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DNA Methylation of the CYP1A1 Promoter Region is Associated with Anti-tuberculous Drug-induced Hepatic Injury

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ABSTRACT

CYP1A1 is a typical one-phase drug metabolizing enzyme, and imbalances of its expression may cause liver tissue damage. At present, few studies on CYP1A1 methylation and anti-tuberculosis drug-induced liver injury are available. Studying the relationship between CpG island methylation in the CYP1A1 promoter region and drug-induced liver injury is, therefore, of great importance. Anti-tuberculosis drugs induced hepatotoxicity in HL-7702 cells was investigated. Dehydrogenase activity was detected by a lactate dehydrogenase assay kit; superoxide dismutase (SOD) activity and malonaldehyde (MDA) concentration were detected by a total superoxide dismutase test kit and a malondialdehyde test kit. Genomic DNA and RNA were extracted from cells; Methylation was inhibited by treating HL7702 cells with 5-aza-2'-deoxycytidine (AZA), and the level was analyzed via the methylation-specific PCR (MSP) methods. The increases in the methylation rate of CpG islands in the CYP1A1 promoter region and expression of DNA methyltransferases (DNMTs). And then the CYP1A1 mRNA and protein expression decreased with cell damage, thus suggesting that elevated methylation of the promoter region of the CYP1A1 gene may affect its expression and could be associated with cell damage. After treatment of the cells with AZA, increases in methylation rate and DNMTs expression were reduced, CYP1A1 mRNA and protein levels increased, and cell damage was ameliorated; these results indicate that inhibition of methylation can reduce cell damage. The promoter region hypermethylation of the CYP1A1 gene were related to its low mRNA levels.

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Authors' Contribution
QR, CN and FF designed the study, performed experimental work and analyzed the data. SS, GZ, YC and BL helped in microscopic examinations. CN and YL wrote the article.

Key words
Antituberculosis drug-induced liver injury, HL7702, CYP1A1, DNA methylation, 5-aza-2'-deoxycytidine

INTRODUCTION

Tuberculosis is an infectious disease with high contagious and fatal rates. At present, the number of tuberculosis cases in China accounts for 27% of the total number, ranking the second in the world. The disease has always been a major issue facing human society (Doherty *et al*., 2013). Current tuberculosis treatments are mainly based on drugs (Sotgiu *et al*., 2015). However, the liver damage brought about by anti-tuberculosis drugs influences the treatment process and options of tuberculosis (Metushi, 2016). The combined application of anti-tuberculosis drugs presents a complex metabolic process in the body, and various drug metabolism enzymes are involved in the detoxification process. Cytochrome P450 enzymes are Phase I drug-metabolizing enzymes that can metabolize drugs entering the body into products that can easily be bound to Phase II drug-metabolizing enzymes and eliminated from the body (Sheweita, 2000). During this process, imbalances in enzyme activity could exert a toxic effect on liver tissue (Feng and He, 2013). For example, isoniazid (INH), a first-line anti-tuberculosis drug, may become a toxic product under the action of P450 enzymes (Preziosi, 2007). The liver damage caused by anti-tuberculosis drugs is called anti-tuberculous drug-induced liver injury (Sun *et al*., 2008).

Encoding gene changes and epigenetic regulation affect the expression of drug-metabolizing enzymes, and DNA methylation is an important epigenetic feature of such enzymes. DNA methyltransferases (DNMTs) can transfer methyl groups to normal cytosines, thereby promoting the formation of methylated cytosines. DNA methylation can affect the development of diseases by regulating the biological effects of genes on target genes.

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Abbreviations:
AZA, 5-aza-2'-deoxycytidine; DNMTs, DNA methyltransferases; INH, isoniazid; RIF, rifampicin; PZA, pyrazinamide; MSP, methylation-specific PCR; LDH, lactate dehydrogenase; MDA, malondialdehyde; SOD, superoxide dismutase.

Studies have shown that methylation of CYP1A1 not only affects the expression of the gene (Takahashi *et al*., 1998), but is also associated with the occurrence of certain diseases (Sharma *et al*., 2010; Tekpli *et al*., 2012). At present, reports on the relationship between methylation of P450 enzymes and the liver injury caused by anti-tuberculosis drugs mainly focus on CYP2E1 (Zhang *et al*., 2016); research on CYP1A1 is fairly limited. However, studies on the methylation of the CYP1A1 promoter region in other types of liver diseases (Beedanagari *et al*., 2010; Mah and Lee, 2014), have been reported. Thus, we hypothesize that CpG methylation in the promoter region of CYP1A1 may influence anti-tuberculous drug-induced liver injury by regulating gene expression.

In this study, first-line anti-tuberculosis drugs, including isoniazid (INH), rifampicin (RIF), and pyrazinamide (PZA), were used to induce HL7702 hepatocyte injury, and 5-aza-2'-deoxycytidine (AZA) was used to inhibit DNA methylation. Correlation between CYP1A1 methylation and anti-tuberculosis drugs indeced liver injury were evaluated by detecting hepatocyte injury, CYP1A1 gene expression, and promoter CpG island methylation.

MATERIALS AND METHODS

Cells and reagents

Human hepatocytes (HL7702) were purchased from the Chinese Academy of Sciences in Shanghai, China. RPMI 1640 was purchased from Corning (Australia, Lot No.: 10-040-CVR), fetal bovine serum was purchased from Clark (Australia, lot: FB15015), penicillin and streptomycin were purchased from BI (Israel, Lot: 03 -031-1B), and 0.25% trypsin was purchased from GIBCO (United States, Lot: 25200-072). Cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum. Cultures were maintained at 37 °C humid and 5% CO_2/95% air.

Anti-tuberculosis drug treatment

A pre-experiment was performed based on a relative cell survival rate of 80%–90%. An orthogonal design was used to determine the optimal culture time and drug concentration of each group via the CCK8 method. Cells were cultured separately in medium containing each of the anti-tuberculosis drugs (TCI, Japan) (INH 800 μg/ml, RIF 300 μg/ml, PZA 500 μg/ml, INH 50 μg/ml + RIF 100 μg/ml, INH 85.5 μg/ml + RIF 427.5 μg/ml + PZA 171μg/ml, total 5 groups) and then harvested after 6, 12, or 24 h. Each group contained 6 parallel samples.

LDH detection

LDH activity was detected by a lactate dehydrogenase assay kit following the manufacturer's instructions for use (Nanjing Jiancheng Bioengineering Institute, China). LDH activity was calculated according to the following formula (Tafail *et al*., 2019). LDH activity (U/L) = (treated group OD-control group OD)/ (standard group OD-blank group OD)/standard product concentration ×1000.

SOD and MDA detection

SOD activity and MDA concentration were detected by a total superoxide dismutase test kit and a malondialdehyde test kit (Nanjing Jiancheng Bioengineering Institute, China), respectively. The SOD activity detection method and the activity calculation reference manual operate. According to the formula to calculate the MDA concentration: MDA (nmol/ml) = (treatment group OD - control group OD)/ (standard group OD-blank group OD) × standard product concentration×sample before the test dilution.

Nucleic acid extraction

DNA extraction kit (Zhuangmeng International Biogen Technology Co., Ltd., Beijing, China) was used for extracting DNA. Genomic RNA extraction was performed using Trizol reagent according to the manufacturer's instructions. The concentration and purity of the DNA and RNA were tested via microplate reader.

Quantitative real-time PCR

Reverse transcription of the extracted RNA was first performed following the instructions accompanying the reverse transcription kit (TaKaRa, Dalian, China) using a PCR amplification apparatus (BIO-RAD, USA) with the following conditions: 37 °C for 15 min, 85 °C for 5 s, and 4°C for 1 min. The reverse-transcripted samples were then processed using a SYBR® Premix Ex TaqTM II PCR kit (TaKaRa, Dalian, China), and mRNA expression was detected by a 7500 Fast Real-Time PCR System (Applied Biosystems, USA) using the following conditions: pre-denaturation at 95 °C for 30 s, denaturation at 95 °C for 5s, and 40 cycles of annealing at 60 °C for 34 s. GAPDH was used as the internal reference gene, the final data was calculated according to the formula $2^{-\Delta\Delta Ct}$=2-((experimental group Ct-experimental group internal reference Ct)-(control group Ct-control internal reference Ct)).

Enzyme-linked immunosorbent assay for protein and enzyme activity

The instructions accompanying a standard ELISA kit were followed. Standard sample wells were established, and a plot of standard product concentration (abscissa) versus optical density (ordinate) was produce. A standard linear regression curve was drawn, and the DNMTs protein expression in each sample was calculated according to curve equation obtained.

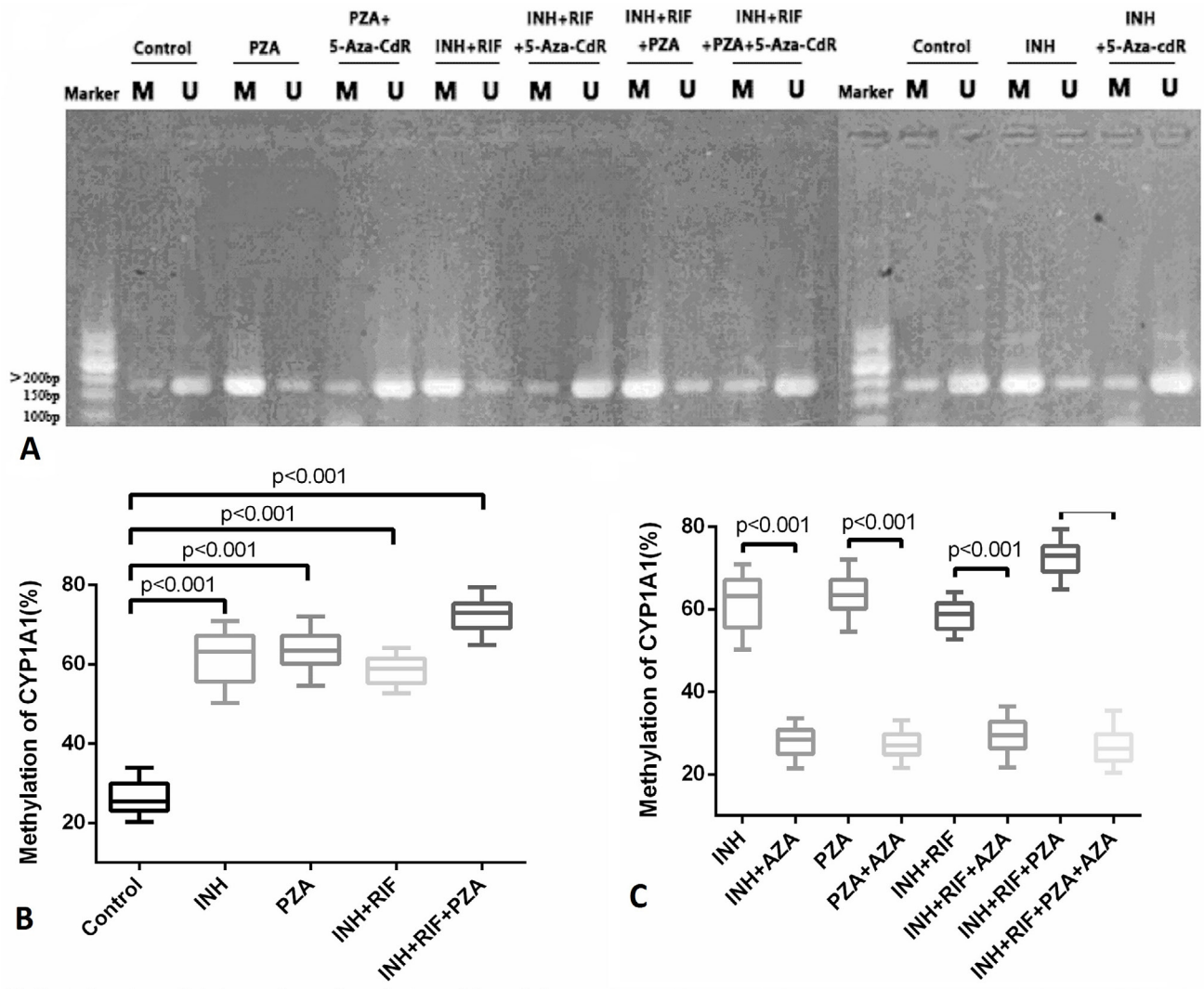

Fig. 1. Methylation of CpG islands in the promoter region of the CYP1A1 gene in different drug groups. (A) Methylation of CpG islands in the CYP1A1 promoter region of each group was detected by methylation-specific PCR (MSP). Here, M represents a methylated band and U represents an unmethylated band. Both bands are 194 bp in length. (B, C) MSP methylation, % = methylation OD/ (methylated OD + non-methylated OD) × 100%. Each group included 6 parallel samples.

5-Aza-2'-deoxycytidine (AZA) treatment

Methylation was inhibited by treating HL7702 cells with AZA. AZA was combined with each of the anti-tuberculosis drugs (INH, PZA, INH + RIF, INH + RIF + PZA) and added to new medium. The concentration of AZA in each well was 10 μM/L (selected from three concentrations of 0.1, 1, and 10 μM/L in a preliminary experiment), and the concentrations of the anti-tuberculosis drugs were kept the same. Cells were harvested after 12 h of incubation (selected from three time points of 6, 12, and 24 h).

DNA methylation analysis

DNA methylation was analyzed via the MSP methods. Whole genomic DNA (200–500 ng) was modified with sodium bisulfite using an EZ DNA Methylation-Gold kit (ZYMO Research Corporation, Irvine, CA, USA), and specific primers for CYP1A1 methylation and non-methylation were designed by the Meth Primer program (http://itsa.ucsf.edu/~urolab/methprimer). The methylation-specific primers were 5'-GATTATTTTTGGTTTGGATTAGC-3' (sense strand) and 5'-TAACCTAACTACCTACCTCCGACG-3' (antisense strand), and the non-methylation-specific

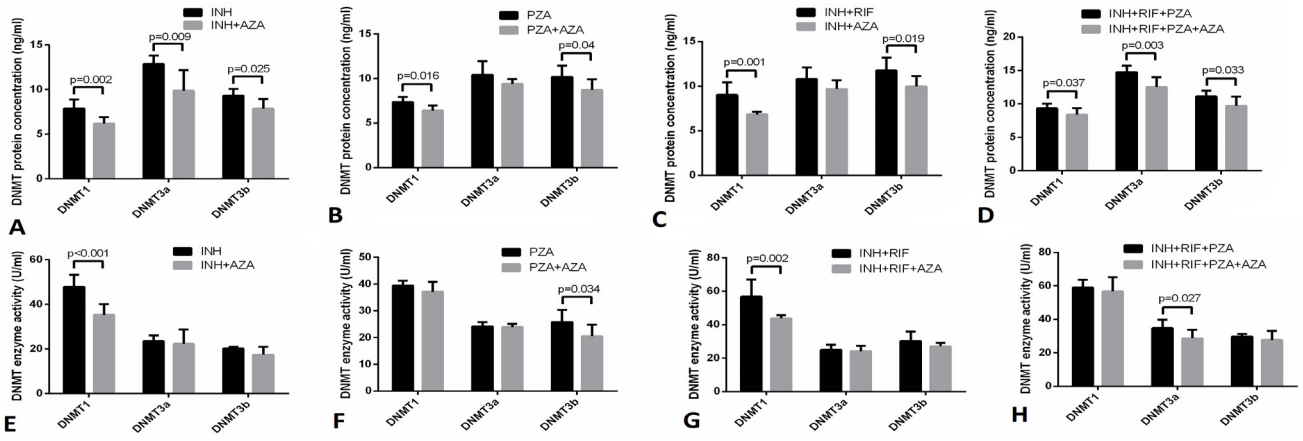

Fig. 2. Changes in DNMT1, DNMT3a, and DNMT3b protein expression and enzyme activity in various drug groups with AZA. The cells were incubated with each of the anti-tuberculosis drugs (isoniazid, INH; pyrazinamide, PZA; INH + rifampicin, RIF; or INH + RIF + PZA) or their combination with AZA (10 μM) for 12 h. Protein concentration and enzyme activity were detected by ELISA. Each group included 6 parallel samples, each of which was measured three times.

primers were 5'-GGATTATTTTTTGGTTTG-GATTAGT-3' (sense strand) and 5'-AACCTAAC-TACCTACCTCCAACACT-3' (antisense strand). The amplified sequences were all 194 bp in length. The specific detection and analysis methods are described according to He's study (He *et al*., 2015).

Statistical analysis

SPSS17.0 software was used for data processing and analysis. LDH activity, SOD activity, MDA content, and CYP1A1, DNA methyltransferase mRNA and protein of liver injury were analyzed by one-way ANOVA. Correlation analysis of DNA meyhylation of CYP1A1 and the liver injury markers LDH, SOD, and MDA was performed using Spearman correlation analysis. The MSP results were analyzed using the t-test, and $P < 0.05$ indicated the statistically significant differences.

RESULTS

Changes in the methylation levels of CpG islands in the promoter region of the CYP1A1 gene after treatment with anti-tuberculosis drugs and 5-Aza-CdR

After incubation with anti-tuberculosis drugs for 12 h in cell culture plates, CpG island methylation in the promoter region of the CYP1A1 gene was detected via methylation-specific PCR (MSP) (Figs. 1A, 1B). The results showed that the methylation rates of the INH, PZA, INH + RIF, and INH + RIF + PZA groups were 61.8% ± 7.2%, 63.5% ± 5.7%, 58.6% ± 3.9%, and 72.5 ± 4.7%, respectively; all of these values are clearly higher than that of the control group (26.3 ± 4.6%). While the RIF group methylation rate was 31.4 ± 3.9%, with no significant increase (P=0.074). Therefore, RIF group was excluded from subsequent analysis.

To observe the inhibitory effect of AZA on CYP1A1 hypermethylation, we combined 10 μM AZA with each of the anti-tuberculosis drugs (INH, PZA, INH+RIF, INH+RIF+PZA) or their combination and cultured cells in the resulting treatments for 12 h. Methylation of CpG islands in the promoter region of CYP1A1 was detected by MSP. As can be seen from Figure 1C, the methylation rate of the promoter region of the CYP1A1 gene decreased significantly after addition of AZA, thus indicating that AZA exerts demethylation effects.

Effect of AZA on the enzyme activity and protein expression of DNMT1, DNMT3a, and DNMT3b

We detected the protein expression (Figs. 2A, B, C and D) and enzyme activity (Figs. 2E, F, G and H) of DNA methyltransferases, which included DNMT1, DNMT3a and DNMT3b. The results showed that there is a positive correlation between DNA methylation of CYP1A1 and protein of DNA methyltransferases, DNMT1, DNMT3a, and DNMT3b (Spearman $r = 0.59, 0.43, 0.58$; $p < 0.01$), which indicated that these genes may effect on methylation of CYP1A1.

Effects of AZA on the expression of CYP1A1 mRNA and protein in cells

As shown in Figure 3, after culture with anti-tuberculosis drugs (6, 12, or 24 h), the expression of CYP1A1 mRNA significantly decreased treated with INH, PZA, INH + RIF, or INH + RIF + PZA for 12 h by 44%, 38%,

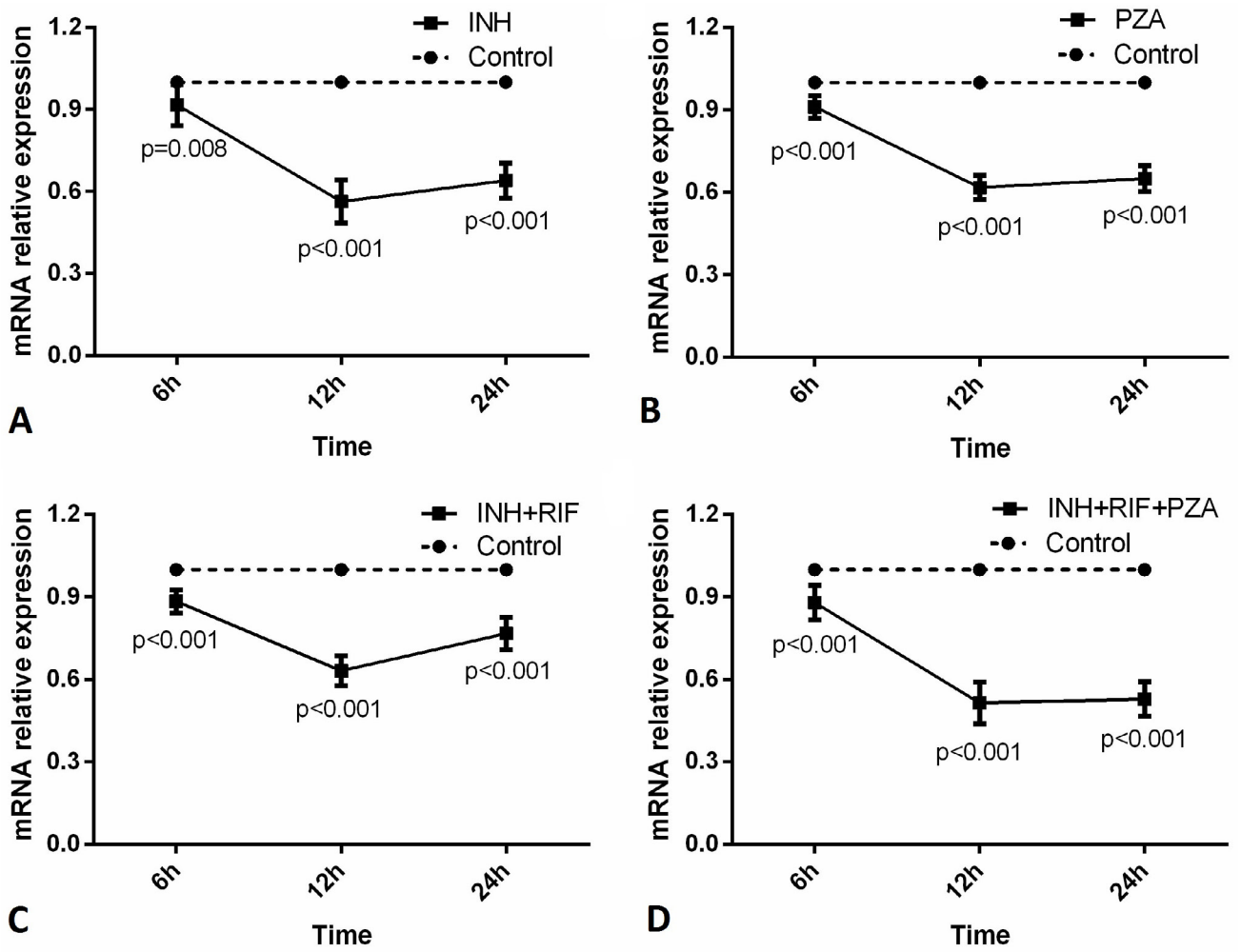

Fig. 3. CYP1A1 mRNA expression in different drug groups at different time points. Quantitative real-time PCR was used to detect mRNA in the drug and control groups (A: isoniazid, INH, B: pyrazinamide, PZA, C: INH + rifampicin (RIF), D: INH + RIF + PZA). Each group included 6 parallel samples, each of which was measured 3 times.

37% and 49%, respectively. Based on these results, 12 h was determined to be the optimal culture time. As shown in Figure 4, AZA could increase CYP1A1 expression obviously in AZA + INH + RIF + PZA group. There was a inversely correlation between DNA methylation and CYP1A1 mRNA levels (Spearman r = -0.74, $p < 0.001$), which indicated that CYP1A1 expression may be regulated by methylation.

Effects of AZA on cell damage and oxidative stress injury

When cells were treated with anti-tuberculosis drugs for 6, 12, or 24 h, lactate dehydrogenase (LDH) activity and malonaldehyde (MDA) concentration increased, while superoxide dismutase (SOD) activity decreased with time ($P < 0.01$), thereby indicating that both cell damage and oxidative stress injury occurred.

Figure 5 reveals that the LDH activity of only the AZA + INH + RIF + PZA group significantly decreased after cells were co-cultured with the anti-tuberculosis drugs plus AZA for 12 h. Oxidative stress is demonstrated by elevated SOD activity (Fig. 6A) and decreased MDA content (Fig. 6B); the AZA + INH + RIF + PZA group, in particular, showed significant changes. CYP1A1 methylation was positively correlated with LDH and MDA levels (Spearman r = 0.412, 0.397; p = 0.002, 0.003). Thus, lower DNA methylation of the CYP1A1 was associated with lower cell damage and oxidative stress injury.

DISCUSSION

This study demonstrated that high levels of DNA methylation of the CYP1A1 CpG islands in liver injury

induced by INH+RIF+PZA were associated with low mRNA expression, high levels of cell injury and oxidative stress injury, and high levels of DNMTs. The results show that the CpG islands methylation level of CYP1A1 promoter region is closely related to the degree of hepatocyte injury induced by INH+RIF+PZA.

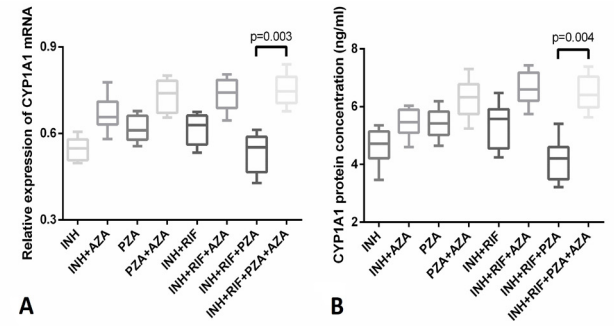

Fig. 4. Changes in CYP1A1 mRNA and protein expression in different drug groups treated with AZA. Cells were cultured for 12 h with each of the anti-tuberculosis drugs (isoniazid, INH; pyrazinamide, PZA; INH + rifampicin, RIF; or INH + RIF + PZA) or their combination with AZA (10 μM). **(A)** CYP1A1 mRNA expression was detected by quantitative real-time PCR. **(B)** CYP1A1 protein expression was detected by ELISA. Each group included 6 parallel samples, each of which was measured 3 times.

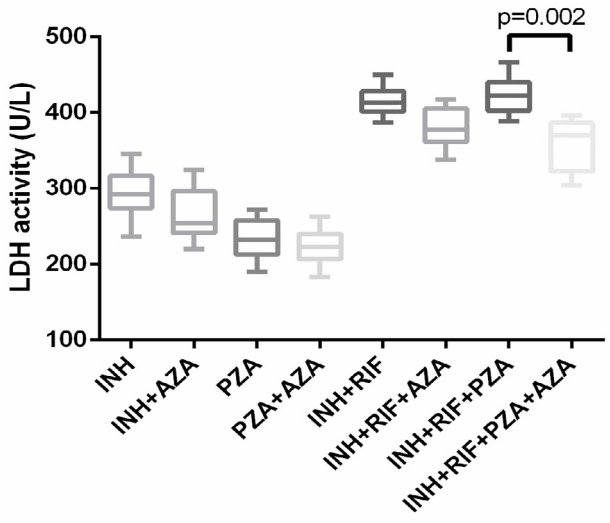

Fig. 5. LDH activity in different drug groups treated with AZA. The cells were cultured for 12 h with each of the anti-tuberculosis drugs (isoniazid, INH; pyrazinamide, PZA; INH + rifampicin, RIF; or INH + RIF + PZA) or their combination with AZA (10 μM). LDH enzyme activity was detected using the micro-enzyme method. Each group included 6 parallel samples, each of which was measured 3 times.

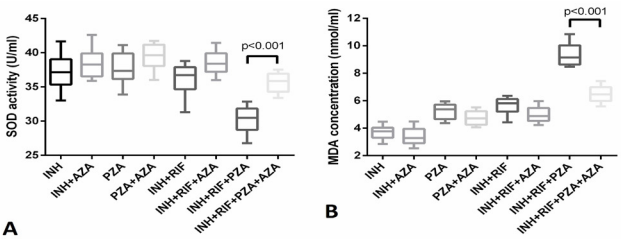

Fig. 6. Changes in SOD and MDA in different drug groups treated with AZA. Cells were cultured for 12 h with each of the anti-tuberculosis drugs (isoniazid, INH; pyrazinamide, PZA; INH + rifampicin, RFP; or INH + RFP + PZA) or their combination with AZA (10 μM). The enzyme activity of SOD was detected by the hydroxylamine method, and the MDA concentration was detected by the TBA method. Each group included 6 parallel samples, each of which was measured 3 times.

A large number of studies have found that changes in methylation of the promoter region can regulate the expression of genes and affect their biological function (Barrow and Michels, 2014; Van *et al*., 2014). Changes in the methylation of oncogenes may play a role in tumors, and the importance of methylation in certain diseases has been observed.

CYP1A1 has recently attracted increased attention due to its role in certain diseases (Ehrlich and Lacey, 2013; Lai *et al*., 2010.). Our study found that the expression of DNMTs and methylation rate of CpG islands in the promoter region of CYP1A1 increased after hepatocytes were cultured with first-line anti-tuberculosis drugs. In particular, methylation increased significantly after treatment with INH, PZA, INH + RIF, or INH + RIF + PZA.

DNMTs can exert methylation by transferring a methyl group to cytosine (Lund *et al*., 2014; Poomipark *et al*., 2016; Sanchez *et al*., 2017). As such, DNMTs changes can reflect methylation levels to a certain extent. When cells were treated with anti-tuberculosis drugs, both DNMTs protein and enzyme activity in the cells increased. We showed here that DNA methylation of CYP1A1 and DNMTs were positively correlated; thus, the DNMTs may participate to anti-tuberculosis drugs-induced DNA methylation of CYP1A1. AZA, as a nucleoside analog, can capture DNMTs by interacting with the DNA of actively proliferating cells and exert demethylation by forming an irreversibly covalent complex with it, thereby inducing the re-expression of silent genes (He *et al*., 2015.). When cells were co-cultured with the anti-tuberculosis drugs in combination with AZA, both the methylation rate of the CYP1A1 promoter region and DNMTs protein and enzyme activity decreased. These observations demonstrate that

hypermethylation of CpG islands in the promoter region of CYP1A1 affects anti-tuberculous drug-induced liver injury, consistent with the findings of He (He *et al*., 2015).

Compared with the results of CYP1A1 expression in anti-tuberculosis drug group, AZA promoted the expression of CYP1A1 mRNA and protein. AZA could obviously increase CYP1A1 mRNA and protein expression in AZA + INH + RIF + PZA group. And our finding showed that DNA methylation and mRNA levels of CYP1A1 were inversely correlated. Considering these results, we believe that low expression of CYP1A1 is regulated by hypermethylation of CpG islands in its promoter region and that AZA stimulates CYP1A1 expression by decreasing CpG methylation.

Addition of AZA to the cultured cells revealed changes in the cell injury index LDH and oxidative stress indicators SOD and MDA. As a glycolytic enzyme, LDH is widely present in different tissues and cells in the body. While LDH activity can be used as a marker of cell damage (Kotoh *et al*., 2011; Lee *et al*., 2011), MDA and SOD are indicators of oxidative damage. Current studies have found that one of the mechanisms of anti-tuberculosis drugs causing liver injury is lipid peroxidation (Du *et al*., 2013; Kumar *et al*., 2014; Wang *et al*., 2016; Wu *et al*., 2015). AZA demonstrated increasing CYP1A1 expression but not effect on LDH, SOD, and MDA levels, which may be related to the mechanism of action of the drug. According to reports, AZA mainly exerts inhibitory effects on methylation (Mitsui *et al*., 2016). The expression of CYP1A1 can be increased by inhibiting the methylation of CpG islands in the promoter region of the gene, which could explain why the effect of AZA on CYP1A1 is fairly obvious. In addition, AZA, as an exogenous chemical, has its own limited ability to repair.

In this work, DNA methylation of CYP1A1 was negatively correlated with injury, thereby suggesting that high levels of DNA methylation of CYP1A1 may aggravate cell injury. Previous research reveals that CYP1A1 expression is regulated by methylation. Therefore, we conclude that hypermethylation of CpG islands in the promoter region of CYP1A1 can lead to low expression of the gene, which may relate to liver injury induced by INH+RIF+PZA. We also found that AZA can alleviate the anti-tuberculous drug-induced liver injury caused by INH + RIF + PZA by reducing CpG island methylation in the CYP1A1 promoter region. In clinical practice, combination therapy is often used to treat tuberculosis, and liver damage caused by this therapy is common. Therefore, our results present important implications for relieving liver injury caused by combination therapy.

CONCLUSIONS

Methylation of CYP1A1 CpG islands was related to its mRNA levels. Hypermethylation of CpG in the promoter region of CYP1A1 inhibited the expression of CYP1A1 in liver injury induced by INH + RIF + PZA. Inhibition of promoter methylation by AZA promoted CYP1A1 expression and reduced the hepatocyte injury induced by INH + RIF + PZA. Therefore, we hypothesize that DNA methylation of the CYP1A1 promoter region might be important in hepatic injury development induced by INH + RIF + PZA.

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Statement of conflict of interest

We declare no conflicts of interest in this study.

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