analyzed by inductively coupled plasma mass spectrometry (ICP-MS) method (ELAN-6000 model, Perkin-Elmer, Sciex, Toronto, Canada) to verify its concentration. Water consumed by control rats and Tr-treated rats was also analyzed to determine the absence of Cd. During the experiment, water consumption and weight gain were measured every day.

2.3. Specimen collection

Following 8 weeks treatment, 12-h fasted rats were killed by cervical decapitation under ether anesthesia. Blood samples were taken from the aortaventralis to obtain the serum by centrifugation at 2000*g* for 15 min. Liver tissues were quickly removed, weighted, dissected out and washed in ice-cold physiological saline solution. Approximately 1 g of liver tissues from each rat was immediately frozen in liquid nitrogen for western blot analysis. Some pieces were quickly fixed with 4% paraformaldehyde for morphological evaluation as described later.

2.4. Measurement of serum biochemical indices

Freshly prepared serum samples were used to detect hepatic enzymes activities (ALT, AST and LDH) and levels of oxidative stress indices (MDA, T-AOC, SOD, GPx, CAT, GSH) using the commercial kits. The detailed detection method of each biochemical assay was performed according to the manufacturer's instructions. T-AOC assay was detected on the basis of the deoxidation ability of Fe^{3+} to Fe^{2+}, which combines with phenanthrene and forms a colored compound that can be detected at 520 nm.

2.5. Histological analysis

Liver specimens in 5-μm-thick paraffin sections were stained with hematoxylin and eosin for conventional morphological evaluation. Slides were examined under a light microscope (Nikon Eclipse E600W, Suzhou, China) by the same pathologist blinded to the treatments. Images were captured near hepatic portal vein.

2.6. TUNEL staining

Apoptosis was analyzed based on terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay. Deparaffinized sections (5-μm-thick) were treated with 10 μg/mL proteinase K in 0.1 M PBS (pH 7.4) for 30 min at 37 °C. Subsequently, these sections were incubated with 0.01 M citrate buffer (pH 6) under microwave irradiation. After thorough washing with PBS, samples were incubated with TUNEL reaction mixture for 1 h at 37 °C in humidified chamber that contains TdT and Biotin-11-dUTP. Biotinylated nucleotides are detected using a streptavidin-horseradish peroxidase (HRP) conjugate. Diaminobenzidine (DAB) reacts with the labeled samples to generate an insoluble colored substrate at the site of DNA fragmentation, followed by counter staining with hematoxylin for characterization of normal and apoptotic cells. Finally, slides were observed by a light microscope. Ten randomly chosen high-power fields from five different slides were selected, without significant necrotic regions, for

counting TUNEL positive cells. Percent of TUNEL-positive cells, indicative of DNA damage, were calculated by dividing the number of TUNEL-positive cells by total number of nuclei.

2.7. Immunohistochemistrical (IHC) staining

Briefly, 5-μm-thick sections on poly-L-lysine-coated slides were deparaffinized in xylene and rehydrated in alcohol, then subjected to antigen retrieval by boiling in 0.01 M sodium citrate buffer (pH 6) in a microwave oven for 15 min. After washing with PBS for three times, slides were incubated with 3% H_2O_2 at room temperature (RT) for 30 min to quench endogenous peroxidase activity. After being washed with PBS for three times and incubated with blocking solution (10% normal goat serum) for 45 min at RT, sections were incubated overnight at 4 °C with the following primary antibodies: Nrf2 (diluted 1:200), SQSTM1/p62 (diluted 1: 500) and LC3B (diluted 1: 200). After washing with PBS for three times, slides were incubated with respective secondary antibodies (HRP conjugated) for 1 h at 37 °C. Then slides were visualized with an Enhanced HRP-DAB Chromogenic Substrate Kit according to the manufacturer's recommendation. Finally, slides were dehydrated and mounted after counter staining with hematoxylin. Images were captured under a light microscope.

2.8. Western blot analysis

Liver specimens were lysed in ice-cold RIPA buffer supplemented with protease inhibitors to prepare total lysates. Also, liver tissues were homogenized in Nuclear Protein Extraction Kit to prepare the nuclear protein extracts. After protein quantification with BCA method, samples were subjected to SDS-PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Darmstadt, Germany). After blocking with 5% skim milk for 50 min at RT, PVDF membranes were incubated overnight at 4 °C with the following primary antibodies: Nrf2 (diluted 1:1000), Keap1 (diluted 1:1000), HO-1 (diluted 1:10000), NQO1 (diluted 1:10000), CAT (diluted 1:1000), GPx (diluted 1:1000), SOD1 (diluted 1:10000), SOD2 (diluted 1:1000), p62 (diluted 1:1000), LC3B (diluted 1:1000), α-tubulin (diluted 1:1000), β-actin (diluted 1:5000), Histone H3 (diluted 1:2000). After washing three times with TBST, PVDF membranes were incubated with appropriate secondary antibodies for 50 min at RT. Then protein bands were detected on a Chemidoc XRS (Bio-Rad, Marnes-La-Coquette, France) using ECL Kit. Proteins levels were determined by computer-assisted densitometric analysis. The density of each band was normalized to its respective loading control (β-actin or α-tubulin or Histone H3). Each test was performed four times using different protein lysates.

2.9. Statistical analysis

Experimental groups were compared using a two-ways analysis of variance (ANOVA) followed by Scheffe's test when data were normally distributed and by the Kruskal-Wallis test when they were not normally distributed. All data were expressed as mean ± SEM ($n = 8$). The statistical significance was evaluated using SPSS 22.0 (SPSS Inc., Chicago, IL, USA) and $P < 0.05$ was regarded as statistically significant.

3. Results

3.1. Tr alleviates Cd-induced hepatic injury in rats

Cd is a well-known hepatotoxic heavy metal, while Tr is a biologically-active substance possessing cytoprotective properties [24,29,30]. To prove whether Tr can alleviate Cd-induced liver damage, both serum markers of hepatic injury (Fig. 1) and pathological assessment (Fig. 2) were detected in this study. Compared with the control group, Cd significantly elevated the activities of liver marker enzymes in serum (ALT, AST and LDH); meanwhile, Cd caused obvious liver pathological changes in the form of irregular hepatic cords arrangement, cytoplasmic vacuolation, ballooning degeneration, coagulative necrosis of hepatocytes and increased hepatic steatosis, confirming successful establishment of Cd-induced hepatotoxicity model. These results indicated that Cd-elevated activities of liver marker enzymes in serum and liver pathological damage were markedly alleviated by Tr treatment. Additionally, Tr alone has no effect on liver enzyme activities in serum and liver pathological changes, suggesting non-toxic effect of Tr. Collectively, these data verified that Tr has the protective effect on Cd-induced liver damage.

3.2. Evaluation of oxidative stress and antioxidant status in rat serum

Oxidative stress is an important factor contributing to Cd-induced hepatotoxicity [11,31–33]. Thus, oxidative stress assays and antioxidant levels in rat serum were determined to explore whether Tr can inhibit Cd-induced oxidative stress in the whole body. As shown in Fig. 3A, Cd-elevated serum MDA level was markedly down-regulated by Tr. Simultaneously, Cd-decreased T-AOC, GSH and antioxidant enzymes levels were significantly improved by Tr administration (Fig. 3B–F). These data strongly proved that Tr can restore Cd-induced oxidative damage and impairment of antioxidant status in whole body.

3.3. Tr inhibits Cd-activated Nrf2 signaling pathway in liver

Nrf2 is a key transcription factor regulating cellular redox homeostasis. Under homeostatic conditions, Nrf2 is anchored by its endogenous inhibitor Keap1 in the cytoplasm, while it is released from Keap1 and translocates into the nucleus when cells encounter an oxidative insult [34]. Thus, we designed this study to investigate the role of Nrf2 signaling pathway in the protective effect of Tr on Cd-induced hepatic injury. First, Cd-induced Nrf2 nuclear translocation in hepatocytes was obviously inhibited by Tr, evidenced by immunohistochemical staining (Fig. 4). Consistently, Tr markedly blocked Cd-mediated Nrf2 nuclear accumulation, assessed by immunoblot analysis (Fig. 5A). Moreover, protein levels of negative regulator of Nrf2 (Keap1) and two Nrf2-downstream target proteins (HO-1 and NQO1) were analyzed to indirectly assess the Nrf2 activation status. Decreased Keap1 protein level and increased HO-1 and NQO1 protein levels in Cd-exposed liver tissues were separately up-regulated and down-regulated by Tr treatment (Fig. 5B–D). It is noteworthy that Tr alone can increase Nrf2 nuclear translocation, demonstrated by localization of Nrf2 by immunohistochemical staining, elevated Nrf2 expression within the nuclear protein extract, decreased Keap1 protein level and increased expressions of Nrf2-downstream target proteins (Figs. 4–5). Collectively, these data verified that Tr can attenuate Cd-activated Nrf2 pathway in liver tissues.

3.4. Tr down-regulates Cd-elevated protein levels of antioxidant enzymes in liver tissues

SOD, CAT and GPx are the classical antioxidant enzymes by Nrf2 activation to resist oxidative stress. Once received redox signals, Nrf2 triggers the transcription of endogenous antioxidant enzymes to prevent oxidative damage. Thus, protein levels of CAT, GPx, SOD1 and SOD2 in liver tissues were examined to further clarify the role of Nrf2 signaling pathway in the protective effect of Tr on Cd-induced liver damage. Data in Fig. 6 indicated that Cd markedly elevated the protein levels of CAT, GPx, SOD1 and SOD2 in rat liver, indicating the self-protective effect of liver in response to Cd exposure. Interestingly, Cd-activated these proteins in liver were significantly abated by Tr treatment, further confirming the inhibitory role of Nrf2 pathway in Tr-mediated protection against Cd-induced hepatotoxicity.

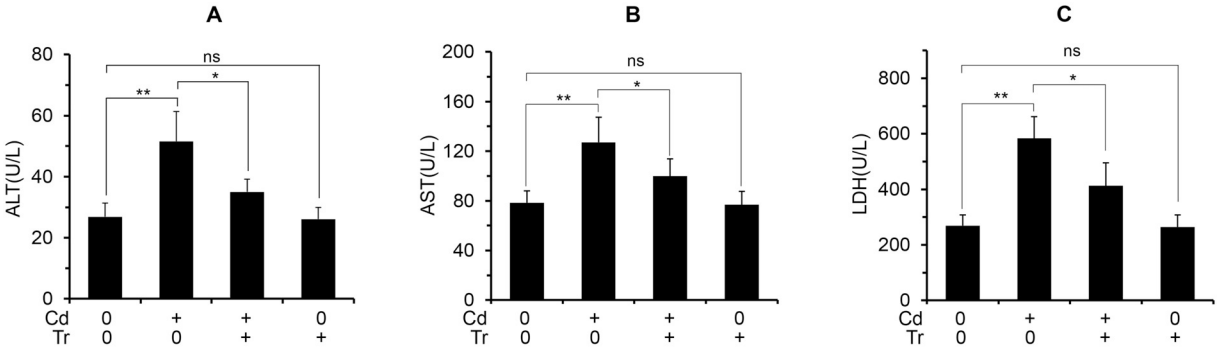

Fig. 1. Hepatic enzymes activity in serum. Rats were treated with $CdCl_2$ (20 mg/L, through drinking water) and/or trehalose (2 g/kg body weight/day, by gavage) for 8 weeks, then serum samples were collected to assess the activities of ALT (A), AST (B) and LDH (C). Data are expressed as mean ± SEM. ($n = 8$). ns, not significant; $^{*}P < 0.05$; $^{**}P < 0.01$.

3.5. Tr restores Cd-induced autophagy inhibition in rat liver

Autophagy is an important cytoprotective mechanism and autophagy inhibition promotes drug-induced hepatotoxicity [35,36]. Tr has been characterized as an effective autophagy inducer in various mammalian cells [37]. Two proteins, LC3 and p62, have been widely used as markers for autophagic activity, and immunoblot analysis of LC3 and p62 has been widely used to monitor the progression of autophagy [38]. To assess whether the protective effect of Tr on Cd-induced hepatotoxicity is related to autophagy, expressions of these two autophagy markers were detected by IHC staining and western blot analysis (Fig. 7), respectively. Cd exposure markedly increased the protein levels of p62 and LC3 (LC3-II), indicating the autophagy inhibition in liver. However, Cd-induced accumulation of p62 and LC3 (LC3-II) was significantly alleviated by Tr administration. Interestingly, Tr treatment alone obviously elevated the protein levels of p62 and LC3 (LC3-II) compared to the control group. These data demonstrate that Tr can restore Cd-induced autophagy inhibition in rat liver.

3.6. Tr blocks Cd-mediated apoptosis in rat liver

Many studies have demonstrated that apoptotic death plays an important role in Cd-induced hepatotoxicity, which is closely related to oxidative stress [11,39,40]. Next, we identified the hepatoprotective effect of Tr via inhibiting Cd-induced apoptosis. Results from Fig. 8 indicated that Cd-elevated TUNEL-positive hepatocytes were markedly attenuated by Tr treatment. Consistently, Tr significantly prevented Cd-induced activation of apoptotic markers, that is, cleaved caspase-9, cleaved caspase-3 and cleaved poly ADP-ribose polymerase (PARP) (Fig. 9). These data verified that Tr inhibited Cd-induced apoptosis in liver via blocking caspase-dependent apoptotic pathway.

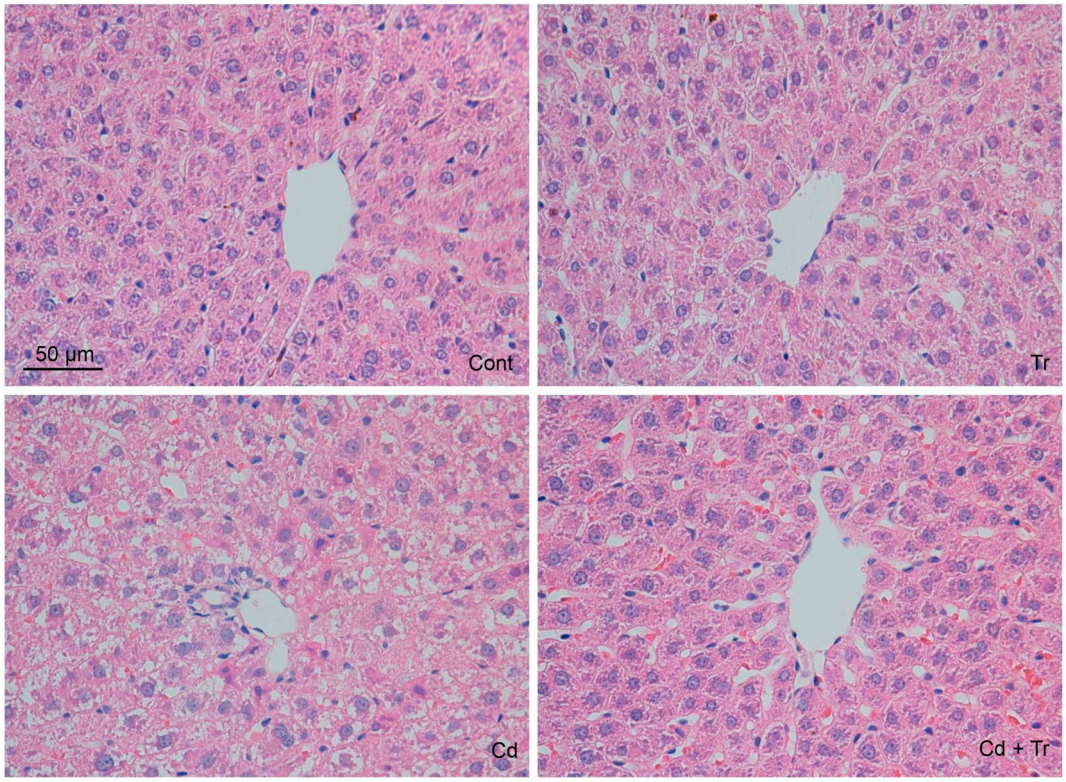

Fig. 2. Pathological changes of liver tissues. Rats were treated with Cd and/or Tr for 8 weeks as mentioned above, and liver sections were stained with hematoxylin and eosin to assess histomorphological changes. Scale bar: 50 μm.

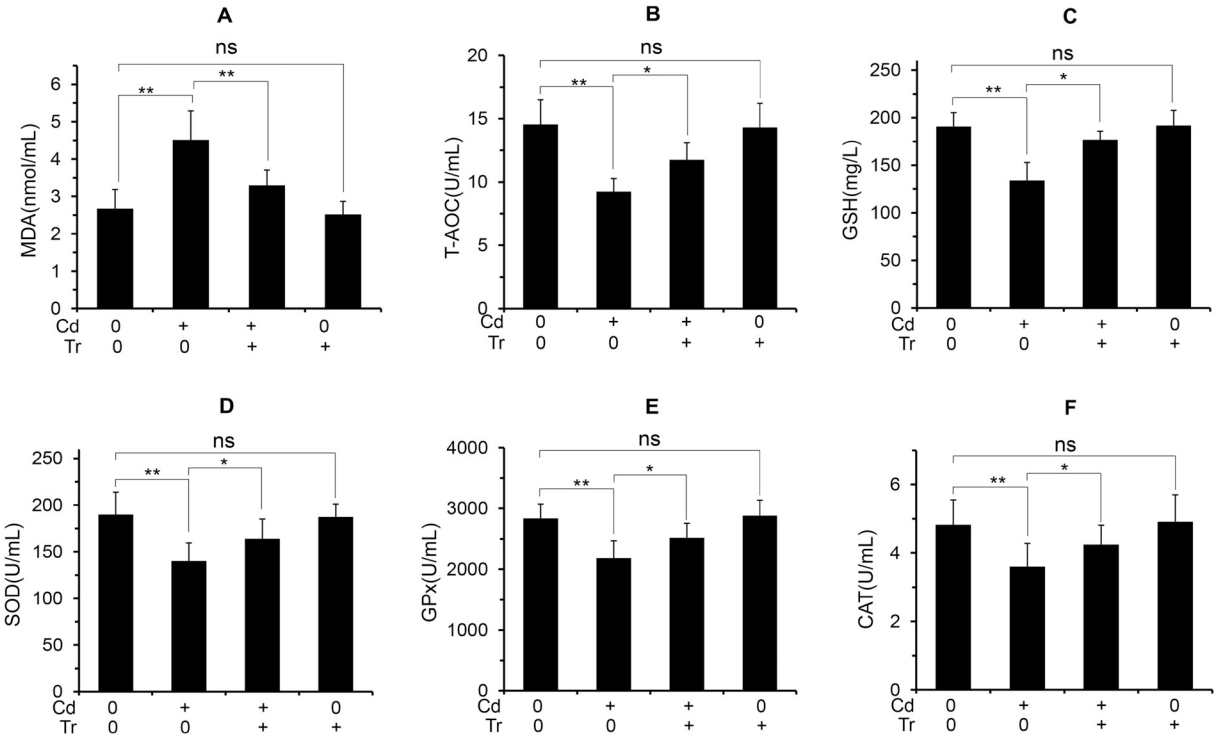

Fig. 3. Oxidative stress assays in serum. Rats were treated with Cd and/or Tr for 8 weeks as mentioned above, then serum samples were collected to examine the levels of MDA (A), T-AOC (B), GSH (C), SOD (D), GPx (E) and CAT (F). Data are expressed as mean ± SEM. ($n = 8$). ns, not significant; $^{*}P < 0.05$; $^{**}P < 0.01$.

4. Discussion

Tr is known for its capability of helping cells cope with potentially cytotoxic changes under various stress conditions such as oxidative damage, dehydration and temperature changes [22]. Consistently, we found that Tr exerts its protective effect against Cd-induced cytotoxicity in primary rat proximal tubular cells [24]. A recent study has shown that Tr can protect hepatic cultured cells from oxidative stress-mediated

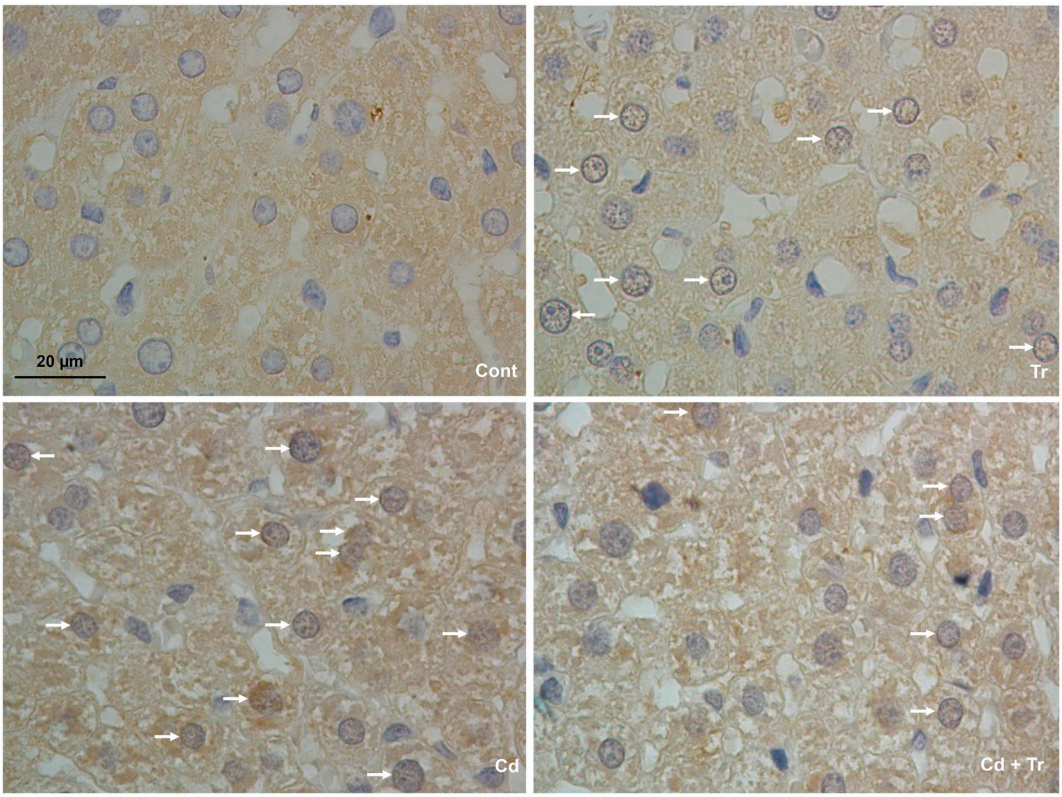

Fig. 4. Tr prevents Cd-induced Nrf2 nuclear translocation in liver tissues. Rats were treated with Cd and/or Tr for 8 weeks as mentioned above, then liver tissues were applied to perform the immunohistochemical staining of Nrf2. Arrows indicate nuclear Nrf2 accumulation. Scale bar: 20 μm.

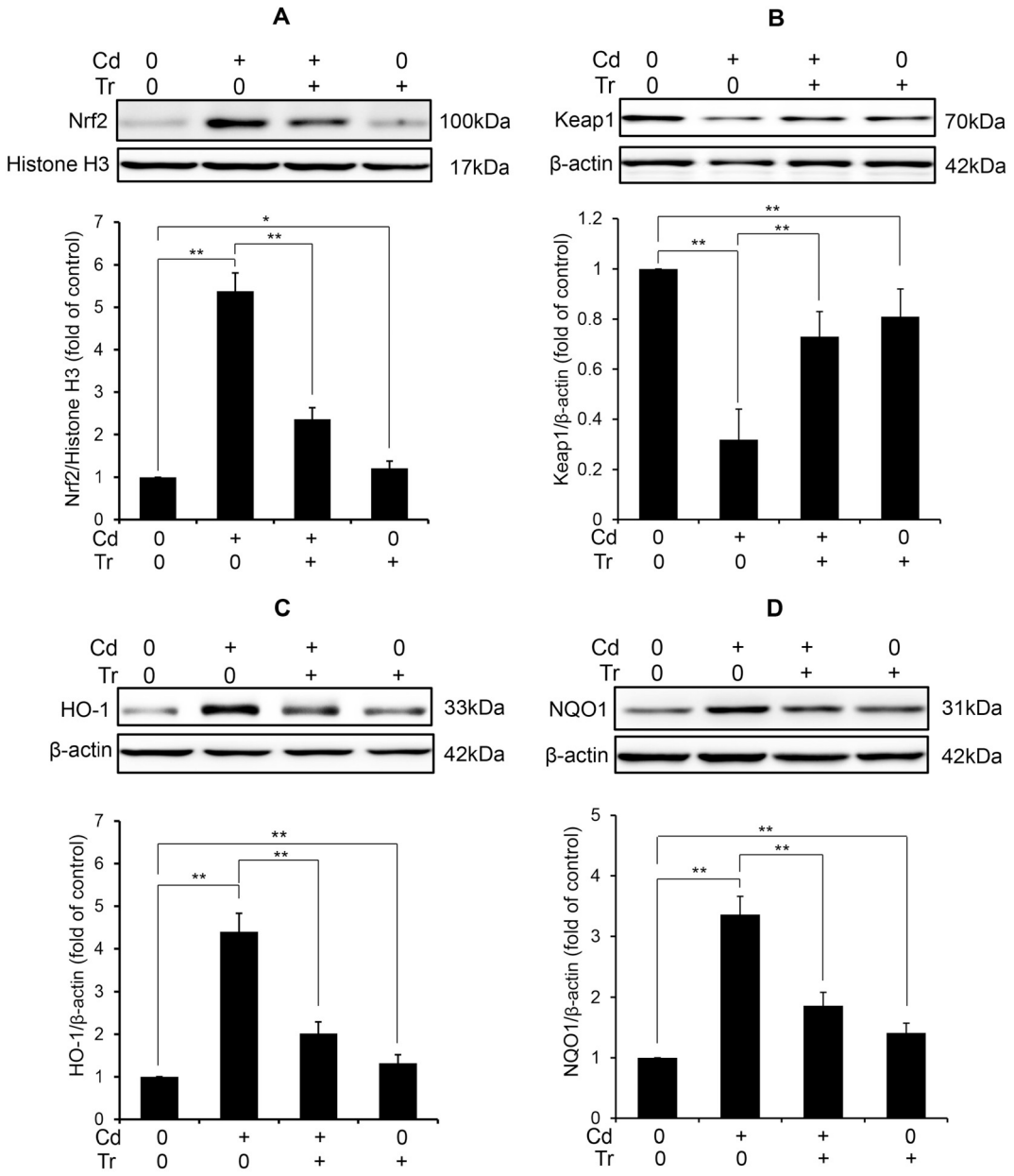

Fig. 5. Inhibitory effect of Tr on Cd-activated Nrf2-Keap1 pathway in liver tissues. Rats were treated with Cd and/or Tr for 8 weeks as mentioned above, then liver tissues were utilized to analyze the protein levels of nuclear Nrf2 (A), Keap1 (B), HO-1 (C) and NQO1 (D) in total cellular lysates by western blot analysis. *Upper panel* representative western blot image; *lower panel* quantitative analysis (mean ± SEM, $n = 4$). *ns*, not significant; $^{*}P < 0.05$; $^{**}P < 0.01$.

cytotoxicity [25]. However, the effect of Tr on Cd-induced hepatic injury remains unknown till now. Here, we investigated the mechanisms underlying the protective action of Tr on Cd-induced hepatotoxicity in rat.

Generally, hepatocellular damage is directly reflected by the elevated serum hepatic enzymes, such as LDH, AST and ALT, and its pathological changes. Data in Figs. 1 and 2 showed that Cd-elevated serum biochemical parameters and concomitant pathological damage in hepatic tissues were obviously alleviated by Tr administration, indicating the hepatoprotective effect of Tr during Cd exposure. Based on the anti-oxidative property of Tr and the role of oxidative stress in Cd-induced toxicity [24], we speculate that oxidative stress may be implicated in the protective effect of Tr on Cd-induced hepatic damage. Thus, serum biomarkers of oxidative stress were firstly assessed to reflect the actual oxidative stress status in whole body (Fig. 3). As expected, Cd-induced lipid peroxidation and impairment of antioxidant defense system were significantly rescued by Tr.

Nrf2 protein has been recognized as the master regulator of a cellular defense mechanism against toxic insults [14]. Its function confers cellular protection to oxidative stress, which plays an important role in the transcriptional activation of an array of antioxidant and detoxification genes [41]. During oxidative stress, the interaction between Nrf2 and Keap1 in the cytoplasm is disrupted [42]. Nrf2 is translocated into the nucleus and interacts with the ARE, which leads to transcriptional induction of several cellular defense genes, such as phase II detoxification enzymes HO-1, NQO1 and direct reactive oxygen species (ROS) scavenging proteins (GPx, SOD, CAT). Numerous studies have previously established that Nrf2 activation prevents Cd-induced liver injury [10,41,43]. Here, our data (Figs. 4–6) gave us a solid evidence that Cd-activated Nrf2 pathway in liver tissues was significantly inhibited by Tr treatment, demonstrating the alleviation of Cd-induced hepatic oxidative stress by Tr via inhibiting Nrf2 pathway.

Interestingly, Cd-decreased serum antioxidant enzyme activities were significantly up-regulated by Tr treatment, but Cd-elevated

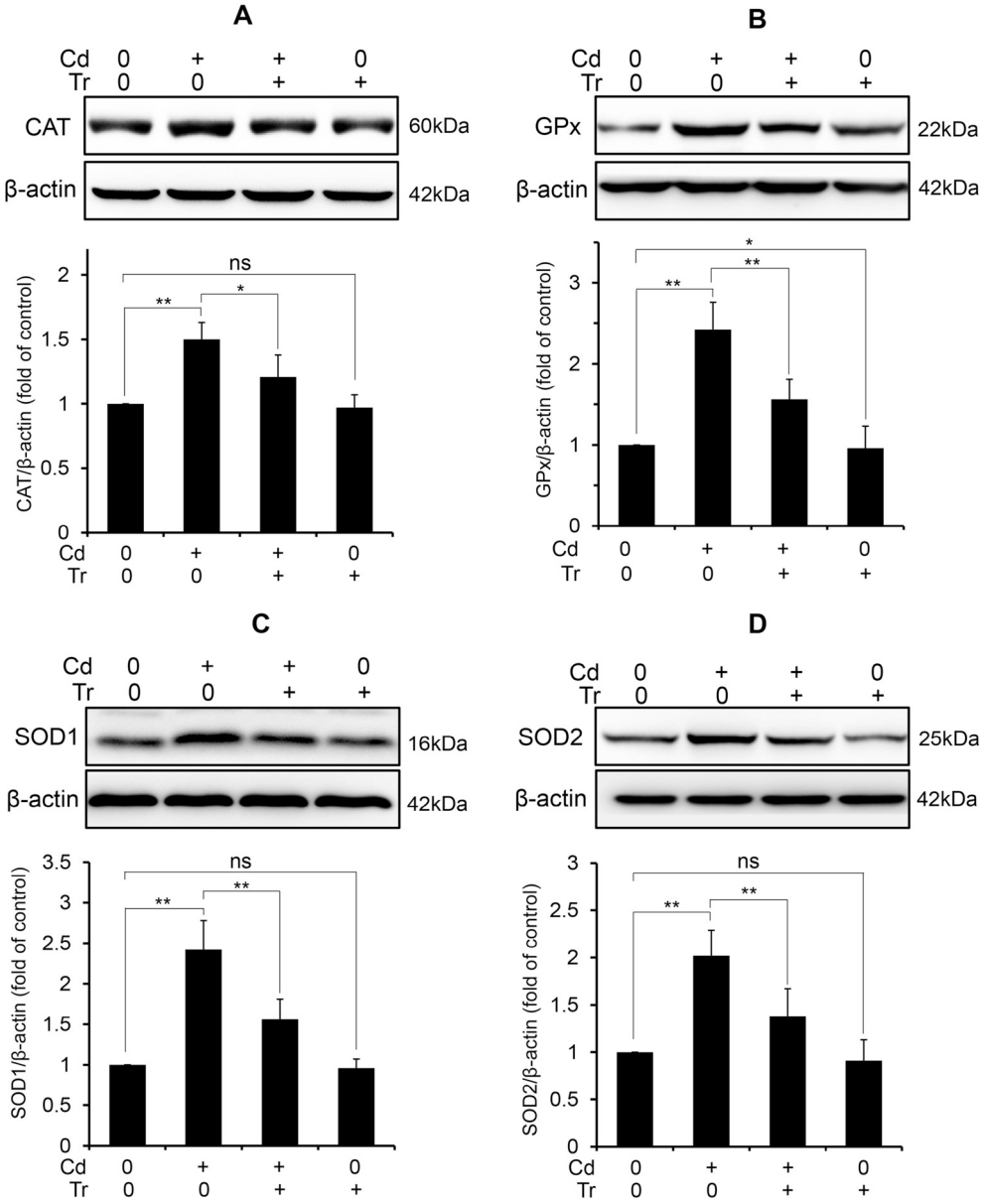

Fig. 6. Tr downregulates Cd-activated protein levels of antioxidant enzymes in liver tissues. Rats were treated with Cd and/or Tr for 8 weeks as mentioned above, then liver tissues were applied to determine the protein levels of CAT (A), GPx (B), SOD-1 (C) and SOD-2 (D) using western blot analysis. *Upper panel* representative western blot image; *lower panel* quantitative analysis (mean ± SEM, $n = 4$). ns, not significant; $^{*}P < 0.05$; $^{**}P < 0.01$.

protein levels of antioxidant enzymes in liver tissues were markedly down-regulated by Tr administration. What is the cause of these changes? Cd exerts multi-organ toxicity following chronic exposure, so its whole-body toxic effect can be reflected by the changes in serum biomarkers of oxidative stress. Meanwhile, Cd-inhibited serum antioxidant enzyme activities were markedly restored by Tr treatment, indicating the antioxidant capacity of Tr upon stress conditions. Moreover, Nrf2 pathway is very important in protecting the liver, while hepatocytes are stimulated to produce self-defense response against Cd exposure by elevating the endogenous antioxidant levels. Tr can also trigger the Nrf2 signaling pathway in rat liver. Once absorbed by the hepatocytes, it may replace endogenous antioxidants to counteract Cd-induced oxidative stress in hepatocytes. Consequently, Cd-elevated protein levels of antioxidant enzymes in rat liver were significantly alleviated by Tr treatment.

Another explanation may be attributed to the autophagy-enhancing effect of Tr. Autophagy is a cellular process whereby the cell adapts to stressful conditions by degrading proteins, aggregates and cellular organelles for energy purposes and, hence, cell survival [44]. Autophagy and oxidative stress are reciprocally linked. Constitutive autophagic activity is especially required to eliminate excessive ROS, while excessive oxidative stress contributes to autophagy inhibition [4,45]. Besides by Keap1, levels of active Nrf2 are regulated by autophagy [46]. The p62 protein (SQSTM1) is commonly used as a marker to study autophagic activity, it accumulates when autophagy is inhibited whereas is degraded when autophagy is induced [38]. Moreover, oxidative stress can up-regulate p62 with resultant sequestration of Keap1 and activation of Nrf2 and Nrf2-dependent antioxidant defense gene expression [46]. In this study, Cd induced autophagy inhibition in liver tissues (Fig. 7), and p62 accumulation may cause a feed-back loop that amplifies the Nrf2 system, leading to persistent hepatic Nrf2 activation and elevated antioxidant enzymes expression. Consistent with the previous result [23], Tr not only activated autophagy, but also upregulated p62 expression, which may be attributed to its mTOR-independent autophagy enhancing-effects. Administration with Tr markedly restored Cd-induced hepatic autophagy inhibition, and p62

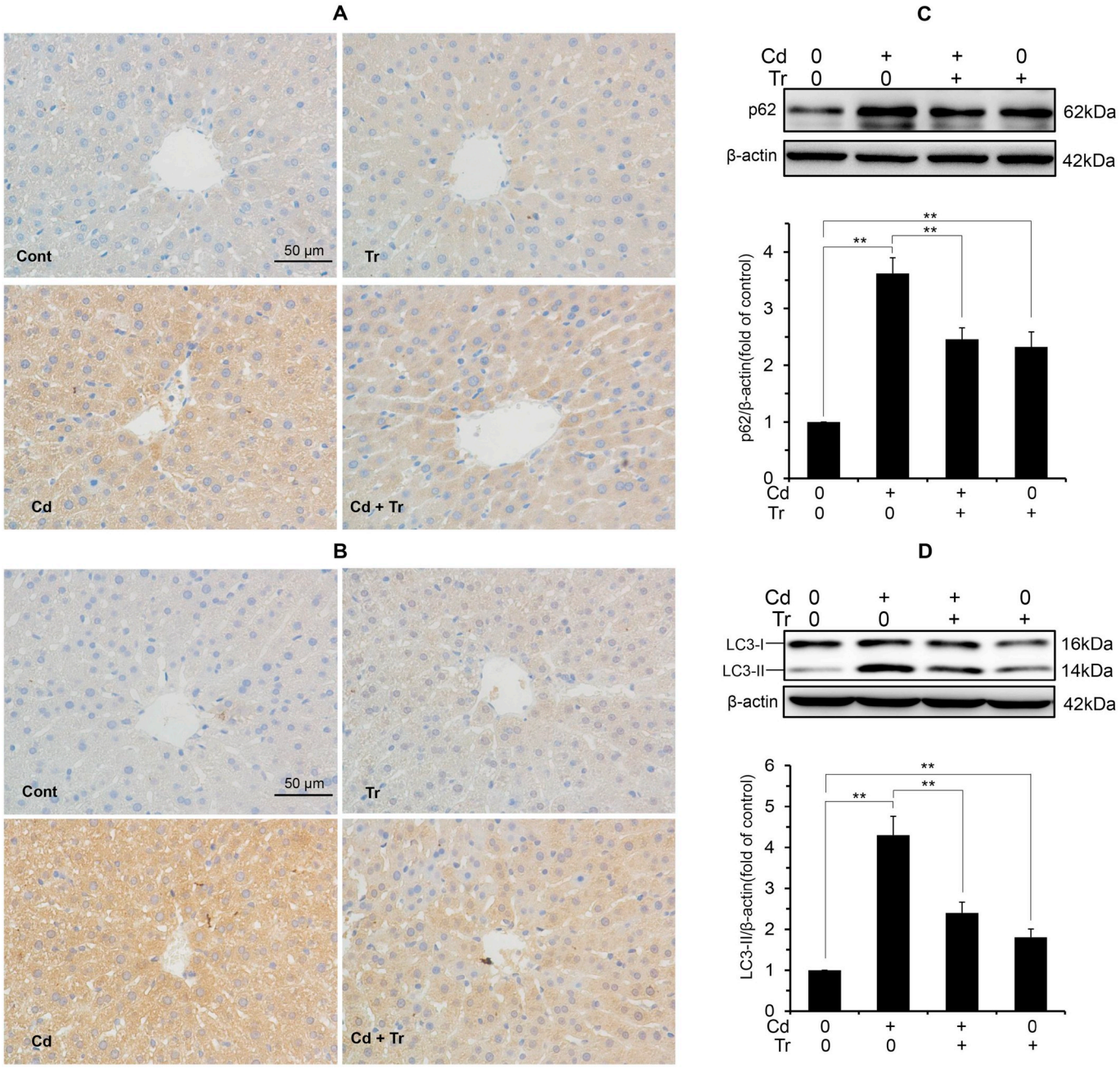

Fig. 7. Inhibitory effect of Tr on Cd-elevated expression of autophagy markers in rat liver. (A, B) Rats were treated with Cd and/or Tr as mentioned above, then liver tissues were used to perform the immunohistochemical staining of p62 (A) and LC3 (B). Scale bar: 50 μm. (C, D) Rats were treated with Cd and/or Tr as mentioned above, then liver tissues were collected to assess the protein levels of p62 (C) and LC3 (D) using immunoblot analysis. Upper panel representative western blot image; lower panel quantitative analysis (mean ± SEM, n = 4). ** $P < 0.01$.

degradation cannot guarantee its binding with Keap1, contributing to functional Keap1-Nrf2 interaction. Resultantly, Cd-induced hepatic Nrf2 activation was significantly inhibited by Tr. These speculations account for the hepatoprotective action of Tr against Cd-induced Nrf2 activation and autophagy inhibition, which needs to be further corroborated.

Moreover, it is noteworthy that oxidative stress-mediated apoptosis plays an important role in Cd-induced hepatotoxicity [5,8,11]. Many studies have demonstrated that Tr exerts its cytoprotective action through preventing apoptosis [24,25,47,48]. For example, it was proved that Tr-based eye drops is effective in the treatment of severe human dry eye by inhibiting apoptosis [49]. Also, Tang et al. (2017) found that Tr has anti-apoptotic effects through the suppression of oxidative stress-induced mitochondrial injury, which is dependent on the promotion of autophagy [22]. Consistent with these previous results, our data (Figs. 8–9) corroborate the protective effect of Tr on Cd-induced apoptosis in liver tissues by inhibiting caspase-dependent pathway; however, whether other apoptotic pathways have a part in this process remains to be further clarified.

Given the intimate relationship between oxidative stress and Cd-induced apoptosis, it indicated that the anti-apoptotic effect of Tr against Cd exposure results from its antioxidant capacity. There is also crosstalk between apoptosis and autophagy. Activation of autophagy protects the cell from apoptosis or delays its initiation, while inhibition of autophagy promotes caspase-mediated apoptosis [50–52]. Thus, the anti-apoptotic effect of Tr may be attributed to its restoration of

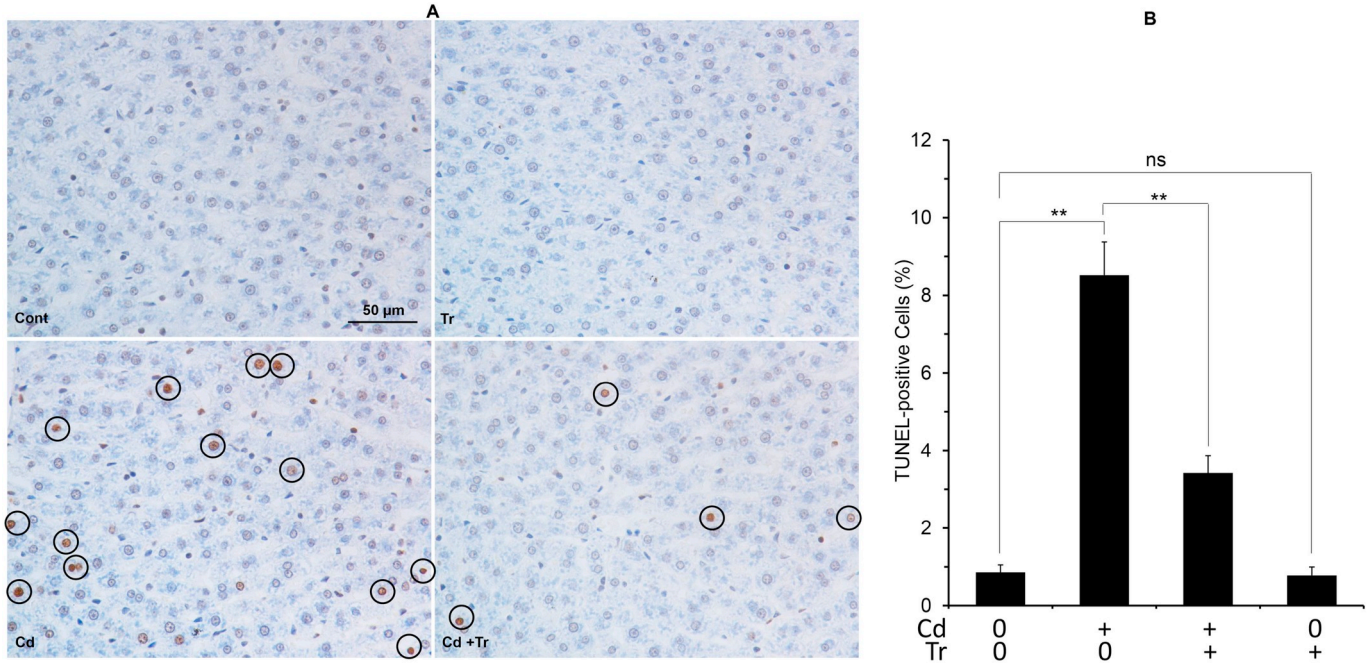

Fig. 8. Tr inhibits Cd-induced apoptosis in liver tissues. Rats were treated with Cd and/or Tr for 8 weeks as mentioned above, then liver tissues were applied to analyze the apoptosis using TUNEL staining. Representative TUNEL staining images are present in (A), and its statistical result of apoptotic rates (B) are expressed as mean ± SEM. ($n = 50$). Typical apoptotic nuclei are circled. Scale bar: 50 μm. *ns*, not significant; ** $P < 0.01$.

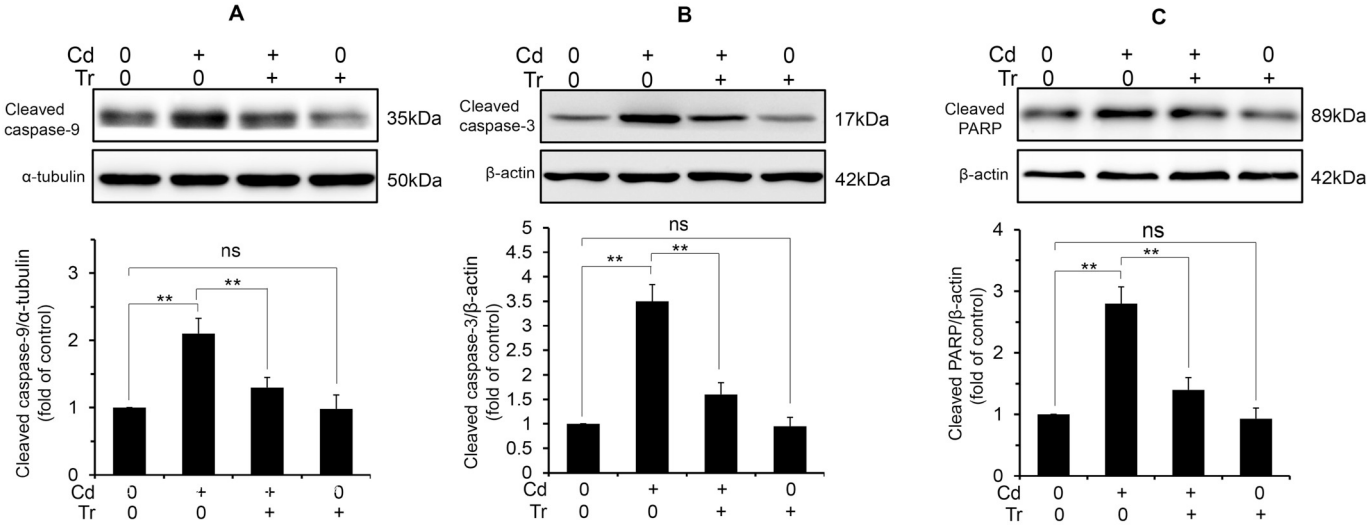

Fig. 9. Effects of Cd and/or Tr on protein levels of apoptotic markers in liver tissues. Rats were treated with Cd and/or Tr for 8 weeks as mentioned above, then liver tissues were applied to analyze the protein levels of cleaved caspase-3 (A), cleaved caspase-9 (B) and cleaved PARP (C) using western blot analysis. *Upper panel* representative western blot image; *lower panel* quantitative analysis (mean ± SEM, $n = 4$). *ns*, not significant; ** $P < 0.01$.

autophagy in Cd-exposed liver tissues. Overall, these findings offer ample evidence to elucidate the protective role of Tr on Cd-induced hepatotoxicity.

In summary, there are several novel findings in this study. First, Tr protects against Cd-induced liver injury via its anti-oxidative activity. Second, Cd-activated Nrf2 signaling pathway in rat liver was markedly attenuated by Tr treatment, indicating that Tr acts as an antioxidant to replace endogenous antioxidant defense ability in rat liver. Third, Cd-induced autophagy inhibition and apoptosis in rat liver was separately restored and inhibited by Tr treatment. Overall, these findings verified that Tr protects against Cd-induced hepatotoxicity via blocking Nrf2 pathway, restoring autophagy and inhibiting apoptosis, suggesting that Tr may serve as potential new candidate drugs to treat Cd-induced hepatotoxicity.

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Conflict of interest

The authors declare that they have no conflict of interest.

Abbreviations

Cd	cadmium
Tr	trehalose
Nrf2	nuclear factor erythroid 2-related factor 2
ARE	antioxidant response element
mTOR	mammalian target of rapamycin
$CdCl_2$	cadmium chloride
MDA	malonaldehyde
T-AOC	total antioxidant capacity
SOD	superoxide dismutase
GPx	glutathione peroxidase
CAT	catalase
GSH	glutathione
AST	aspartate aminoteansferase
ALT	alanine transaminase
LDH	lactic dehydrogenase
TUNEL	terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling
ECL	enhanced chemiluminescence
BCA	bicinchonininc acid
Keap1	Kelch-like epichlorohydrin-associated protein 1
HO-1	heme oxygenase 1
NQO1	NAD(P)H dehydrogenase [quinone] 1
SQSTM1/p62	sequestosome 1
LC3	microtubule-associated protein 1 light chain 3
PARP	poly (adenosine diphosphate-ribose) polymerase
PVDF	polyvinylidene fluoride
HRP	horseradish peroxidase
PBS	Phosphate buffered saline
DAB	diaminobenzidine
IHC	immunohistochemistrical
RT	room temperature
ROS	reactive oxygen species

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