Mechanisms of Detoxification and Anti-Oxidation of Nrf2 − ARE Pathway in Isonicotinic Acid Hydrazide-Induced Mouse Liver Injury

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Abstract

The regulatory mechanisms of nuclear factor erythroid 2-related factor 2 (Nrf2) mainly include detoxification and antioxidation in the progress of isonicotinic acid hydrazide (INH)-induced liver injury. The interaction and protective effects of these two injury mechanisms have not been reported. In this study, eight Kunming mice were administered with INH via gavage at a dose of 90 mg/kg.d. The mice were then killed for 1 d, 3 d, 5 d, 7 d, 2 w, 3 w, and 4 w, and the control groups received gavage of the same volume of distilled water. The pathological changes of liver tissues and location of Nrf2 in liver cell were observed. The superoxide dismutase (SOD) and malondialdehyde (MDA), as well as the expressions of Nrf2, glutathione S-transferase (GST), and SOD mRNAs and proteins were examined. After drug administration for 1–2 weeks, the SOD (total SOD, Cu-ZnSOD, and MnSOD), GST (GSTA1 and GSTM1), and Nrf2 exhibited trough levels, whereas the MDA content reached the peak. These results suggest that Nrf2 nucleocytoplasmic transport occurred in the experimental groups on the seventh day after administration, and then the expressions of mRNAs and proteins of GSTA1, GSTM1, Cu-ZnSOD, and MnSOD were all upregulated with the activation of the Nrf2-antioxidant responsive element (ARE) pathway.

Introduction

Isonicotinic acid hydrazide (INH) is an irreplaceable first-line drug in the standard anti-tuberculosis chemotherapy regimen recommended by the World Health Organization. The incidence of drug-induced liver injury in patients undergoing treatment for multidrug-resistant tuberculosis is 16.5% [1]; thus, the side effects of the treatment have received considerable attention. INH is acetylated by acetyl transferase 2 and catalyzed by cytochrome P450 enzyme system in the hepatocytes. Toxic metabolites, hydrazine, and acetylated derivatives then covalently bind to the macromolecular substances in hepatocytes; binding to the substances results in hepatocyte membrane potential change, nuclear acid damage, protein conformation change, and lipid peroxidation [2]. Meanwhile, electron transfer leads to generation of endogenous superoxide anions, and reactive oxygen species (ROS) is produced as a result. These processes are generally interrelated, forming a vicious circle [3,4]. Toxic metabolites are detoxified mainly by phase II drug metabolism, such as binding to reduced glutathione (GSH) and benzoquinone degradation.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is an important endogenous antidote and an antioxidant transcription factor. Under normal physiological conditions, Nrf2 binds to its adaptor protein Keap1 and is anchored in the cytoplasm. Nrf2 enters the nucleus and binds to the antioxidant responsive element (ARE) through stimulation of oxidative stress or electrophilic substances [5-7]. Subsequently, the transcriptions of a series of target genes of NAD(P)H: quinone oxidoreductase 1, superoxide dismutase (SOD), glutathione S-transferase (GST), catalase, and multidrug resistance-associated protein are initiated, exerting effects, such as detoxification, anti-oxidative damage, and chemical carcinogenesis inhibition [8-12]. Resveratrol, carotene, guava, and selenium can up regulate the activities of antioxidant enzymes (AOEs) to protect hepatocytes by activating the anti-oxidative pathway of Nrf2 [13,14]. Comparatively, the loss of Nrf2 evidently aggravates the occurrence of non-alcoholic fatty liver disease [15].

To date, direct injuries [16] or pure oxidative stress injury [17-19] of drugs are the main focus in the research on the pathogenesis of INH-induced liver injury. The interaction of these two injury mechanisms and the protective effects of the Nrf2–ARE pathway against drug toxicity and oxidative damage have not been reported. Thus, investigating liver detoxification mechanism and different patterns of anti-oxidative stress in INH-induced liver injury is important for the promotion of detoxification and excretion of intermediate active metabolites, enhancement of anti-oxidative damage ability in the body, and prevention of liver injury. In this research, gavage with 90 mg/kg INH was adopted to establish the mouse model of liver injury. The expressions of proteins and mRNAs of Nrf2, SOD, and GST, and the Nrf2 transmembrane transport in the liver tissues of experimental animals were observed. The different patterns of drug-detoxifying enzymes and AOEs in liver injury induced by INH were analyzed. Moreover, the regulatory mechanisms of Nrf2–ARE toward detoxification and oxidative damage in INH metabolism were explored, providing a basis for clinically preventing INH-induced liver injury.

Materials and Methods

Experimental animals and administration regimen

A total of 112 specific pathogen-free Kunming mice weighing between 18 g and 22 g were selected. An equal number of male and female mice were included. The animals were purchased from Beijing HFK Bioscience Co., Ltd. (animal license no.: SCXK (Jing) 2009-0004) and raised in a laboratory with a barrier system in the Medical Laboratory Animal Center of Hebei United University (now combined into North China University of Science and Technology)

(raising environment license no.: SYXK (Ji) 2010-0038). After 1 week adaptive rising, these animals were randomly divided into 14 groups, with 8 individuals each. INH was administered to the mice in seven experimental groups at a dose of 90 mg/kg.d (calculated according to the dose of adult clinical administration) for 1 d, 3 d, 5 d, 7 d, 2 w, 3 w and 4 w, respectively. Gavage was performed at a fixed time point daily. For the seven control groups, gavage administration of the same volume of distilled water was provided on the same days and time as that of INC in the experimental groups. This research was approved by the Animal Ethics Committee of Hebei United University (No: 2013-028).

Collection of animal samples

At 24 h after final gavage administration, the mice in all groups were weighed, and their eyeballs were enucleated for blood sampling. Blood samples were placed at room temperature for 30 min and then centrifuged at 3000 r/min for 10 min. Serum was isolated for biochemical tests. Mice were killed via cervical dislocation to collect liver tissues. A transection was made at the porta hepatis; half of the liver tissues being used for pathological observations was preserved in 10% formalin, and the other half was stored at −80°C, which being used for mRNA extraction and protein analysis.

Measurement of biochemical parameters

Plasma was stored at −80°C until assayed. The plasma was used for the estimation of ALT, AST. Estimations were carried out as automatic biochemical analyzer.

Measurement of GST and SOD activities and GSH and MDA contents in liver tissues

Liver tissues were stored at −80°C until assayed. Liver tissues were prepared to make 1:10 homogenates, the activities of GST and SOD and GSH and MDA content in hepatic homogenate were measured using assay kits according to the instructions.

Measurement of proteins of Nrf2, GST, and SOD and observation of mRNA in liver tissues

Liver tissues were prepared to make 1:5 homogenates, the Measurement of proteins of Nrf2, GST, and SOD content in hepatic homogenate were measured using assay kits according to the instructions.

RNA was isolated from mice liver tissues using the RNeasy mini Kit following the manufacturer's instructions. RNA integrity was electrophoretically verified by ethidium bromide staining and by OD_{260} / OD_{280} nm absorption ratio at 1.8 - 2.0. 2 μg RNA of each sample was reverse transcribed to cDNA in 20 μl reactions using M-MLV first strand synthesis Kit.

Quantitative real-time PCR was performed in a 20 μl final volume. The cDNA was used for PCR amplification under the following conditions: preheating at 95°C for 5 min, denaturing at 95°C for 30 sec, annealing at 60°C (Nrf2, GST and SOD) for 30 sec, and extension at 72°C for 30 sec. The reaction was repeated for 40 cycles followed by incubation at 72°C for 5 min. β-actin as a reference gene, was used to normalize each sample and each gene. Relative mRNA expression in each sample was calculated as the ratio of target gene concentration to GAPDH concentration. Primers used are listed in Table 1.

Statistical analysis

Data analysis was performed with SPSS 17.0 software. Measurement data were expressed as average ± standard deviation. Normality and homogeneity-of-variance tests were sequentially conducted on the data. Comparison of homogeneity of variance among the groups was performed using one-way ANOVA. The Student–Newman–Keuls method was used to analyze the differences among the groups (α = 0.05).

The changes of the liver function indexes in mouse liver tissues in the control groups did not indicate statistical significance at the seven time points. Thus, the measured values at the second week were used as a representative of the control group for the comparison with experimental groups in the result analysis.

Results

Analysis of liver histopathology and liver function indexes ALT and AST in serum

No apparent pathological changes were found in the mouse liver tissues in the normal control groups (Figure 1A). For the experimental groups, hepatocyte swelling was occasionally found on the third day, accompanied by visible liver injury (Figure 1C). With the extension of administration time, liver injury was aggravated (Figures 1D–1G). In the 4 week group, necrotic lesions were observed. Fibrous tissues proliferated and became fascicular. Moreover, dikaryocytes and regenerated hepatocytes were observed, and the structure of hepatic lobules was relatively clear (Figure 1H).

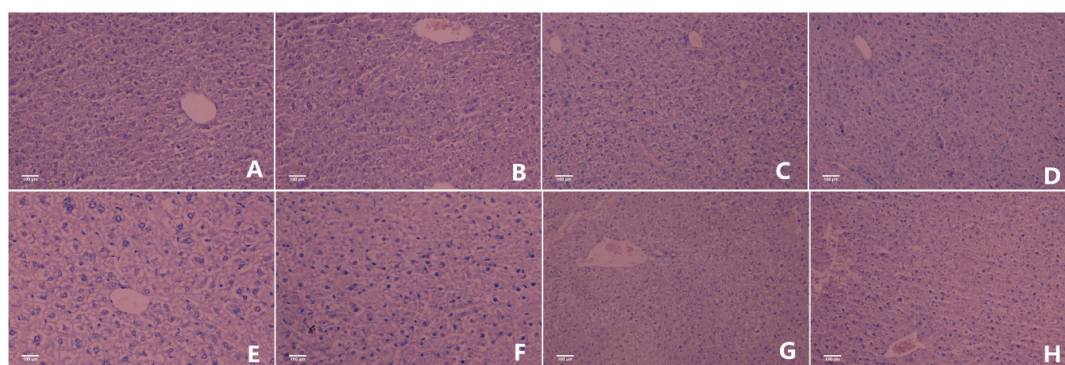**Figure 1:** Effect of isoniazid (INH) on liver histopathological changes in mouse (HE × 100). The mouse were ig given INH 99 mg·kg⁻¹·d⁻¹ or vehicle once per day for 1 d, 3 d, 5 d, 7 d, 2 w, 3 w, 4 w respectively and were scarificed. The liver tissue samples were collected from the control (A) group, or INH-treated for 1 d (B), 3d (C), 5 d (D), 7 d (E), 2 w (F), 3 w (G), 4 w (H) group respectively.

After gavage administration, the ALT contents in mouse serum in the experimental groups were higher than those in the control groups (28.88 U/L ± 2.24 U/L) in the second (43.13 U/L ± 4.32 U/L), third (57.88 U/L ± 4.82 U/L), and fourth weeks (55.88 U/L ± 3.64 U/L). Meanwhile, the AST contents were apparently higher than those in the control groups (104.13 U/L ± 8.72 U/L) in the fourth week (150.25 U/L ± 10.29 U/L); the differences indicate statistical significance. Liver function indexes can reveal the progressive development of mouse liver injury, but the timeliness is unsatisfactory.

GST and SOD activities and GSH and MDA contents in liver tissues

Compared with the control groups, total SOD and GST activities in the experimental groups were decreased at the fifth day, seventh day, and second week; the differences indicate statistical significance. In particular, the activity of Cu-ZnSOD was the lowest in the second week. However, after four weeks of administration, the enzyme activity was higher than that in the two-week group; the result indicates that Nrf2 played an important role in the regulation. No differences of MnSOD activity were observed among different groups (Table 2).

Note: * compared with the control group, $P < 0.05$; # compared with the 2-week group, $P < 0.05$.

The GSH contents in the mouse liver tissues in the 5 day, 7 day, 2 week and 3 week experimental groups were lower than those in the control groups. The differences exhibited statistical significance. However, the MDA contents in the 7 day, 2 week and 4 week experimental groups were higher than those in the control groups, and the differences showed statistical significance (Table 3).

Note: * compared with the control group, $P < 0.05$.

Expressions of mRNAs and proteins of Nrf2, GST, and SOD in liver tissues

With the extension of administration time, the expressions of Nrf2, GST, and SOD mRNAs in the experimental groups showed a general increasing trend. The Nrf2 mRNA expression was up regulated early, starting from the fifth day. GSTA1 mRNA showed high expression in the second week, whereas GSTM1, Cu-ZnSOD and MnSOD mRNA expressions upregulated in the third week. Compared with the control groups, the changes described above exhibited statistical significance (Figure 2).

As shown in Figure 3, the second week is a critical time point. The contents of Nrf2, GSTA1, GSTM1, Cu-ZnSOD and MnSOD proteins in the mouse tissues in the experimental groups gradually decreased with the extension of administration time and then reached trough levels in the second week. With continuous administration, the protein contents gradually increased. Compared with the control groups, these differences showed statistical significance. As observed from the results in Figures 2 and 3, the upregulation of mRNA expressions of relative genes led to the simultaneous upregulation of protein expressions. However, under the continuous action of INH, protein expressions could not return to the normal levels.

Nrf2 protein distribution in liver tissues

Immunohistochemical analysis showed that the Nrf2 expression sites in hepatocytes in the control groups were different from those in all INH administration groups (Figure 4 and Table 4). Nrf2 was expressed in the cytoplasm in the control groups, and yellow staining was not observed in the nucleus. After gavage administration for 7 days, 2 weeks, 3 weeks, and 4 weeks, stained nucleus was observed, indicating the expression of Nrf2. Moreover, with the extension of administration

Gene		Primer Sequences(5'-3')	length
Nrf2	Forward	5'-CACATTCCCAAACAAGATGC-3'	374
	Reverse	5'-TCTTTTTCCAGCGAGGAGAT-3'	
Cu-ZnSOD	Forward	5'-ATGGCGATGAAAGCGGTGTG-3'	465
	Reverse	5'-TTACTGCGCAATCCCAATCACTC-3'	
MnSOD	Forward	5'-ATGTTGTGTCGGGCGGCG-3'	669
	Reverse	5'-TCACTTCTTGCAAGCTGTGTATCTTTCA-3'	
GSTA1	Forward	5'-GACTGCTTTCTTCAGGGTTCAAG3'	111
	Reverse	5'-TCTGTGTAATTCATGGCTGATTCC-3'	
GSTM1	Forward	5'-CGACGCTCCCGACTATGACA-3'	182
	Reverse	5'CACGAATCCGCTCCTCCTCT-3'	
GAPDH	Forward	5'-ACCACAGTCCATGCCATCAC-3'	452
	Reverse	5'-TCCACCACCCTGTTGCTGTA-3'	

Groups	GST (U/mg prot)	Total SOD (U/mg prot)	Cu-ZnSOD (U/mg prot)	MnSOD (U/mg prot)
Control groups	36.42 ± 3.69	317.95 ± 19.32	281.92 ± 24.52	41.42 ± 9.35
1 d	32.81 ± 4.63	302.06 ± 35.03	261.51 ± 27.16	40.55 ± 11.34
3 d	39.94 ± 8.63	287.24 ± 32.33	246.82 ± 23.62	33.82 ± 12.52
5 d	23.26 ± 4.55*	272.63 ± 22.43*	247.94 ± 13.05	38.08 ± 7.38
7 d	25.97 ± 6.46*	276.14 ± 20.90*	261.86 ± 16.84	24.75 ± 10.57
2 w	24.03 ± 7.19*	271.00 ± 27.43*	242.53 ± 21.48*	31.05 ± 8.65
3 w	34.08 ± 6.07	307.48 ± 19.72	253.49 ± 25.13	41.32 ± 11.69
4 w	36.64 ± 4.58	302.07 ± 24.94	277.11 ± 18.87#	28.57 ± 12.58
F	3.365	3.769	3.499	1.312
P	0.005	0.002	0.004	0.262

Table1: The sequences of primers and amplification length of amplified genes.

Groups	GSH (µmol/g prot)	MDA (nmol/mg prot)
Control groups	17.92 ± 5.35	0.54 ± 0.09
1 d	17.16 ± 3.44	0.63 ± 0.14
3 d	11.98 ± 3.12	0.69 ± 0.30
5 d	5.93 ± 1.21*	0.76 ± 0.21
7 d	4.67 ± 2.01*	0.94 ± 0.28*
2 w	6.39 ± 2.43*	0.91 ± 0.23*
3 w	8.89 ± 3.29*	0.73 ± 0.22
4 w	11.60 ± 3.62	0.80± 0.15*
F	11.869	3.816
P	< 0.001	0.002

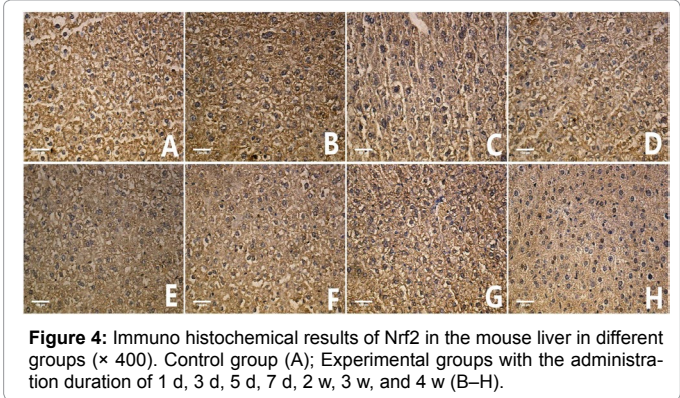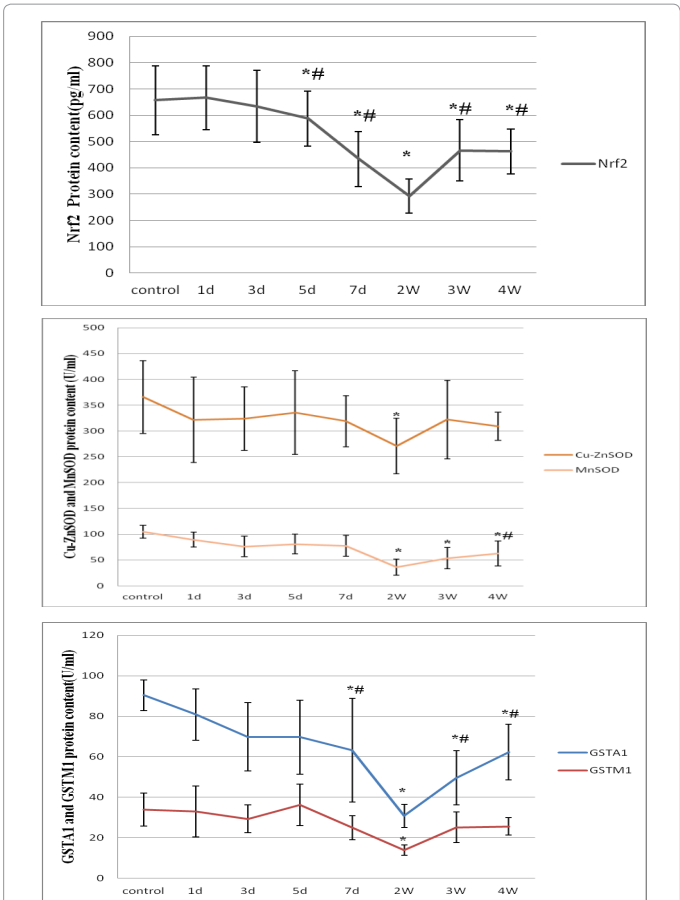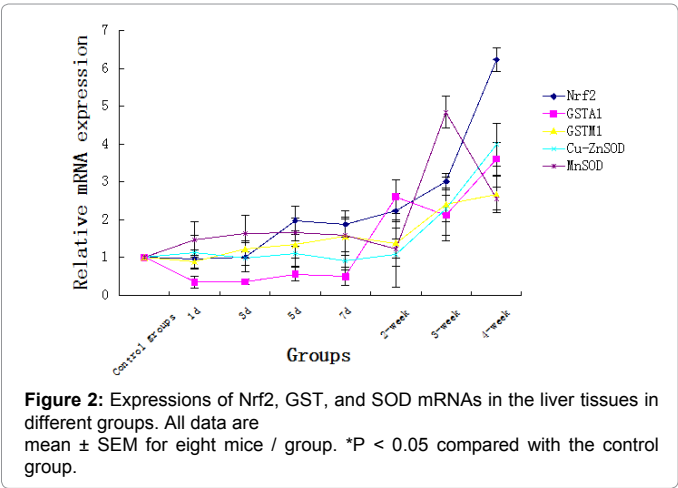**Table 2:** Comparison of GST and SOD activities in the mouse liver tissues in different groups ($\bar{x}$ ± s, n = 8).

Groups	n	AOD
Control groups	3	0.086 ± 0.008
1 d	3	0.090 ± 0.007
3 d	3	0.101 ± 0.018
5 d	3	0.107 ± 0.016
7 d	3	0.171 ± 0.014*
2 w	3	0.229 ± 0.023*#
3 w	3	0.238 ± 0.024*#
4 w	3	0.249 ± 0.033*#
F		161.477
P		< 0.001

Table 3: GSH and MDA contents in the mouse liver tissues in different groups ($\bar{x}$ ± s, n = 8).

Groups	n	AOD
Control groups	3	0.086 ± 0.008
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7 d	3	0.171 ± 0.014*
2 w	3	0.229 ± 0.023*#
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4 w	3	0.249 ± 0.033*#
F		161.477
P		< 0.001

Note: * compared with the control group, $P < 0.05$.

Table 4: Immunohistochemical results of Nrf2 in the mouse liver in different groups ($\bar{x}$ ± s)

Figure 2: Expressions of Nrf2, GST, and SOD mRNAs in the liver tissues in different groups. All data are mean ± SEM for eight mice / group. *$P < 0.05$ compared with the control group.

Figure 3: Expressions of Nrf2, GST, and SOD proteins in the liver tissues in different groups. All data are mean ± SEM for eight mice / group. * $P < 0.05$ compared with the control group; # $P < 0.05$ compared with the 2-week group.

Figure 4: Immuno histochemical results of Nrf2 in the mouse liver in different groups (× 400). Control group (A); Experimental groups with the administration duration of 1 d, 3 d, 5 d, 7 d, 2 w, 3 w, and 4 w (B–H).

time, the Nrf2 expression in the nucleus was increased. The significant differences with the control groups were confirmed, which suggests the transmembrane transport of Nrf2 proteins.

Discussion

In this research, the effects of the Nrf2–ARE pathway on INH-induced mouse liver injury was explored from two perspectives, i.e., detoxification of drug metabolites and antioxidant capacity. The results indicated that oxidative damage occurred in the mouse liver tissues through INH gavage. Subsequently, the Nrf2–ARE pathway was

activated and exerted protective effects via upregulating the expressions of total GST, GSTA1, GSTM1, total SOD, Cu-ZnSOD, and MnSOD. This finding indicates that the Nrf2–ARE pathway strengthened detoxification and regulated antioxidation in a dynamic pattern. When these two functions are exerted successively, the protective effects on hepatocytes are more significant. Thus, these data clearly illustrate the contribution of the Nrf2-ARE signaling pathway to against INH-induced hepatotoxicity.

The GST mRNA expression in the mammal liver can be used to evaluate the detoxification ability of the organism, and the mRNA expression of the antioxidant enzyme SOD can be used to estimate the antioxidant ability. After administration with INH for 5 days, 7 days, 2 weeks, 3 weeks, and 4 weeks, the Nrf2 protein in the mouse liver tissues showed a lower expression, indicating that the protein itself was also attacked by the drug. However, the Nrf2 mRNA expression gradually increased. This result indicates that the body could up regulate the expression of damaged proteins via compensatory regulation. The overexpression of Nrf2 possesses protective effects against microcystin-induced mouse liver injury [20]. Moreover, the Nrf2 mRNA expression was up regulated in the fourth week, suggesting that the organism itself had a certain protective mechanism. However, the experimental design ends at the 4th week after administration. Thus, after the Nrf2 protein content was increased from the trough level, its effects on the expression of its downstream target gene was not observed. The regulatory effects of Nrf2–ARE in INH-induced liver injury could be further demonstrated through the extension of drug administration time, as well as the intervention toward detoxification and anti-oxidation.

Nrf2 is anchored in the cytoplasm under normal circumstances. The Nrf2 expression in the rat hepatocytic nucleus could be significantly increased because of the effects of curcumin, and the expressions of its downstream proteins GSH and SOD are expected to be up regulated accordingly [21]. After 7 days of INH administration, the positive expression of Nrf2 in the nucleus could be observed at each time point, which manifested the transmembrane transport. After the transport, the expressions of GST and SOD mRNAs in the tissues were both up regulated. The up regulation suggests that in the liver injury induced by INH, Nrf2 entered the nucleus through nucleocytoplasmic transport, activating the Nrf2–ARE pathway. The expressions of the downstream target genes GST and SOD were then up regulated, and protective factors in the body were activated; this phenomenon promotes elimination of INH and its intermediate toxic metabolites and repairs damaged tissues [22,23].

INH can form hydrazine and acetylated derivatives after the metabolism in the liver. These toxic metabolites are mostly eliminated through binding to GSH under the action of GST [24]. After 2 weeks of administration, GSH content and activity remained at the lowest level. This finding suggests that the generation rate of GSH was lower than its consumption rate with continuous administration and accumulation of metabolites. Excessive consumption of GSH, which is the main substance responsible for eliminating exogenous chemical toxins, led to GSH deficiency. However, the activity and content of GST in the 3rd and 4th week groups were higher than those in the 2 week group, suggesting that protective factors in the body were activated, such as Nrf2. Detoxifying enzymes showed an increased expression and resisted the attack of exogenous toxins. Thus, if phase II detoxification ability in the body can be enhanced timely at an early stage, and the active intermediate metabolites of drugs in the tissues can be eliminated as far as possible, the INH-induced liver injury may be avoided.

In animal experiments on alcoholic liver injury, SOD activity is significantly decreased, and the expressions of Cu-ZnSOD and MnSOD proteins and their mRNA expressions are lower than those in the control groups [25]. In this research, the total SOD activities in the 5 day group, 7 day group and 2 week group were lower than those in the control groups, and it was continuously decreased in the liver tissues during gavage administration. The decrease suggests that SOD proteins were attacked by active metabolites and ROS molecules, leading to the decrease of enzyme activity. Meanwhile, the changes of MDA content can indirectly indicate free radical level in cells and the extent of oxidative damage in tissues [8]. On the seventh day, its content was the highest, and was also maintained at a high level in the second and fourth weeks.

Conclusion

In summary, this study clearly demonstrated that Nrf2-ARE pathway can protect against INH-induced liver injury. After nucleocytoplasmic transport of Nrf2 protein, the expressions of its downstream target genes GST and SOD were promoted, and the up regulation time of SOD was at a later time point than that of GST mRNA, suggesting that the Nrf2–ARE pathway may first regulate the detoxification ability in the body, and then regulate the antioxidant ability.

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