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Hepatotoxic effect of Rifampicin as an Anti-Tuberculosis drug on male Albino rat

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ABSTRACT

Tuberculosis is one of the serious airborne infectious diseases. Rifampicin is commonly used as anti-tuberculosis drug which creates drug-induced hepatotoxicity. Physiologically, liver maintains metabolic homeostasis and also regulates the detoxification process. The study of rifampicin mediated hepatotoxicity had been performed on male albino rat after its oral administration with a dose of 50 mg/kg body weight/day for 14 days. Several biochemical markers like serum glutamate pyruvate tranaminase (AST), serum glutamate oxaloacetate transaminase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), serum total protein, serum bilirubin, serum cholesterol were considered to evaluate the toxicity. Significant elevation of level of AST (115.89%), ALT (134.40%), ALP (46.15%), serum cholesterol (91%) and bilirubin content (119.44%) had been observed in treated group compared with control group. High level of MDA content as lipid peroxidation marker was also been noticed in drug induced group. Histopathological studies had shown the disintegrated hepatolobular structure with dilated central vein. All these findings indicated that the selected dose of rifampicin is hepatotoxic; proper monitoring and care are essential during the treatment of tuberculosis.

Keywords: rifampicin; hepatoxicity; anti-tuberculosis

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Abbreviations

ABCB1: ABC transporter subfamily B member 1; ALP: alkaline phosphatase; ALT: alanine transaminase; AST: aspertate transaminase; CP: continuation phase; DILI: drug-induced liver injury; HRZE: isoniazid, rifampicin (RIF), pyrazinamide, and ethambutol; IP: intensive Phase; LDH: lactate dehydrogenase; LPO: lipid peroxidation; MDA: malondialdehyde; PPARγ: proliferators activated receptor gamma; PXR: pregnane X receptor; RIF: rifampicin; ROS: reactive oxygen species:

INTRODUCTION

Liver is the "metabolic factory" of the body and plays central role to control the metabolism of every nutrient as well as foreign substances including drugs. Hepatic cytochrome P-450 enzyme system is essential for biotransformation of drugs through oxidative pathways followed by conjugation with glucuronide/sulphate/glutathione which convert the molecules to hydrophilic metabolites those are excreted by the kidney or through the gastrointestinal tract.[1] Owing to these properties, liver is the main target of drug toxicity and drug-induced liver injury (DILI) is the most common side effect in clinical.[2] Currently, over 1000 drugs are known to cause DILI, and the list is continuously growing up.[3] Zhou et al.[4] reported that anti-tuberculosis drugs were the leading agents of DILI. Tuberculosis is one of the top curable infectious diseases and creates serious public health problem in developing countries. According to World Health Organization, 9.6 million people were suffering from tuberculosis and 1.5 million had been died in 2014.[5] In developed countries, the incidence of tuberculosis increases due to immunodeficiency disease like HIV (human immunodeficiency virus) infection.[6] Currently, four major pharmacological agents (isoniazid, rifampicin, pyrazinamide,

and ethambutol) are used as anti-tuberculosis drug. The regimen for adult respiratory tuberculosis treatment includes a combined preparation of isoniazid, rifampicin (RIF), pyrazinamide, and ethambutol (HRZE; H=75mg / R=150mg / Z=400mg / E=275mg) for 2 months as Intensive Phase (IP) of treatment, followed by additional four months of Continuation Phase (CP) of treatment with HRE (H=75mg / R=150 mg / E=275 mg).[7] Among these drugs, rifampicin (RIF) is the main initiator of hepatotoxicity.[8] It causes hepatocellular dysfunction followed by hepatic lesions, cellular changes, lobular necrosis and hyperbilirubinemia.[9]

Sensi et al.[10] had isolated rifamycin from the culture of *Streptomyces mediterranei* which is the derivative [3-(4-methyl-1-piperazinyl)-iminomethyl] of rifamycin. Rifampicin is a complex semisynthetic macrocyclic antibiotic[11] with empirical formula $C_{43}H_{58}N_4O_{12}$ and molar mass 822.953 g/mol. This polyketide compound belongs to ansamycins class of molecule containing napthoquinone core in the heterocyclic structure that is spanned by an aliphatic ansa chain. The napthoquinonic chromophore gives red-orange crystalline colour of rifampicin. This drug is well absorbed from the stomach and then metabolized in the liver by deacetylation followed by hydrolysis to give 3-formyl rifampicin. Deacetyl rifampicin is more polar than the parent compound, and microbiologically active.[12, 13] Rifampicin binds to the β subunit of RNA polymerase through hydrogen bonds between hydroxyl groups of the ansa bridge and the napthol ring containing amino acid residues of RNA polymerase.[14] The outcome is inhibition of bacterial DNA-dependent RNA synthesis.

.In the present scenario, multi-drug therapy is the best choice for the treatment of tuberculosis instead of isoniazid mono-drug therapy. However, hepatotoxicity is one of the serious problems, especially for RIF. The present study has primarily focused on the mechanism of RIF-induced liver injury in rat model.

MATERIAL AND METHODS

Chemicals

Sodium chloride (NaCl), Potassium dihydrogen phosphate (KH_2PO_4), Dipotassium hydrogen phosphate (K_2HPO_4), Sodium hydroxide (NaOH), Trichloro acetic acid (TCA), Thiobarbituric acid (TBA), Potassium hydroxide (KOH), Alcohol, and other chemicals had been procured from Merck Ltd., SRL Pvt. Ltd., Mumbai, India and rifampicin had been purchased from HiMedia Laboratories, Pvt. Ltd., Mumbai, India.

Selection of animals and maintenance

The study was performed on 18 healthy Wister strain male albino rats, having a body weight of 100 ± 15 g, supplied by Saha Enterprise, Kolkata (CPSEA, Govt. of India registered farm). They were acclimatized in laboratory condition for a period of 2 weeks. Proper care for the experimental animals was provided according to the guidelines of the "Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA)", India and all experimental procedures were approved by Institutional Animal Ethical Committee (Reg No. 1617/GO/Re//S/12/CPCSEA). Experimental animals were housed (three rats per cage) in a room having temperature 22 ± 2 °C, humidity 50 ± 10% with 12 ± 1 h light and 12 ± 1 h dark cycle. To carry out the experiments, the experimental animals were divided into three groups and each group comprises 6 rats (n=6/gr). Group-I (control group) received normal diet and water ad libitum, Group-II (rifampicin induced treated group) received normal diet, water ad libitum and oral supplementation of rifampicin with a dose of 50 mg/kg body weight/day[15] for 14 days, and Group-III (Sham treated group) received normal diet, water ad libitum and oral supplementation of riboflavin with a dose of 10 mg/kg body weight/day as placebo. Previously, riboflavin was also used as placebo by Low et al.[16]

Sacrifice of animals and collection of blood and tissues

Over the treatment schedule of 14 days, body weight of all the experimental animals were taken by using animal's weighing machine. Then, the animals were sacrificed (as per guideline of CPSEA, Govt. of India) to evaluate the rifampicin mediated hepatoxicity. Blood sample was collected from the aorta, and hepatic tissue was taken for different biochemical and histological studies. Before preservation of hepatic tissue, the weight of liver of all three groups was recorded. The tissues were stored into −20 °C until preparation of tissue homogenates. For histological examination, liver was preserved in 10% neutral formaldehyde solution till processed.

Histological study

Hepatic tissue was washed in ethanol for dehydration and the portion of the tissue was embedded in paraffin wax. Histological slides were made by cutting the section in 6 µm thickness. Eosin and hematoxylin stain were used to observe the histo-architecture of the hepatic tissue. The histopathological changes were recorded by using scoring system.

Separation of serum and preparation of liver homogenate

Serum was separated by centrifugation (1500×g for 15 min) of blood samples and then kept in −20 °C for biochemical estimation of different parameters. Similar type method was also followed by Tripathy et al.[17] Tissue homogenate was prepared through the following process: 1.5 g hepatic tissue was washed initially in 0.9% normal saline and made homogenate in ice-cold buffer (0.25 M sucrose, 1 mM EDTA, and 1 mM Tris-HCl, pH 7.4). The homogenate was centrifuged at 6000×g for 10 min in 4 °C.[18] Then supernatant was separated and stored at −20 ^{0}C for biochemical study.

Study of biochemical markers of hepatotoxicity

The extent of hepatotoxicity was determined by measuring the activities of several important intracellular hepatic enzymes like aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) in serum and tissue homogenate. Serum bilirubin, total protein and albumin concentration were also estimated. All these assays were performed by using assay kits of Span Diagnostics Ltd., India.

Assessment of lipid peroxidation

The degree of lipid peroxidation (LPO) in tissue homogenate was measured by estimating the formation of thiobarbituric acid reactive substances (TBARS) as malondialdehyde (MDA) content according to the method of Ohkawa et al.[19] The reaction mixture contained tissue homogenate (200 µl) 20% TCA (1.5 ml) and 1.34% TBA (1.5 ml) mixture followed by boiling for 30 minutes, then allowed to cool by addition of 2.5 ml butanol. The whole mixture was centrifuged at 2000×g for 5 minutes and then optical density of supernatants was measured at 535 nm. The amount of malondialdehyde (MDA) content was expressed as nmol of MDA/mg of protein.

RESULTS

Measurement of body weight and liver weight

The alterations of body weight and liver weight were measured and the results were furnished in Table 1. The rate of increase of mean body weight was very slow in treated group compared to control group and sham treated group. The liver weight had proportionately increased in control group and sham treated group along with body weight. While, the treated animals showed minimum increase of liver weight; this might be accumulation of lipid.

Table 1. Effect of rifampicin on body weight and liver weigh.

Group	Body weight (gm)			Liver weight (gm)
	0 days	7 days	14 days	14 days
Control	125.5 ± 2.33	132.0 ± 2.58	138.5 ± 2.67	6.4 ± 0.93
Treated	122.5 ± 3.9	123.7 ± 4.1	129.0 ± 1.98 *	5.2 ± 0.53*
Sham treated	124.22 ± 2.83	136.0 ± 4.58	139.6 ± 1.67	6.2 ± 0.72
Values are expressed as Mean ± SEM, n=6; * indicates significant difference (P < 0.001) compared to control Group.				

Biochemical markers of hepatotoxicity

In this present study, hepatotoxicity was started after administration of rifampicin (50 mg/kg body wt/rat/day). The mean value of serum AST, ALT, ALP, LDH, of hepatic tissue had been increased significantly (p < 0.001) by 115.89%, 134.40%, 46.15% and 173.94%, respectively in rifampicin treated Group compared to control group (Fig. 1); but, any significant changes did not observed in placebo receiving Group. However, the activities of these enzymes in hepatic tissue homogenate were decreased by 26.47%, 41.55%, 7.65% and 25.38% respectively in treated group. The activity of these enzymes in sham treated group was very nearer to the control group (Fig. 1). The total protein and albumin content in serum were decreased significantly (p < 0.001) by 36.11% and 53.38% respectively in rifampicin treated group in respect of control group. Albumin-globulin ratio (A/G) had been dropped to 50% of its original value after treatment (Table 2). An insignificant change in protein concentration had been found in sham treated group. Beside these, the serum cholesterol level significantly increased upto 91.0% in the treated group. Administration of rifampicin showed a significant (P < 0.001) elevation of serum bilirubin (total, conjugated and unconjugated) by 119.40%, 47.82% and 148.0% respectively (Table 3). In this study, MDA content was measured to evaluate the lipid peroxidation and degree of membrane damage. MDA content was significantly (P<0.001) increased by 194.0% in treated group compared to control group (Fig. 2). However, no such significant changes were observed in sham treated group.

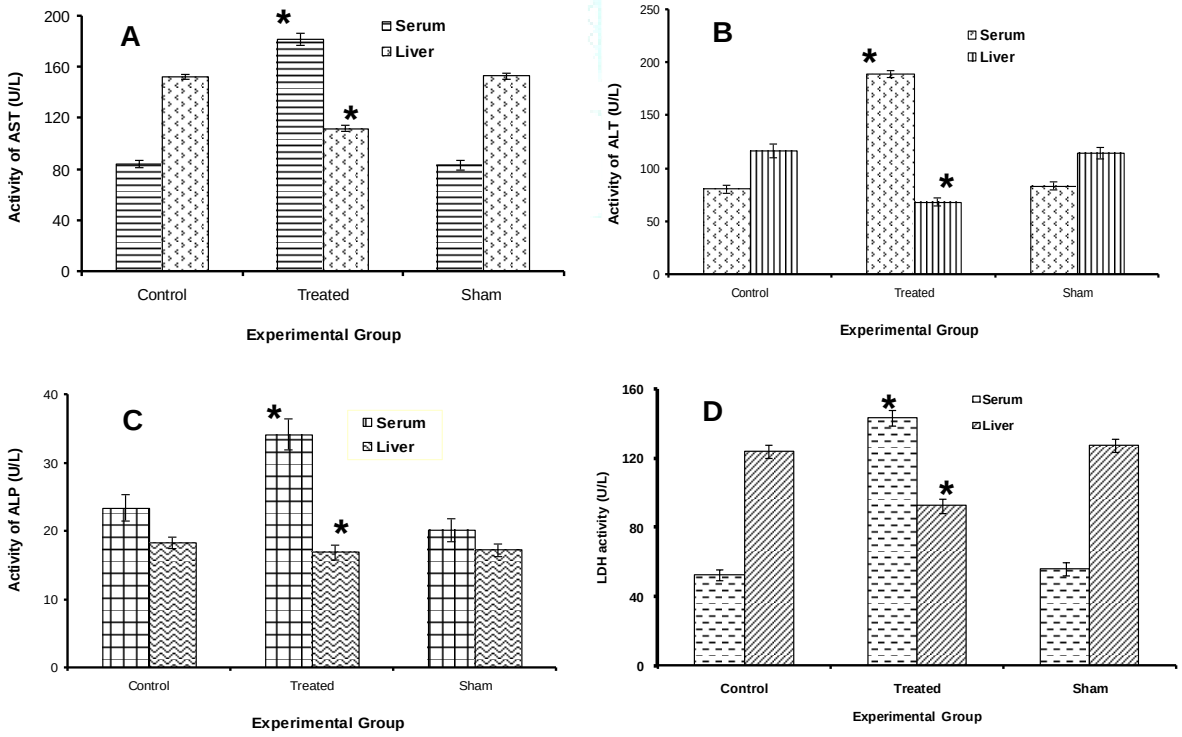

Figure 1. Graphical presentation represents the effect of rifampicin on AST (A), ALT (B), ALP (C) and LDH (D) activity in control, treated and sham treated animals. Values are expressed as mean ± SEM, n =6. * indicates significant difference (P < 0.001) compared to control Group.

Table 2. Effect of rifampicin on serum total protein, albumin and albumin-globulin ratio.

Group	Serum total protein (mg/dl)	Serum albumin (mg/dl)	Albumin-globulin ratio
Control	9.22 ± 1.14	4.72 ± 0.86	1.04
Treated	5.89 ± 0.6*	2.2 ± 0.35*	0.56
Sham treated	8.56 ± 0.98	4.82 ± 0.86	1.28
Values are expressed as Mean ± SEM, n=6; * indicates significant difference (P < 0.001) compared to control Group.			

Table 3. Effect of rifampicin on serum cholesterol and bilirubin.

Group	**Serum cholesterol (mg/dl)**	**Serum bilirubin (mg/dl)**		
		Total	Cojugated	Unconjugated
Control	72.17 ± 4.12	0.36 ± 0.014	0.115 ± 0.007	0.25 ± 0.016
Treated	138.2 ± 3.57*	0.79 ± 0.038*	0.17 ± 0.005	0.62 ± 0041*
Sham treated	73.57 ± 3.47	0.34 ± 0.016	0.120 ± 0.005	0.27 ± 0.019
Values are expressed as Mean ± SEM, n=6; * indicates significant difference (P < 0.001) compared to control Group.				

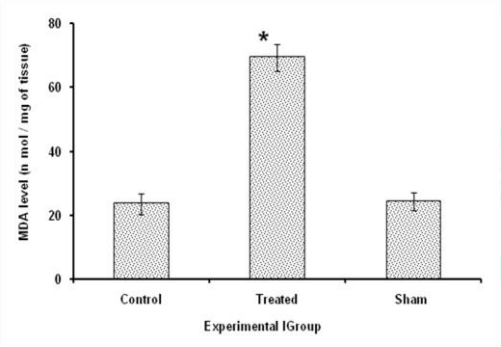

Figure 2. Graphical presentation represents the effect of rifampicin on MDA content in control, treated and sham treated animals. Values are expressed as mean ± SEM, n =6. * indicates significant difference (P < 0.001) compared to control Group.

Histological examinations

Marked changes had been found in rifampicin treated group compared to control group and placebo supplemented sham treated group. The, histological study of the liver sections of control animals showed normal hepatocellular architecture without any sign of necrosis along with well preserved hepato-lobular pattern and normal size of central vein. These findings were also very similar in sham treated group. However, liver sections of rifampicin treated group had shown the lipid accumulation, massive cellular necrosis, enlargement of central vein and sinusoidal space, and portal vein disruption which indicates loss of cellular architecture due to excessive intracellular lipid deposition (Fig. 3 Table 4).

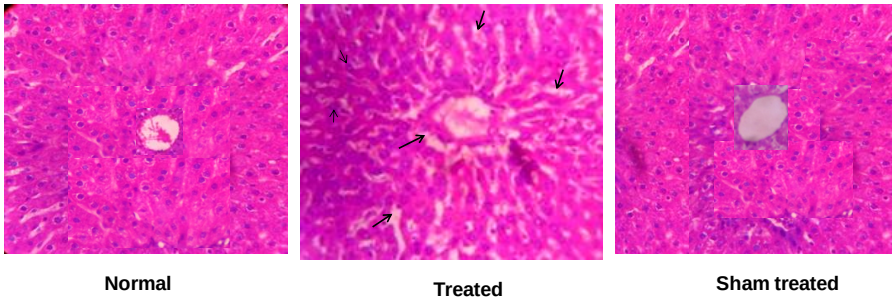

Figure 3. Histological structure of liver of Control, rifampicin and Sham treated group. The sections were stained by eosin and hematoxylin and observed under 40× magnification.

Table 4. Histological changes in liver. The scoring was made in six-point scale according to Ishak et al. 1995.[36]

Parameters	**Control group**	**Rifampicin treated group**	**Sham treated group**
Cellular necrosis	0	5	0
Hepatocyte degeneration	0	5	1
Portal vein disruption	2	4	2
Lipid Accumulation	0	5	0

DISCUSSION

Drug induced liver injury is very common during the treatment of tuberculosis. The rate of DILI is approximately 58% and 5–22% cases has been linked to acute liver failure.[20] In the treatment regimen of tuberculosis, RIF is the first-line drug, but exerts sever hepatototoxicity after its administration.[21] The present study indicated that all the toxicity related marker enzymes like ALT, AST. ALP and LDH (Fig. 1) increased significantly in serum of treated animals. The leaching of the intracellular enzymes occurred due to oxidative stress induced LPO mediated membrane damage. Similar type findings were also reported by Rana et al.[15] and Kim et al.[9] A scheme of proposed mechanism of rifampicin induced liver injury has been given in Figure 4 which indicates that hepatotoxicity is directly associated to cytochorome P450 dependent drug metabolism. Rifampicin is an agonist of xeno sensing pregnane X receptor (PXR) which is a member of nuclear receptor superfamily of ligand dependent transcription factors.[13] RIF induces the over expression of pregnane X receptor (PXR); the result is more amount of CYP3A4 subset of cytochorome P450 enzyme, responsible for drugs/xenobiotics metabolism.[22] The PXR mediated inducible enzymes, are CYP2B6, 2C9, 2C19, and 3A4. PXR also increases the transcriptional activity of ATP dependent ABCB1 transporter. Beside these, PXR regulates the metabolism of bile acid, bilirubin, steroid hormone, glucose and lipid.[23] Decreased value of serum total protein and albumin (Table 2) are very common during any type of hepatoxicity. Generally, liver is the site for synthesis of most of the plasma proteins except gamma globulin and any injury/toxicity of the hepatocytes reduces the rate of formation of plasma proteins.

Rifampicin had tended to increases the lipid peroxidation marker such as MDA (Fig. 2) which is associated with oxidative stress (OS). Chowdhury et al.[24] reported that RIF stimulates oxidative stress (OS) mediated lipid peroxidation (LPO) in hepatic cells. Oxidative stress promotes excess production of ROS ($^{\bullet}O_2^-$, $^{\bullet}OH$, H_2O_2) which starts LPO mediated membrane damage. ROS binds with unsaturated units of the polyunsaturated fatty acids of membrane lipids. This reaction converts the membrane lipids (RH) to lipid macro radical (ROO•) in presence of oxygen and promotes continuation of the chain reactions of lipid peroxidation. Finally, ROO• was modified to hydroperoxide (ROOH) or endoperoxide followed by malondialdehyde.[18] Accumulation of MDA indicates the imbalance of redox homeostasis as well as tissue damage.

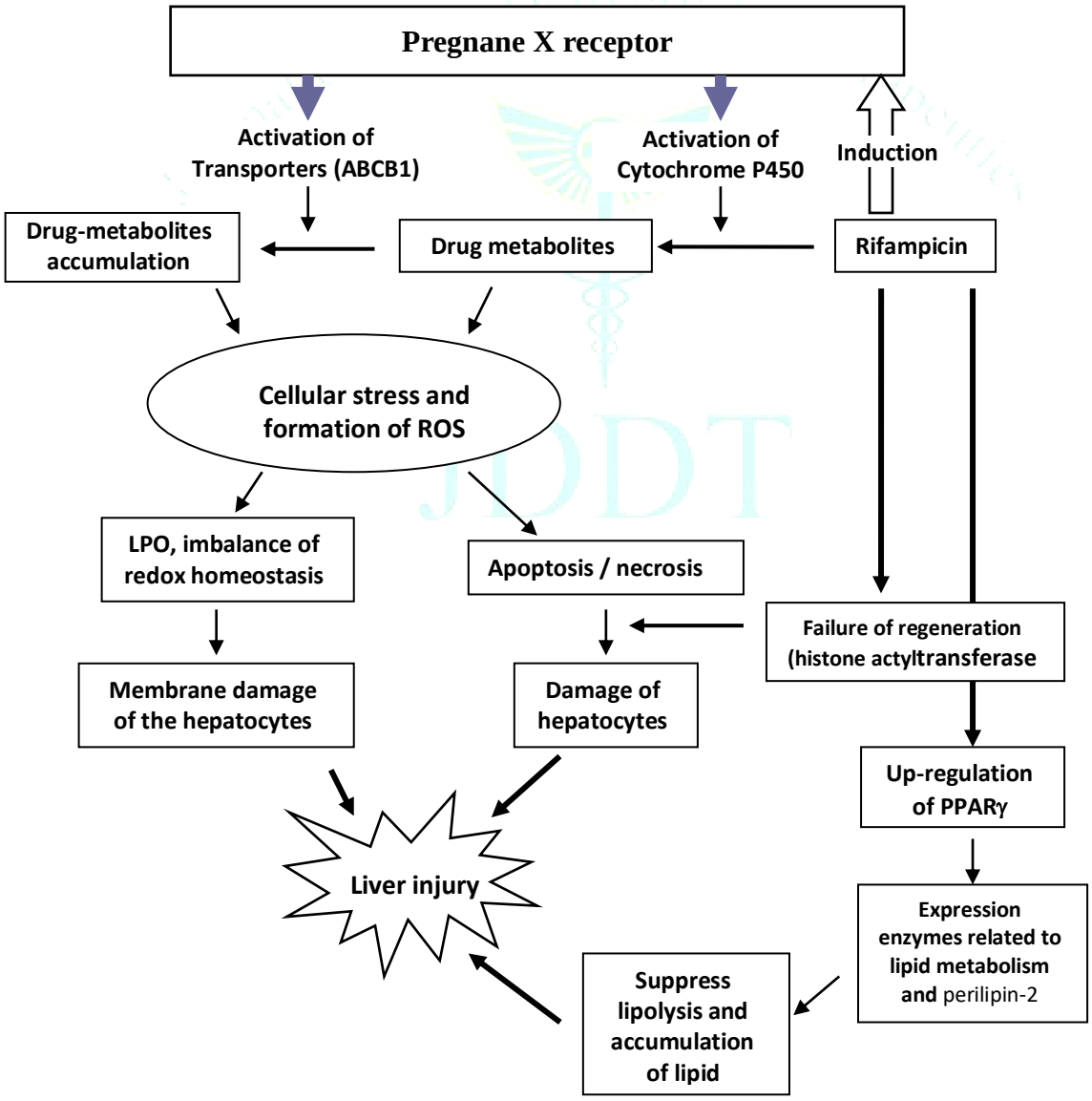

Figure 4. Proposed hypothetical model of mechanism of rifampicin induced liver injury.

The significant increase of serum total bilirubin (both conjugated and unconjugated) was observed after administration of RIF (Table 3). Jussi et al.[25] reported that RIF hampered the bilirubin uptake; the result is subclinical unconjugated hyperbilirubinemia. Conjugated hyperbilirubinemia was due to inhibition of the bile salt exporter pump.[26] Moreover, improper bilirubin clearance at the sinusoidal membrane or impeded secretion at the canalicular level may also enhance the serum bilirubin level.[27, 28].

The results of histological studies of rifampicin treated group had revealed that there were marled changes in cellular disintegration, lipid accumulation, alteration of cytoarchitecture and necrosis of the hepatic cells (Fig. 3) along with significant elevation of serum cholesterol level (Table 3). Previously, it was reported that RIF mediated liver damage is done by increasing oxidative stress in mitochondria, apoptotic response of liver cell, cholestasis effects, and hepatic lipid accumulation in rodent.[21] The accumulation of lipid in hepatic cells is made via up-regulation of peroxisome proliferators activated receptor gamma (PPARγ). Recently, Kim et al.[9] had observed that up-regulation of PPAR stimulates the expression of five proteins (apolipoprotein C-III, acyl-CoA-binding protein, 3-ketoacyl-CoA thiolase A and B, and perilipin-2) related to lipid metabolism. Actually, perilipin coats the lipid droplets in adipocytes with phospholipid monolayer and maintains the maturation and metabolism of lipid droplets.[29, 30] The coating of perilipin suppresses lipolysis and promotes accumulation of lipid droplets in hepatic tissue.[31, 32]

The overall findings indicated that rifampicin mediated hepatotoxicty was a complex process. The multi-drug (isoniazid, RIF, pyrazinamide, and ethambutol) therapy against tuberculosis enhances the potential effects of hepatotoxic.[33] RIF induces the activity of CYP3A4 which leads to increases the metabolism of isoniazid, yielding toxic metabolites like isonicotinic acid and hydrazine by activating isoniazid hydrolases.[34, 35] At the later stage, hydrazine is further metabolized to more toxic components such as N-hydroxy acetyl hydrazine, acetyl diazine, acetyl onium ion, acetyl radical.[13] Thus, RIF amplifies the hepatotoxic effects of anti-tuberculosis drugs.

CONCLUSION

In conclusion, it can be stated that anti-tuberculosis drug, rifampicin alters the level of serum protein, serum bilirubin, MDA content, AST, ALT, ALP and LDH enzymes activity in albino rat when orally administered for 14 consecutive days. Till now, except these drugs there are no alternative medicines for the treatment of tuberculosis. Thus, proper monitoring and care must be given during the period of treatment of tuberculosis.

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CONFLICT OF INTEREST STATEMENT

All authors have none to declare as conflicts of interest.

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