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RESEARCH ARTICLE

Developing Animal Model for Analysing Liver Toxicity of Isoniazid and Rifampicin Combination

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Abstract

Background: Liver toxicity often results from drug administration, such as antituberculosis. The prevalence of liver toxicity in tuberculosis patients remained high in the last decade. Liver toxicity induced by isoniazid (INH) and rifampicin (RIF) combination might have different features and mechanisms with liver toxicity induced by other substances or drugs (acetaminophen, carbontetrachloride, d-galactosamine, ethanol or dimethylnitrosamine). The goal of this study was to investigate the toxic dose and duration of administration of combined isoniazid and rifampicin that contributed to the hepatic injury in rats. Methods: A combination of INH-RIF contained 100 mg of INH and 100 mg of RIF. The induction of INH-RIF was given for 28 consecutive days. Twenty rats were divided into five groups: control, group 1 (dose 50 mg), group 2 (dose 100 mg), group 3 (dose 200 mg), group 4 (dose 400 mg),and group 5 (dose 800 mg). At the end of induction, serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured, and liver histopathology was evaluated. Data were analyzed with one-way Anova test. Results: Serum AST and ALT levels were significantly higher in groups 1 and 2 compared to control ($p<0.001$). Post-hoc analysis revealed that 50 mg and 100 mg dose significantly increased the serum AST and ALT. Conclusion: There were significant differences in serum AST and ALT, as well as histopathological scores, among rats induced by INH and RIF combination.

Keywords: *Isoniazid; Rifampicin; Liver; Animal.*

Introduction

For years, tuberculosis had become a major problem in many countries. However, tuberculosis treatment remains difficult since it has to be given in a relatively long duration and often results in side effects, as well as drug resistance. Tuberculosis regiment recommended in Indonesia is a fixed-combination of first-line antituberculosis drugs, namely isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA) and ethambutol (ETB) [1]. Antituberculosis use often leads to the occurrence of some unwanted side effects, mostly is hepatic injury. The prevalence of liver damage related to antituberculosis administration among tuberculosis patients remained high in the last decade. It was ranged between 14.7% to 48.4% in many populations. Hepatic damage induced by antituberculosis may result in severe and serious diseases in patients. It was also reported to responsible for up to 50% of acute liver failure cases in the USA. More than 75% of the cases were considered as fatal diseases and required liver transplantation for the management [2, 4]. Some previous evidence had already performed an induction of liver toxicity by administering antituberculosis drugs. However, the toxic

dose for each drug, as well as the duration of administration, were inconsistent.

It was crucial to develop a specific model for studying hepatic damage induced by isoniazid and rifampicin (INH-RIF) in animals, particularly rats. As we know, isoniazid and rifampicin are the main antituberculosis drugs that should be administered for long-duration protocols of tuberculosis treatment. Moreover, liver toxicity related to combined isoniazid and rifampicin use may not precisely have the same feature and mechanism like hepatic injury induced by other substances or drugs, namely acetaminophen, ethanol, carbontetrachloride, d-galactosamine and dimethylnitrosamine [5, 9]. Therefore, we conducted this research to investigate the toxic dose and duration of administration of combined isoniazid and rifampicin that contributed to the hepatic injury in rats.

By developing the animal model for liver toxicity induced by isoniazid and rifampicin combination, we could study more about the mechanism of liver damage induced by combined isoniazid and rifampicin. Subsequently, this may also help us to explore herbal or synthetic preparations for the treatment of hepatic injury induced by combined isoniazid and rifampicin.

Methods

Samples Selection

This study was an experimental study with a randomized post-test only control group design. This study was performed in the Integrated Biomedical Laboratory, Faculty of Medicine, Udayana University. Our study protocol was approved by the Animals Ethics Committee of Veterinary Faculty, Udayana University, Indonesia. Healthy male Wistar rats (*Rattus norvegicus*), aged 8-12 weeks, weight 150-200 grams were included in our study.

Induction of Liver Toxicity

Approximately 20 rats were randomly divided into five groups:

The control group (not received INH-RIF),

Group 1 (Received INH-RIF suspension, dose 50 mg per 200 grams rat per day),

Group 2 (Received INH-RIF suspension,dose 100 mg per 200 grams rat per day),

Group 3 (Received INH-RIF suspension, dose 200 mg per 200 grams rat per day),

Group 4 (Received INH-RIF suspension,dose 400 mg per 200 grams rat per day)

Combined isoniazid and rifampicin used in our study was a fixed combination of isoniazid and rifampicin in oral dosage form (tablet) which was consist of 150 mg isoniazid and 150 mg rifampicin. For induction of hepatic injury, combined INH-RIF suspension was prepared by diluting the tablet in aquabidest for oral administration. The INH-RIF combinations were administered for 28 consecutive days (4 weeks) intragastrically. Rats were also given foods and water ad libitum. At the end of the study, the rats were sacrificed. Blood and liver samples were collected for evaluating biomarkers for liver injury.

Measurement of Transaminase Enzyme Concentration

We assessed serum concentration of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), as well as liver histopathology, to evaluate liver toxicity. Serum AST and ALT concentration were measured with spectrophotometry technique.

Liver Histopathological Examination

Liver tissues were fixed in 10% formalin and embedded into paraffin. The liver sections were stained with hematoxylin-eosin (HE) for histopathological analysis under the light microscope. Semiquantitative analysis of liver histopathology was performed using a scoring system that defined the grade of congestion, degeneration and necrosis in five visual fields. Each feature then scored in the range 0 to 3:

0: no congestion, degeneration or necrosis in 5 visual fields

1: there was congestion, degeneration or focal necrosis in 5 visual fields

2: there was congestion, degeneration or multifocal necrosis in 5 visual fields

3: there was congestion, degeneration or diffuse necrosis in 5 visual fields

Statistical Analysis

Data analysis was performed with One Way Anova test for comparing serum AST and ALT levels among groups. Liver toxicity scores were analyzed using the Chi-Square test. The p-value below 0.05 was considered statistically significant.

Results

Concentration of Transaminase

In group 3, which received 200 mg of combined INH-RIF, all rats died on day 28. In group 4, after received INH-RIF combination (contained 400 mg of isoniazid and 400 mg of rifampicin), all rats in this group died on day 14. The serum level of AST and ALT in the control group, groups 1 and 2, were demonstrated in Table 1. All data were homogenous and normally distributed. There was a significant difference in the serum concentration of AST and ALT among the three groups ($p<0.001$).

Post-hoc analysis revealed that dose 50 mg and 100 mg of combined INH-RIF were significantly increased serum concentration of AST and ALT ($p<0.001$). One hundred mg of combined INH-RIF significantly produced higher serum concentration of AST and ALT than dose 50 mg of combined INH-RIF ($p<0.001$).

Table 1: The AST and ALT level after combined INH-RIF administration

Variable	Control group (mean ± SD)	Group 1 (mean ± SD)	Group 2 (mean ± SD)	*p*
AST concentation (U/L)	37.38 ± 0.485	53.41 ± 0.485	60.85 ± 1.009	<0.001
ALT concentration (U/L)	18.93 ± 0.485	34.15 ± 1.564	50.82 ± 1.838	<0.001

Table 2: Post-hoc analysis for AST and ALT level after combined INH-RIF administration

Variable	Compared group	Mean difference	95%CI		*p*
			Lower bound	Upper bound	
AST concentation (U/L)	Control *vs.* Group 1	-16.02333	-17.4305	-14.6162	<0.001
	Control *vs.* Group 2	-23.46667	-24.8738	-22.0595	<0.001
	Group 1 *vs.* Group 2	-7.44333	-8.8505	-6.0362	<0.001
ALT concentration (U/L)	Control *vs.* Group 1	-15.21333	-18.0525	-12.3741	<0.001
	Control *vs.* Group 2	-31.88333	-34.7225	-29.0441	<0.001
	Group 1 *vs.* Group 2	-16.67000	-19.5092	-13.8308	<0.001

Liver Histopathological Feature

Liver histopathological features observed in the control group, groups 1 and 2, were represented in Table 3. The histopathological scores of the liver in all groups were shown in Table 4.

Table 3: Liver histopathological features related to combined INH-RIF administration

Group	Liver Histopathological		
	Congestion	Degeneration	Necrosis
Control group	None	None	None
Group 1 (50 mg of INH-RIF)	Diffuse	None	None
Group 2 (100 mg of INH-RIF)	Diffuse	Diffuse	Multifocal

Table 4: Histopathological score of the liver after combined INH-RIF administration

Group	Histopathological Score		
	Congestion	Degeneration	Necrosis
Control group	0	0	0
Group 1 (50 mg of INH-RIF)	3	0	0
Group 2 (100 mg of INH-RIF)	3	3	2

Discussion

Liver toxicity influenced by antituberculosis administration might manifest as mild to severe liver damage. The clinical manifestations of antituberculosis hepatic injury are commonly similar to general drug-induced liver injuries, like fever, icterus, malaise, fatigue, nausea, vomiting, eosinophilia, as well as enhanced serum concentration of AST and ALT. The histopathological features of hepatic injury induced by antituberculosis generally include hepatocellular steatosis, necrosis, and inflammatory infiltration. These features might be different for other hepatotoxic drugs or substances [10]. The mechanisms for hepatic damage related to antituberculosis, particularly isoniazid and rifampicin, had not fully understood yet. Previous studies exploring molecular pathways contributed to

antituberculosis liver injury, only administered isoniazid as the principal component of the antituberculosis regimens.

Otherwise, in medical practice, rifampicin was also administered together with isoniazid for a long duration during the intensive and continuation phase of tuberculosis treatment. There had been no specific study assessing the effect of combined isoniazid and rifampicin on the liver. Unlike other hepatotoxic substances, stress oxidative, inflammation and apoptosis had a role in the occurrence of antituberculosis hepatic damage. Moreover, specifically on the isoniazid-induced hepatic injury, CYP2E1 enzyme contributes an important role in the occurrence of liver toxicity induced by isoniazid [10, 14].Many evidence had stated that isoniazid was strongly correlated with liver toxicity. This was related to the pharmacological characteristics of isoniazid. It has been well-established that isoniazid undergoes several phases of metabolism and produces some toxic metabolites, namely hydrazine dan acetylhydrazine[13].

The CYP2E1 enzyme catalyzes the creation of those toxic metabolites. Isoniazid itself is also a CYP2E1 inducer that could induce CYP2E1 activity. A previous study conducted by Hassan *et al*. represented that isoniazid enhanced the expression of CYP2E1 protein [11]. Physiologically, the CYP2E1 enzyme catalyzes phase I metabolism (via oxidation reaction). In this process, CYP2E1 could alter oxygen molecules into very reactive components, namely reactive oxygen species (ROS) such as superoxide anion, singlet oxygen, hydrogen peroxide, and hydroxyl radical. In the existence of the CYP2E1 inducer, ROS formation may continuously increase. Oxidative stress results from this process subsequently leads to lipid peroxidation and DNA damage [11, 14, 15]. Rifampicin is also an enzyme inducer. A combination of isoniazid and rifampicin would highly promote cytochrome P450 activities that would contribute to more serious hepatic damage.

This may result from the enhanced production of isoniazid toxic metabolites [16, 17]. The reactive metabolites of isoniazid might also create covalent adduct with liver macromolecule, thus would react with lysine residues on hepatic proteins. This reaction will subsequently promote immune response and inflammations. Some researches had stated that the administration of the INH-RIF combination resulted in enhanced concentrations of proinflammatory marker (TNF-α, IL-1ß, IL-6, NFκB), as well as histopathological changes of the liver toward inflammation features [11, 16, 20]. ROS creation was also assumed to induce apoptosis related to hepatic damage regarding INH and RIF use. Some in vivo studies had proved that INH and RIF combination contributed to apoptosis,which manifested as enhanced apoptosis index and other apoptosis markers namely Bax, caspase-3, p53 and decreased Bcl-2 [11, 15, 16, 21]. Animal models developed for investigating liver toxicity influenced by antituberculosis in some evidence generally utilized rats or mice. The majority of rat strain used for evaluating hepatic damage related to antituberculosis was Wistar [17, 18, 20, 22, 24] Researches performed by Sankar *et al*. and Shih *et al*. utilized Sprague-Dawley strain for assessing hepatic injury on rats [16, 25].

Some other *in vivo* studies exploring liver toxicity associated with combined INH and RIF using Balb/c mice and Knock Out (KO) mice [21, 26]. Our study used Wistar rats for assessing liver toxicity on animals. Not only the strain of animal, but the dose of each antituberculosis used in the study was also varied in some animal studies. Some evidence applied a proportional dose for each antituberculosis drug, but others administered different doses for each drug. Our research revealed that 100 mg of combined INH-RIF (consist of 100 mg INH and 100 mg RIF) per rat per day for 28 days induced liver injury in Wistar rats. Our finding was different from other studies. Our study demonstrated a higher dose of INH and RIF for inducing liver damage in rats. The majority of other evidence for investigating liver toxicity regarding INH-RIF administration had stated that the administration of 50 mg INH-RIF (contained 50 mg of INH and 50 mg of RIF) per kg rat per day intragastrically for 21-30 days would produce hepatic injury [16, 19, 20, 22].

Other researches administered 100 mg INH and 100 mg of RIF per kg per day for 14 consecutive days [21]. Several studies had conducted toxicity studies for antituberculosis that applied different dose

for each antituberculosis drug [17, 18, 23, 26]. Study conducted by Adaramoye *et al.* revealed that the dose of INH, RIF, PZA, and ETB applied in rats for inducing hepatic injury were 5 mg, 10 mg, 15 mg, 15 mg per kg rat per day, respectively, for eight weeks [18]. Research performed by Lian *et al.* applied 150 mg of INH and 300 mg of RIF per kg rat per day [26].

A study by Shih *et al.* administered 50 mg of INH and 100 mg of RIF per kg rat per day for three weeks [25].A study by Miglani *et al.* used 27 mg, 54 mg, and 135 mg of INH, RIF, and PZA, respectively, per kg rat per day for 30 days for inducing liver injury [24]. A study by Hussain *et al.* applied 7.5 mg, 10 mg, and 35 mg of INH, RIF, and PZA, respectively, per kg rat per day for 35 consecutive days.[23] A study done by Liu *et al.* administered 100 mg, 250 mg, and 50 mg of INH, RIF, and PZA, respectively, per kg rat per day for 30 days [17].

Conclusions

In conclusion, 100 mg of the INH-RIF combination showed significant liver toxicity. A 100 mg of combined INH-RIF resulted in significantly enhanced serum concentration of AST and ALT, as well as significant hepatic damage (represented on the histopathological feature of the liver).

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