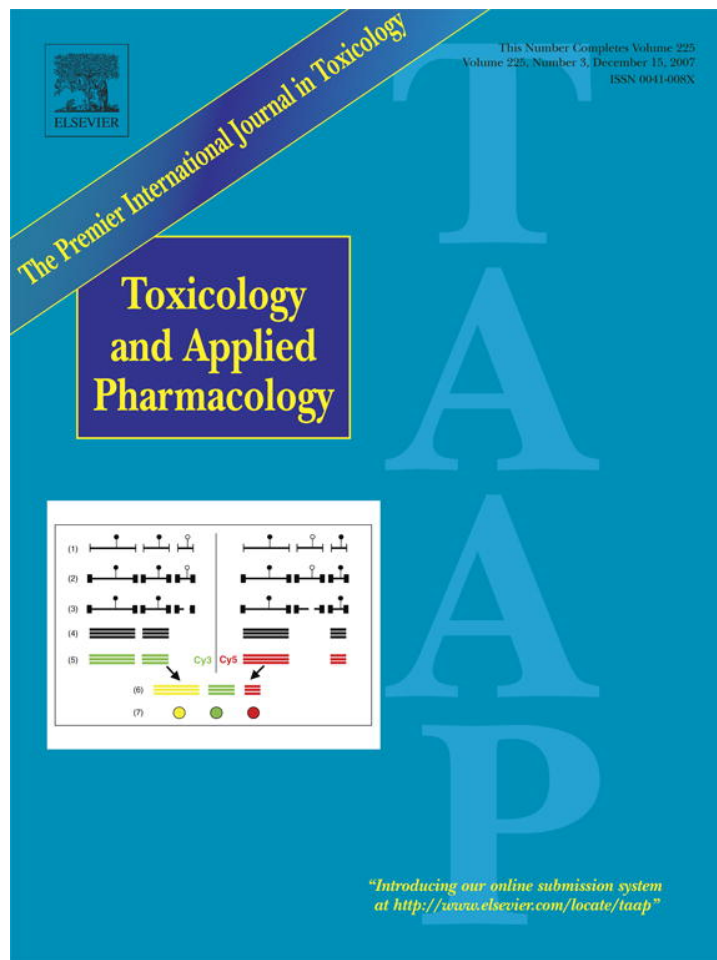


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## Epigenetic changes in the rat livers induced by pyrazinamide treatment <sup>☆</sup>

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### Abstract

Drug-induced liver injury, including drug-induced hepatotoxicity during the treatment of tuberculosis infection, is a major health problem with increasingly significant challenges to modern hepatology. Therefore, the assessment and monitoring of the hepatotoxicity of antituberculosis drugs for prevention of liver injury are great concerns during disease treatment. The recently emerged data showing the ability of toxicants, including pharmaceutical agents, to alter cellular epigenetic status, open a unique opportunity for early detection of drug hepatotoxicity. Here we report that treatment of male Wistar rats with antituberculosis drug pyrazinamide at doses of 250, 500 or 1000 mg/kg/day body weight for 45 days leads to an early and sustained decrease in cytosine DNA methylation, progressive hypomethylation of long interspersed nucleotide elements (LINE-1), and aberrant promoter hypermethylation of placental form glutathione-*S*-transferase (*GSTP*) and *p16<sup>INK4A</sup>* genes in livers of pyrazinamide-treated rats, while serum levels of bilirubin and activity of aminotransferases changed modestly. The early occurrence of these epigenetic alterations and their association with progression of liver injury specific pathological changes indicate that alterations in DNA methylation may be useful predictive markers for the assessment of drug hepatotoxicity.

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**Keywords:** DNA methylation; Pyrazinamide; Liver injury; Rat

### Introduction

Drug-induced severe hepatotoxicity is a major health problem with increasingly significant challenges to modern hepatology (Holt and Ju, 2006; Björnsson, 2006). It can arise at various stages during the development of a drug or, in many cases, even after its approval. Drug-induced liver injury has been a long-standing concern in the treatment of tuberculosis infection, especially during treatment of latent tuberculosis (Durand et al., 1996; Lenaerts et al., 2005; McElroy et al., 2005; Younossian et al., 2005; Ijaz et al., 2006; Saukkonen et al., 2006). The hepatotoxic effects, including fatal liver injury, of most commonly used antituberculosis drugs, such as isoniazid, rifampicin, and pyrazinamide, resulted in the revision of the US guidelines for treatment of latent tuberculosis (McCarthy, 2001).

Therefore, the assessment and monitoring of the hepatotoxicity of antituberculosis drugs for prevention of liver injury are great concerns during disease treatment. Additionally, isoniazid and rifampicin are considered as non-genotoxic rodent hepatocarcinogens (Nie et al., 2006), though they have not been recognized as carcinogens for humans (Skakun and Tabachuk, 1991).

Current clinical diagnosis of drug-induced liver injury depends on non-specific elevation in liver test results, in particular, increases in serum aminotransferases activity and bilirubin levels (Björnsson, 2006). This proven approach is successfully employed in the assessment of hepatotoxicity of newly developed drugs; however, the accuracy of this method is limited for monitoring liver injury induced by pharmacological agents during treatment of chronic diseases and for determining long-term prognosis. Furthermore, this methodology does not allow the elucidating and understanding of the underlying pathophysiological and biochemical mechanisms of drug-induced liver dysfunction and the prediction of the outcome of drug-induced liver injury. This illustrates clearly the crucial need for uncovering underlying mechanisms of drug-induced liver injury for

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improvement of its diagnostic accuracy. More importantly, further studies are needed to determine the long-term prognosis of drug-induced hepatotoxicity (Björnsson, 2006).

The recently emerged data showing the ability of toxicants, including pharmaceutical agents, to alter cellular epigenetic status open a unique opportunity for early detection of drug hepatotoxicity. Importantly, it has been suggested that drug-induced epigenetic changes, especially alterations in global DNA methylation status, as well as changes in methylation status of particular genes, may be more sensitive indicators of liver toxicity than classic parameters used for toxicity assessment, and they may be useful predictive biomarkers for toxicities (Moggs et al., 2004; Watson et al., 2004). Furthermore, the early appearance of epigenetic changes may give an advantage for timely detection of toxic potential compared to the currently used indicators.

Based on these considerations, the present study was undertaken to determine (a) whether or not the drug-induced hepatotoxicity is associated with alteration in DNA methylation status, (b) whether the assessment of alteration in DNA methylation may be used as a biomarker for detection and monitoring of drug-induced liver injury.

We demonstrate that exposure of male Wistar rats to antituberculosis drug pyrazinamide for 45 days leads to an early and sustained decrease in cytosine DNA methylation, progressive hypomethylation of long interspersed nucleotide elements (LINE-1), and aberrant promoter hypermethylation of placental form glutathione-S-transferase (*GSTP*) and *p16<sup>INK4A</sup>* genes in livers of pyrazinamide-treated rats. Importantly, these changes were associated with the progression of pathomorphological changes indicative for liver injury.

## Materials and methods

**Animals, treatment, and tissue preparation.** Male Wistar rats were obtained from the Institute of Pharmacology and Toxicology of the Academy of Medical Sciences of Ukraine breeding facility (Kyiv, Ukraine), housed in a temperature-controlled room (24°C), and given *ad libitum* access to water and laboratory diet. In the preliminary study, the rats (body weight 160–200 g) were allocated randomly and treated with pyrazinamide at doses of 250, 500, or 1000 mg/kg/day body weight or vehicle by oral gavage for 45 days. The low dose corresponded to human therapeutic dose taking into account species sensitivity according to U.S. Food and Drug Administration Guidance available at <http://www.fda.gov/cder/guidance/index.htm>, and the second and third doses exceeded human dose by 2- and 4-fold, respectively. Bedding, feed, water, and animal maintenance were identical for all rats. Five rats per group were sacrificed after 45 days of pyrazinamide initiation; prior to sacrifice, blood was taken from narcotized rats. The livers were excised, frozen immediately in liquid nitrogen, and stored at –80 °C for subsequent analysis. The liver slices from median lobe were fixed in 10% neutral buffered formalin for 48 h, processed, embedded in paraffin, sectioned at 4 µm, and mounted on glass slides. The sections were stained with hematoxylin and eosin for histopathological examination.

For the main study, a concentration of 250 mg/kg/day of pyrazinamide and treatment for 15, 30 or 45 days were selected. After exposure, 5 rats per control or treated groups were sacrificed at indicated time intervals, and blood and liver tissues samples were collected as described above. Additionally, 5 rats from control and treated groups were sacrificed 30 days after the rats received the last pyrazinamide treatment.

All animal experimental procedures were carried out in accordance with animal study protocol approved by the Institutional Animal Care and Use Committee.

**Analyses of serum bilirubin content and aminotransferase activities.** The total serum bilirubin, alanine aminotransferase (ALT), and aspartate amino-

transferase (AST) were determined by standard methods according to the manufacturer's instructions (PLIVA-Lachema, Brno, Czech Republic).

**Determination of global DNA methylation status.** The extent of global DNA methylation was evaluated with a radiolabeled [<sup>3</sup>H]dCTP extension assay as described previously (Pogribny et al., 1999).

**Methylation analysis of LINE-1 repetitive elements.** Methylation status of LINE-1 was determined by the combined bisulfite restriction analysis (COBRA) as described previously (Eads and Laird, 2002), including bisulfite modification of genomic DNA, PCR amplification, and digestion of PCR products with restriction enzymes *Bst*UI or *Rsa*I (Tryndyak et al., 2007).

**Determination of the *p16<sup>INK4A</sup>* methylation status by methylation-specific PCR (MSP).** The methylation status of the promoter and first exon of the *p16<sup>INK4A</sup>* gene was determined by MSP analysis; the primer sequences and PCR conditions have been previously described (Swafford et al., 1997). The PCR products were separated on 3% high resolution agarose gels (Sigma, St. Louis, MO), stained with ethidium bromide, and photographed. Negative control PCR amplifications were performed by using both sets of modified primers with untreated DNA. The absence of PCR products confirmed that unmodified DNA could not be amplified with modified sets of primers.

**Determination of the *GSTP* promoter methylation status by methylation-sensitive PCR.** Genomic DNA was digested with *Hpa*II or *Bst*UI cytosine-methylation-sensitive restriction endonuclease followed by PCR amplification of a 168 bp fragment of the *GSTP* promoter region. In our previous study, we have shown that CpG sites located within this fragment are differentially methylated in normal and injured liver tissues (Steinmetz et al., 1998). By using primers that flank the *Hpa*II or *Bst*UI cleavage sites within the 5' region of the *GSTP* gene, quantitative recovery of PCR product will vary directly with the extent of *Hpa*II- or *Bst*UI-induced DNA breaks at unmethylated CCGG or CGCG sites, respectively. One microgram of genomic DNA was treated with 20 units of *Hpa*II or *Bst*UI restriction endonuclease (New England Biolabs, Ipswich, MA) for 16 h according to the manufacturer's instructions. The primer sequences were: the sense primer 5'-TCATCGTCCACGCAGCTTTGA-3' and the antisense primer 5'-CCTTCTCTGTTTTGTCCCCAGAAC-3'. The semi-quantitative aspect of the procedure was verified by a linear increase in PCR product recovery with increasing cycle number and DNA template concentration. The PCR products were separated on 3% agarose gels (Sigma), stained with ethidium bromide, photographed, and the band intensity was analyzed by ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The results are presented as ratio of PCR product recovery after digestion DNA with the appropriate restriction endonuclease to undigested DNA.

**Statistical analysis.** Results are presented as mean±S.D. Statistical analyses were conducted by two-way ANOVA, using treatment and weeks as fixed factors. *P*-values<0.05 were considered significant.

## Results

### *Effect of different doses of pyrazinamide on the serum bilirubin levels, activity of aminotransferases, and liver DNA methylation in pyrazinamide-treated rats*

In the present study, rats were exposed to pyrazinamide in order to determine association between the drug-induced liver pathology and perturbation in genome-wide and region-specific DNA methylation. Increased levels of serum bilirubin and activities of ALT and AST are established clinical indicators of liver damage (Durand et al., 1996; Lenaerts et al., 2005). Treatment of rats with pyrazinamide in doses of 250, 500 or 1000 mg/kg/day body weight led to minor changes in serum ALT and AST activities (Fig. 1, panels B and C). After 45 days of

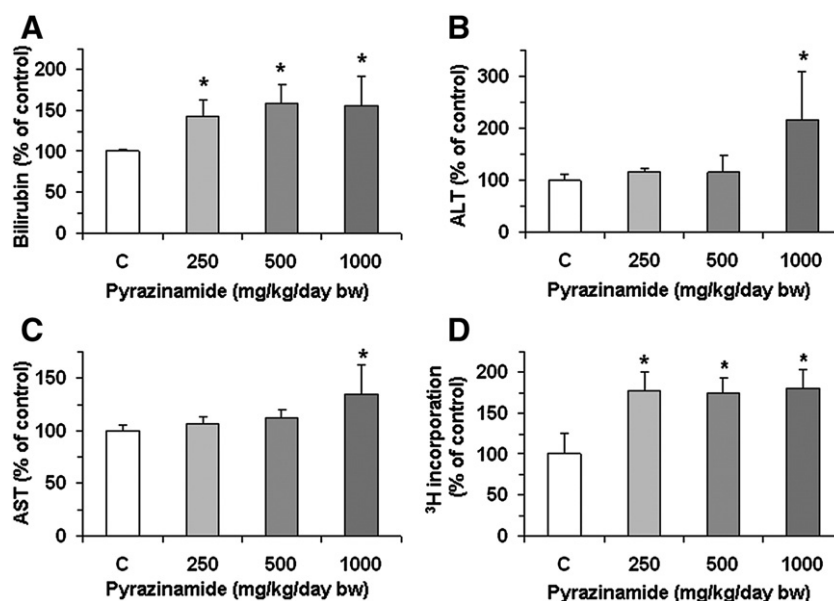


Fig. 1. Effect of pyrazinamide at different concentrations after 45 day treatment on the serum bilirubin levels (A), serum ALT activity (B), and AST activity (C) and DNA methylation in the rat livers (D). The extent of [<sup>3</sup>H]dCTP incorporation is directly proportional to the number of unmethylated CCGG sites. \*Significantly different from control group.

pyrazinamide treatment, only serum bilirubin content was elevated by ~50% in all experimental groups (Fig. 1, panel A), while only the maximal dose of 1000 mg/kg resulted in the significant increase of ALT and AST activities (Fig. 1, panels B and C).

The DNA methylation status in the liver of pyrazinamide-treated rats was assessed with a sensitive cytosine extension assay that measures the proportion of unmethylated CpG sites in DNA. The assay is based on the ability of methylation-sensitive restriction endonuclease *HpaII* to cleave unmethylated CCGG sequences and leave a 5' guanine overhang that can be used for the subsequent single nucleotide extension with labeled [<sup>3</sup>H]

dCTP (Pogribny et al., 1999), and the extent of [<sup>3</sup>H]dCTP incorporation is directly proportional to the number of unmethylated CpG sites. The pyrazinamide treatment resulted in the dose-independent increase in serum bilirubin levels and hepatic DNA hypomethylation (Fig. 1, panels A and D); however, changes in DNA methylation were more pronounced. The extent of hepatic DNA methylation in rats treated with 250, 500, or 1000 mg/kg/day of pyrazinamide was ~1.7–1.8 times greater compared to the age-matched control rats. Therefore, for the further study we used the concentration of 250 mg/kg/day of pyrazinamide, the lowest dose that leads to prominent DNA hypomethylation.

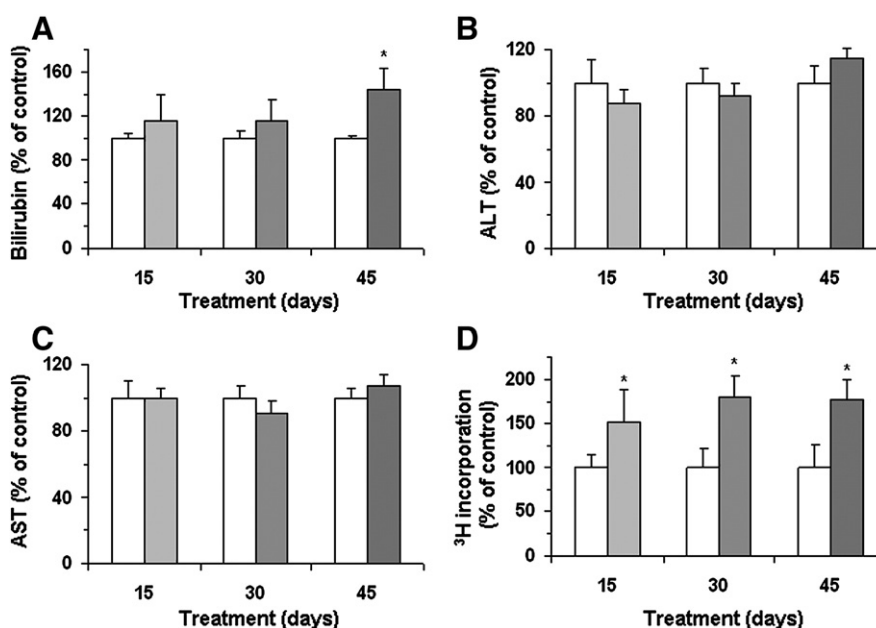


Fig. 2. Effect of pyrazinamide at a concentration of 250 mg/kg/day on the serum bilirubin levels (A), serum ALT activity (B), and AST activity (C) and hepatic DNA methylation (D). \*Significantly different from control group.

*Effect of pyrazinamide on the serum bilirubin levels and activity of aminotransferases*

Fig. 2 shows that treatment of rats with 250 mg/kg/day of pyrazinamide did not change serum ALT and AST activities over 45 days of the experiment (panels A and B). The level of serum bilirubin was increased by 44% only after 45 days after pyrazinamide initiation (panel C).

*Effect of pyrazinamide on global DNA methylation in liver*

The extent of DNA methylation in the liver of control rats did not change over the 45 day period (Fig. 2, panel D). In contrast, in the rat livers treated with pyrazinamide at a concentration of 250 mg/kg/day, DNA became substantially hypomethylated after 15 days of treatment. At that time, the incorporation of [<sup>3</sup>H]dCTP into *Hpa*II-digested DNA isolated from livers of pyrazinamide-treated rats was 1.5 times greater than in the age-matched control animals. At later times (30 and 45 days), the extent of DNA hypomethylation in livers of pyrazinamide-treated rats increased and was 1.8 times greater compared to untreated rats, and the hypomethylated state of DNA remained at this level 30 days after the last pyrazinamide treatment.

*Effect of pyrazinamide on LINE-1 methylation in liver*

It is known that the majority of cytosine methylation in mammals resides in repetitive DNA elements and exons other than the first exon (Rollins et al., 2006) and that most of this methylation is located in transposons (Yoder et al., 1997). Transposons are interspersed repetitive DNA sequences that constitute almost 50% of the human genome and about 40% of the rat genome. One of the primary functions of DNA methylation in normal cells is maintenance of the genome stability by silencing the expression of these repetitive DNA elements (Yoder et al., 1997; Schulz et al., 2006). It has been suggested that genome-wide DNA hypomethylation largely affects transposons (Yoder et al., 1997). In view of this, we measured the effect of pyrazinamide treatment on the methylation status of LINE-1, which constitute almost 23% of rat genome.

Fig. 3A shows the extent of LINE-1 regulatory region methylation in livers of control and pyrazinamide-treated rats, as determined by the COBRA assay. The ratio of undigested and *Bst*UI-digested PCR products did not change in liver of control rats over 45 days of the experiment indicating stable LINE-1 methylation. In contrast, treatment with pyrazinamide resulted in progressive LINE-1 hypomethylation, which was evidenced by the increased undigested/*Bst*UI-digested PCR product ratio.

*Effect of pyrazinamide on promoter methylation of GSTP and p16<sup>INK4A</sup> genes*

Changes in DNA methylation induced by drug exposure may not be limited to repetitive DNA sequences only, but they may

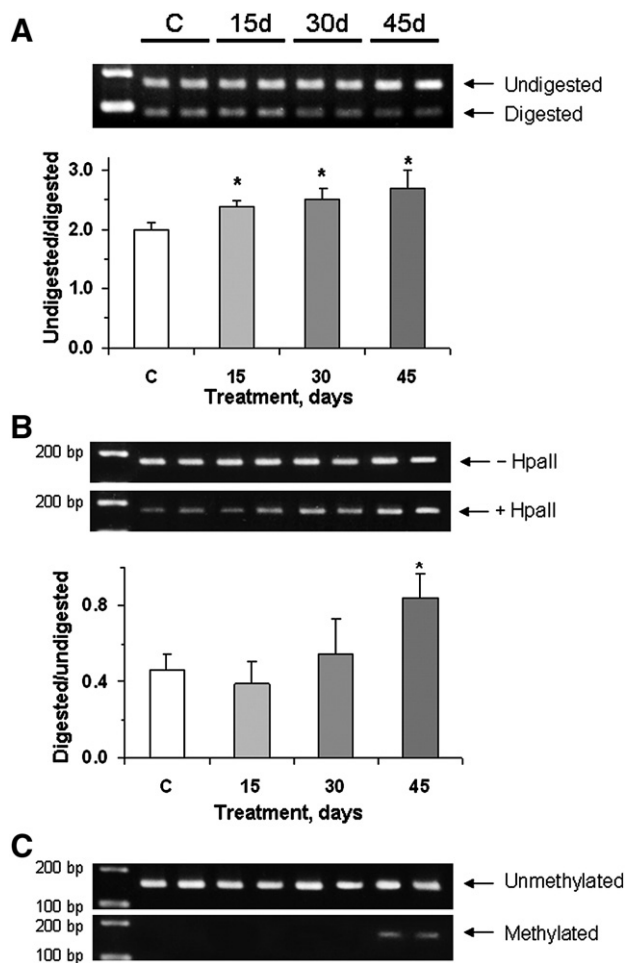


Fig. 3. Effect of pyrazinamide at a concentration of 250 mg/kg/day on the methylation status of LINE-1 repetitive elements and *GSTP* and *p16<sup>INK4A</sup>* genes in the rat livers. (A) LINE-1: representative photograph of the COBRA assay with restriction by *Bst*UI and the percentage of cut bands (methylated). \*Significantly different from control group. (B) *GSTP*: representative photograph after treatment with *Hpa*II and the percentage of digested (methylated) DNA. \*Significantly different from control group. (C) *p16<sup>INK4A</sup>* representative photograph of methylation-specific PCR products.

also affect genes playing an important role in the regulation of cellular metabolic pathways. Fig. 3B shows substantial hypermethylation of promoter region of the *GSTP* gene in livers of pyrazinamide-exposed rats. More importantly, pyrazinamide treatment resulted in hypermethylation of the key cell cycle regulator *p16<sup>INK4A</sup>* gene as evidenced by the presence of methylated alleles after 45 days of pyrazinamide exposure (Fig. 3C).

*Histological analysis of liver after pyrazinamide treatment*

Treatment of rats with pyrazinamide caused dose-independent and time-dependent pathological changes in rat livers. Fig. 4 shows prominent pathological signs of liver injury in rats treated with 250 mg/kg/day of pyrazinamide. Histopathological analysis of livers of rats treated with pyrazinamide for 15 days only revealed prominent morphological changes in livers characterized by heavy inflammatory reaction, fatty

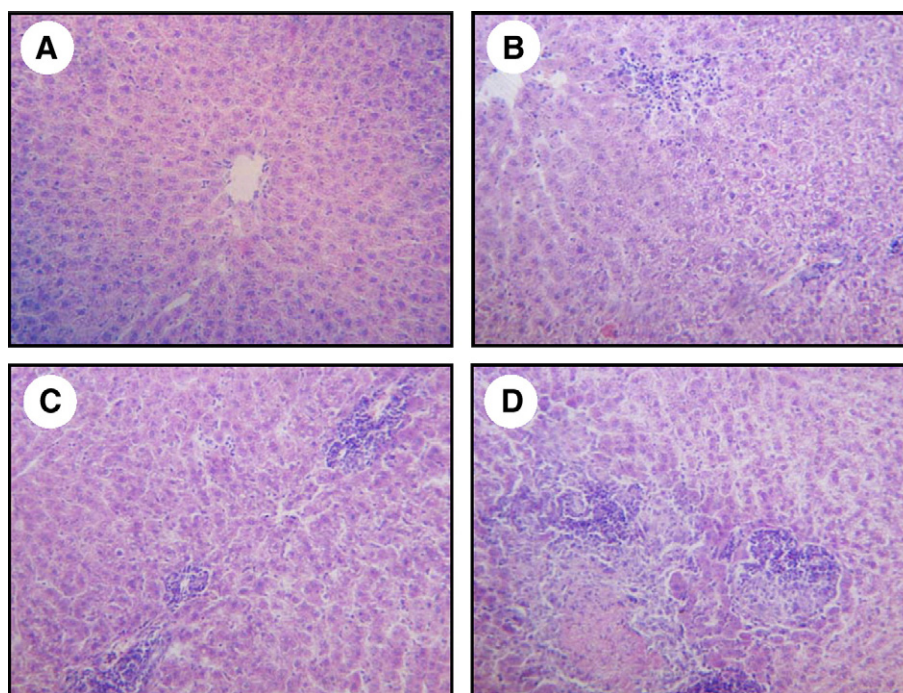


Fig. 4. Histological changes in the livers of rats treated with pyrazinamide at a concentration of 250 mg/kg/day. (A) Control group (B, C, and D) after 15, 30, and 45 days of pyrazinamide treatment, respectively (original magnification  $\times 400$ ).

infiltration of hepatocytes, and nonzonal necrosis (Fig. 4B). At later times, 30 and 45 days of treatment, liver tissue of pyrazinamide-treated rats displayed prominent signs of fibrosis development (Figs. 4C and D).

## Discussion

Drugs used for treatment of latent tuberculosis infection frequently induce acute liver injury (McElroy et al., 2005; Yew and Leung, 2006). In this report we demonstrate that liver hepatotoxicity caused by treatment with antituberculosis agent pyrazinamide is associated with prominent changes in DNA methylation characterized by global DNA and LINE-1 hypomethylation, and aberrant promoter hypermethylation of *GSTP* and *p16<sup>INK4A</sup>* genes. Typically, drug-induced liver toxicity is assessed mainly by measuring serum levels of bilirubin and activity of aminotransferases, which are commonly used methods in clinical practice. In general, the increased values of all these three parameters are associated with and reflect the status of liver injury (Tasduq et al., 2006), although in many cases of liver injury they remain unchanged (Kramer et al., 2004; Lenaerts et al., 2005). It has been shown that pyrazinamide at doses of 500 and 1500 mg/kg/day body weight for 2 weeks did not affect the ALT and AST activities, and the dose of 2500 mg/kg/day merely increased their activities (Slivka, 1988). Similarly, the results of our study showed that only maximal dose of pyrazinamide caused significant changes in serum ALT and AST after 45 days of pyrazinamide treatment. However, histological examination revealed the pathological signs indicative of severe liver injury (acute inflammation, necrosis and steatosis) as early as 15 days after treatment initiation. These changes progressed to fibrosis after 45 days of

pyrazinamide exposure reflecting enhancement of pathological alterations in livers. Recently, steatohepatitis, including drug-induced steatohepatitis, has been increasingly recognized as a life-threatening fatty liver disease and a premalignant condition for subsequent development of hepatocellular carcinoma (Farrell, 2002; Stravitz and Sanyal, 2003; Grieko et al., 2005; Moradpour and Blum, 2005; McKillop et al., 2006). This further illustrates the crucial need of reliable biomarkers for early diagnosis of drug-induced liver injury, especially during long-time treatment of chronic pathologies as tuberculosis.

In contrast to modest changes in serum bilirubin levels and enzymes activities, pyrazinamide treatment resulted in rapid and prominent alterations in hepatic DNA methylation patterns. The extent of global DNA hypomethylation, as well as hypomethylation of LINE-1 repetitive DNA elements in the liver of pyrazinamide-treated rats, depended on neither dose of pyrazinamide nor treatment duration and occurred at early time points of exposure. Similar observations were reported in respect to other pharmaceutical agents, such as tamoxifen, phenobarbital, 5-fluorouracil, staurosporine, etc. (Watson and Goodman, 2002; Watson et al., 2004; Tryndyak et al., 2007). Recent reports have indicated clearly the importance of stable DNA hypomethylation in genomic instability as one of the major contributing factors to neoplastic cell transformation (Pogribny et al., 2006a,b). Hypomethylation of DNA occurs at very early preneoplastic stages of hepatocarcinogenesis leading to the establishment of epigenetically reprogrammed cell population with high potential of progression into tumor cells (Pogribny et al., 2006a,b). Furthermore, it has been hypothesized that sensitivity to hepatocarcinogenesis may be related inversely to the capacity to maintain DNA methylation (Goodman and Watson, 2002). Our finding that stable DNA remained

hypomethylated after pyrazinamide treatment may indicate the formation of such epigenetically reprogrammed cells. One of the additional critical features of these epigenetically reprogrammed cells is hypermethylation and silencing of critical genes, especially genes involved in cellular defense, cell cycle arrest, and DNA repair (Karpinets and Foy, 2005). Hypermethylation of two of these genes, *GSTP* and *p16<sup>INK4A</sup>*, was induced by pyrazinamide treatment.

Glutathione-S-transferases are the family of enzymes protecting cells against oxidants and electrophilic carcinogens and, hence, are critical determinants of cancer pathogenesis (Tchou et al., 2000). It is well established that silencing of the *GSTP* gene expression is associated with its promoter hypermethylation, which often occurs in hepatocellular carcinoma and even at preneoplastic stages of liver carcinogenesis (Tchou et al., 2000; Yang et al., 2003). More importantly, a tumor suppressor gene *p16<sup>INK4A</sup>* that regulates cyclin-dependent kinases is also frequently inactivated in liver cancer through promoter methylation (Jasmer et al., 2000; Jin et al., 2000). Similar to *GSTP* gene hypermethylation, *p16<sup>INK4A</sup>* gene promoter hypermethylation occurs also at early stages of carcinogenesis, in preneoplastic liver cirrhosis, and increases with progression of hepatocarcinogenesis (Fukai et al., 2005; Matsuda and Ichida, 2006). In light of these considerations, the pyrazinamide-induced appearance of promoter hypermethylation of *GSTP* and *p16<sup>INK4A</sup>* genes along with hypomethylation of genomic DNA and LINE-1 repetitive elements may be considered as early epigenetic events indicative of formation of epigenetically reprogrammed liver cells.

The association between the appearance of aberrant DNA methylation in the livers and induction of liver injury caused by pyrazinamide treatment suggests that liver injury is a main causative factor resulting in alterations in DNA methylation. It is well established that inflammation-mediated cytosine damage may substantially alter DNA methylation patterns resulting in hypo- and hypermethylation events. Recent studies have shown that inflammation-mediated damage to cytosine may direct enzymatic DNA methylation, especially within CpG-rich regions in DNA, resulting in an inappropriate *de novo* methylation, whereas certain inflammation-induced oxidative damage to methyl-CpG sequences inhibits DNA methylation (Valinluck and Sowers, 2007). Considering the fact that inflammation is one of the main events in the chain of drug-induced liver injury-specific pathological changes, we hypothesize that alteration in DNA methylation patterns is a sensitive drug-independent indicator of hepatotoxicity. Additionally, because of the steadiness of DNA methylation changes, the same aberrant DNA methylation patterns specific for drug-injured liver can be identified in plasma and serve as a potential valuable non-invasive diagnostic marker for liver injury and as a prognostic factor for monitoring of the disease progression. Similar approach to the epigenetic changes detection in plasma has shown promising results for prenatal and cancer diagnosis (Chim et al., 2006; Levenson, 2007). More importantly, the potential reversibility of epigenetic changes offers a unique opportunity for chemoprevention of drug-induced liver injury.

In conclusion, the present study shows for the first time that pyrazinamide treatment causes notable epigenetic alterations in

the rat livers, characterized by global DNA and LINE-1 hypomethylation and gene-specific hypermethylation. The early occurrence of these epigenetic alterations and their association with progression of liver injury specific pathological changes indicate that alterations in DNA methylation may be useful predictive markers for the assessment of drug hepatotoxicity.

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