Nicotinic Acid against Acetaminophen-Induced Hepatotoxicity via Sirt1/Nrf2 Antioxidative Pathway in Mice

Da Hu\(^1\), Li Zhang\(^1\), Rong Jiang\(^2\), Cuiting Liao\(^1\), Juanjuan Xu\(^1\), Shifang Jiang\(^1\), Yongqiang Yang\(^3\), Lin Lin\(^1\), Jiayi Huang\(^1\), Yi Shen\(^1\), Li Tang\(^1\) and Longjiang Li\(^1,2,*\)

\(^1\)Department of Pathophysiology, and \(^2\)Department of Histology and Embryology, Chongqing Medical University, No.1 Yixueyuan Road, Chongqing, 400016, China

(Received October 23, 2020)

**Summary**  Acetaminophen (N-acetyl-p-aminophenol, APAP) overdose causes hepatotoxicity, even liver failure, and oxidative stress plays pivotal role in its pathogenesis. Nicotinic acid (NA) is one form of vitamin B3, which has been used to treat a series of diseases in clinic for decades. To date, several studies have evidenced that NA has anti-oxidative property. Therefore, NA may have the hepatoprotective potential against APAP-induced toxicity. Here, our aim was to investigate the beneficial effect of NA against hepatotoxicity induced by APAP and its mechanism in vivo. BALB/c mice were intraperitoneally injected with NA (100 mg/kg) 3 times at 24, 12 and 1 h before APAP (600 mg/kg or 400 mg/kg) challenge. The results showed that pretreatment of NA markedly improved the survival rate, alleviated serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels and mitigated the histopathological injuries compared to APAP-exposed mice. Furthermore, NA significantly elevated the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) content, while reduced malondialdehyde (MDA) level. Finally, the signaling pathway was probed. The western blot revealed that NA up-regulated Sirtuin1 (Sirt1), nuclear factor erythroid 2-related factor 2 (Nrf2) and NAD(P)H quinone dehydrogenase-1 (NQO-1) expression and down-regulated Kelch-like ECH-associated protein 1 (Keap1) level in liver followed APAP exposure, implying Sirt1/Nrf2 axis exerted an essential role in the protective mechanism of NA on APAP toxicity. In brief, pretreatment of NA effectively protects liver against hepatotoxicity due to overdose of APAP through an antioxidant dependent manner modulated by Sirt1/Nrf2 signaling pathway.

**Key Words**  acetaminophen, nicotinic acid, Sirt1, Nrf2, oxidative stress

Acetaminophen (N-acetyl-p-aminophenol, APAP) is one of the most commonly used antipyretic and analgesic agent worldwide. As an over-the-counter drug, it is easy to access and the overdose can cause severe hepatotoxicity (1). Up to now, APAP is the leading etiology of drug-induced liver failure in western countries. The proportion of acute liver injury caused by APAP in the US is as high as 46%, and it causes 500 deaths a year in the UK (2), which is an obstacle to the more efficient application of APAP for patients. Generally, APAP (85–90%) is mainly metabolized into non-toxic product such as glucuronic acid conjugate by phase II conjugating enzymes (uridine diphosphate-glucuronosyltransferase, UGT or sulfotransferase, SULT) or excreted in the urine (only 2%) and bile. Moreover, about 10% of APAP is degraded to N-acetyl-para-benzoquinoneimine (NAPQI) by CYP2E1 in hepatocytes, which is a strong oxidative metabolite (3). Under physiological conditions, NAPQI is cleared by glutathione (GSH). APAP overdose causing exhaustion of GSH that leads to oxidative stress. Non-detoxified NAPQI can bind to mitochondria, triggering mitochondria damage and subsequently cell death. In general, oxidative stress accounts for the main reason for APAP-induced liver injury.

N-Acetylcysteine (NAC) is the precursor for synthesis of GSH, which is a powerful antioxidant and is clinically approved in practice. However, NAC has its limits, such as the short administration period post APAP toxicity (6–8 h). In recent years, a lot of effort has been made to investigate the preventive and therapeutic method for APAP toxicity, especially Chinese herbal medicines (4–6) and some accepted old drugs.

Nicotinic acid (NA) is one form of vitamin B3, which has been applied to clinical therapy for several decades, especially as a lipid-lowering drug. At present, it has been confirmed that NA has the property of attenuating oxidative stress in rat model of chronic renal failure (7) and improving acute lung injury by suppressing the reactive oxygen species (ROS) dependent pathways (8). Meanwhile, NA also inhibits the activity of nicotinamide adenine dinucleotide phosphate oxidase in human primary hepatocytes (9), which implies its anti-oxidative ability in liver (10). Besides, the depressant prooxidant process by NA administration alleviates the liver fibrosis of rats (11).

In addition, as an important precursor, NA can be synthesized to nicotinamide adenine dinucleotide
(NAD+) through Preiss-Handler pathways (12), and orally administration of NA could elevate hepatocellular NAD+ level (13). It is well known that NAD+ is an essential coenzyme that is involved in various pivotal biological processes and is also a substrate for Sirtuin1 (Sirt1). As a class-III histone deacetylase, Sirt1 has been evidenced in improving glucose tolerance, anti-inflammatory effect and also anti-oxidative effect by activating nuclear factor erythroid 2-related factor 2 (Nrf2) pathway (14). Intriguingly, several studies have reported that the expression level of Sirt1 was down-regulated in mouse model of APAP (15, 16), and increased level of Sirt1 could attenuate APAP induced liver injury (6, 17).

Based on the roles of antioxidant and precursor of NAD+, NA could have the beneficial effects against APAP-induced hepatotoxicity, and Sirt1/Nrf2 signaling pathway may be involved in it.

MATERIALS AND METHODS

Treatment of animals. Six to eight weeks old male BALB/c mice (18–22 g) were purchased from the department of Experimental Animal Center of Chongqing Medical University. All animals were raised in an air-conditioned room (temperature: 20–25°C) with a 12 h light/dark cycle, and fasting for solids and liquids for 24 h to exhaust GSH before APAP administration. The experimental procedures were approved by Animal Care and Use Committee of Chongqing Medical University.

APAP (Yuanye Bio-Technology Company, Shanghai) was dissolved in normal saline (NS) to establish the animal model. For complete dissolution, the solution was warmed up to 60˚C and cooled down to room temperature. Meanwhile, NA was dissolved in NS for further experiments. The dose of NA and APAP were determined according to references (18, 19) and our previous results. Animals were divided into four groups: control group, NA (100 mg/kg) treated group, APAP treated group (400 mg/kg), NA+APAP group, NA was intraperitoneally injected at 24, 12 and 1 h before APAP challenge, three times totally. Then, 8 h after APAP injection, mice were anesthetized by pentobarbitone (100 mg/kg, intraperitoneal injection) and sacrificed to collect ocular venous blood and liver tissue samples for further experiments.

Determination of survival rate. To investigate the effect of NA on overall survival rate of mice after APAP challenge and to make sure the majority of mice in APAP group could sacrifice, we conducted experiments using higher dose of APAP and found 600 mg/kg was a reasonable choice in the survival test according to our results. Mice were separated into three groups (n = 30): APAP (600 mg/kg) group, NA (100 mg/kg)+APAP, NA (200 mg/kg)+APAP group. After APAP treatment, mice were observed every half-hour for 24 h and the deaths were recorded as well.

Measurement of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT). The serum was extracted from the ocular venous blood by centrifugation at 1,500 ×g for 10 min at 4°C. Transaminases activities were determined spectrophotometrically with ALT/AST assay kit (Nanjing Jiancheng Institute of Biotechnology, China). All procedures were conducted according to the instructions. Enzymatic activities of serum ALT/AST were measured by detecting the absorbance of phenylhydrazone catalyzed by ALT/AST at 505 nm.

Liver index calculation and hematoxylin and eosin (H&E) staining. Weights of mice livers were recorded and then extracted and weighed for the calculation of liver index (liver weight/total weight). Fresh liver tissues were fixed using 10% paraformaldehyde. After a series of operations including anhydrous ethanol dehydration and paraffin embedding, the embedded wax was sectioned into thin slices, generally 5–8 μm thickness, the slices were then mounted on glass slides and dried. Finally, the slices were stained with H&E using standard methods. Histology analysis of liver sections was implemented using a light microscope (Olympus, Tokyo, Japan), and the necrotic area of the liver sections were observed to determine the severity of the damage and scored according to the following formula: normality marks 0, minimal impairment (<1%, the percentage of degeneration or necrosis area in the microscopic field, the same below) marks 1, mild impairment (1–25%) marks 2, moderate impairment marks 3 (26–50%), and 4 (51–75%), 5 (76–100%) expressed severe/high.

Measurement of hepatic total superoxide dismutase (SOD) activity. There are only two types of SOD in mice: Cu/Zn-SOD and Mn-SOD. According to the commercial kit protocol (Jiancheng Bioengineering Institute, China), total SOD activity of liver was detected using a competitive inhibition mechanism by measuring the reduced formation of nitrites for its specific inhibitory effect on superoxide anion radical. Under the impact of chromogenic agents nitrite showed an amaranthine color and the absorbance of nitrite-reagent compound was measured at 550 nm by visible ultraviolet spectrophotometer. Data were expressed as SOD U/mg protein as compared with the standard.

Measurement of hepatic catalase (CAT) activity. Reagent was purchased from Nanjing JIancheng Institute of Biotechnology. According to the instructions, liver tissue was accurately weighed and nine times the weight of normal saline was added to prepare the homogenate. According to the reagent specification, hydrogen peroxide was added and catalyzed by CAT within liver homogenate, then the reaction was terminated subsequently by adding ammonium molybdate, the remaining hydrogen peroxide reacts with ammonium molybdate to form a yellowish compound, activity of CAT was determined by detecting the absorbance at 405 nm.

Measurement of hepatic malondialdehyde (MDA) formation. An appropriate amount of liver tissue (40 mg) was thawed with normal saline, following by ultrasonication, the homogenate was centrifuged at 1,000 ×g, and the supernatant was collected for subsequent detection. Protein quantification was calculated by BCA assay kit (Thermo Scientific, USA). Then according to the operating table of kit, absorbance value was finally
detected at 523 nm to determine the MDA level, for MDA is a lipid peroxidation degradation product which can bind with thiobarbituric acid (TAB) to form a red product with a maximum absorption peak at 523 nm. The data are expressed as nmol MDA per mg protein of liver tissue (nmol/mg protein).

**Measurement of hepatic glutathione (GSH).** Ten percent homogenate of liver tissue was prepared using normal saline and the concentration of homogenate was measured according to the method mentioned above. Reagent was purchased from Nanjing Jiancheng Institute of Biotechnology. According to the instructions, GSH reacts with dithionitrobenzoic acid to form a yellow compound which reflects the GSH content by detecting the absorbance at 405 nm. The data are expressed as μmol GSH per mg protein of liver tissue (μmol/mg protein).

**Western blot analysis.** Appropriate amount of liver samples were cut up with ophthalmic scissors and the tissues were fully lysed with RIPA lysis-buffer (containing PMSF and phosphorlyase inhibitor). Then the supernatant was separated by high speed centrifuge (12,000 ×g) and protein concentration was detected by BCA assay kit. Equal amounts of denatured protein (40 μg) samples were then separated by electrophoresis on 10% polyacrylamide–SDS gel. Then the protein was transferred on to nitrocellulose membrane, the membrane was then blocked with 5% skimmed milk in Tris-buffered saline (TBST) for 2 h at room temperature and the membranes were incubated overnight at 4°C with primary antibody against β-actin (dilution 1 : 1,000, 4A BIOTECH), Sirt1 (dilution 1 : 500, Bioss Antibodies), Kelch-like ECH-associated protein 1 (Keap1) (dilution 1 : 1,500, Wanleibio), NAD(P)H quinone dehydrogenase-1 (NQO-1) (dilution 1 : 1,000, abcam) and Nrf2 (dilution 1 : 1,000, proteintech). After washing off the unbound antibodies, blots were then incubated with anti-rabbit (dilution 1 : 2,000) or anti-mice (dilution 1 : 4,000) horseradish peroxidase conjugated secondary antibodies for 2 h at room temperature. Then blots were developed using an enhanced chemiluminescence system (Advanta, USA) and the ChemiDoc Touch Imaging System (Bio-Rad). The gray values of blots were also calculated.

**Statistical analysis.** Data are expressed as the mean ± standard deviations. Statistical significance was determined by one-way ANOVA or log-rank test with GraphPad Prism 6, p value <0.05 was considered statistically significant.

**RESULTS**

**NA improved survival rates in mice exposed to APAP overdose**

A rapid increase of deaths occurred in group following APAP treatment within first 12 h, by contrast, deaths in NA (100 mg/kg) pretreatment group were quite moderate. After 24 h, the difference between the two groups reached its summit, and the survival rate in APAP treated group was 30% and 80% in NA (100 mg/kg) pretreatment group. However, NA (200 mg/kg) pretreatment group was only 20% (Fig. 1). Statistics analysis (log-rank) showed that there was a significant difference between NA (100 mg/kg) pretreatment and APAP treated group, which strongly indicated the preventive effect of NA on APAP toxicity.

**NA decreased the elevation of serum ALT/AST of mice induced by APAP**

There was a remarkably increasing of serum ALT and AST in APAP treated group compared with that of the control group. Pretreatment of NA (100 mg/kg) significantly reduced ALT and AST levels in APAP group (Fig. 2), suggesting that NA was involved in preventing the APAP induced liver toxicity.

**NA protected the liver from histological and general morphology injury**

Livers of mice were weighed and taken pictures in each group, figuring out liver index (liver weight/total weight, Fig. 3(a)). Due to hyperemia and swelling caused by APAP, liver index was significantly increased in APAP group and NA partially reversed the elevation of liver index. Histology analysis of liver was also conducted by H&E staining, liver H&E staining showed nor-
mal hepatic lobular structure in control and NA treated group, while in APAP treated group, centrilobular necrosis and edema of hepatocytes was obviously observed (Fig. 3(b)). Herein, mice of NA pretreatment group had attenuated liver injury, which was corresponding with other results exhibited above. Pretreatment of NA mitigated oxidative stress caused by APAP in liver

Malondialdehyde (MDA) is an index reflecting the severity of lipid peroxidation. Pretreatment of NA significantly suppressed the level of MDA elevation in liver, but NA treated group did not change the MDA level compared with that of the control group (Fig. 4(a)), implying NA protected against APAP toxicity through decreasing oxidative stress. Antioxidant parameters were also tested in order to comprehensively investigate the mechanism: total SOD (Fig. 4(b)) and CAT (Fig. 4(c)) activity in liver were measured and results suggested

Fig. 2. Enzymatic evidence of NA in alleviating liver injury. NA was administrated in the way described above, serum was collected 8 h after APAP injection and serum ALT/AST were measured immediately. CON: mice were treated with NS. NA: mice were treated with NA. APAP: mice were treated with NS and exposed to APAP. NA+APAP: mice were treated with NA and exposed to APAP. Values of each group (n=5) were expressed as the mean±SD. ** p<0.01.

Fig. 3. NA pretreatment significantly prevented liver from histopathological and general morphology damage. Mice livers of the four groups were extracted and weighted immediately to calculate the liver index (a). Then livers were cut into slices and liver sections were stained with hematoxylin and eosin for histological examination (b) to assess the severity of liver injury and scores were counted according to methods described above. CON: mice were treated with NS. NA: mice were treated with NA. APAP: mice were treated with NS and exposed to APAP. NA+APAP: mice were treated with NA and exposed to APAP. Values of each group (n=5) were expressed as the mean±SD. ** p<0.01.
that NA had the ability of increasing activities of antioxidant enzymes. The results were also consistent with the liver GSH content (Fig. 4(d)), which was closely related to susceptibility to liver injury.

NA activated Sirt1/Nrf2 signaling pathway

Sirt1 is a critical enzyme studied in the mouse model of APAP and several researches had reported that Sirt1 was down regulated in APAP toxicity (15, 20) and its down regulation was mediated by interleukin-1β (IL-1β)/nuclear factor kappa B (NF-κB) signaling pathway. Our experimental results were consistent with the conclusions (Fig. 5(a)). Intriguingly, NA restored the level of Sirt1 and Nrf2 in APAP group. However, NA could not up-regulate Sirt1 or Nrf2 level in mice liver of control group (Fig. 5(b)), implying NA has the different role to regulate the expression of Sirt1 and Nrf2 in physiological and pathological conditions. These results are consistent with other reports (21). In addition, NA pretreatment down regulated the expression level of Keap1 in APAP group (Fig. 5(c)), which has the capacity of inhibiting Nrf2 and its downstream activation of antioxidant responses elements (ARE) and subsequently transcription of antioxidant enzymes such as NQO-1 (Fig. 5(e)), as expected, the consequences revealed a significant increase in NQO-1 of NA+APAP group (Fig. 5(d)).

**DISCUSSION**

NA is one of the most stable structures of B-vitamins family and belongs to vitamin B3. It is mainly abundant in natural foods, such as milk, vegetables, and meats (22). In this study, we discovered NA, a long-approved drug in clinic as a lipid lowering drug and also for cure of pellagra, had reveals significant beneficial impact on liver injury caused by APAP over dose as evidenced by H&E staining and plasma ALT and AST, etc. NA also increased the level of liver GSH content, which is essential to antioxidant stress in liver. In this research, we mainly explored the role of NA on antioxidant stress in APAP-induced liver injury. In fact, evidences suggest that supplementation of NA protected male C57BL/6 mice from hepatocytes deaths and alcohol-induced liver injury by reducing oxidative stress in a protein kinase B (Akt) dependent manner (23). NA also inhibited mitochondrial oxidative stress evoked by methylmercury (MeHg) poisoning in male Wistar rats (24). Other scholars reported that dietary NA administration was sufficient to resist oxidative damage in rat liver under tert-butyl hydroperoxide challenge and improved hepatic zinc uptake as well (25).

Here, we found that antioxidative enzymes were significantly activated by NA administration, and Sirt1/ Nrf2 axis was involved in the mechanism of liver protection. Several studies had illuminated in detail that activation of Nrf2 was mediated by Sirt1 (14, 26, 27). In physiological states, Nrf2 was conjuncted with Keap1, inhibiting the nuclear translocation of Nrf2 and subsequently transcription of ARE, which triggers the
Fig. 5. NA significantly increased Sirt1/Nrf2/NQO-1 expression level in mice liver suffering APAP overdose (a, b, d) but had a reduced Keap1 level (c). The representative pictures of blots were shown above and the gray value of blots were calculated and expressed as the mean±SD. CON: mice were treated with NS, NA: mice were treated with NA, APAP: mice were treated with NS and exposed to APAP, NA+APAP: mice were treated with NA and exposed to APAP. *p<0.05, **p<0.01.
activation of antioxidant enzymes, for instance, NQO-1 is one of these downstream transcription product, and is closely related to antioxidant stress (28, 29). Our results verified that NA up-regulated NQO-1 expression in mice model of APAP with increased Nr2 and also decreased Keap1 protein level as expected.

In consistent with our discovery, it is reported that hepatocellular level of Sirt1 was reduced in APAP treatment (15, 16) and IL-1β/NF-κB was involved in the process (4), which is the possible reason for down-regulation of Sirt1. However, how does NA restore Sirt1 level in liver exposed to APAP is still unknown. Researches by other scholars have drawn conflicting conclusion, and they found that Sirt1 level did not alter in mice model of APAP, this may owe to the different nutritional status of mice. For instance, a reference (30) indicated that excessive APAP resulted in notable increase in Sirt1 expression in mice liver of normal diet, and decreased Sirt1 level acting as a resistant factor of APAP damage by increasing liver’s antioxidant capacity, which seemed to contradict our experimental results. However, this paper also pointed out this conclusion did not contradict with others’ due to the different nutritional status. For example, basal GSH content varies from different period of fasting, which influences the sensitivity of mice exposed to APAP thus altering the expression of Sirt1. But the detailed regulatory mechanism of Sirt1 in different nutritional statuses is largely unknown. All in all, pretreatment of NA alleviated APAP induced liver injury was an established fact.

However, treatment of NA only 1 h before APAP administration revealed a quite limited effect, and post treatment of NA had no protective effect. We hypothesized that two reasons may contribute to this phenomenon: (1) In the experiments with NA pretreatment, 24 h in advance, and a total of three times of NA pretreatments were required to alleviate hepatotoxicity, obviously, treatment of NA only 1 h post APAP was not sufficient to reduce liver injury. (2) oxidative stress caused by APAP may reduce the key enzymatic activity for NA transforming into NAD⁺ (31), which acted as a coenzyme of Sirt1 thus activating the downstream targets. Although we found that Sirt1 and Nr2 increased in NA pretreatment group, we did not verify the causal relationship between the two proteins. Indeed, many studies had confirmed that activation of Nr2 was mediated by Sirt1 activation (32, 33) and knockout of Sirt1 partly inhibited the up-regulation of Nr2 (17). Further study should focus on Sirt1 and its downstream impact on Nr2 by down-regulation of Sirt1 level.

Disclosure of state of COI
The authors declare that they have no conflicts of interest concerning this article.

Acknowledgments
We would like to thanks Kerui Fan and Xianwen Peng for your advices and helps in our experiments.

REFERENCES
14) da Cunha M, Arruda S. 2017. Tucum-do-Cerrado (Baccharis setosa Mart.) may promote anti-aging effect by upregulating SIRT1-Nrf2 pathway and attenuating oxi-


