Hepatoprotector effects of press needle acupuncture of GB34 and BL18 against isoniazid and rifampicin induced liver injury in Wistar rats

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Abstract. Treatment for tuberculosis typically consists of a combination of 5 drugs, of which isonicotinylhydrazide (INH) and rifampicin are the most significant ones. However, they are also associated with serious hepatotoxicity and fatal liver injury. The purpose of this study was to examine the hepatoprotector effect of press needle acupuncture on drug induced liver injury caused by INH and rifampicin by measuring the histopathological changes and variations in the SGOT, SGPT and MDA levels. This single-blinded, experimental, randomised control trial included 28 Wistar rats that were randomly allocated into four groups [control group (n = 7), negative control group (n = 7), press needle group (n = 7) and Sham acupuncture group (n = 7)]. Press needle acupuncture was administered using Pyonex press needle at Acupoints GB34 Yanglingquan and BL18 Ganshu. Significant differences in the mean histopathological scores were observed between the groups. The press needle acupuncture group exhibited lower steatosis (1.57 ± 0.787) compared to the negative control (2.33 ± 0.516) and the Sham acupuncture groups (2.29 ± 0.488) (p < 0.05). Therefore, it was concluded that press needle acupuncture had a hepatoprotector effect on histopathological changes associated with drug induced liver injury caused by isoniazid and rifampicin.

1. Introduction
Tuberculosis (TB) is one of the most common health problems globally, with approximately 9.6 million incident cases and 1.5 million deaths occurring in 2015, as per the WHO Global Tuberculosis Report [1]. Currently, Indonesia ranks fifth in terms of prevalence globally, being preceded by only India, China, South Africa and Nigeria [1,2]. According to the Indonesian Health Profile, published by the Ministry of Health in 2014, the prevalence rates of TB in Indonesia were approximately 0.4%, while the incidence rates in 2013, as per the Global Tuberculosis Control, were 343 cases per 100,000 population [3]. The TB prevention plan, developed by the Indonesian government’s National Program
for TB control in collaboration with the World Health Organization, included the Directly Observed Treatment Short course (DOTS) strategy consisting of 5 components, one of which was the administration of anti-TB drugs [4]. However, some of the first-line drugs included in the DOTS program, such as isoniazid, rifampicin, pyrazinamide, streptomycin and ethambutol, are known to have several side effects. For example, isoniazid and rifampicin are known to be hepatotoxic drugs and can cause drug-induced liver injury (DILI), which is characterised by the elevation of liver transferase enzymes [e.g. serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT)] [5] and resembles hepatitis virus infections histopathologically. Although the mechanism through which isoniazid and rifampicin causes liver injury is still unclear, it has been suggested that oxidative stress associated with increased lipid peroxidation and decreased endogenous antioxidants may have a role to play.

DILI treatment due to anti-TB drugs is symptomatically for the mild case and drug cessation for severe case. Several studies have examined the hepatoprotector effects of some drugs and herbal medications that are known to improve heart disorders that occur subsequent to hepatotoxicity caused by anti-TB drugs such as isoniazid and rifampicin [6-8]. In addition, the evidence suggests that acupuncture may have a hepatoprotector effect by affecting the levels of liver transferase enzymes and endogenous antioxidants in patients experiencing heart failure caused by chemicals. However, there is limited evidence on the hepatoprotector effects of acupuncture in patients with liver failure caused by isoniazid and rifampicin. In recent studies examining the effects of pharmacopuncture techniques in animals diagnosed with liver failure caused by ethanol, acupuncture was seen to affect the levels of liver transferase enzymes, which are indicators of liver injury, and endogenous antioxidants in the animals. Histopathological features of liver showed the difference between animals in treatment and control group [9-11].

2. Methods
This single-blinded, experimental, randomized control trial was carried out in the Laboratory of Pathological Anatomy and the Laboratory of Biochemistry and Molecular Biology in the Faculty of Medicine, Universitas Indonesia, Jakarta. The study sample consisted of adult male Wistar rats. The Wistar rats were randomly divided into four groups, as follows: a) control group: did not receive any drugs or acupuncture treatment; b) negative control group: received isoniazid and rifampicin; c) press needle group: received press needle acupuncture treatment at the GB34 and BL18 points + isoniazid and rifampicin and d) Sham acupuncture group: received acupuncture at the Sham point located caudal to the GB 34 point + isoniazid and rifampicin. The findings of the sample size calculations suggested a minimum of 6 rats per group. Therefore, after taking a dropout rate of 10% into consideration, the current study included 7 rats in each group. Isoniazid and rifampicin were administered for ten days at a dosage of 100 mg/kg of body weight per day. The needles in the acupuncture groups (press needle and Sham acupuncture groups) were attached for 10 days.

The histopathological features of the liver tissues were compared between the groups, and the mean SGOT, SGPT and MDA levels before and after administration of the drugs and acupuncture treatment were compared within and between the groups. The statistical procedures selected for analysis were dependent on the type of data. Numerical variables that were normally distributed were compared using the ANOVA test, while the Kruskal–Wallis test was used for data that did not exhibit a normal distribution. Moreover, normally distributed paired data were compared using t-tests, while the non-parametric equivalent was the Wilcoxon test. The level of statistical significance was set at p<0.05, and SPSS, version 15 was used to carry out all the data analyses.

The study protocol was approved by the Health Research Ethics Committee, Faculty of Medicine, Universitas Indonesia-dr.Cipto Mangunkusumo Hospital (928/UN2.F1/ETHICS/2016) and the research was performed in laboratory of Pathology and Anatomy Department of Faculty of Medicine, Universitas Indonesia with number: 424/UN2.F1.D/PA/PPM.00.02/2016.
3. Results
The initial study sample consisted of 28 rats; however, the study was terminated on day 4 as the rats exhibited a tendency to die. Eight rats dropped out of the study, of which 7 rats died and one was excluded as the blood sample exhibited lysis. Table 1 shows the sample demographics.

Table 1. Sample demographics by group.

| Variables          | Normal Control | Negative Control | Acupuncture | Sham | P Value |
|--------------------|----------------|------------------|-------------|------|---------|
| Gender:            |                |                  |             |      |         |
| Male               | 6              | 4                | 6           | 4    |         |
| Female             | 0              | 0                | 0           | 0    |         |
| Body Weight [gram (SD)] | 300.17        | 299.75           | 298.33      | 303.50 | 0.859* |
| Early SGOT [U/L (SD)] | 66.445        | 52.817           | 52.865      | 50.343 | 0.556* |
| Early SGPT [U/L (SD)] | (30.570)      | (12.843)         | (12.248)    | (14.236) |         |
| Early MDA [nmol/ml (SD)] | 0.123         | 0.093            | 0.232       | 0.611 | 0.000* |
|                     | (0.073)        | (0.098)          | (0.195)     | (0.089) |         |

* ANOVA Test

No significant differences in gender, body weight and early SGOT and SGPT levels were observed between the groups (Table 1). However, early MDA levels were seen to significantly differ between the four groups.

The findings of the current study showed that the mean SGOT and SGPT levels after treatment were lower than those observed before treatment, although this difference was not statistically significant. In the press needle acupuncture group, the mean early SGOT (U/L) levels were seen to drop from 52.865 ± 12.248 to 42.507 ± 31.196 after treatment (Wilcoxon test p-value >0.05), while the mean early SGPT levels decreased from 42.995 ± 22.881 to 22.770 ± 6.646 (Wilcoxon test P > 0.05). This downward trend in SGOT and SGPT levels was also observed in the other groups, although once again this was not statistically significant.

Although a large number of rats died, the pathological anatomy of their livers could still be examined (n = 27) (Table 2).

Table 2. Comparison of pathological anatomy images between groups.

| Group        | Mean (SD) | P     |
|--------------|-----------|-------|
| Pathological | Normal Control | 0    | 0.000* |
| Anatomy      | Positive Control | 2.30 (0.516) |     |
|              | Acupuncture       | 1.57 (0.787) |     |
|              | Sham             | 2.29 (0.488) |     |

*Kruskal–Wallis test

A significant difference in the pathological anatomy images scores was observed between the four groups (Table 2), with the press needle acupuncture group exhibiting the lowest values compared to the other groups (P = 0.000)
The pathological anatomy image scores of the press needle acupuncture group were lower than that of the negative control and Sham acupuncture groups (p = 0.002) (Table 3). Between the four groups, the changes in the mean SGOT, SGPT and MDA levels were not significantly different.

Histopathological Images Scoring can be seen in Figure 1-4. The histopathological images were scored based on the size of the fatty liver tissue, and statistical analysis showed that the press needle acupuncture group exhibited the lowest scores compared to the positive control and Sham acupuncture groups. The mean score of the acupuncture group was 1.57 ± 0.787, while those of the positive control group and Sham group were 2.30 ± 0.516 and 2.29 ± 0.488, respectively. This difference was statistically significant (P < 0.05).

Table 3. Comparison of changes in the levels of SGOT, SGPT and MDA and the Pathological Anatomy scores between the four groups.

| Variable          | Group               | Mean (SD)  | P      |
|-------------------|---------------------|------------|--------|
| Pathological      | Normal Control      | 0          | 0.002**|
| scores            | Negative Control    | 2.25 (0.5) |        |
|                   | Press Needle        | 1.67 (0.816) |    |
|                   | Sham                | 2.25 (0.5) |        |
| SGOT Changes      | Normal Control      | −26.239 (32.462) | 0.833*|
|                   | Negative Control    | −17.096 (35.712) |    |
|                   | Press Needle        | −10.358 (29.215) |    |
|                   | Sham                | −19.133 (15.765) |    |
| SGPT Changes      | Normal Control      | −1.747 (11.761) | 0.830**|
|                   | Negative Control    | −2.328 (25.587) |    |
|                   | Press Needle        | −3.055 (21.341) |    |
|                   | Sham                | −20.225 (29.503) |    |
| MDA Changes       | Normal Control      | (0.136 0.396) | 0.089**|
|                   | Negative Control    | (0.671 0.369) |    |
|                   | Press Needle        | (0.282 0.904) |    |
|                   | Sham                | (0.394 0.701) |    |

*ANOVA Tests **Kruskal–Wallis test
Figure 1. H.E staining of samples from normal control group shows fatty vesicles (micro-moderate < 5%, centrilobular, scoring 0) (a) 40× magnification and (b) 100× magnification.

Figure 2. H & E staining of samples from press needle group shows fatty vesicles (micro-moderate 5%–33%, centrilobular, scoring 1) (a) 40× magnification and (b) 100× magnification.

Figure 3. H & E staining of samples from Sham acupuncture group shows fatty vesicles (micro-moderate 34%–66%, centrilobular, scoring 2) (a) 40× magnification and (b) 100× magnification.
Figure 4. H & E staining of samples from negative control group shows fatty vesicles (micro-moderate > 66%, centrilobular, scoring 3) (a) 40× magnification and (b) 100× magnification.

4. Discussion

The initial duration of the administration of INH and rifampicin was 10 days, based on previous evidence, and the rats were expected to survive until the study was complete. However, 8 rats dropped out of the study, of which one was excluded because of lysis of the blood samples, preventing examination of SGOT, SGPT and MDA levels, and 7 rats died. On the second day of the study, 1 rat in the negative control group died, and its blood and liver samples could not be collected. Five rats died on day 3 of the study, of which 2 were from the negative control group, 1 was from the press needle acupuncture group and 2 were from the Sham acupuncture group. Only liver samples were collected for examination. On day 4 of the study, 1 rat from the Sham group died and, once again, only liver samples could be collected. Moreover, 2 rats (1 from the positive control group and 1 from the acupuncture group) experienced convulsions upon the administration of the drugs on day 4 and were euthanised immediately. Blood samples were collected using the intracardial technique, along with liver tissue samples. Given the high number of deaths observed, a decision was made to euthanise all of the rats on day 4 and to collect blood and liver tissue samples. Acute INH poisoning may cause nervous system disorders by decreasing the GABA levels in the brain tissues. Clinical symptoms include repeated convulsions, peripheral neuropathy and coma. Based on the observation of convulsions in two of the rats upon administration of INH and rifampicin on day 4, it was concluded that the deaths were caused by either central nervous system disorders secondary to INH toxicity or secondary acute liver failure attributable to INH and rifampicin toxicity. Further investigations are necessary to confirm the causes of death.

The ANOVA test showed no significant differences in gender, body weight (p = 0.859), early SGOT (P = 0.556) and SGPT levels (P = 0.0467), although the difference in early MDA levels between the four groups was statistically significant (P ≤ 0.000). The mean early MDA plasma level was higher in the acupuncture and Sham groups (0.232 nmol ± 0.195 and 0.611 nmol ± 0.089, respectively) compared to the normal control and positive control groups (0.123 ± 0.073 and 0.093 ± 0.098, respectively) (Table 1). MDA is the end product of lipid peroxide reactions that occur naturally in the body as a result of reactive oxygen species and phagocytic activity. The body defends itself against oxidants with the help of enzymatic (catalase and superoxide dismutase) and non-enzymatic (vitamin A and E) antioxidants. During inflammation, cells such as neutrophils, monocytes, eosinophils and lymphocytes release cytokines and chemokines, which stimulate phagocytic and non-phagocytic activity through protein kinase signalling activation. IL 1β, TNF-α and IFN-γ stimulate the formation of ROS in the epithelial cells during inflammation, resulting in an increase in the lipid peroxidase and
MDA levels. Prior to treatment, the rats in the acupuncture and Sham groups were shaved to expose the skin in the area where acupuncture would be administered. This may have caused inflammation in the skin, resulting in elevated MDA levels [12].

In the current study, the administration of INH and rifampicin resulted in oxidative stress that may have led to damage of the liver cells. We hypothesised that acupuncture would decrease MDA plasma levels, which is a marker of oxidative stress. However, the results of this study showed an increase in the mean MDA plasma levels in all the groups after treatment, and this may have been caused by a number of confounding factors. Although the level of MDA increased, the possibility of endogenous antioxidant enzymes level increased can also happened, so that oxidant antioxidants balanced.

Oxidative stress can affect the performance of the mitochondria and cause stress, leading to the disruption of energy production and metabolism-related disorders. The increased MDA levels observed in this study resulted in oxidative stress and steatosis, suggesting fat metabolism disorders caused by mitochondrial stress. Although the MDA levels were elevated in the press needle acupuncture group, the scoring for the histological characteristics of steatosis were lower than the other groups. Preece et al. [13] reported that the administration of hydrazine, which is an active metabolite of INH, in rats resulted in hepatotoxicity and a decrease in the levels of adenosine triphosphate (ATP). ATP is the primary source of intracellular tissue energy in the human body, and its key role is cellular biosynthesis. It is produced during respiration, one of them through the Krebs cycle. In this cycle, adenosine diphosphate (ADP) is a precursor of ATP by phosphate addition which occurs in mitochondria. Decreased levels of ATP can cause disturbances in cell metabolism and subsequent cell death. Secretion of ATP can be achieved by mechanical, thermal or photostimulation. Acupuncture point stimulation through the nervous system can reach the target organ and influences the inflammatory factors and the regulation of organ function [13,14].

The markers of hepatocellular damage include liver transaminase enzymes such as SGOT and SGPT. Liver cell damage accompanied by disruption of the cell membrane can cause the release of the transaminase enzymes into the bloodstream. Previous evidence suggests that the administration of INH and rifampicin may cause damage to the liver cells, resulting in increased SGOT and SGPT levels, and the stimulation of the acupuncture points GB34 and BL18 results in a decrease in SGOT and SGPT levels. Although some studies have reported that the administration of INH and rifampicin in rats may cause liver cell damage and increased SGOT and SGPT levels, others have reported that INH can inhibit the liver enzymes [15]. The use of suitable animal subject for liver injury model due to INH and rifampicin may be considered for further research. According to the research conducted by Surich et al. [16], rabbit was a suitable animal for INH and rifampicin poisoning model.

The histopathological characteristics of drug induced liver injury caused by isoniazid and rifampicin include liver cell tissue necrosis, cholestatic or steatosis (fatty liver). Although a large number of rats included in this study died, histopathological examinations of their liver tissues could still be carried out. The findings showed existence of micro-moderate vesicular fatty livers in 27 rats. Distribution of centrlobular predominant was more dominant in areas that are far from the blood vessels. DILI caused by isoniazid and rifampicin is a complex condition. Factors that may have a role to play include the dosage of INH and rifampicin administered; variations in study duration; differences in individual antioxidant capacity, which may affect the resilience of the individual against oxidative reactions; existence of inflammatory factors from previous treatment, which can affect laboratory assessment; animal models with physiological conditions that resemble human conditions.

Although the histopathological image scores observed in this study suggested that acupuncture exerted a hepatoprotector effect, its efficacy in significantly improving liver injury through the endogenous antioxidant system remains unclear. Although changes in the mean levels of various markers of liver injury and those related to the balance between oxidants and antioxidants were observed, these were not statistically significant. Future studies should include preliminary tests, suitable animal models such as rabbits, and assessment of endogenous antioxidant markers (catalase and super oxide dismutase) to better understand the hepatoprotector effects of acupuncture.
against the balance of oxidant and antioxidant that affected the liver injury caused by INH and rifampicin.

5. Conclusion
The results of this study suggest that press needle acupuncture treatment exerts hepatoprotector effects on animal subjects diagnosed with drug induced liver injury due to isoniazid and rifampicin.

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