Therapeutic Efficacy of Wuzhi Tablet (*Schisandra sphenanthera* Extract) on Acetaminophen-Induced Hepatotoxicity through a Mechanism Distinct from *N*-Acetylcysteine

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Received November 12, 2014; accepted December 22, 2014

ABSTRACT

Acetaminophen (APAP) hepatotoxicity is the most common cause of drug-induced liver injury and *N*-acetylcysteine (NAC) is the primary antidote of APAP poisoning. Wuzhi tablet (WZ), the active constituents well identified and quantified, is a preparation of an ethanol extract of *Schisandra sphenanthera* and exerts a protective effect toward APAP-induced hepatotoxicity in mice. However, the clinical use of WZ to rescue APAP-induced acute liver injury and the mechanisms involved in the therapeutic effect of WZ remain unclear. Therefore, the effect of WZ on APAP hepatotoxicity was compared with NAC in mice, and molecular pathways contributing to its therapeutic action were investigated. Administration of WZ 4 hours after APAP treatment significantly attenuated APAP hepatotoxicity and exerted much better therapeutic effect than NAC, as revealed by morphologic, histologic, and biochemical assessments. Both WZ and NAC prevented APAP-induced

c-Jun N-terminal protein kinase activation and mitochondrial glutathione depletion in livers. The protein expression of nuclear factor erythroid 2-related factor 2 target genes including *Gclc*, *Gclm*, *Ho-1*, and *Nqo1* was increased by WZ administration. Furthermore, p53 and p21 levels were upregulated upon APAP exposure, which were completely reversed by postdosing of WZ 4 hours after APAP treatment over 48 hours. In comparison with NAC, WZ significantly increased the expression of cyclin D1, cyclin D-dependent kinase 4, proliferating cell nuclear antigen, and augmenter of liver regeneration in APAP-injured livers. This study demonstrated that WZ possessed a therapeutic efficacy against APAP-induced liver injury by inhibiting oxidative stress and stimulating a regenerative response after liver injury. Thus WZ may represent a new therapy for APAP-induced acute liver injury.

Introduction

Acetaminophen (APAP) is a commonly used analgesic and antipyretic drug, which is relatively safe and effective when used at therapeutic doses. However, unintentional and/or intentional overdose of APAP usually results in hepatic toxicity, which has become the most common cause of drug-induced liver injury and a persistent important public health concern in the United States and Great Britain (Larson et al., 2005). An overdose of APAP saturates the glucuronidation and sulfation pathways, leading to massive accumulation of the reactive intermediate *N*-acetyl-*p*-benzoquinone imine (NAPQI). Normally, NAPQI is detoxified by conjugation to glutathione (GSH) and rapidly excreted in urine. However, excess NAPQI after APAP overdose depletes GSH and binds to cellular proteins, which triggers initiation of the injury process, ultimately leading to hepatocellular necrosis (Saito et al., 2010a).

This work was supported by the Natural Science Foundation of China [Grants 81373470, 81320108027]; the Science and Technology Ministry of China [Grant: 2012ZX09506001-004]; and the Fundamental Research Fund for the Central Universities [No. 13ykpy08].

dx.doi.org/10.1124/dmd.114.062067.

N-acetylcysteine (NAC) has been the primary antidote of APAP poisoning in clinical practice for several decades (Prescott et al., 1977; Kanter, 2006). NAC ameliorates APAP hepatotoxicity by replenishing GSH when administered before or early after APAP, because NAC provides cysteine as a precursor for GSH synthesis (Saito et al., 2010b). However, its efficacy, related to the time delay between APAP overdose and therapy, is limited to the early stages of APAP intoxication (Whyte et al., 2010). Moreover, prolonged treatment with a high dose of NAC would impair liver regeneration after liver injury induced by APAP (Athuraliya and Jones, 2009; Yang et al., 2009). Therefore, it would be of great value to develop more effective and safe drugs for prevention and therapeutic intervention strategies to treat APAP-induced hepatotoxicity.

Schisandra sphenanthera, the dried ripe fruit of *Schisandra sphenanthera* Rehd. et Wils, has been widely used as a restorative and tonic in Asia and is now indexed in the Pharmacopoeia of China. *Schisandrae sphenanthera* exerts a beneficial effect in improving the body's resistance to disease, stress, and other debilitating processes (Panossian and Wikman, 2008). Moreover, *Schisandra sphenanthera* was found to possess numerous pharmacological activities such as hepatoprotective effects against chemical hepatitis, viral and various

ABBREVIATIONS: ALR, augmenter of liver regeneration; ALT, alanine aminotransferase; APAP, acetaminophen; ARE, antioxidant response element; AST, aspartate aminotransferase; CDK4, cyclin D-dependent kinase 4; GCLC, glutamate-cysteine ligase catalytic subunit; GCLM, glutamate-cysteine ligase modifier subunit; GSH, glutathione; H&E, hematoxylin and eosin; HO-1, heme oxygenase-1; JNK, c-Jun N-terminal protein kinase; NAC, *N*-acetylcysteine; NAPQI, *N*-acetyl-*p*-benzoquinone imine; NQO1, NAD(P)H:quinone oxidoreductase 1; NRF2, nuclear factor erythroid 2-related factor 2; PCNA, proliferating cell nuclear antigen; WZ, Wuzhi tablet.

hepatotoxins, and improvement of heart and kidney function (Zhu et al., 2000; Xie et al., 2010). Wuzhi tablet (WZ), a preparation of an ethanol extract of *Schisandra sphenanthera*, is widely used in the clinical practice in China to protect liver function in chronic hepatitis and liver dysfunction patients. Chemical fingerprint of WZ and the content of six lignans, including schisandrin A, schisandrin B, schisandrin C, schisandrol A, schisandrol B, and schisantherin A, was determined in our previous study (Qin et al., 2014a,b). The contents (mg/g tablet) of schisandra lignans in WZ were as follows: schisantherin A, 12.97 ± 1.20 mg/g; schisandrin A, 12.57 ± 1.59 mg/g; schisandrin B, 0.027 ± 0.0031 mg/g; schisandrin C, 0.047 ± 0.0054 mg/g; schisandrol A, 0.077 ± 0.0086 mg/g; and schisandrol B, 0.89 ± 0.10 mg/g, respectively. Schisantherin A and schisandrin A were the two highest bioactive lignans in WZ, and other lignans were much lower than the above contents. Recently, WZ was found to possess a protective effect against APAP hepatotoxicity when administrated 3 days before APAP treatment (Bi et al., 2013; Fan et al., 2014b). However, whether WZ could rescue APAP-induced hepatotoxicity and the mechanisms involved remain unknown. The purpose of this study was to investigate whether WZ exerted a therapeutic effect against APAP-induced acute hepatotoxicity and, if so, how it compared with NAC and what molecular pathways contributed to its therapeutic action.

Materials and Methods

Chemicals and Reagents. Acetaminophen and *N*-acetylcysteine were obtained from Sigma-Aldrich (St. Louis, MO). Wuzhi tablets were manufactured by Fanglue Pharmaceutical Company (Guangxi, China) and have 7.5 mg schisantherin A per tablet. Antibodies against total JNK, p-JNK, and GAPDH were from Cell Signaling Technology (Danvers, MA). Glutamate-cysteine ligase catalytic subunit (GCLC), p53, and glutamate-cysteine ligase modifier subunit (GCLM) antibodies were obtained from Abcam (Cambridge, UK). NAD(P)H:quinone oxidoreductase 1 (NQO1), heme oxygenase-1 (HO-1), and p21 antibodies from Santa Cruz Biotechnology (Santa Cruz, CA). All other antibodies including p-c-jun, PCNA, cyclin D1, cyclin D-dependent kinase 4 (CDK4), and augmentor of liver regeneration (ALR) were provided by Shanghai Sangon Biotech Co., Ltd. (Shanghai, China). The secondary antibody was obtained from Cell Signaling Technology.

Animals. Male C57BL/6 mice (6–8 weeks old) were obtained from Laboratory Animal Center of Sun Yat-sen University (Guangzhou, China). The mice were acclimatized 1 week before the experiment, maintained under controlled conditions (22–24°C, 55–60% humidity and 12-hour light/dark cycle) and fed with standard food and water ad libitum. All experimental procedures were in accordance with the Regulations of Experimental Animal Administration issued by the Ministry of Science and Technology of the People's Republic of China (<http://www.most.gov.cn>). All animal protocols were approved by the Ethics Committee on the Care and Use of Laboratory Animals of Sun Yat-sen University.

Mice were fasted overnight before APAP administration. WZ and APAP solutions were prepared as described previously (Bi et al., 2013). The NAC solution was made fresh in 0.9% saline at 10 mg/ml (pH 7.4). The dosage of WZ (350 mg/kg) used in the current study was derived from the dose of humans in clinical practice with correction for body surface difference between humans and mice. In the initial experiments, the mice were administered a single dose of 400 mg/kg APAP by intraperitoneal injection. Blood was collected from the retrobulbar venous plexus before APAP dosing and 2, 4, 24, 48, 72 hours after APAP treatment to observe the dynamic change of liver injury. In subsequent experiments, mice were randomly divided into five groups: 1) untreated control group, 2) WZ (350 mg/kg)-treated group, 3) APAP-treated group, 4) APAP/WZ (350 mg/kg)-treated group, and 5) APAP/NAC (200 mg/kg)-treated group. Four hours after APAP dosing (400 mg/kg i.p.), mice were administered WZ (350 mg/kg), NAC (200 mg/kg), or saline solution by gavage with an interval of 12 hours for 2 consecutive days. Mice were killed at 3, 12, 24, 36, and 48 hours after WZ or NAC administration. Serum and liver tissues were harvested. A portion of the liver was immediately fixed in 10% buffered formalin for histologic sections, and the remaining tissues were flash frozen in liquid nitrogen and stored at -80°C for further use.

Histologic and Biochemical Assessment. Liver tissues fixed in neutral buffered formalin were embedded in paraffin, cut into 3- μm -thick sections, and stained with hematoxylin and eosin (H&E) according to a standard protocol.

H&E-stained liver sections were examined using a LEICA DM5000B microscope (Leica, Heidelberg, Germany) and used for necrosis scoring. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined to evaluate APAP-induced liver injury with a commercial AST or ALT assay kit (Kefang Biotech, Guangzhou, China) on a Beckman Synchron CX5 Clinical System. Reduced glutathione (GSH) in mitochondria was measured using commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Western Blot Analysis. Liver whole cell, cytosolic, or mitochondrial extracts were prepared from frozen liver tissues, and protein concentrations were determined using the Bradford method. Equivalent amounts of protein extracts from three different mouse liver tissues in each group were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then blotted onto polyvinylidene fluoride membranes (Millipore, Bedford, MA). Phosphorylated proteins were blocked with 5% bovine serum albumin in Tris-buffered saline, and other proteins with 5% nonfat milk in Tris-buffered saline. Membranes were incubated overnight at 4°C with primary antibodies, followed by secondary antibodies conjugated with horseradish peroxidase for 1 hour at room temperature. Protein-antibody complexes were detected using an electrochemiluminescence (ECL) kit (Engreen Biosystem, Beijing, China) and exposed to an X-ray film (GE Healthcare, Piscataway, NJ). The intensity of protein bands was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD).

Statistical Analysis. Data are presented as the mean $\pm$ S.E.M. To determine statistically significant difference between two groups, two-tailed unpaired Student's *t* test was carried out using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA). For multiple comparisons, one-way analysis of variance followed by with Bonferroni post hoc test was applied. The difference was considered statistically significant at $P < 0.05$.

Results

Dynamic Change of APAP-Induced Liver Injury in Mice. Male C57BL/6 mice treated with a single dose of 400 mg/kg APAP were used to observe the dynamic change of APAP-induced liver injury to choose the right time point for WZ or NAC dosing. Time courses of serum ALT and AST activities from APAP-treated mice were investigated over 72 hours to characterize APAP hepatotoxicity. Treatment with 400 mg/kg APAP resulted in significant hepatic toxicity as revealed by increased aminotransferase activity (Fig. 1A). ALT and AST levels after APAP exposure were rapidly elevated with a peak at 4 hours, subsequently reduced at later time points, and then tended toward baseline by 72 hours. Furthermore, centrilobular hepatocellular necrosis was clearly evident in APAP injured livers by 4 hours as demonstrated by histopathological analysis of H&E-stained liver sections (Fig. 1B). These results indicated that severe liver injury was already evident at 4 hours after APAP treatment. ALT and AST activities in mice treated with APAP were increased within 2 hours compared with those of 0 hour, but the difference of AST levels between 0 hour and 2 hours was not statistically significant. Thus, 4 hours was chosen as the time point for WZ or NAC dosing to rescue APAP hepatotoxicity in mice.

Therapeutic Effects of WZ or NAC on APAP-Induced Hepatotoxicity in Mice. The therapeutic effects of WZ or NAC on APAP-induced acute liver injury in mice were directly compared. Four hours after APAP treatment, mice were administrated WZ or NAC. APAP-treated mice exhibited centrilobular necrosis, loss of nuclei, and cellular swelling in the central vein region at 12 and 24 hours (Fig. 2A). Compared with the first 24 hours, much less hepatocellular injury and necrosis was observed at 48 hours but was not yet completely resolved consistent with a previous report (Fan et al., 2014a). Treatment with WZ 4 hours after APAP markedly diminished hepatic damage that was limited to small, localized hepatic necrosis around the centrilobular areas, whereas extensive necrotic region similar to saline treatment in APAP-treated group was evident in the APAP/NAC-treated group. Additionally, APAP/NAC-treated mice showed massive hemorrhage at 12 and

Fig. 1. Dynamic change of APAP-induced liver injury in mice. (A) Dynamic change of serum ALT and AST activities from APAP-treated mice over a time course of 0–72 hours after a dose of 400 mg/kg APAP challenge. Data are expressed as the mean $\pm$ S.E.M. ($n = 5$). (B) H&E-stained liver sections from mice before or 4 hours after APAP treatment. * $P < 0.05$, *** $P < 0.001$ versus mice before APAP exposure.

24 hours after NAC dosing, which was not observed in APAP-treated and APAP/WZ-treated mice.

Dynamic changes of serum ALT and AST activities were also measured over 48 hours (Fig. 2B). There was no significant difference in ALT and AST activities between APAP-treated and APAP/NAC-treated mice over a time course of 0–48 hours. Compared with NAC, WZ significantly inhibited the elevation of ALT and AST levels induced by APAP. Notably, 3 hours after WZ administration, ALT and AST levels in APAP/WZ-treated mice had already declined relative to APAP-treated and APAP/NAC-treated mice, which may be due to the absorption of active compounds of WZ *in vivo* (Qin et al., 2014b). Beyond 24 hours, there were no significant differences among the injured livers in AST levels, whereas mice treated with WZ after APAP exhibited relatively lower ALT levels than those treated with saline or NAC. This suggested that ALT activity was preferentially reduced over AST upon WZ treatment. Furthermore, administration of WZ 4 hours after APAP increased the survival rate of mice with APAP-induced liver injury compared with NAC treatment (Fig. 2C). Taken together, these observations indicated that WZ had a therapeutic potential to rescue APAP hepatotoxicity when liver injury was evident; however, postdosing of NAC 4 hours after APAP treatment had no effect in alleviating APAP-induced liver injury in mice.

Effects of WZ or NAC on JNK Activation Induced by APAP. Western blot analysis of total and phosphorylated JNK indicated JNK activation in APAP-treated mice at 24 hours (Fig. 3, A and B). Elevated p-JNK levels were consistent with increased p-c-jun levels, suggesting that p-JNK was correlated with enhanced activity. Administration of WZ or NAC 4 hours after APAP treatment significantly inhibited APAP-induced JNK phosphorylation. However, WZ exerted a stronger effect

than NAC by inactivating JNK and suppressing JNK activity to normal levels in response to APAP treatment.

Effects of WZ or NAC on GSH Depletion Induced by APAP. It is known that hepatic GSH depletion by NAPQI generation is a key event in APAP toxicity (Reid et al., 2005). Thus the levels of mitochondrial reduced GSH in uninjured and injured livers were examined. Mitochondrial GSH levels in APAP-treated mice were still lower than that of control mice by 12 hours (Fig. 3C), indicating that oxidative stress still existed. WZ treatment resulted in a significant elevation of mitochondrial GSH content in uninjured livers and a recovery of GSH in APAP injured livers at 12 hours. Administration of NAC 4 hours after APAP treatment could completely reverse the decrease of GSH induced by APAP. GSH levels in APAP-treated mice were restored to baseline by 24 hours, as previously described (Bi et al., 2013). Compared with APAP-treated mice, APAP/WZ-treated mice also exhibited increased GSH levels at 24 hours. The results demonstrated that WZ and NAC attenuated GSH depletion induced by APAP.

Effects of WZ or NAC on NRF2 Target Genes. The expression levels of proteins involved in NRF2-antioxidant response element (ARE) pathway were measured to explore whether WZ or NAC activated this pathway to alleviate APAP-induced liver injury. Western blot analysis of NRF2 target proteins indicated that APAP treatment inhibited expression of GCLM, GCLC, NQO1, and HO-1 at 12 and 24 hours (Fig. 4). Mice treated with WZ after APAP showed a higher GCLM, GCLC, NQO1, and HO-1 levels during the first 24 hours compared with those of APAP-treated and APAP/NAC-treated mice. However, WZ had no significant effect on expression of NRF2 target proteins at 48 hours in uninjured livers. At 48 hours, levels of these four proteins in APAP-injured livers recovered to normal levels.

Fig. 2. Therapeutic effects of WZ or NAC on APAP-induced hepatotoxicity in mice. (A) H&E-stained liver sections, (B) serum ALT and AST activities from control, WZ (350 mg/kg)-treated, APAP-treated, APAP/WZ (350 mg/kg)-treated, APAP/NAC (200 mg/kg)-treated mice over 48 hours after WZ or NAC administration. Data are expressed as the mean $\pm$ S.E.M. ($n = 5$). (C) Kaplan-Meier survival curve for APAP-treated, APAP/WZ (350 mg/kg)-treated mice, and APAP/NAC (200 mg/kg)-treated mice with a hepatotoxic dose of APAP (400 mg/kg) over 72 hours after saline, WZ, or NAC administration. Data are the mean $\pm$ S.E.M. ($n = 7$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control mice, # $P < 0.05$, ## $P < 0.01$ versus APAP-treated mice, $^{\$}P < 0.05$, $^{ss}P < 0.01$ versus APAP/NAC-treated mice.

Effects of WZ or NAC on Liver Regeneration after APAP Hepatotoxicity. In a previous study, the p53/p21 signaling pathway was reported to participate in compensatory liver regeneration after APAP-induced liver injury (Fan et al., 2014a). Thus, p53 and p21 protein expression was investigated to explore whether WZ or NAC could block p53/p21 signaling to promote liver repair in mice after APAP-induced toxicity. The results showed that p53 and p21 levels were low or undetectable in uninjured livers (Fig. 5). As expected, APAP treatment markedly induced p53 and p21 expression with the peak p53/p21 at 12 hours after by a decline to normal levels at 48 hours. The upregulation of p53 and p21 expression induced by APAP during the first 24 hours was significantly suppressed by WZ treatment and recovered to baseline over 48 hours after WZ dosing. NAC treatment also clearly increased p53 protein level at 12 hours, which was not significantly different with that of APAP-treated mice. However, at 24 and 48 hours after NAC administration, p53 protein expression was reduced near to normal levels. Consistent with p53, p21 level in APAP-injured livers was also upregulated by NAC with peak expression at 12 hours followed by a time-dependent decline to baseline, which was still higher than that of APAP/WZ-treated mice at 48 hours. These results indicated that NAC had no significant effect in preventing the aberrant increase of p21 in injured livers. To evaluate cell cycle activation, the dynamic changes of cell proliferation-associated proteins including CDK4, cyclin D1, PCNA, and ALR in uninjured and injured livers were determined. CDK4 and PCNA protein levels showed a significant downregulation upon APAP treatment over 48 hours relative to those of

uninjured livers. WZ treatment significantly reversed the decrease of CDK4 and PCNA levels by APAP and restored CDK4 and PCNA expression to baseline at 48 hours, whereas NAC had no effect on the decreased CDK4 and PCNA expression induced by APAP. Cyclin D1 levels in APAP-injured livers were similar to baseline at 12 hours, but slightly decreased at 24 hours after APAP treatment, which were significantly reversed by WZ administration compared with NAC. At 48 hours, cyclin D1 in APAP-treated mice returned to normal level and exhibited a slight increase after WZ or NAC treatment. APAP also slightly inhibited ALR expression compared with that of control mice, which was restored by WZ over 48 hours. Additionally, WZ alone showed no obvious effect on CDK4 and ALR protein levels, but clearly upregulated cyclin D1 expression and slightly increased PCNA level in uninjured livers. Taken together, these data indicated that WZ treatment led to the timely onset of liver repair processes by inhibiting p53/p21 signal and activating cell cycle to limit liver injury and promote regeneration after APAP hepatotoxicity.

Discussion

WZ pretreatment protected against APAP-induced liver injury in our previous studies (Bi et al., 2013; Fan et al., 2014b). Pretreatment with hepatoprotective drugs for alleviating toxicity of hepatotoxic compounds is an attractive strategy for future drug development but is not directly applicable to patients seeking treatment after ingestion of drugs such as APAP at hepatotoxic doses (Patel et al., 2012). Whether WZ

Fig. 3. Effects of WZ or NAC on mitochondrial oxidative stress induced by APAP. (A) Western blot analysis of p-JNK and JNK levels using mitochondrial extracts, p-c-jun, and GAPDH levels using liver total extracts from control, WZ (350 mg/kg)-treated, APAP-treated, APAP/WZ (350 mg/kg)-treated, APAP/NAC (200 mg/kg)-treated mice at 24 hours after WZ or NAC treatment. (B) Densitometric analysis of Western blots. Data are expressed as the mean $\pm$ S.E.M. ($n = 3$). (C) Mitochondrial GSH levels from control, WZ (350 mg/kg)-treated, APAP-treated, APAP/WZ (350 mg/kg)-treated, APAP/NAC (200 mg/kg)-treated mice at 12 and 24 hours after WZ or NAC treatment. Data are expressed as the mean $\pm$ S.E.M. ($n = 5$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control mice, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus APAP-treated mice, $^sP < 0.05$ versus APAP/NAC-treated mice.

could rescue APAP-induced hepatotoxicity and the mechanisms involved remain unknown. Therefore, the current study aimed to investigate the therapeutic value of WZ on APAP-induced hepatotoxicity and to compare its effect with NAC. In clinical practice, antidotes are usually given after the occurrence of APAP toxicity. In this study, NAC and WZ were administrated 4 hours after APAP when aberrant elevation of ALT and AST activities and evident hepatic necrosis were observed, which should be more relevant to the clinical situation. The results indicated that rescue therapy of WZ significantly limited hepatocellular damage and necrosis induced by APAP, reduced serum ALT and AST activities, and increased survival rate of APAP-injured animals. However, mice treated with NAC 4 hours after APAP administration still showed massive hepatic toxicity similar to that in APAP-treated mice. These findings demonstrated that WZ successfully rescued mice from APAP hepatotoxicity and might provide clinically useful means to treat liver injury associated with APAP. NAC had no therapeutic effect on APAP hepatic toxicity when administrated 4 hours after APAP challenge, which was consistent with previous studies (James et al., 2003; Park et al., 2013). Several studies have demonstrated that NAC prevented APAP toxicity when administered before or early (such as 1 or 2 hours) after APAP (Salminen et al., 1998; James et al., 2003). The efficacy of NAC is inversely dependent on its time of administration relative to APAP

overdose in the clinical setting, which is well established in APAP intoxication patients (Schmidt et al., 2002).

It is reported that APAP has approximate half-life of 1 hour in mice (Fischer et al., 1981), indicating that APAP was fully metabolized before NAC or WZ administration in the current study (Latchoumycandane et al., 2007; Ghosh et al., 2010). The same amount of NAPQI and protein adducts was generated by 4 hours in mice treated with APAP. Thus the mechanism of WZ for its therapeutic action was not through inhibition of cytochrome P450-catalyzed APAP bioactivation (Jaeschke et al., 2011). Instead, WZ is likely to influence the excretion of APAP conjugates, to reduce oxidative stress, or to promote liver repair to alleviate APAP-induced liver injury.

Activation of JNK is known to be a crucial signaling component of APAP-induced hepatotoxicity (Gunawan et al., 2006; Saito et al., 2010a). NAC and WZ significantly inhibited JNK phosphorylation induced by APAP, but WZ further suppressed JNK activity as evidenced by decreased p-c-jun levels. NAC, as a GSH precursor, increased the mitochondrial GSH level in mice after APAP challenge as previously described (Saito et al., 2010b). The decline of GSH by APAP was completely restored by WZ and NAC. Because NAPQI covalent binding and APAP protein adducts have already been formed before 4 hours after APAP exposure, GSH replenishment is not a prerequisite event for

Fig. 4. Effects of WZ or NAC on protein expression of NRF2 target genes. (A) Western blot analysis of GCLC, GCLM, HO-1, NQO1, and GAPDH proteins in livers from control, WZ (350 mg/kg)-treated, APAP-treated, APAP/WZ (350 mg/kg)-treated, APAP/NAC (200 mg/kg)-treated mice. (B) Densitometric analysis of Western blots. Data are expressed as the mean $\pm$ S.E.M. ($n = 3$). * $P < 0.05$, ** $P < 0.01$ versus control mice, # $P < 0.05$ versus APAP-treated mice, § $P < 0.05$ versus APAP/NAC-treated mice.

ameliorating toxicity. Thus administration of NAC 4 hours after APAP exposure had no beneficial effect on rescue of APAP hepatotoxicity.

NRF2 regulates the expression of a battery of cytoprotective genes encoding intracellular detoxifying enzymes and antioxidant proteins that are responsible for GSH synthesis, antioxidant defense, conjugation, transport, and excretion of the metabolites through ARE (Chan et al., 2001). NRF2 plays a critical role in the multiple steps related to alleviation of APAP toxicity and is identified as a protective target for resistance to liver injury (Gum and Cho, 2013). WZ reversed the decreased protein expression of NRF2 target genes including *Gclm*, *Gclc*, *Nqo1*, and *Ho-1* by APAP and promoted the recovery of these proteins compared with saline or NAC treatment during APAP-induced liver injury. This suggests that WZ may activate the NRF2-ARE pathway to induce detoxification and antioxidation and to inhibit the deleterious effects of oxidative stress by APAP.

Extensive studies have demonstrated that cell division and tissue repair occur simultaneously in response to tissue injury induced by xenobiotics such as APAP, carbon tetrachloride, or thioacetamide (Chanda and Mehendale, 1996; Mehendale, 2005). The ability of remaining hepatocytes to regenerate and replace the dead or dying cells determines survival after hepatocellular injury (Itoh and Miyajima, 2014). If hepatocellular regeneration and tissue repair are timely stimulated after liver damage by a therapeutically compatible mechanism, it should be possible to prevent death arising from serious liver injury (Dalhoff et al., 2001; Fausto et al., 2006). p53 is a tumor suppressor that plays an important role in regulating cell growth, DNA repair, and apoptosis (Shams et al., 2013). Under severe DNA damage by APAP overdose, p53 and its target gene *p21* are activated to inhibit cell proliferation or trigger cell apoptosis (Zhao et al., 2012). WZ significantly inhibited the activation of p53 and p21 by APAP, serving

Fig. 5. Effects of WZ or NAC on liver regeneration after APAP-induced liver injury. (A) Western blot analysis of p53, p21, cyclin D1, CDK4, PCNA, ALR, and GAPDH proteins in livers from control, WZ (350 mg/kg)-treated, APAP-treated, APAP/WZ (350 mg/kg)-treated, APAP/NAC (200 mg/kg)-treated mice. (B) Densitometric analysis of Western blots. Data are expressed as the mean $\pm$ S.E.M. ($n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control mice, # $P < 0.05$, ## $P < 0.01$ versus APAP-treated mice, \$\$\$ $P < 0.05$ versus APAP/NAC-treated mice.

to promote hepatocyte proliferation and liver repair after APAP-induced liver injury. In addition, the expression of cell proliferation-associated proteins including CDK4, cyclin D1, PCNA, and ALR downregulated by APAP was markedly reversed by WZ treatment and returned to

normal levels in advance compared with those of APAP-treated and NAC-treated mice. This indicated that WZ could promote the timely onset of liver repair, which arrested the accelerated progression of liver injury and led to a faster recovery from toxicity. The onset of protein

expression of cyclin D1, CDK4, PCNA, and ALR was clearly delayed in APAP-treated and APAP/NAC-treated mice in comparison with that of APAP/WZ-treated mice. Delayed recovery of cell proliferation-associated protein expression accounts for reduced hepatocyte proliferation and contributes to exaggerated hepatotoxicity. Beyond 24 hours, there were no significant differences in aminotransferase activities and protein expression of p53, p21, cyclin D1, CDK4, and ALR among APAP-treated, APAP/NAC-treated, and APAP/WZ-treated animals. In fact, some of the animals with extensive hemorrhage and hepatocellular necrosis died and were excluded in the recovery phase. Thus only the more robust survivors was contained in the 48-hour groups of the APAP-treated and APAP/NAC-treated animals, suggesting that timely onset of liver regeneration plays a crucial role in recovery from injury and survival of an animal exposed to APAP challenge. The current findings demonstrated that WZ inhibited p53/p21 signaling mainly serving to ameliorate APAP hepatotoxicity by timely stimulation of liver regeneration after liver injury. However, the specificity of WZ action mechanisms needs further investigation, as hepatoprotection might be occurring by means of various mechanisms, including, but not limited to, reducing oxidative stress and promoting liver regeneration.

Additionally, pretreatment with WZ before APAP administration was found to not only restore APAP impaired fatty acid β -oxidation to normal levels through blocking the increase in serum palmitoylcarnitine and oleoylcarnitine, but also maintenance normal fatty acid metabolism, which potentially contributed to the hepatic protection of WZ against APAP-induced hepatic toxicity (Bi et al., 2013). However, whether WZ administration after APAP treatment could also alter the fatty acid β -oxidation status and fatty acid concentrations and then play a role in the therapeutic effect of WZ still need to be further investigated.

In summary, this study clearly demonstrated that APAP hepatotoxicity was alleviated and rescued by WZ treatment 4 hours after administration of a toxic dose of APAP, and the therapeutic effect of WZ was much better than that of NAC. The therapeutic efficacy of WZ against APAP-induced liver injury may be due to the regulation of NRF2 and p53/p21 pathways to inhibit oxidative stress and to stimulate the regenerative response after liver injury. WZ may represent a promising therapy for APAP-induced acute liver injury.

Authorship Contributions

Participated in research design: Fan, Gonzalez, Huang, Bi.

Conducted experiments: Fan, Chen, Jiang, Yi, Wang, Tan, Zeng, Yo, Wang.

Performed data analysis: Fan, Qu.

Wrote or contributed to the writing of the manuscript: Fan, Gonzalez, Bi.

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