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Full Length Article

High Acetylation of Histones in the Promoter Region of CYP2E1 Gene Increases Hepatocyte Apoptosis Induced by Isoniazid

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Abstract

To investigate the effects of isoniazid-induced changes in histone acetylation in the hepatocyte CYP2E1 promoter region on hepatocyte apoptosis, the protein changes and the mRNA expressions were detected by ELISA, Western blot and real-time fluorescent quantitative PCR respectively. The acetylation of H3K56 and H4K5 in the CYP2E1 promoter region was detected by the Chip technique. Pathological results and changes in ALT and AST levels indicate that isoniazid caused liver cells damaged. In the isoniazid group, the HAT activity, the anti-apoptotic index Bcl_2 mRNA expression and total acH3K56 and acH4K5 expression levels were decreased; The HDAC activity, the acH3K56 and acH4K5 expression levels of the CYP2E1 gene promoter region, and the mRNA expression of apoptosis index JNK and Bax were increased. In the isoniazid + Garcinol group, HAT activity, the total acH3K56 and acH4K5 expression levels, the CYP2E1 gene promoter region acH3K56 and acH4K5 expression levels and its mRNA and protein expression were reduced. And this group showed decreased expression of JNK and Bax mRNA and increased expression of Bcl_2 mRNA. Inhibition of CYP2E1 gene promoter acetylation in hepatocytes can not only reduce CYP2E1 expression but also reduce hepatocyte apoptosis induced by isoniazid.

Keywords: Cytochrome P450 2E1; Histone acetylation; Liver injury; Isoniazid

Introduction

Tuberculosis is an infectious disease caused by Mycobacterium tuberculosis (Fattorini *et al.* 2013). There are three main types of Mycobacterium tuberculosis, which are *Mycobacterium bovis* (bovine type), *M. tuberculosis* (human type), and *M. avium* (avian type). In addition, there are two types of strains, cold-blooded and mouse, but they are not pathogenic to humans and animals. China has 1 million new cases each year and 130,000 deaths (Yang and Gao 2018). It can be seen that tuberculosis remains an important public health problem worldwide, so the prevention and treatment of tuberculosis is particularly important. In the anti-tuberculosis treatment program, isoniazid, as a first-line anti-tuberculosis drug, can not only inhibit the synthesis of Mycobacterium tuberculosis but also bind to the coenzyme of Mycobacterium tuberculosis and interfere with the synthesis of DNA and ribonucleic acid. However, its metabolism in the liver can cause liver cell damage, resulting in the interruption of tuberculosis treatment, which can easily lead to the occurrence of drug-resistant tuberculosis (Sonika and Kar 2012; Castelli and Sulis 2017). Therefore, understanding the mechanism of hepatic injury caused by isoniazid is particularly important.

CYP2E1 is an important member of the CYP450 family, is important for the metabolism of endogenous and exogenous compounds, and is associated with chemical toxicity and carcinogenesis of the liver (Toussirot *et al.* 2014). The anti-tuberculosis drug, isoniazid, is catalyzed by acetyltransferase 2 and cytochrome P450 enzymes in hepatocytes (Abdelmegeed *et al.* 2017). Its ultimate toxic metabolites and acetyl derivatives can directly covalently bind to macromolecular substances in hepatocytes to cause liver damage and at the same time generate an endogenous superoxide anion; these damages can further promote the production of reactive oxygen species (Singh *et al.* 2009; Metushi *et al.* 2016). Excessive oxidative stress can activate the JNK pathway, leading to hepatocyte apoptosis, which in turn promotes liver damage (Wang *et al.* 2011; Jiménez-Jiménez *et al.* 2016).

Histone acetylation, as the main form of histone modification, is a critical mode of action for chromatin remodeling in the tail of histones. The lysine residues of the four core histones H2A, H2B, H3, and H4 can be produced

from histone acetylation. Histones H3 and H4 are more easily modified. Histone acetylation is a reversible dynamic process regulated by histone acetyltransferase (HAT) and histone deacetylase (HDAC). HAT acetylates a histone lysine, resulting in relaxation of the chromatin structure, easy binding to transcription factors, and promotion of gene activation. In contrast, HDAC removes acetyl groups from highly acetylated histones, resulting in a tight chromatin structure and inhibition of gene transcription (Hassan *et al.* 2015). Yang *et al*. (2010) found that the histone deacetylase inhibitor TSA can acetylate H3 in the promoter region of the CYP2E1 gene in HepG2 cells and increase the expression of CYP2E1 to promote apoptosis of hepatoma cells. However, the acetylation of histones is regulated by both acetylase and deacetylase. The study of deacetylase inhibitors alone does not reflect the overall acetylation of histones.

Garcinia indica, a plant found extensively in tropical regions. The extract from the fruit of *Garcinia indica*, popularly known as Kokum or Mangosteen, has been valued in the Indian subcontinent, Africa, and China for its sweet and sour taste and has traditionally been used as a seasoning, a snack, or steeped in syrup for a refreshing drink (Padhye *et al.* 2009). Garcinol is a polyisoprenylated benzophenone present in *Garcinia indica* fruit rind that also inhibits both CBP/p300 and PCAF HAT activities. Therefore, this study will compare the enzyme activities of HAT and HDAC in hepatocytes when isoniazid, garcinol, and the two drugs are used in combination, and hoping to further explain the mechanism of liver injury caused by antituberculosis drugs.

Materials and Methods

Cells and reagents

The cell line used was the human liver cell line HL-7702, which was purchased from the Shanghai Academy of Sciences. A medium containing 90% RPMI 1640 (CORNING), 10% fetal calf serum (CLARK), and 1% double antibody was used as a medium for HL-7702 cells, and the cells were cultured in 5% CO_2 and 37°C environment. Isoniazid was purchased from TCI Corporation of Japan, Garcinol was purchased from Cayman.

Cell treatment

Isoniazid was diluted to 600, 800, 1000, and 1200 μg/mL, Garcinol was diluted to 1, 5, and 10 μmol/L to act on hepatocytes, and the optimal experimental drug concentration and reagent concentration were determined by the CCK8 method. After collecting the cells, the cell suspension was adjusted to have a cell density of 1 × 10 5 /mL and seeded in a 96-well plate, and 100 μL of the suspension was added to each well. After culturing for 24 h in an incubator in 5% CO_2 at 37°C, the old culture solution was aspirated, 100 μL of the culture solution was added to each of the control wells, and different concentrations of the drug solution were added to each test well to continue the culture. After 24 h, the liquid in the well was aspirated, the wells were washed with a sterile PBS solution, the residual liquid in the well was aspirated and 100 μL of CCK8 dilution was added to each well. A blank well not seeded with cells was used as a negative control well to which only 100 μL of CCK8 dilution was added. After incubating, the 96-well plate at 5% CO_2 and 37°C for 1 h. The 24-h survival rate of the cells was calculated as the ratio of the absorbance of the experimental wells to that of the control wells.

Real-time fluorescent PCR

Total RNA was extracted with Trizol. The reverse transcription reaction system was 20 μL, in which the reverse transcriptase was 4 μL and 1000 ng of content corresponded to the volume of RNA, which was complemented with dH_2O. The reverse transcription reaction conditions were as follows: 37°C for 15 min, 85°C for 5 s, and 4°C for 1 min. The real-time PCR system was 20 μL, of which SYRB Green was 10 μL, dH2O was 6.8 μL, the upstream and downstream primers were each 0.4 μL, Rox was 0.4 μL, and cDNA was 2 μL. The quantitative PCR reaction conditions were as follows: pre-denaturation at 95°C for 30 s, denaturation at 95°C for 5 s, and annealing at 60°C for 30 s for a total of 40 cycles. The mRNA expression was calculated via $2^{-\Delta\Delta ct}$ using GAPDH as an internal reference.

Enzyme linked immunosorbent assay

The cultured cells were collected for 24 h and operated on in strict accordance with the HAT and HDAC activity ELISA kits. The kits were purchased from Beijing Dongge Weiye Biotechnology Co., Ltd.

Western immunoblotting

The cells were lysed with RIPA protein lysate and the sample protein concentration was quantified with a BCA kit. According to the molecular weights of histones H3K56 (17 kD), H4K5 (11 kD), and CYP2E1 (57 kD), 15% and 8% of separation gel and 5% of concentrated gel were respectively disposed. After transfer, 5% skim milk powder was blocked at room temperature for 2 h. After washing, they were incubated overnight at 4°C with acetylated histone H3K56 (1:10000), H4K5 (1:10000), and CYP2E1 (1:1000) antibodies. The secondary antibody was incubated for 2 h at room temperature and developed after rinsing the next day. Six replicates were examined in each sample.

Chromatin immunoprecipitation

The Chip detection method was used for

immunoprecipitation by formaldehyde cross-linking and cell sonication, and specific DNA was collected. The q-PCR was used to quantitatively analyze the acetylation of H3K56 and H4K5 in the promoter region of the CYP2E1 gene strictly following the instructions in the kit. The kit was purchased from Wuhan Aibotek Biotechnology Co., Ltd.

Statistical analysis

Data were analyzed by using SPSS software (version 23.0). Mean ± standard deviation was used to express data. One-way analysis of variance was used to compare the differences between data and indicators. The SNK-q test was used for comparison.

Results

Establishment of hepatocyte injury model

After isoniazid was diluted to different concentrations and applied to hepatocytes, the cell survival rate of the CCK8 method was selected to be between 80 and 85%, and the concentration of isoniazid (1000 μg/mL) was determined. At this drug concentration, preliminary experiments showed that the changes of core indicator CYP2E1 mRNA was obvious, and could inhibit hepatocytes injury combined with histone acetylase inhibitor, it was indicating that the drug concentration was reasonable. The maximum non-toxic dose was chosen to be the inhibitor concentration (Garcinol 5 μmol/L). At the experimental concentrations of isoniazid for 6 h, 24 h, and 48 h, the results showed that CYP2E1 mRNA expression increased after 6 h of dosing, so the time was determined to be 6 h. Fig. 1 shows that the activity of ALT and AST in the hepatocyte culture medium of the isoniazid group was significantly higher than that of the control group. The pathological changes in the cells in the experimental group showed that the hepatocyte injury model was successfully established.

Histone acetylation level, CYP2E1 mRNA, and protein expression changes under isoniazid

Compared with the control group, HAT activity decreased, HDAC activity increased, and the total acH3K56 and acH4K5 expression levels decreased (Fig. 2), indicating that isoniazid caused a decrease in the overall acetylation level of hepatocytes.

Furthermore, we used Chip PCR to detect the expression levels of acH3K56 and acH4K5 in the promoter region of CYP2E1 gene. The results showed that isoniazid increased the histone acetylation level of the CYP2E1 gene promoter region compared with the control group, which was opposite to the overall acetylation level (Fig. 3).

The mRNA and protein expression of the CYP2E1 gene were detected by real-time fluorescent PCR and Western blot. Compared with the control group, the expression of CYP2E1 mRNA (Fig. 4A) and protein (Fig. 4B) was increased in hepatocytes in the isoniazid group.

Effects of isoniazid combined with inhibitor on histone acetylation level and CYP2E1 mRNA and protein expression

To determine the effect of the histone acetylase inhibitor Garcinol combined with isoniazid on hepatocyte histone acetylation levels, we examined HAT activity in the Garcinol and INH + Garcinol groups. Compared with the control group, the HAT activity of the Garcinol group was decreased and the HAT activity of the INH + Garcinol group was also lower than that of the INH group (Fig. 5A), indicating that Garcinol has an inhibitory effect on HAT activity. The expression levels of acH3K56 and acH4K5 were detected. Compared with the control group, the expression levels of acH3K56 and acH4K5 in the inhibitor control Garcinol group did not change much. Compared with the INH group, the expression level of the INH + Garcinol group was significantly decreased (Fig. 5B). This indicates that inhibition of HAT causes a decrease in the overall histone acetylation level of hepatocytes caused by isoniazid.

Furthermore, we examined the expression levels of acH3K56 and acH4K5 in the promoter region of the hepatocyte CYP2E1 gene by isoniazid combined with Garcinol. Compared with the isoniazid group, the expression levels of acH3K56 and acH4K5 in the promoter region of the CYP2E1 gene in the INH + Garcinol group were decreased, indicating that Garcinol activated the CYP2E1 gene (Fig. 6). The acetylation level of the sub-region also has an inhibitory effect.

We examined the mRNA and protein expression of the CYP2E1 gene in the Garcinol group, INH + Garcinol group. Compared with the INH group, the mRNA (Fig. 7A) and protein (Fig. 7B) expression of the CYP2E1 gene in the INH + Garcinol group decreased (Fig. 7), indicating that inhibition of histone acetylation in the promoter region of the CYP2E1 gene can be reduced.

To determine the effect of isoniazid and isoniazid combined with inhibitors on the expression of apoptosis markers, we examined the mRNA levels of the JNK, Bax, and Bcl2 genes. Compared with the control group, the mRNA levels of the JNK and Bax genes in the INH group increased. The mRNA level of the Bcl2 gene was decreased. Compared with the isoniazid group, the levels of JNK and Bax mRNA were decreased, and the mRNA level of the Bcl2 gene was increased in the isoniazid plus inhibitor group (Fig. 8), indicating that hepatic cell apoptosis was caused by isoniazid. Additionally, isoniazid in combination with the inhibitors can inhibit apoptosis. Compared with the control group, the mRNA levels of the JNK, Bax, and Bcl2 genes in the inhibitor control groups did not change much, indicating that the inhibitor alone did not cause hepatocyte apoptosis.

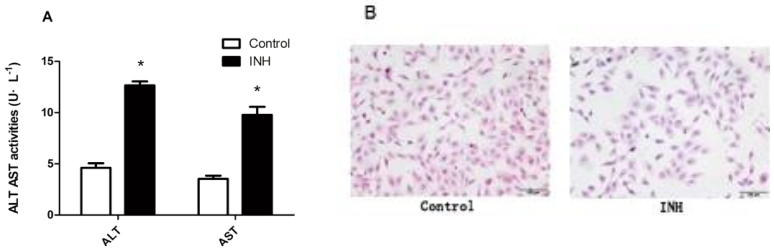

Fig. 1: ALT and AST activity and hepatocyte morphology of hepatocytes after hepatic cells were treated with isoniazid. (**A**) ALT and AST activity of hepatocyte supernatants treated with isoniazid. *The difference was statistically significant at $P < 0.05$ compared with the control group. (**B**) Hepatocyte morphological changes (HE, ×100) for isoniazid

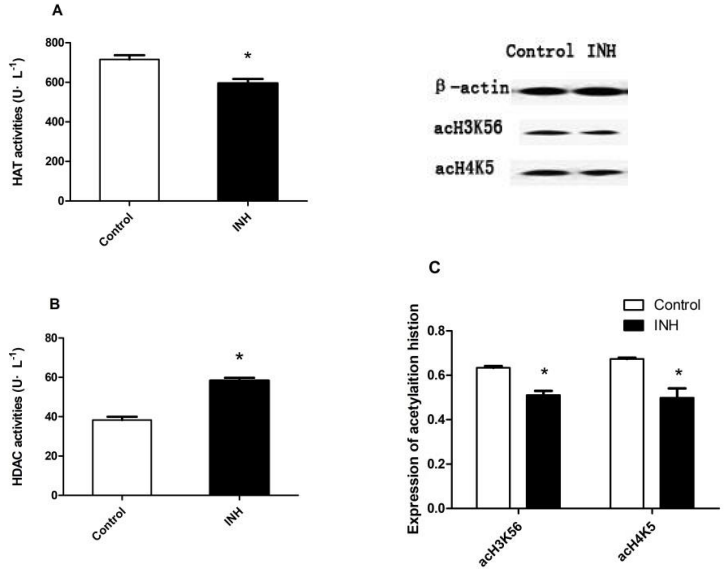

Fig. 2: Hepatocyte HAT and HDAC enzymatic activity and total acH3K56 and acH4K5 expression. *$P < 0.05$ was considered statistically significant compared with the control group

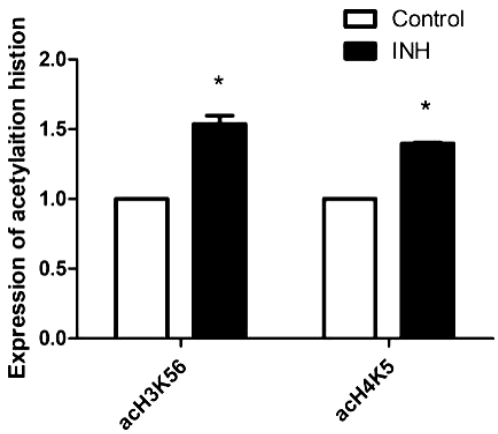

Fig. 3: Expression of acH3K56 and acH4K5 in the promoter region of the hepatocyte CYP2E1 gene. *$P < 0.05$ was considered statistically significant compared with the control group

The variation trend in liver cell morphology and the apoptosis index were the same. On the hepatocyte morphology, it did not change much in the inhibitor control group than the control group, but it showed a long fusiform and triangular shape and decreased cell number in the isoniazid group. Hematocytosis and triangular hepatocytes were decreased when inhibitor Garcinol was added into the isoniazid treated cell group (Fig. 9).

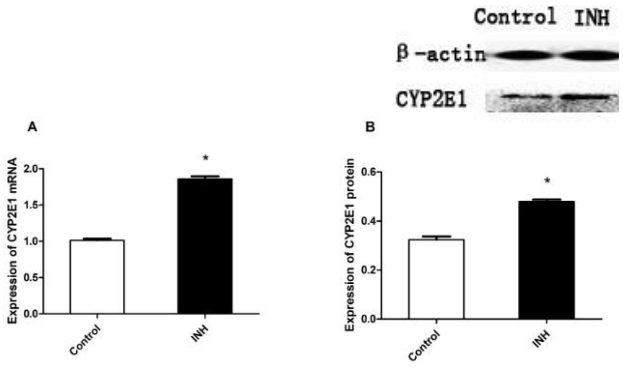

Fig. 4: Expression of CYP2E1 mRNA and protein in hepatocytes. Expression of CYP2E1 mRNA $^{*}P < 0.05$ was considered statistically significant

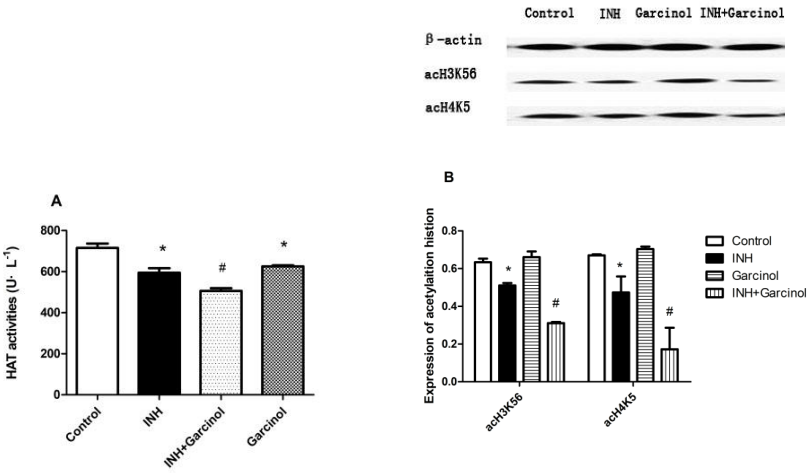

Fig. 5: Hepatocyte HAT and HDAC enzymatic activity and overall acH3K56 and acH4K5 expression. $^{\#}P < 0.05$ was statistically significant compared with the isoniazid group; $^{*}P < 0.05$ was considered statistically significant compared with the control group

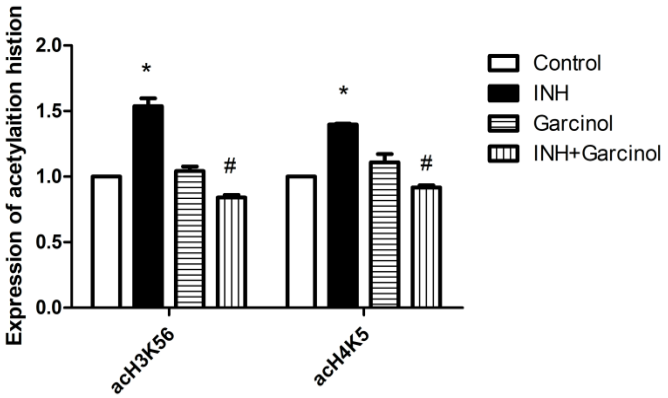

Fig. 6: Expression of acH3K56 and acH4K5 in the promoter region of the hepatocyte CYP2E1 gene. $^{\#}P < 0.05$ was statistically significant compared with the isoniazid group; $^{*}P < 0.05$ was considered statistically significant compared with the control group

Discussion

Isoniazid, as a first-line anti-tuberculosis drug, can increase serum alanine aminotransferase levels in 20% of patients and obvious liver toxicity in 2% of patients (Chen *et al.* 2015). Our data showed that hepatocytes were damaged after 6 h in hepatic cells treated with 1000 μg/mL isoniazid, which showed increased activity of ALT and AST in the

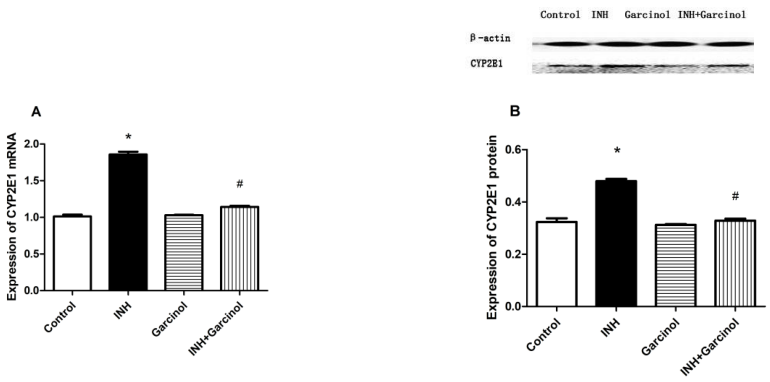

Fig. 7: Changes in CYP2E1 mRNA and protein in hepatocytes. $^{\#}P < 0.05$ was statistically significant compared with the isoniazid group; $^{*}P < 0.05$ was considered statistically significant compared with the control group

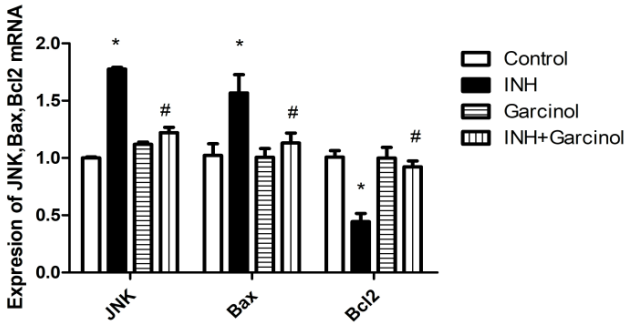

Fig. 8: Changes in mRNA of the JNK, Bax, and Bcl2 genes in hepatocytes after treatment with isoniazid combined with inhibitors. $^{*}P < 0.05$ was considered statistically significant compared with the control group, and $^{\#}P < 0.05$ was considered to be statistically significant compared with the isoniazid group

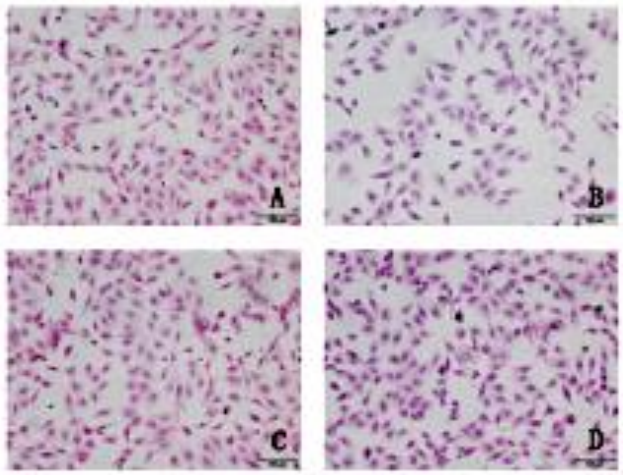

Fig. 9: Comparison of hepatocyte morphology in different treatment groups (HE ×100). (**A**) is a blank control group, (**B**) is an INH group, (**C**) is a Garcinol group, (**D**) is an INH + Garcinol group

cell supernatant, and long, spindle-shaped, and triangular hepatocytes in a reduced number, which proved that the cell damage model was successful.

Our data showed that HAT activity decreased and HDAC activity increased after the application of isoniazid in hepatocytes, and the overall acH3K56 and acH4K5 expression decreased, but the acH3K56 and acH4K5 expression in the CYP2E1 gene promoter region increased. Contrary to the overall trend, CYP2E1 gene mRNA and protein expression both increased. Cytochrome P450 is one

of the most important phase I metabolic enzymes in the human body (Massabayeva *et al.* 2019). The CYP2E1 subtype is mainly distributed in the liver. In humans, CYP2E1 is encoded by the CYP2E1 gene, which is located at the 10q24.3 qter (Wang *et al.* 2016). CYP2E1 is an important drug-metabolizing enzyme that oxidizes the INH intermediate acetyl hydrazine to hepatotoxins, such as acetyltransferase and ammonium acetylate (Liu *et al.* 2015). Studies have shown that different genotypes of the CYP2E1 gene cause changes in CYP2E1 enzyme activity or levels, which affect liver damage by affecting the chemical modification of the drug in the liver (Neafsey *et al.* 2009; Davydova *et al.* 2019). Yang's results indicate that low expression of CYP2E1 in HepG2 cells is associated with histone hypoacetylation in the promoter region of CYP2E1 gene, suggesting that histone acetylation in the promoter region of the gene affects the expression of the CYP2E1 gene (Yang *et al.* 2010). Histones are regulated by HAT and HDAC, and HAT acetylates histone lysine, resulting in a loose chromatin structure, which is beneficial for binding to transcription factors and promoting gene transcription. HDAC deacetylates highly acetylated histones, and the chromatin structure becomes tight, inhibiting gene transcription. In this study, isoniazid caused decreased HAT activity and increased HDAC activity in HL-7702 cells, and overall acH3K56 and acH4K5 expression levels decreased, but the CYH2E1 promoter region acH3K56 and acH4K5 expression levels increased, indicating that the transcription and expression of the CYP2E1 gene may be activated with the CYP2E1 gene. Sub-regional histone acetylation is associated with increased acH3K56 and acH4K5 expression levels in the promoter region of the hepatocyte CYP2E1 gene.

To further determine the effect of histone acetylation on CYP2E1 expression, we used the histone acetylase inhibitor Garcinol in combination with isoniazid to change the level of histone acetylation. The results showed that the HAT activity of the Garcinol group was lower than that of the control group, indicating that Garcinol had an inhibitory effect on HAT activity. Compared with the INH group, the HAT activity of the INH + Garcinol group was decreased, the overall acH3K56 and acH4K5 expression levels were decreased, the CYH2E1 promoter region acH3K56 and acH4K5 expression levels were decreased, and the CYP2E1 mRNA and protein expression levels were decreased, indicating that lowering the CYP2E1 gene promoter region histone acetylation level can inhibit CYP2E1 transcription and translation. HAT includes p300, etc. Garcinol is a potent inhibitor of histone acetyltransferase p300 and PCAF in vivo and in vitro (Yamaguchi *et al.* 2000; Padhye *et al.* 2009; Yuan *et al.* 2012). Garcinol has been shown to have anti-inflammatory and anti-oxidative effects as an acetyltransferase inhibitor, and has shown a dose-dependent tumor-cell-specific growth inhibitory effect in some tumor cells. The above results indicate that changing the level of histone acetylation in the promoter region of CYP2E1 gene can change the expression of CYP2E1 gene.

To determine the effect of CYP2E1 expression changes on cell damage, we examined the expression of JNK, Bax, and Bcl_2 mRNA and cell morphology. Our data showed that compared with the control group, the expression of JNK and Bax mRNA in the experimental group increased, and the expression of Bcl_2 mRNA decreased. Studies have shown that increased expression of CYP2E1 leads to an increase in endogenous superoxide anions, further promoting the production of reactive oxygen species, thereby activating the JNK pathway. Compared with the isoniazid group, isoniazid combined with Garcinol inhibited the expression of CYP2E1, decreased the expression of JNK and Bax mRNA, and increased Bcl_2 mRNA expression.

Conclusion

This experiment confirmed that increased expression of CYP2E1 induced by isoniazid aggravated hepatocyte injury, and increased expression of CYP2E1 during hepatocyte injury induced by isoniazid were associated with changes in histone acetylation levels. Garcinol has protective effects against hepatocyte damage caused by isoniazid. The protective effect of the histone acetylase inhibitor Garcinol on hepatocytes is worthy of attention. It is hoped that the results will provide a basis for the prevention and treatment of anti-tuberculosis drug-induced liver injury.

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Author Contributions

Fumin Feng and Qi Ren conceived and designed research. Jinfeng Li, Yiyang Zhang and Yingshu Li conducted experiments. Xue Wang, Mi Zhang, Dongxue Wu, and Yingzhi Chong contributed new reagents or analytical tools. Yingshu Li analyzed data. Qi Ren wrote the manuscript. All authors read and approved the manuscript.

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