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Original Article

Regulation of hepatobiliary transporters during cholestasis may mediate the phenomenon of adaptation to cholestasis induced by rifampicin in rats

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Abstract: Background: It is known that antituberculosis drugs initially induce liver injury, and then the liver progressively adapts to the drug and mitigates the drug-induced liver injury. This study aimed to explore the molecular mechanisms responsible for liver adaptation to Rifampicin (RFP). Methods: RFP was administered at a dose of 100 mg/kg·d to rats for 7 weeks. On days 0, 7, 14, 21, 28, 35, 42, and 49, 0.5 ml of blood was drawn for use in liver biochemistry assays. Liver tissues were collected on days 7, 14, and 49 for examination of the expression of multidrug resistance protein 2 (Mrp2), bile salt export pump (Bsep), multidrug resistance protein 4 (Mrp4), and sodium-dependent taurocholate cotransporting polypeptide (Ntcp) by quantitative RT-PCR and Western blotting. Results: The serum alkaline phosphatase, total bilirubin, and direct bilirubin levels and liver TBA level began to rise at day 7, reached peak levels in week 1 or 2, and then declined gradually over time in RFP-treated rats. MRP4 mRNA expression was significantly increased on day 49. The expression of Ntcp protein was significantly decreased on day 49. The expression of Mrp4 and Bsep protein increased, reaching peak levels on days 14 and 49, respectively. Conclusions: Liver adaptation to RFP for mitigation of liver injury is likely mediated through the regulation of hepatobiliary transporters.

Keywords: Adaptation, rifampicin, liver biochemistry, bile acids, cholestasis, hepatobiliary transporter

Introduction

Rifampicin (RFP), a semisynthetic antibiotic of the rifamycin family, is widely prescribed for treating tuberculosis, and hepatotoxicity is one of the most serious adverse effects of RFP. However, 10% to 20% of patients who are treated with RFP experienced only mildly elevated levels of liver biochemical markers, which then gradually returned to the normal range over the treatment course (http://www.livertox.nih.gov/Rifampin.htm). This observation suggests that the liver possesses the mechanisms to respond to drug insult and to minimize hepatotoxicity. This capacity is termed adaptation to injury [1-3]. A better understanding of the mechanisms underlying adaptation to the drugs will not only help in reducing unnecessary drug withdrawal [4] but also will provide insight into cholestasis caused by other factors.

The mechanisms of adaptation remain largely unknown. One of suggested mechanisms is elevation of hepatic transporters that can transport hepatotoxicants out of liver. Hepatic transporters are essential for the uptake and excretion of a variety of organic anions including bile acids and bilirubin (BIL). Bile acid excretion is mainly mediated by the bile salt export pump (Bsep) and multidrug resistance associated protein 2 (Mrp2), which are located at the canalicular membrane of hepatocytes. Increased expression of both Bsep and Mrp2 was observed in rodents after bile acid feeding [5]. The sodium-dependent taurocholate cotransporter (Ntcp), which is located at the basolateral membrane, mediates hepatic uptake of bile acids, and expression of Ntcp is reduced in the cholestatic liver disease and in a rodent model of cholestasis and bile acid overload [5, 6]. Multidrug resistance protein 4 (Mrp4) at the

Table 1. Primer sequences used for RT-PCR

Gene	Forward primer (5eq')	Reverse primer (5eque)
Bsep	TTTTCCAGAGGCAGCTATCG	ATGGCTGCACTCAAAGATCC
Mrp2	CCATTATCCGTGCCTTTGAG	ACGACCAAGTTTCCAACCAG
Mrp4	GAACGCTACGAGAAAGTCATC	GCCCGTGCCAAGTTCAC
Ntcp	AGGCATGATCATCACCTTCC	AAGTGGCCCAATGACTTCAG
GAPDH	ACAGCAACAGGGTGGTGGAC	TTTGAGGGTGCAGCGAACTT

basolateral membrane mediates the alternative basolateral bile acid excretion, and expression of Mrp4, which is usually expressed at low levels, is dramatically induced during cholestasis [5, 7].

In our previous study, a mice model of the hepatic adaptation to the administration of rifampicin was developed. We observed the initial elevation of cholestasis-related biochemical changes and then the decline of those markers, including alkaline phosphatase (ALP), total BIL (TBIL), and total bile acids (TBA) in mice [8]. This model provides a good system in which to study the mechanisms underlying the phenomenon of adaptation to cholestasis induced by RFP. In this study, we investigated how the liver adapts to RFP by using this animal model of cholestasis generated by long-term administration of rifampicin. We characterized the dynamic changes in liver biochemistry and examined the cellular and subcellular histopathology. Finally, we determined the expression profiles of the main hepatobiliary transporters for bile acids, Mrp2, Bsep, Mrp4, and Ntcp.

Materials and methods

Animals and animal treatment

Male Wistar rats (weighing 160-180 g and aged 6-8 weeks) were purchased from Beijing Vital River, Beijing, China. The rats were housed in stainless-steel cages in an animal room maintained at 20-25°C with a 12-h light/dark cycle (lights on at 7:00 A.M. and off at 7:00 P.M.). Food and water were supplied without restriction.

All animal experiments were approved by the Animal Experiment Administration Committee at Anhui Medical University institution.

After 1-week of adaptive breeding, 36 rats were randomly divided into the RFP and control group. Those in the RFP group received 100 mg/kg RFP (manufactured by Shanghai Xinyi) in 0.5% carboxymethyl cellulose (20 mg/ml), by gavage between 9:00 A.M. and 10:00 A.M. every morning for 7 weeks. The control group was administered with the same amount of 0.9% saline. Rats were fed ad libitum during this period. On days 0, 7, 14, 21, 28, 35, 42, and 49, 0.5 ml blood was collected from the orbital venous plexus. The rats in the RFP group were sacrificed at weeks 1, 2, and 7, respectively, for collection of their blood and liver samples. The nine rats in the control group were sacrificed at week 7.

Blood biochemistry and liver TBA assay

Serial blood samples were obtained at the end of every week. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), TBIL, direct bilirubin (DBIL), and ALP were analyzed using spectrophotometric colorimetry following the respective instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The total bile acids level in the liver was measured using an enzymatic assay with a commercial kit (R&D Systems, USA).

Histopathology

Liver samples were fixed in 10% neutral buffered formalin for 24 h and embedded in paraffin. The tissue block was sectioned at 5 μm, and the sections were stained with hematoxylin-eosin (H&E) for histological assessment.

Hepatic ultrastructure under electron microscopy

Liver tissues were placed in 3% glutaraldehyde and then postfixed in 1% osmium tetroxide. The tissue was dehydrated in ethanol-acetone solution and embedded in Epon 618. Sections were cut from the epoxy block, stained with uranyl acetate and lead citrate, and examined using a *JEM21230SX* transmission electron microscope.

Quantitative real-time polymerase chain reaction (RT-PCR)

Total RNA was isolated from each liver sample using Trizol (Invitrogen) according to the manufacturer's instructions. Complementary DNA was synthesized using a reverse transcription

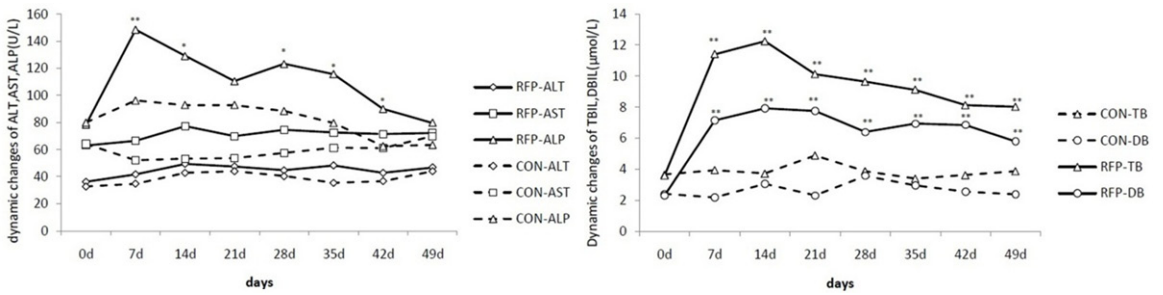

Figure 1. Dynamic changes in ALT, AST, ALP, TBIL, and DBIL levels over 8 time points spanning 7 weeks. (*P < 0.05, **P < 0.01 compared with the normal group at the same time points).

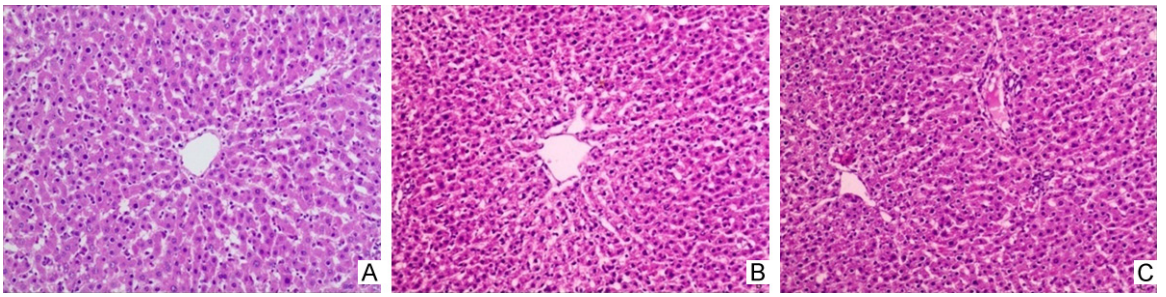

Figure 2. Hematoxylin-eosin staining of liver sections. A: Control group; B, C: RFP group. B: Steatosis was observed in a small number of hepatocytes. C: A small number of inflammatory cell infiltrates were present in the portal areas.

kit from Promega. The quantitation of specific messenger RNA (mRNA) was performed using a kit (SYBR green, Bio-Rad Laboratories) and the ABI Step One Plus real-time PCR system (ABI, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as reference control. Specific primers for transporter and housekeeping genes were synthesized by Sangon Biotech (Shanghai, China). The primer sequences are listed in **Table 1** [9, 10]. After normalizing the expression of the target gene to GAPDH expression, specific mRNA expression relative to the control value in each sample was calculated and presented.

Western blot analysis

Liver membrane fractions were prepared as described by Gant et al [11]. Membrane proteins were separated in 7.5% sodium dodecyl sulfate (SDS) polyacrylamide gels with a 4.4% stacking gel and blotted onto polyvinylidene fluoride membranes. The membranes were blocked with 5% w/v of non-fat dry milk dissolved in TBS-T and incubated overnight at 4°C with the following primary antibodies: anti-Bsep antibody (1:400, Santa Cruz Biotechnology), anti-Mrp2 antibody (1:400, Santa Cruz Biotechnology), anti-Ntcp antibody (1:400, Santa Cruz Biotechnology), and anti-Mrp4 antibody (1:400, MyBiosource, MA). Then the membranes were incubated for 1 h with species-appropriate secondary antibodies conjugated with horseradish peroxidase (Jackson Immuno Research Laboratories). Immunoreactive complexes were detected using the ECL™ Western Blotting Detection System. The band intensity was semi-quantified by densitometry (ImageJ software). After normalizing the individual protein bands to an internal reference band, protein expression relative to the control value was determined.

Statistical methods

Statistical analysis was performed using the SPSS 16.0 program. The results are presented as the means ± standard deviation. Comparisons between groups were undertaken using an unpaired Student *t* test. A value of $P < 0.05$ was considered statistically significant.

Results

Dynamic changes in liver biochemical markers over time

No significant changes in the ALT, AST, ALP, TBIL, and DBIL levels were detected over time in the control group. As shown in **Figure 1**, both

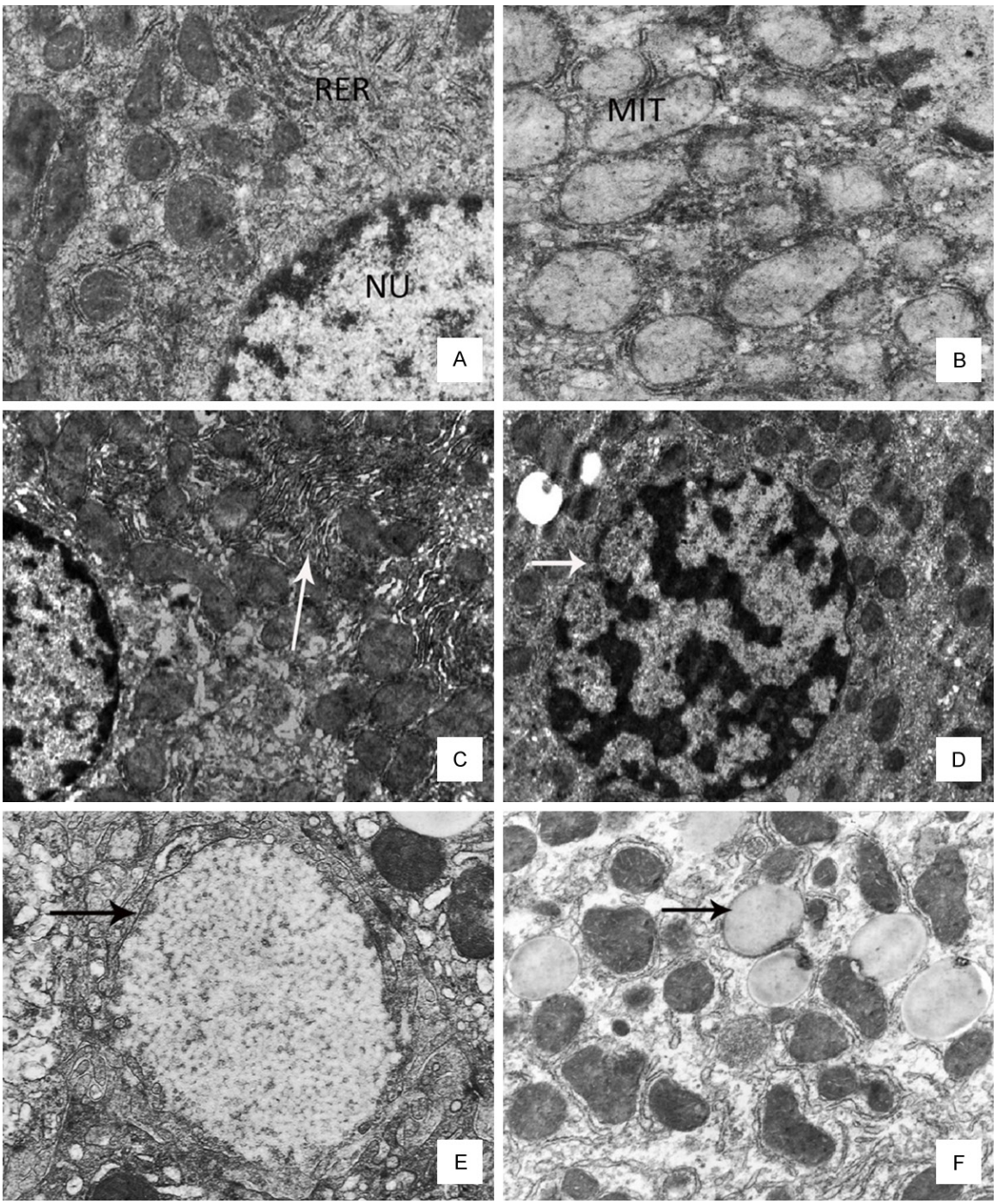

Figure 3. Normal and altered hepatocyte ultrastructures. A, B: Control group: The big and round cell nucleus, evenly distributed chromatin and mitochondria with clear cristae and membrane are shown, and the rough endoplasmic reticulum is also clear. NU: nucleus. MIT: mitochondrion. RER: rough endoplasmic reticulum. (Magnification factors: × 5000, × 10000). C-F: RFP group. C: The mitochondrial membrane is blurred and the endoplasmic reticulum is dilated. (× 10000); D: Chromatin margination and wavy nuclear membrane (× 5000); E: Capillary bile duct dilatation and cholestasis can be seen (× 10000); F: Larger and enriched lipid droplets in cytoplasm (× 10000).

the ALT and AST levels in the RFP group were mildly increased, but there were no significant differences compared with those of the normal group ($P > 0.05$). However, the ALP level reached the highest level at day 7 ($P < 0.01$) and then declined with extended administra-

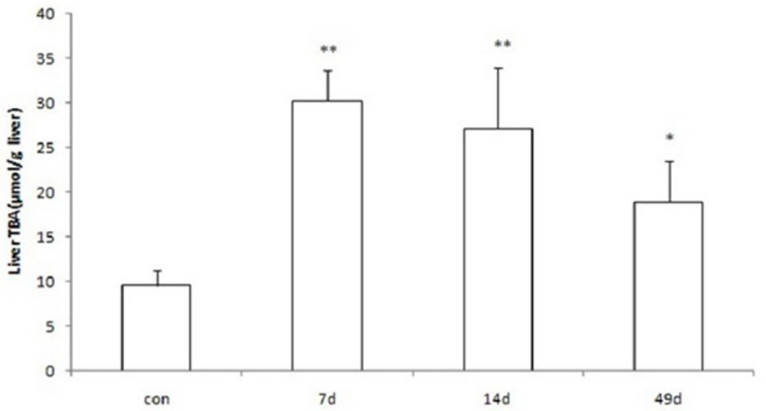

Figure 4. Total bile acids in the liver on days 7, 14, and 49 in the RFP group and the control group (compared with the normal group: *P < 0.05).

tion of RFP for 7 weeks. The serum TBIL and DBIL were increased and reached the peak values at day 14 (P < 0.01) before showing a slow progressive decrease until the end of the experiment, although the values were still significantly higher than those of the control group at the same time points. However, the TBIL, DBIL, and ALP levels in the RFP group on day 49 were statistically lower than their respective peak values (P < 0.05; **Figure 1**).

Liver histopathological changes

In the control group, under light microscopy, the lobule structure including the central vein and portal area was clear. The hepatic cord was arranged in order, and no cytopathic changes were observed. The lobule structure in the RFP group was basically intact on days 7, 14, and 49, but we noticed cytopathic changes in the livers. A small number of hepatocytes with steatosis and inflammatory cell infiltration were present in the portal areas (**Figure 2A-C**). There was no significant difference in the pathological changes in this group over three time points.

Changes in ultrastructure of hepatocytes

The electron microscopy images showed oval nuclei with evenly distributed chromatin, mitochondria with clear cristae, cells with abundant rough and smooth endoplasmic reticula, rich glycogenosomes, and a small number of lysosomes and lipid droplets in control group (**Figure 3A**, **3B**). In the RFP group (**Figure 3C-F**), swelling of the mitochondria and blurred mitochondrial membrane was observed. The dilatation of endoplasmic reticula, diminished glycogen granules, and enriched and expanded lipid droplets in cytoplasm were detected. Marginized chromatin and wavy nuclear membrane were noted. Additionally, the capillary bile duct was dilated to differing extents (mostly to a diameter of 1-3 μm), and the lumen was filled with irregular-shaped, granular, and lamellar materials of different electron densities.

TBA accumulation in the liver

As shown in **Figure 4**, the TBA level in the liver was significantly higher in the RFP group on days 7, 14, and 49 compared to that in the control group (P < 0.05). In addition, the TBA level peaked on day 14 and then was significantly decreased on day 49 (P < 0.05).

Changes in organic anion transporter Mrp2, Bsep, Ntcp, and Mrp4 mRNA levels

As shown in **Figure 5**, Mrp4 mRNA levels were increased over days 7, 14, and 49 in the RFP group, and the level on day 49 was 481 ± 44% higher than that in the control group. In contrast, the mRNA levels of the canalicular export pumps Bsep and Mrp2 showed no significant difference between the control and RFP groups. The mRNA level of the basolateral export pump Ntcp was increased on days 7 and 14 and decreased on day 49 in the RFP group, but showed no significant difference from levels in the control group.

Changes in Mrp2, Bsep, Ntcp, and Mrp4 protein levels

To examine the Bsep, Mrp4, Ntcp, and Mrp2 protein levels, Western blotting was performed using membrane fractions prepared from livers in the RFP group. As shown in **Figures 6** and **7**, the Mrp4 level was significantly increased to 166 ± 14.6% and 248 ± 20.1% on days 7 and 14, and the increase was reduced to 178 ± 22.8% on day 49 compared to expression in the control sample. The Ntcp protein level was decreased to 34.7 ± 5.7% of the control on day

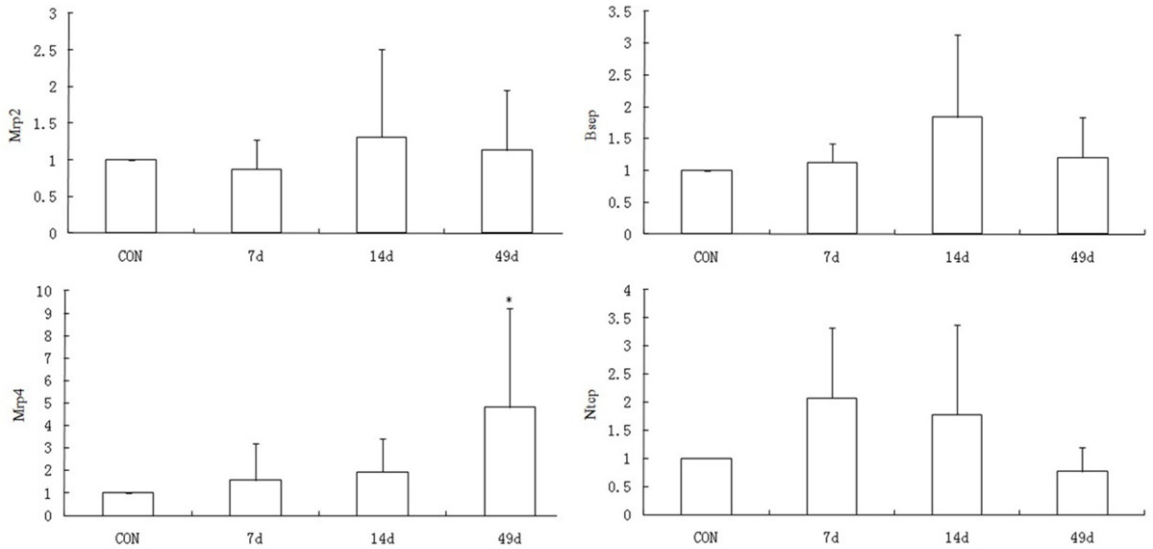

Figure 5. The quantitation of mRNA expression of Bsep, Mrp2, Mrp4, and Ntcp over time. The mRNA levels in the RFP group are expressed relative to the values of individual mRNAs in the control group, which were set as 1: *P < 0.05, **P < 0.01 as compared with the normal group.

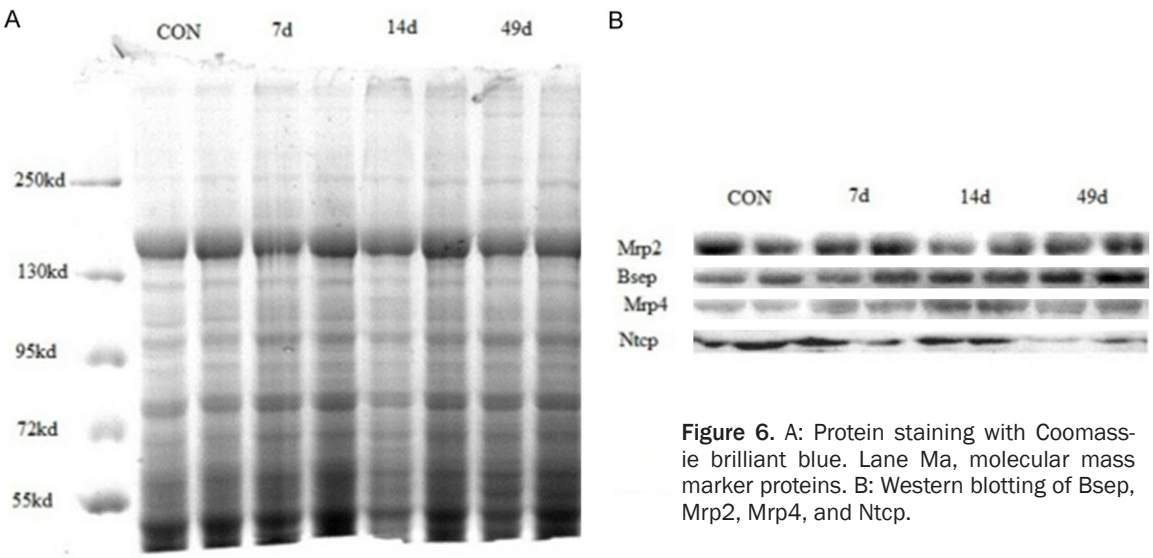

Figure 6. A: Protein staining with Coomassie brilliant blue. Lane Ma, molecular mass marker proteins. B: Western blotting of Bsep, Mrp2, Mrp4, and Ntcp.

49. Mrp2 protein expression did not significantly change during cholestasis. Bsep protein expression was significantly increased to 160 ± 19.4% and 194 ± 7.1% of the control level on days 14 and 49, respectively.

Discussion

RFP is a semisynthetic antibacterial drug of the rifamycin family and it is usually used jointly with isoniazide as an effective regimen for antituberculous therapy [12]. The challenge is that administration of RFP often causes liver injury [13], resulting in elevation of transaminase and the appearance of jaundice or cholestasis that could lead to early cessation of RFP-based treatment. However, approximately 10% to 20% of patients only experience minor transient elevations of liver biochemical markers in serum during long term RFP therapy. Such abnormalities do not require dose adjustment or discontinuation [14-17]. These clinical observations suggest that the liver has the capacity to cope with RFP insult and can adapt to long-term administration of RFP in a portion of treated patients. This adapting process is marked by

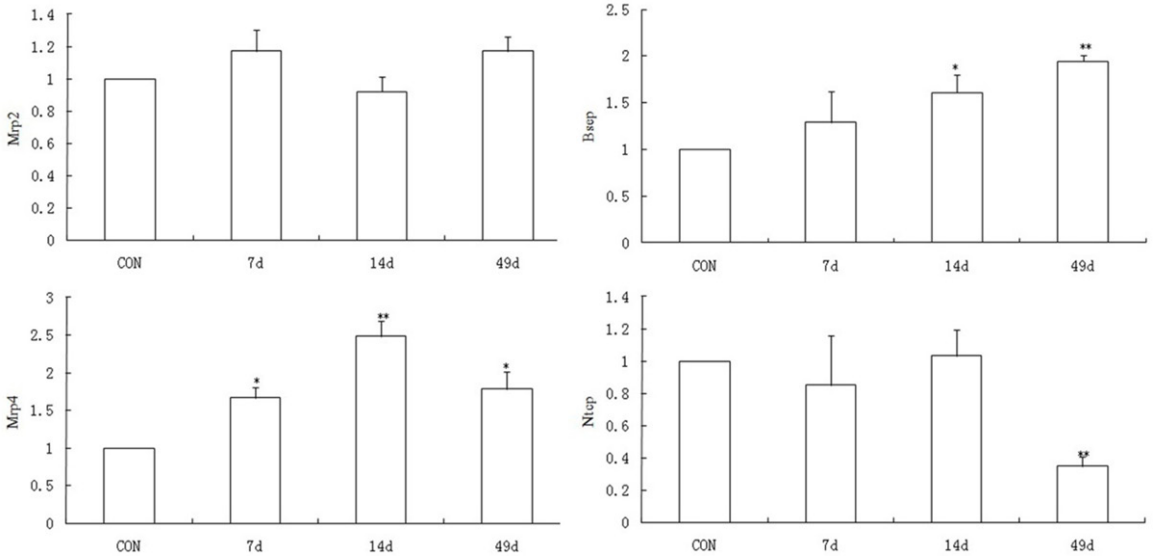

Figure 7. Quantitation of Mrp2, Bsep, Mrp4, and Ntcp protein expression. The Western blotting bands were quantified with a scanning densitometer and normalized as described in the Materials and Methods. The levels of proteins on days 7, 14, and 49 are expressed relative to the values of individual proteins in the control group, which were set as 1: *P < 0.05, **P < 0.01 as compared with the normal group.

initial drug-induced hepatic injury followed by a decline in the elevated serum liver biochemistry levels and even a return to normal levels despite extended administration of the same dosage of drug [18]. Understanding the hepatic adaptation is of clinical importance and a better understanding can be established through studying an animal model of the phenomenon of adaptation to cholestasis induced by RFP in rats. Our previous study [8] showed that the administration of RFP induced hepatic injury including cholestasis in mice. We reasoned that this animal system can be further developed into an animal model of hepatic adaptation to RFP therapy, which is useful for studying the mechanisms underlying how the liver responds to RFP insult and mitigates the liver injury at the molecular level.

In this study, we successfully established a model of the phenomenon of adaptation to cholestasis induced by RFP in rats. We observed two distinctive profiles of liver biochemical indicators between the RFP-treated and control groups. Serum ALP, TBIL, and DBIL levels were basically stable over a 7-week course in the control group, but they were elevated and reached peak values on day 7 or 14 before slowly, progressively declining after the peak in the group with daily administration of RFP for 7 weeks. It was reported that serum BIL was increased on day 3 and then decreased in rats treated with consecutive administration of RFP for 7 days [19]. Our model with a 7-week RFP treatment course is closer to the clinical adaptation to RFP than the 7-day model is. This dynamic change in liver biochemistry in our model rats recapitulated the manifestations observed in clinical cases with RFP adaptation. Histopathological changes in the liver during the course of adaptation were limited in both RFP-treated patients and animals. However, we found the direct evidence of cholestasis in RFP-treated rats under electron microscopy. The capillary bile duct was mostly dilated to 1-3 μm in diameter. The lumen was filled with irregular-shaped, granular, and lamellar materials at different electron densities. We also found that the histopathology of the observed mild liver injury was not aggravated over time despite continuous administration of RFP. Those patients who established RFP adaptation also showed mild hepatitis or mild liver-cell damage [20]. This histological evidence further supported the validity of our model.

Previous studies indicated that a complex but coordinated adaptive response was triggered to reduce cholestasis or excessive retaining and depositing of the bile acids in the liver and other organs when the bile acids accumulated to a high level within liver [5]. It is conceivable

that hepatocytes carry potential reserve capacity that can be initiated to handle increases in bile acids and derived products that are toxic. As suggested, possible steps to eliminate cholestasis include repression of hepatic bile acid uptake, induction of bile acid and BIL detoxification systems, and elevation of bile acid excretion through orthograde canalicular and alternative basolateral membranes. These adaptive responses are possibly mediated by transporter proteins. Up-regulation of both Bsep and Mrp2 expression during cholestasis is deemed an important adaptive mechanism to prevent or mitigate bile acid accumulation within hepatocytes. Repression of Ntcp expression during cholestasis helps limit hepatic bile acid uptake, which reduces hepatocellular bile acid overload. Mrp4 expression is also induced during cholestasis. All of these changes appear to be orchestrated to accelerate the removal of the bile acids and derived products from the liver.

Previous study of the effects of RFP treatment on transporter expression showed that RFP induced Mrp2 mRNA expression in human hepatocytes [21]. Significantly higher levels of Mrp2 mRNA and protein expression were detected in RFP treated healthy gallstone patients compared with controls [22]. However, the expression levels of Mrp2 in RFP treated rats did not significantly differ from controls in this study. Rifampicin increased the expression of Mrp4 and Bsep mRNA and their proteins in glycochenodeoxycholic acid (GCDCA)-treated human hepatocytes and bile duct-obstructed rats [23], but the expression levels of *BSEP* and MRP4 were not changed in healthy gallstone patients [22]. Our results showed changes in the levels of these transporter proteins in the RFP-treated group. The Bsep protein was upregulated on days 7 and 49. The Mrp4 mRNA and protein levels were elevated, and the Ntcp protein level was reduced on day 49. These results provide indirect evidence that the induction or inhibition of organic anion transporters may mediate recovery from the accumulation of bile acids in the liver insulted by RFP.

Certainly, an adaptive response to RFP insult is a very complex process that also likely involves the action of other organs to accelerate the elimination of the toxic parent drug or its metabolites from the body [24]. There are several adaptive pathways including upregulation of antioxidant pathways, development of immune tolerance by the adaptive immune system, and induction of mitochondria adaptation and regeneration of cells [25, 26]. Further studies are required to clarify additional adaptive pathways for mitigation of the cholestasis.

In summary, we established a rat model that resembles the clinical adaptation to RFP-induced cholestasis in this study. We also presented the findings that the hepatic adaption to RFP insult was possibly mediated through the induction of Mrp4 and Bsep to increase the export of bile acids and through inhibition of Ntcp to limit the uptake of bile acids.

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Disclosure of conflict of interest

None.

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