

Original Article

Inhibition of mitochondrial complex I by rotenone protects against acetaminophen-induced liver injury

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Abstract: Acetaminophen (APAP) is widely used as an antipyretic analgesic in clinic. However, overdose-related severe liver injury is a major concern of this drug. Recently, accumulating evidence indicated an important role of mitochondrial abnormality in the pathogenesis of APAP hepatotoxicity. Thus, the present investigation was undertaken to evaluate the effect of mitochondrial complex I inhibition by rotenone on APAP hepatotoxicity. In this study, male BALB/c mice were pretreated with 250 ppm of rotenone in food for 3 days, then the animals were intraperitoneally injected with 300 mg/kg APAP. After 24 h APAP administration, animals developed severe liver injury as shown by the remarkable elevation of ALT and AST and hepatic centrilobular necrosis in line with the reduced liver GSH content. Strikingly, rotenone treatment markedly attenuated liver injury as shown by the improved liver enzyme release and liver morphology and enhanced liver GSH content. Meanwhile, rotenone ameliorated mitochondrial abnormality, inflammatory response and oxidative stress. Moreover, the downregulation of NOX4, a documented protector against APAP hepatotoxicity, was significantly restored by rotenone. However, mitochondrial complex III inhibitor AZOX failed to protect liver against APAP-induced injury. Together, these results suggested that inhibition of mitochondrial complex I but not mitochondrial complex III played a potent role in protecting against APAP hepatotoxicity.

Keywords: Rotenone, acetaminophen, hepatotoxicity, mitochondria, inflammation

Introduction

Acetaminophen (APAP) is strongly recommended as an analgesic and antipyretic drug in clinic [1, 2]. For children, it is one of two recommended antipyretic drugs. However, acute overdose can cause severe liver injury in both humans [3] and animals [4, 5]. In the United States, around 30,000 patients are admitted to hospitals every year because of APAP hepatotoxicity, accounting for more than 50% of acute liver failure and approximately 20% of cases of liver transplantation [6].

It is known that therapeutic dose of APAP is mainly metabolized in liver by UDP-glucuronosyltransferases (UTGs) and sulfotransferase (SULTs). However, excessive intake of APAP saturates the glucuronidation and sulfation routes, resulting in the formation of large amount of NAPQI (NADH Coenzyme Q oxidoreductase) converted by CYP450, which is then detoxified

by the conjugation with GSH [7]. GSH is essential for hepatic function [8]. Once GSH is depleted, NAPQI covalently binds to critical cellular proteins, with the formation of reactive oxygen species (ROS), causing hepatocellular necrosis and apoptotic cell death, and eventually resulting in severe centrilobular hepatotoxicity and acute liver failure [9].

In the past years, mitochondrial dysfunction was documented as one of the critical mechanisms mediating the onset and progression of APAP hepatotoxicity [10]. Thus, targeting mitochondria has been considered as a potential strategy for the therapy of APAP-induced liver injury. Actually, literatures demonstrated that proper inhibition of mitochondrial activity under pathological conditions could improve organ injury [11-16]. Rotenone, a lipophilic natural compound, mainly derived from the roots and stems of *Lonchocarpus* and *Derris* species, is the prototype of a number of synthetic insecti-

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cides/acaricides [17]. It acts as a strong inhibitor of complex I of the mitochondrial respiratory chain (MRC) [9]. In the present study, to define the role of mitochondrial complex I inhibition in APAP-induced liver injury, we treated the mice with a mitochondrial complex I inhibitor rotenone before they were exposed to APAP and found a striking protective effect of rotenone against APAP hepatotoxicity possibly through inhibiting the activity of those abnormal mitochondria and subsequent amelioration of mitochondrial oxidative stress and inflammation.

Materials and methods

Rotenone treatment in model of APAP-induced liver injury in mice

Eight-week-old male BALB/c mice weighing approximately 20-23 g were purchased from Animal Core Facility of Nanjing Medical University. Animals were fed with standard chow ad libitum and allowed free access to water. The mice were housed in a temperature-controlled ($25\pm 1^\circ\text{C}$) facility and maintained on a 12-h:12-h light-dark cycle (lights on at 6:00 a.m. and lights off at 6:00 p.m.). After 1 week of adaption, mice were randomly divided into three groups: (1) Vehicle (n=5); (2) APAP (n=7); (3) APAP + Rotenone (n=7, APAP and rotenone were both from Sigma-Aldrich). Group (1) and (2) were fed with jelly diet with vehicle and group (3) was fed with the jelly diet with rotenone at a dose of 250 ppm for 3 days, respectively. Then all the animals were fasted for 12 h but kept free access to water before APAP administration. After 12 h fasting, group (2) and (3) were given a single dose of APAP (300 mg/kg) by intraperitoneal injection and group (1) were injected with equal volume of saline as control. All the animals were fed with the food and water as before after APAP injection. The animals were sacrificed after 24 h of APAP treatment. Blood and liver samples were harvested immediately for further test. All animal experiments procedures were approved by the Nanjing Medical University Institutional Animal Care and Use Committee.

Azoxystrobin treatment in model of APAP-induced liver injury in mice

The feeding conditions of the mice used to study the effect of Azoxystrobin (AZOX) on APAP-induced liver injury were consistent with the rotenone experiment. The mice were ran-

domly divided into four groups (N=8 per group): (1) Vehicle group, (2) AZOX group, (3) APAP group, and (4) APAP + AZOX group. The mice in AZOX group and APAP + AZOX group were treated with 25 mg/kg/d AZOX mixed in the jelly diet for 3 days, then animals were fasted for 12 h but kept free access to water before intraperitoneal injection of a single dose of APAP (300 mg/kg, dissolved in saline). Vehicle and APAP groups were only fed with jelly diet with vehicle for control. All the animals were fed with the same food and water as before after APAP injection. After 24 h APAP injection, the mice were sacrificed, and the blood and liver tissues were harvested for further analyses.

Histological analysis

Harvested liver tissues were cut into small pieces and fixed in 4% paraformaldehyde (PFA) and embedded in paraffin according to the standard procedure for further histological analysis. Then the embedded liver tissues were cut into 5 μm thick sections and stained with hematoxylin-eosin (H&E) for morphological analysis.

Dihydroethidium (DHE) staining

Cryosections from frozen liver tissues (5 μm) were prepared with a Leica CM1900 Scryostat (Leica, Germany). The sections were stained with DHE solution (2 μM) (S0063, Beyotime, China) for 30 minutes in the dark at 37°C , then washed 3 times with PBS. Finally, LSM710 laser confocal microscope (Zeiss, Germany) was used to photograph under 543 nm excitation light.

Determination of liver enzymes

Blood obtained from the mice was collected into the tubes treated with Heparin Sodium Solution (Qianhong Bio-pharma, China) (1 IU/ml) for anticoagulation. After centrifugation at 1,500 rpm for 10 min at room temperature, the plasma was carefully collected into new tubes. The plasma enzyme levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assessed by Hitachi 7600 modular chemistry analyzer according to the manufacturer's instructions (Hitachi Ltd, USA).

Hepatocellular GSH measurement

Liver tissues were homogenized in 1 mL of ice-cold PBS containing 1 mM EDTA (pH7.5). The supernatants were obtained from the tissue

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Table 1. Primers used for real-time PCR

Primer	Sequence
IL-6_F	5'-GGCAATTCTGATTGTATG-3'
IL-6_R	5'-GACTCTGGCTTTGTCTTT-3'
IL-1 β _F	5'-CAACCAACAAGTGATATTCTCCATG-3'
IL-1 β _R	5'-GATCCACACTCTCCAGCTGCA-3'
MCP-1_F	5'-CTTCTGGGCCTGCTGTTC-3'
MCP-1_R	5'-CCAGCCTACTCATTGGGATCA-3'
ICAM-1_F	5'-CGCTCCGCTACCATCAC-3'
ICAM-1_R	5'-GGCGGCTCAGTATCTCCTC-3'
SOD1_F	5'-AGCCCGCGGATGAAG-3'
SOD1_R	5'-CCTTCCAGCAGTCACATTGC-3'
SOD2_F	5'-CGGCCTACGTGAACAATCTC-3'
SOD2_R	5'-GATAGCCTCCAGCAACTCTCC-3'
SOD3_F	5'-TTCTTGTCTACGGCTTGCTAC-3'
SOD3_R	5'-CTCCATCCAGATCTCCAGCACT-3'
NOX4_F	5'-TGCTGCATGGTGGTGGTATT-3'
NOX4_R	5'-ACCTGAAACATGCAACAGCAG-3'
mtDNA_F	5'-ATCCTCCAGGATTTGGAAT-3'
mtDNA_R	5'-ACCGGTAGGAATTGCGATAA-3'
mtCytb_F	5'-TTCTGAGGTGCCACAGTTATT-3'
mtCytb_R	5'-GAAGGAAAGGTATTAGGGCTAAA-3'
mtND1_F	5'-GCTTTACGAGCCGTAGCCGT-3'
mtND1_R	5'-CGGTTTGTCTGCTAGGGT-3'
mtND2_F	5'-CCATCAACTCAATCTCACTTCTATG-3'
mtND2_R	5'-GAATCCTGTAGTGGTGAAGG-3'
mtND3_F	5'-CCCCAAATAAATCTGTA-3'
mtND3_R	5'-CTCATGGTAGTGGGAAGT-3'
mtND4_F	5'-GCTTACGCCAAACAGAT-3'
mtND4_R	5'-TAGGCAGAATAGGAGTGAT-3'
mtND4L_F	5'-GCCATCTACCTTCTTCA-3'
mtND4L_R	5'-TAGGGCTAGTCTACAGC-3'
mtND5_F	5'-GCCAACAACATATTCAACTTTTC-3'
mtND5_R	5'-ACCATCATCCAATTAGTAGAAAGGA-3'
mtND6_F	5'-GGGAGATTGGTTGATGTA-3'
mtND6_R	5'-ATACCCGCAACAAGAT-3'
mtCOX1_F	5'-CAGACCGCAACCTAAACACA-3'
mtCOX1_R	5'-TTCTGGGTGCCAAAGAAT-3'
mtCOX2_F	5'-GCCGACTAAATCAAGCAACA-3'
mtCOX2_R	5'-CAATGGGCATAAAGCTATGG-3'
mtCOX3_F	5'-CGTGAAGGAACCTACCAAGG-3'
mtCOX3_R	5'-ATTCCTGTTGGAGTCCAGCA-3'
mtATP6_F	5'-CCATAAATCTAAGTAGCCATTCCAC-3'
mtATP6_R	5'-AGCTTTTTAGTTTGTGTGCGGAAG-3'
mtATP8_F	5'-ACATTCCCACTGGCACC-3'
mtATP8_R	5'-GGGGTAATGAATGAGGC-3'
GAPDH_F	5'-GTCTTCACTACCATGGAGAAGG-3'
GAPDH_R	5'-TCATGGATGACCTTGCCAG-3'

homogenization by centrifugation at 10,000 rpm for 10 min at 4°C. A GSH quantification kit (Cat#: A061-1, Jiancheng, Nanjing, China) was used for the determination of GSH content in liver according to the manufacturer's instructions.

Hepatic lipid peroxidation (MDA) assay

Lipid peroxidation of liver in mice was evaluated by measuring thiobarbituric acid (TBA) according to the modified method by Ohkawa [19]. Liver tissue (~100 mg) was homogenized in 1 mL PBS containing 1 mM EDTA and centrifuged at 10,000 × g for 10 min at 4°C. MDA content was determined by MDA quantification kit (Cat#: A003-1, Jiancheng, Nanjing, China) according to the manufacturer's instructions. MDA value was normalized to the hepatic cell protein content determined by a BCA kit (Cat#: P0010, Beyotime, China). The amount of lipid peroxidation was expressed as nmol/mg protein.

Western blotting

The homogenized liver tissue was lysed by RIPA buffer (Cat#: P0013B, Beyotime, China) and protein concentration was determined by BCA protein concentration assay kit (Cat#: P0012S, Beyotime, China). Proteins from whole liver lysates were denatured in 95°C water bath for 5 min, separated by SDS-polyacrylamide gel electrophoresis, and transferred onto PVDF membranes. The blots were blocked for 1 h with 5% nonfat dry milk in tris-buffered saline (TBS) at room temperature, followed by shaking incubation overnight with primary antibodies against SOD2 (Cat#: BS6734, Bioworld, USA), NOX4 (Cat#: ab133303, Abcam, USA), and β -actin (Cat#: AP0060, Bioworld, USA) at 4°C. After being washed with TBS-T buffer for three times, blots were incubated with secondary antibody (Cat#: A0208, Beyotime, China) and visualized with ECL kits (Cat#: PA112, Tiangen, China).

Quantitative RT-PCR (qRT-PCR) analyses

The mRNA and mtDNA copy numbers were detected by qRT-PCR. Total RNA was isolated from liver tissue using the Trizol reagent according to manufacturer's instructions (Takara, Japan). cDNA was generated from 1 μ g total RNA using PrimeScript RT reagent Kit (Cat#:

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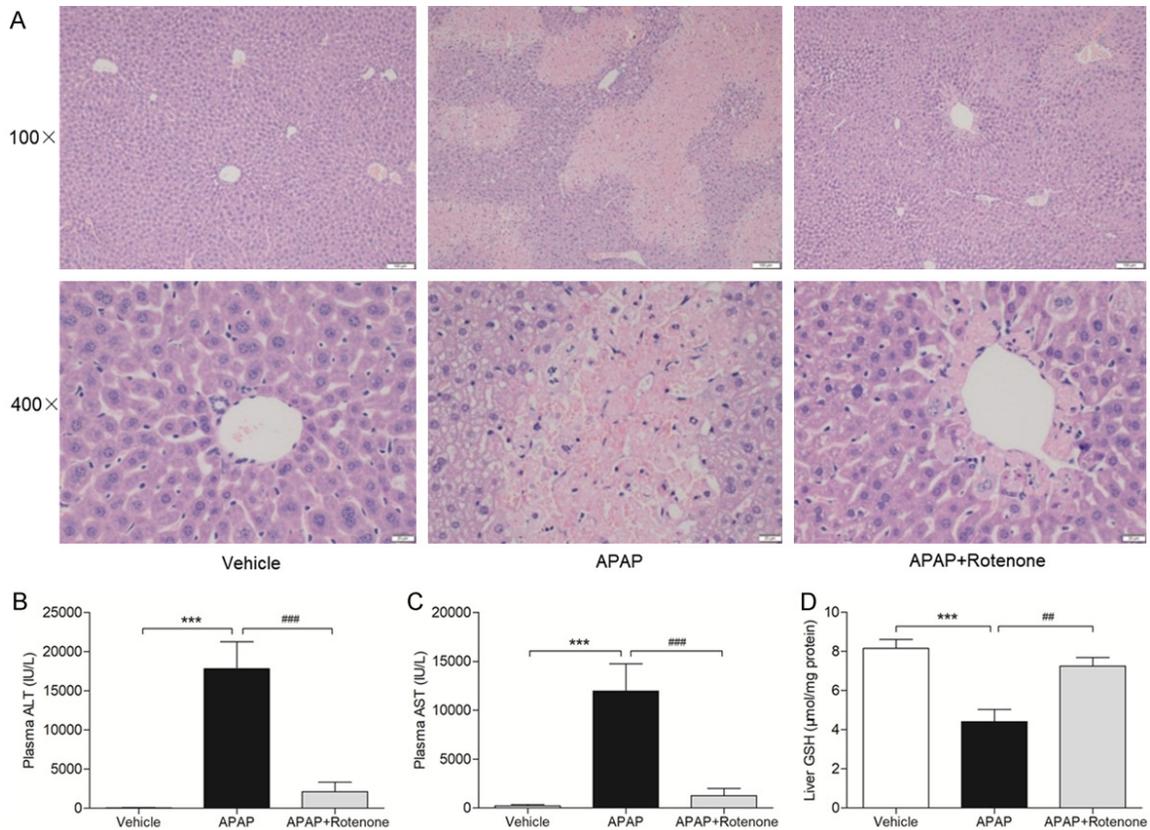


Figure 1. Rotenone treatment significantly attenuated APAP-induced liver injury. (A) Liver sections were stained with H&E and evaluated by microscope ($\times 100$ and $\times 400$). (B, C) The plasma levels of ALT (B) and AST (C) in mice. (D) GSH levels in liver was assayed using a commercial kit. Data were expressed as mean \pm SD, $n=5-7$ in each group. *** $P<0.001$ compared to vehicle group, ** $P<0.01$ compared to APAP group, ### $P<0.001$ compared to APAP group.

RR036, TaKaRa, Japan). The PCR primers were designed using primer 3 software (available at <http://frodo.wi.mit.edu/primer3/>) and the sequences were shown in **Table 1**. Quantitative RT-PCR was carried out using an ABI Prism 7500 Sequence detection system (Applied Biosystems, Foster City, CA) with SYBR Green dye as the fluorogenic probe. Cycling conditions were 95°C for 10 min, followed by 40 repeats of 95°C for 15 s and 60°C for 1 min. mRNA was normalized to GAPDH, and were calculated using delta method from threshold cycle numbers.

Statistical analysis

All data from the experiments were expressed as a mean \pm SD. Statistical significance was determined by Student's t test for comparisons of two groups. Multigroup comparisons were performed using ANOVA multiple comparisons followed by Turkey's post hoc test. Results were considered statistically significant when $P<0.05$.

Results

Effect of rotenone treatment on liver injury induced by APAP

We established an APAP hepatotoxicity mouse model by IP injection of APAP at a dose of 300 mg/kg. After 24 h APAP administration, the plasma levels of ALT and AST were robustly increased, indicating a severe liver injury. Strikingly, pretreatment with rotenone markedly blunted the elevation of ALT and AST in blood (**Figure 1B, 1C**). Furthermore, we performed H&E staining and observed that APAP treatment resulted in broad hepatic centrilobular necrosis, which was significantly attenuated by rotenone therapy (**Figure 1A**). In agreement with the amelioration of liver enzyme release and morphological damage, APAP-induced reduction of GSH content was significantly restored in the liver of rotenone-treated animals (**Figure 1D**). These results demonstrated a protective role of rotenone treatment against APAP-induced liver injury.

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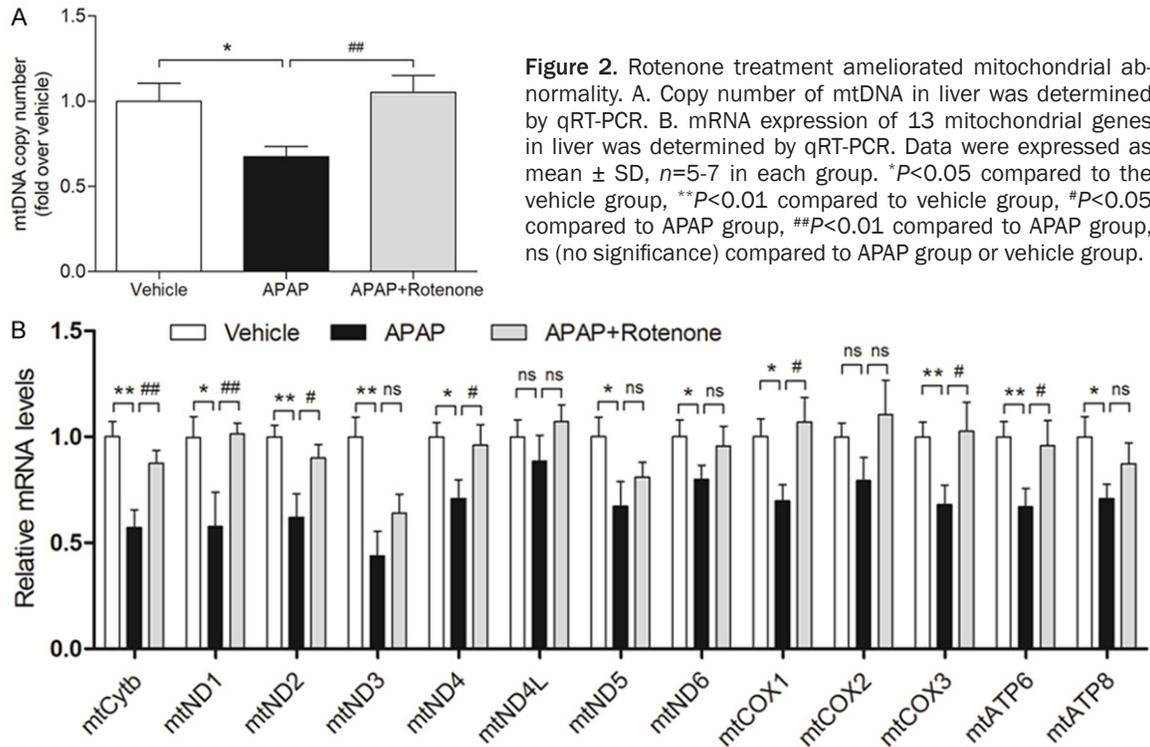


Figure 2. Rotenone treatment ameliorated mitochondrial abnormality. A. Copy number of mtDNA in liver was determined by qRT-PCR. B. mRNA expression of 13 mitochondrial genes in liver was determined by qRT-PCR. Data were expressed as mean \pm SD, $n=5-7$ in each group. * $P<0.05$ compared to the vehicle group, ** $P<0.01$ compared to vehicle group, # $P<0.05$ compared to APAP group, ## $P<0.01$ compared to APAP group, ns (no significance) compared to APAP group or vehicle group.

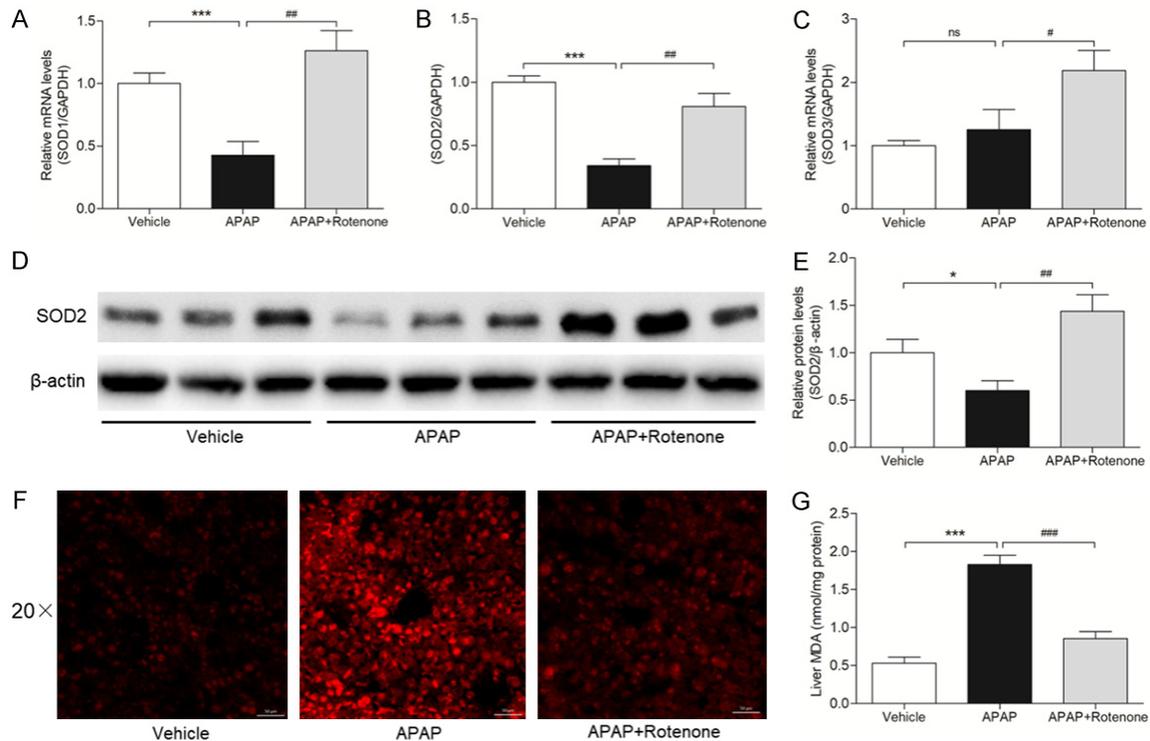


Figure 3. Rotenone blunted oxidative stress in the liver of mice received APAP treatment. A. mRNA levels of SOD1 were determined by qRT-PCR. B. mRNA levels of SOD2 were determined by qRT-PCR. C. mRNA levels of SOD3 were determined by qRT-PCR. D. Western blot of SOD2. E. Quantitative analysis of SOD2 Western blots. F. Dihydroethidium (DHE) staining of liver tissues ($\times 200$). G. Oxidative stress marker MDA in liver was measured using a commercial kit. Data were expressed as mean \pm SD, $n=5-7$ in each group. * $P<0.05$ compared to vehicle group, *** $P<0.001$ compared to vehicle group, # $P<0.05$ compared to vehicle group, ## $P<0.01$ compared to APAP group, ### $P<0.001$ compared to APAP group.

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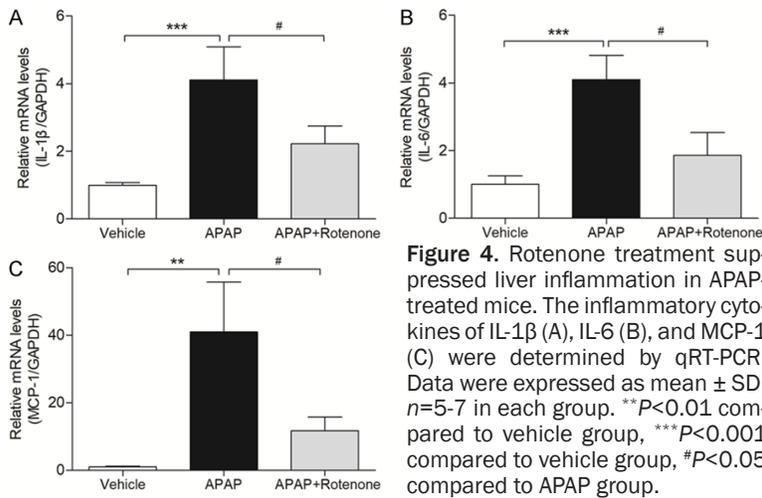


Figure 4. Rotenone treatment suppressed liver inflammation in APAP-treated mice. The inflammatory cytokines of IL-1 β (A), IL-6 (B), and MCP-1 (C) were determined by qRT-PCR. Data were expressed as mean \pm SD, $n=5-7$ in each group. ** $P<0.01$ compared to vehicle group, *** $P<0.001$ compared to vehicle group, # $P<0.05$ compared to APAP group.

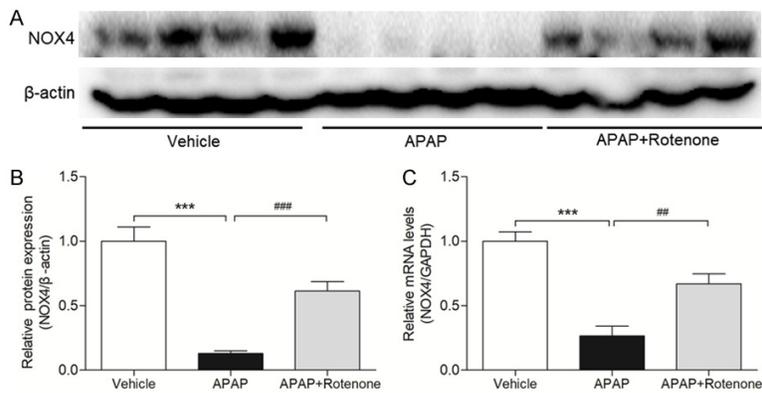


Figure 5. Rotenone treatment significantly reversed the decrement of NOX4 in liver exposed to APAP. A. NOX4 protein level was determined by Western blotting, and β -actin was used as loading control. B. Densitometry of the Western blots in A. C. mRNA levels of NOX4 were determined by qRT-PCR. Data were expressed as mean \pm SD, $n=5-7$ in each group. *** $P<0.001$ compared to vehicle group, ### $P<0.001$ compared to APAP group.

Rotenone ameliorated mitochondrial abnormalities and suppressed oxidative stress in the liver of mice received APAP treatment

Recent evidence suggested a critical role of mitochondrial dysfunction in the pathogenesis of APAP-induced liver injury. Here we observed the status of mitochondria by the examination of mtDNA copy number and the expressions of 13 mitochondrial genes (Figure 2A, 2B). As expected, 24 h APAP treatment strikingly lowered the levels of mtDNA copy number and the mitochondrial gene expressions (Figure 2A, 2B), suggesting the occurrence of mitochondrial abnormality. Furthermore, we examined the expressions of antioxidant enzymes SOD1, SOD2, and SOD3. Strikingly, APAP administration resulted in a remarkable reduction of SOD1

and SOD2 at mRNA levels, which was largely normalized by rotenone treatment (Figure 3A, 3B). The mRNA expression of SOD3 was unaltered by APAP, while it was significantly enhanced after rotenone treatment (Figure 3C). The protein expression of SOD2 was further confirmed by Western blotting (Figure 3D, 3E). Next, DHE staining was applied to monitor intracellular ROS. As shown in Figure 3F, enhanced ROS in liver tissue of APAP-treated mice was largely abolished by rotenone. Similarly, the increased oxidative stress marker MDA in liver was also blocked by rotenone (Figure 3G). These data indicated that rotenone treatment improved mitochondrial abnormality and blocked oxidative stress in liver induced by APAP challenge.

Rotenone suppressed APAP-induced inflammatory response

Inflammation is always a dominant phenomenon in APAP hepatotoxicity. Here we also found that APAP-induced up-regulation of inflammatory cytokines of IL-6, IL-1 β , and

MCP-1 was significantly blocked by rotenone as determined by qRT-PCR (Figure 4A-C), which might also contribute to the protective effect of rotenone against APAP-induced liver injury to some extent.

Effect of rotenone treatment on NADPH oxidase 4 expression in the liver of APAP-treated mice

There have been reported that NADPH oxidase 4 (NOX4) could regulate homocysteine metabolism and protect against acetaminophen-induced liver damage in mice [18]. In this study, APAP significantly downregulated the protein and mRNA levels of NOX4 in mice liver, which was strikingly reversed by rotenone treatment (Figure 5A-C). These results suggested

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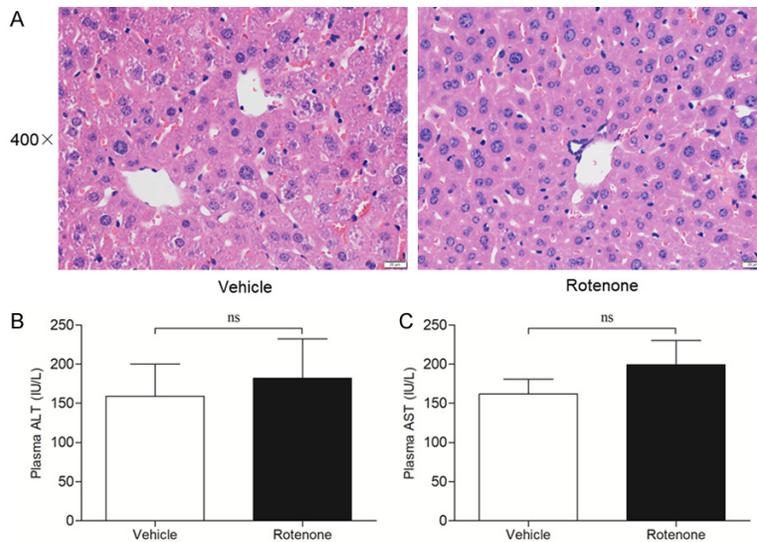


Figure 6. Rotenone at the dose of 250 ppm in food had no obvious hepatotoxicity in mice. (A) Liver sections were stained with H&E and evaluated by microscope ($\times 400$). (B, C) The plasma levels of ALT (B) and AST (C) in mice. Data were expressed as mean \pm SD, $n=8$ in each group. ns (no significance) compared to vehicle group.

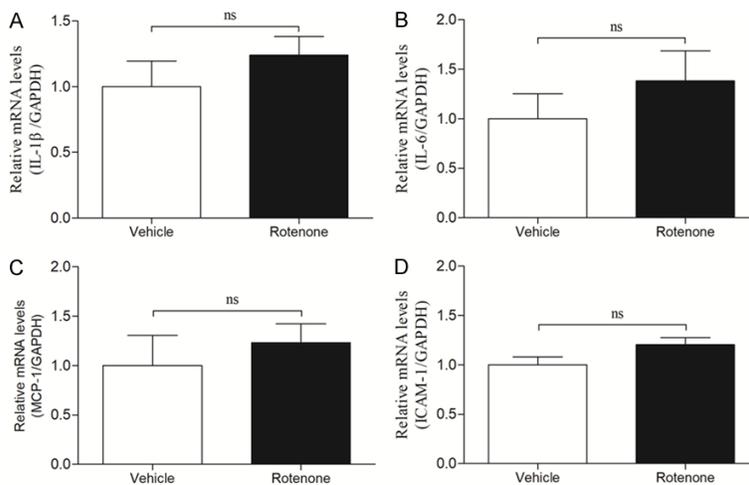


Figure 7. Rotenone did not significantly affect the mRNA levels of inflammatory mediators in the liver of mice. The inflammatory cytokines of IL-1 β (A), IL-6 (B), MCP-1 (C), and ICAM-1(D) were determined by qRT-PCR. Data were expressed as mean \pm SD, $n=5-7$ in each group. ns (no significance) compared to vehicle group.

that the restoration of NOX4 after rotenone treatment might be beneficial in this experimental setting.

Evaluation of rotenone toxicity in the liver of mice

In order to evaluate the hepatotoxicity of rotenone at the dose used in this study, we com-

pared some indexes of liver function in the mice with or without rotenone treatment. H&E staining of liver tissues showed no difference between groups in morphology (Figure 6A). Moreover, compared with the vehicle group, rotenone had no significant effect on serum ALT and AST levels (Figure 6B, 6C). In addition, we detected the expressions of inflammatory cytokines by qRT-PCR and found that rotenone did not significantly affect the mRNA levels of IL-1 β , IL-6, MCP-1, and ICAM-1 in liver (Figure 7A-D). These data indicated that rotenone at the dose of 250 ppm in food had no obvious hepatotoxicity in mice.

Azoxystrobin treatment failed to improve APAP-induced hepatotoxicity in mice

We know that MRC is composed of five complexes. Thus, we additionally used an inhibitor of mitochondrial complex III, azoxystrobin (AZOX), to pretreat the mice with or without APAP challenge. The dose of azoxystrobin used in this study has been shown to be safe and effective in a mouse model [19]. The H&E staining showed that AZOX did not reverse liver necrosis and oxidative stress caused by APAP (Figure 8A). Moreover, enhanced plasma levels of ALT and AST were not blocked by AZOX (Figure 8B, 8C). These data suggested that

inhibition of mitochondrial complex III could not protect against APAP hepatotoxicity.

Discussion

In the liver, the majority (~90%) of APAP is metabolized by UDP-glucuronosyltransferases (UTGs) and sulfotransferase (SULTs), with a few (2%) is excreted in the urine [20]. Another

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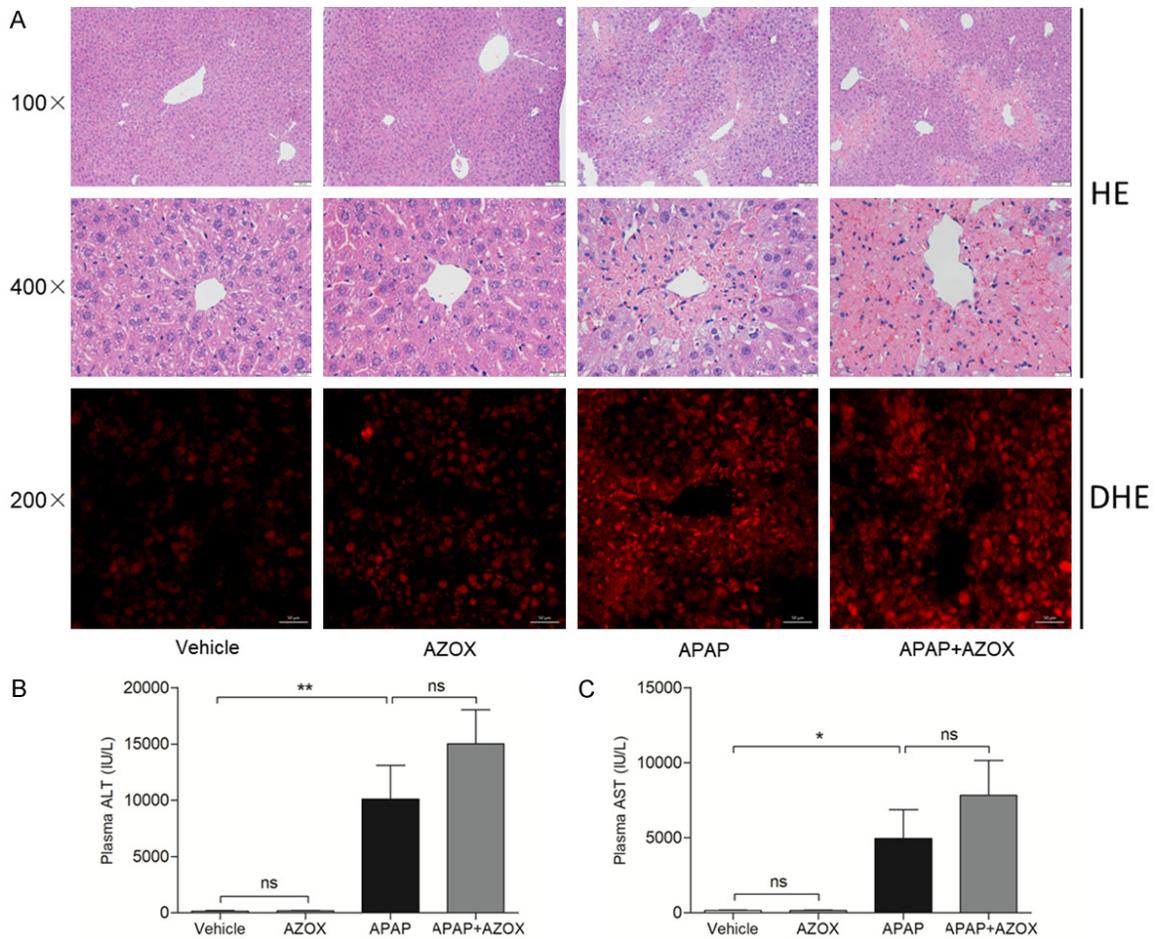


Figure 8. Azoxystrobin treatment failed to improve APAP-induced hepatotoxicity of mice. (A) Liver sections in each group were stained with H&E ($\times 100$ and $\times 400$) and DHE ($\times 200$). (B, C) The plasma levels of ALT (B) and AST (C) in each group of mice. Data were expressed as mean \pm SD, $n=8$ in each group. * $P<0.05$ compared to vehicle group, ** $P<0.01$ compared to vehicle group, ns (no significance) compared to APAP group or vehicle group.

tion of APAP is shunted by hepatic cytochrome CYP 2E1 (to a lesser extent with CYP 1A2 and 3A4) to phase I oxidation, in which a highly reactive toxic metabolite, N-acetyl-para-benzo-quinone imine (NAPQI), is formed [21, 22]. This minor metabolite is typically harmless, since it is mostly conjugated with GSH and excreted in bile. However, when large amount of APAP saturates sulfation pathway, and the glucuronidation cannot keep up any longer, there is an excess generation of reactive NAPQI metabolite which consumes GSH, resulting in the depletion of GSH stores [23-25]. The excessive NAPQI could bind to mitochondrial proteins to change the morphology and function of liver mitochondria [26, 27], leading to mitochondrial oxidative stress and apoptosis [4] and liver failure [28]. In the present study, we found that GSH was significantly depleted by overdose of APAP. Meanwhile, the mtDNA copy number and

the expressions of mitochondrial genes were decreased, indicating a mitochondrial abnormality. Mitochondrial dysfunction has a known role in mediating APAP-induced hepatotoxicity [29]. Under pathological conditions, abnormal mitochondria could release massive ROS and proapoptotic factors to promote the organelle injury and cell death. Thus, in theory, an appropriate inhibition of the activity of these injured mitochondria might be helpful in protecting organelles including mitochondria themselves. In agreement with this notion, inhibition of mitochondrial complex I activity by rotenone strikingly improved the liver injury, as well as the mitochondrial abnormality.

Oxidative stress has been found to play an important role in APAP-induced hepatocellular toxicity, and antioxidant shows protective effect against APAP hepatotoxicity [30, 31]. Here we

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found that both SOD1 and SOD2 were significantly down-regulated by APAP exposure. It is known to us that SOD2 is chiefly located in mitochondria [32], with a potent antioxidant activity [33]. In addition to the damage to mitochondrial DNA, oxidative stress also causes damage to lipids and cell membranes [34]. Free radicals act on the lipid peroxidation reaction. The final product of oxidation is malondialdehyde (MDA) which could affect the mitochondrial respiratory chain complex and the key enzyme activity in mitochondria [35]. MDA can also exacerbate the damage of membrane via opening the mitochondrial permeability transition (MPT) pore [36], which can lead to cell necrosis because of impaired Ca^{2+} homeostasis [37]. Therefore, MDA content is a marker of the degree of the oxidative damage to the membrane system. Here we found a remarkable reduction of SOD1 and SOD2 accompanied with an enhanced MDA level in liver after APAP treatment, suggesting APAP-associated dysregulation of SOD1 and SOD2 may lead to the increment of ROS production, which in turn caused oxidative damage of cell membrane. Treatment with rotenone to inhibit the activity of the injured mitochondria could reduce ROS production and ameliorate membrane damage.

Previous studies have also shown the increased proinflammatory mediators including IL-1 β , IL-6, and MCP-1 in liver after the challenge of overdose APAP [27, 38]. Also, a number of evidence demonstrated that oxidative stress could trigger the inflammatory response in many pathological processes [39, 40]. As a mitochondrial complex I inhibitor, the anti-inflammatory property of rotenone was also found in a mouse model of LPS-induced acute lung injury [41]. In agreement with these findings, we found that the enhanced mRNA levels of IL-1 β , IL-6, and MCP-1 in liver tissues were significantly suppressed by rotenone, indicating attenuated inflammation.

The main biological function of the NOX family protein is to produce ROS. Under the normal condition, the ROS produced by this pathway can maintain normal physiological activity of cells [42]. NOX4 has a physiological role in regulating Hcy flux to remethylation and transsulfuration pathways by redox-dependent regulation of BHMT activity [18]. NOX4 deficiency could cause the depletion of hepatic GSH stores, leading to liver injury under the challenge of

APAP. In agreement with this concept, we found that NOX4 in liver were significantly downregulated by APAP at both mRNA and protein levels, which was restored by rotenone administration, suggesting that NOX4 might contribute to the protective effect of rotenone in this experimental setting. Particularly, the enhanced NOX4 might contribute to the restoration of liver GSH content to some extent.

It is known that mitochondrial complex I and III are the primary sources of O_2^- -production in mitochondria [43-45]. The superoxide is released into the matrix from the complex I, and the superoxide produced by the complex III is released into both the matrix and the intermembrane space [46, 47]. In the absence of ADP, electrons from succinate (FADH₂-linked Complex II substrate) can flow back to complex I to produce more O_2^- . Thus, complex I is considered the major ROS-generating site in mitochondria under some pathological conditions [46, 48]. This may be the reason that using AZOX to inhibit the activity of mitochondrial complex III failed to improve APAP-induced liver injury.

In summary, the present study demonstrated that inhibiting the activity of the dysfunctional mitochondria in liver exposed to overdose of APAP through a mitochondrial complex I inhibitor significantly attenuated liver injury in parallel with the blockade of oxidative stress and inflammation. These results suggested a clinical potential in treating APAP hepatotoxicity via the inhibition of mitochondrial complex I in the future.

Acknowledgements

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Disclosure of conflict of interest

None.

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