Pharmaceutical Sciences Asia

Pharm Sci Asia 2022; 49(6), 534-542

DOI:10.29090/psa.2022.06.22.212

Research Article

Carbon tetrachloride-induced acute liver toxicity: selecting dosage and biomarkers for evaluating hepatoprotective drugs in ICR outbred mice

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ABSTRACT

Evaluating effects of putative chemical or herbal agents against a single intraperitoneal administration of carbon tetrachloride (CCl_4) in rodents is a widely used model for studying hepatoprotective potency. Since the toxic effects of CCl_4 is dependent on individual species; therefore, our study aimed to demonstrate a procedure to select the optimal dosage of CCl_4 and types of liver damage-associated biomarkers for testing hepatoprotective drugs in ICR mice. To include inter-individual genetic variation, the test was conducted in outbred mice. Silymarin and rebamipide were applied as the representative tested agents. We revealed that 15-150 µL/kg of CCl_4 induced liver damage including hepatocyte vacuolation and ballooning with infiltration of inflammatory cells, centrilobular necrosis, and increased serum alanine aminotransferase and aspartate aminotransferase, in a dosage-dependent manner. Nonetheless, serum levels of bilirubin were not significantly increased at 15 µL/kg of CCl_4. On the other hands, the level of alkaline phosphatase was not parallel with the increased dosage of CCl_4. Most importantly, as observed using liver histology and serum biomarkers, rebamipide and silymarin showed hepatoprotective effects against 15 µL/kg of CCl_4 merely, whereas both drugs were unable to protect liver injury against 150 µL/kg of CCl_4. In conclusion, this study demonstrated how to design an experiment to select the optimal dosage of CCl_4 for evaluating hepatoprotective effects of putative agents in a specific tested species. In addition, we revealed choices of serum biomarkers which could be associated with the severity of liver damage.

Keywords:
CCl_4, Carbon tetrachloride, Liver toxicity, Hepatoprotective, Biomarker

1. INTRODUCTION

Carbon tetrachloride (CCl_4) is a widely used hepatotoxin for studying potential benefits of putative chemical or herbal agents in rodents[1]. CCl_4 induces liver damage via the toxic effects of its metabolite, trichloromethyl free radical ($CCl_3^{\cdot}$), which altered cellular integrity leading to swelling, cytolysis, and death of hepatic cells[2]. Currently, although its mechanisms have not been fully elucidated, long-term exposure of low-dosage CCl_4 can establish liver fibrosis and cirrhosis, and this well-validated model serves as a tool for studying anti-fibrotic drugs[3]. In contrast, short and relatively high-dosage administration of CCl_4 is used as a model to evaluate hepatoprotective drugs. Following a pretreatment period with the tested drug, this acute model requires a single CCl_4 application to the rodents prior to euthanization for assessing liver injury[4-5]. Together with histological changes after CCl_4 exposure, liver injury-associated parameters; for example, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and bilirubin, could be used to evaluate the hepatoprotective effects.

Since to the sensitivity against CCl_4 is dependent on individual species, the optimal dosage of this hepatotoxin

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to induce liver toxicity in a certain type of animal is the most essential determinant to be taken into consideration. To illustrate, FVB/N mice are relatively less susceptible to the toxicity of CCl_4 when compared to C57BL/6 mice[6]. Conversely, BALB/c mice appeared to be very vulnerable to CCl_4 causing occasional mortality in long-term exposure[3]. According to this variation, a universal dosage of CCl_4 could not be valid to all experiments. Therefore, our study aimed to demonstrate a procedure to select the optimal dosage of CCl_4 for testing hepatoprotective drugs in ICR mice, a mouse strain which its sensitivity against CCl_4 was unrecognized. Furthermore, although inbred mice are preferred in most biomedical research because of their less genetic variability[7], our experiment was conducted in outbred mice to include inter-individual genetic variation. Silymarin and rebamipide were selected to be the representative tested drugs in this study. Silymarin is a mixture of various flavonolignan isomers isolated from seeds of *Silybum marianum* (milk thistle). Currently, silymarin is a well-known cytoprotective agent used in patients with liver-related disorders[8], and is vastly used as a reference to compare its effects with a putative agent on CCl_4 exposure[5,9]. Rebamipide is a gastroprotective drug for the treatment of gastric ulcer which possesses multiple cytoprotective properties including antioxidant effect[10]. Although hepatoprotective effects of rebamipide against acute toxicity of CCl_4 was unclear, its attractive mechanism of action could be beneficial to protect liver damage. Besides the protocol optimization, a reliable liver-damage parameter to signify the effect of hepatoprotective agents could be primarily identified. Therefore, we additionally demonstrated how to select the associated serum biomarkers which reflected the severity of liver damage in ICR outbred mice.

2. MATERIALS AND METHODS

2.1. Animals

Male ICR mice (National Laboratory Animal Center, Nakhon Pathom, Thailand), 6-weeks old were used for the study. The mice were housed under a 12-hours light/dark cycle in a temperature/humidity-controlled room with standard rodent diet and filtered water *ad libitum*. The study was performed after acclimatization for at least 7 days. The study protocol (PYR002/2019 and PYR001/2020), which complied with the ARRIVE guidelines (Animal Research: Reporting of *In Vivo* Experiments)[11], was approved by the Animal Ethical Committee of the Faculty of Pharmacy, Mahidol University.

2.2. Chemicals and reagents

CCl_4 was purchased from Shanghai Seasonsgreen Chemical (Shanghai, China). Rebamipide (Mucosta™) was kindly provided by Thai Otsuka Pharmaceutical (Bangkok, Thailand). Silymarin was purchased from Indena (Settala, Italy). Other unspecified chemicals and reagents used in this study were purchased from Sigma (Wisconsin, USA) or Himedia (Mumbai, India).

2.3. Experimental design

First, we evaluated dosage-dependent effects of a single intraperitoneal administration of CCl_4. The mice were randomly divided into 5 groups to receive CCl_4 at the dosage of 0, 15, 30, 75, and 150 µL/kg. Due to volatility and small volume, CCl_4 was diluted in olive oil to yield 0, 1, 2, 5, and 10% volume/volume before given with an equivalent volume according to individual body weight. Second, another set of mice was pretreated with rebamipide (300 mg/kg) or silymarin (100 mg/kg) to evaluate their hepatoprotective effects against the lowest (15 µL/kg) and highest (150 µL/kg) tested dosage of CCl_4. The drugs were dispersed in 0.5% sodium carboxymethylcellulose and given to the mice at -72, -48, -24, and -2-hours by oral gavage before intraperitoneal injection of CCl_4 (0-hour). The control mice were given 0.5% sodium carboxymethylcellulose which was used as a drug vehicle. At 24-hours after receiving CCl_4 in both studies, the mice were anesthetized using carbon dioxide before cardiac puncture for blood collection. Subsequently, the liver was perfused using 0.9% sodium chloride solution via portal vein until no residual blood and harvested for examining liver damage. The timeframe for evaluating hepatoprotective effects of drugs was summarized in Figure 1.

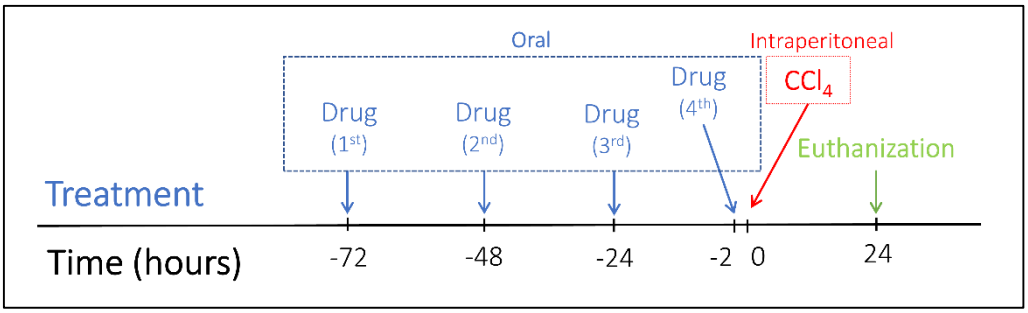

Figure 1. Timeframe for evaluating hepatoprotective effects of drugs against CCl_4 in ICR outbred mice. The drugs were given by oral gavage 4 times while CCl_4 was given once via intraperitoneal injection. The euthanization was performed at 24-hours after CCl_4 administration.

2.4. Macro- and microscopic appearances

Full of harvested livers were immediately examined for macroscopic appearances. For microscopic evaluation, the livers were fixed in 10% neutral buffered formalin for at least 48 hours, dehydrated in a series of ethanol, cleared in xylene, and embedded in paraffin. The 4 μm liver sections were stained with hematoxylin/eosin (H&E) for examination of hepatic lesions. The images were visualized using an Olympus IX-81 microscope (Tokyo, Japan) with 10x and 40x objective lens and analyzed for 5 random frames per liver.

2.5. Measurements of serum biomarkers

The clotted blood was centrifuged at 10,000 g for 10 minutes to collect serum. The levels of ALT, AST, ALP, total and direct bilirubin, creatinine, and blood urea nitrogen (BUN) were immediately quantified using Olympus AU400 Chemistry Analyzer (Tokyo, Japan) with the standard diagnosis reagent kits.

2.6. Statistic

Besides individual values, the bar graphs are expressed as means±standard error of the mean (SEM) of numerical results among the same treatment. Due to the data was not normally distributed, the non-parametric analysis of statistical significance was performed using unpaired Mann-Whitney test to compare between individual treatment with the corresponding reference. The statistical calculation was conducted using Prism 6.01. A *p*-value less than 0.05 was considered significant.

3. RESULTS

3.1. Dosage-dependent effects of CCl_4

After liver perfusion, small nodules on the surface of liver were markedly seen in all CCl_4-treated groups (Figure 2A-E). This nodule formation was not found in the liver of CCl_4-free mice. Nevertheless, size, amount, and localization of these nodules appeared to be independent on the dosage of CCl_4. In addition, hemorrhagic areas were found in the liver of mice received 75 and 150 μL/kg of CCl_4.

Microscopically, H&E staining showed that CCl_4 caused centrilobular necrosis in a dosage-dependent manner (Figure 2F-O). At 15 μL/kg of CCl_4, hepatocyte vacuolation and ballooning with infiltration of inflammatory

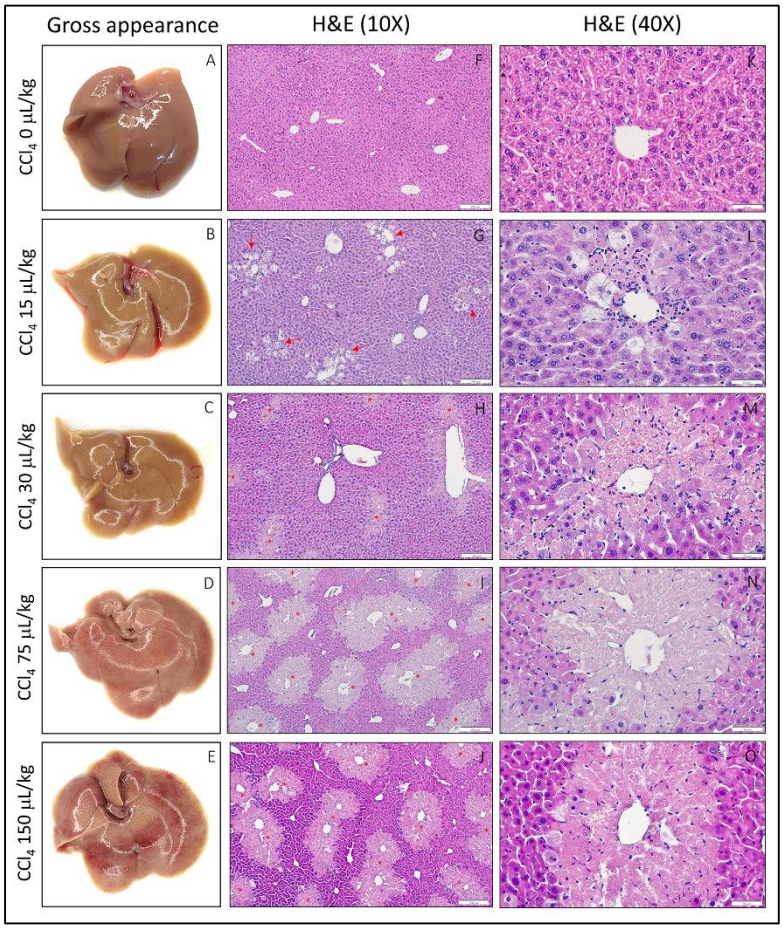

Figure 2. Representative gross appearances (A-E), hematoxylin/eosin (H&E) staining at 10X (F-J, scaled bar=200 μM), and at 40X (K-O, scaled bar=50 μM) in the liver of ICR outbred mice received various dosages of CCl_4. A, F, K = 0 μL/kg; B, G, L = 15 μL/kg; C, H, M = 30 μL/kg; D, I, N = 75 μL/kg; and E, J, O = 150 μL/kg. Arrows (↑) and asterisks (*) indicate hepatocyte vacuolation/ballooning and necrotic region, respectively.

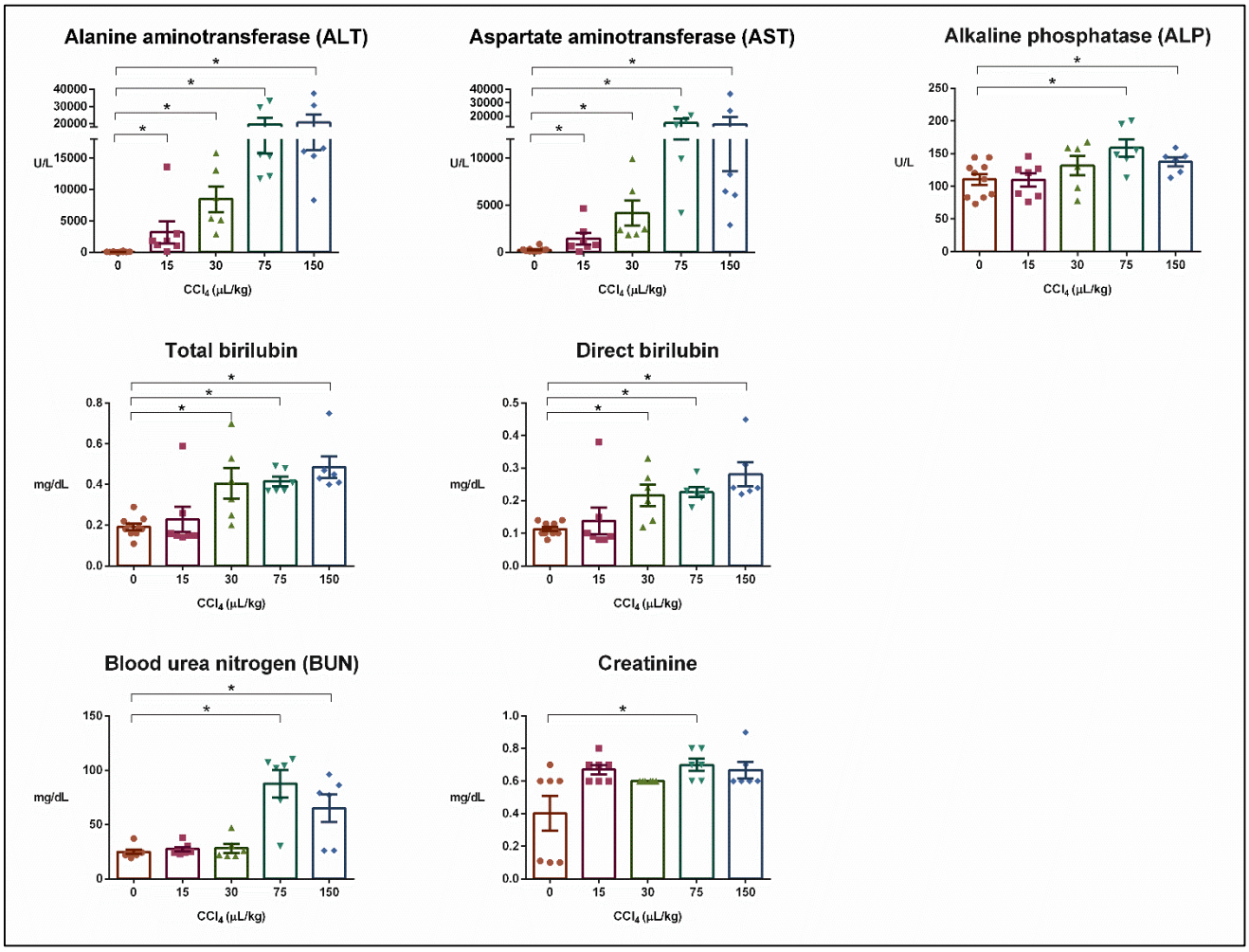

Figure 3. Serum biomarkers in ICR outbred mice received various dosages of CCl_4. Scatter plot represents individual values. Bar graphs and corresponding error bars indicate means and SEM among the same treatment, respectively (n=6-10). * indicates $p<0.05$ when compared to 0 μL/kg CCl_4. U = units.

cells, implicating a sign of tissue inflammation, were appeared adjacently to the central veins. We could not be able to detect necrotic region, a fused area of hepatocyte death at this lowest tested dosage of CCl_4. At 30 μL/kg of CCl_4, necrotic regions which indicated the dissemination of death hepatocytes implicating a high degree of liver damage could be seen around central veins with infiltrated inflammatory cells. The necrotic regions were evidently remarked at 75 and 150 μL/kg CCl_4.

In line with the histological damage against CCl_4, increased levels of serum ALT and AST appeared to be in a dosage-dependent manner (Figure 3). However, the level of ALT and AST at 75 μL/kg (19,531 U/L and 15,068 U/L, respectively) were close to the level at 150 μL/kg (20,738 U/L and 14,047 U/L, respectively). Although the levels of ALP were significantly increased at 75 and 150 μL/kg, it should be noted that the increased ALP were in a lower magnitude when compared to those of ALT and AST. At 15 μL/kg of CCl_4, the levels of bilirubin, both quantified as total (conjugated and unconjugated) and direct (conjugated) were not obviously raised when compared to other higher dosages. At 75 μL/kg and 150 μL/kg, the levels of BUN were significantly increased. In contrast, the levels of creatinine were not consistently raised.

Since the liver damage resulting from CCl_4 could be observed at 15 μL/kg, we selected this lowest tested dosage to compare with 150 μL/kg in the next step. In fact, CCl_4 at both 75 and 150 μL/kg could induce liver damage in a comparable magnitude; however, CCl_4 at 150 μL/kg (10-fold higher than 15 μL/kg) was selected to assure that the tested conditions were totally different.

3.2. Effects of reference drugs against low dosage of CCl_4 (15 μL/kg)

Rebamipide and silymarin showed hepatoprotective effects against 15 μL/kg of CCl_4, the lowest tested dosage (Figure 4). Although the gross appearances were indistinguishable since nodule formation could be observed in all treatments, H&E staining showed that the hepatocyte vacuolation and ballooning around central veins in mice received rebamipide or silymarin appeared to be seen at a limited region only. In contrast, these abnormal

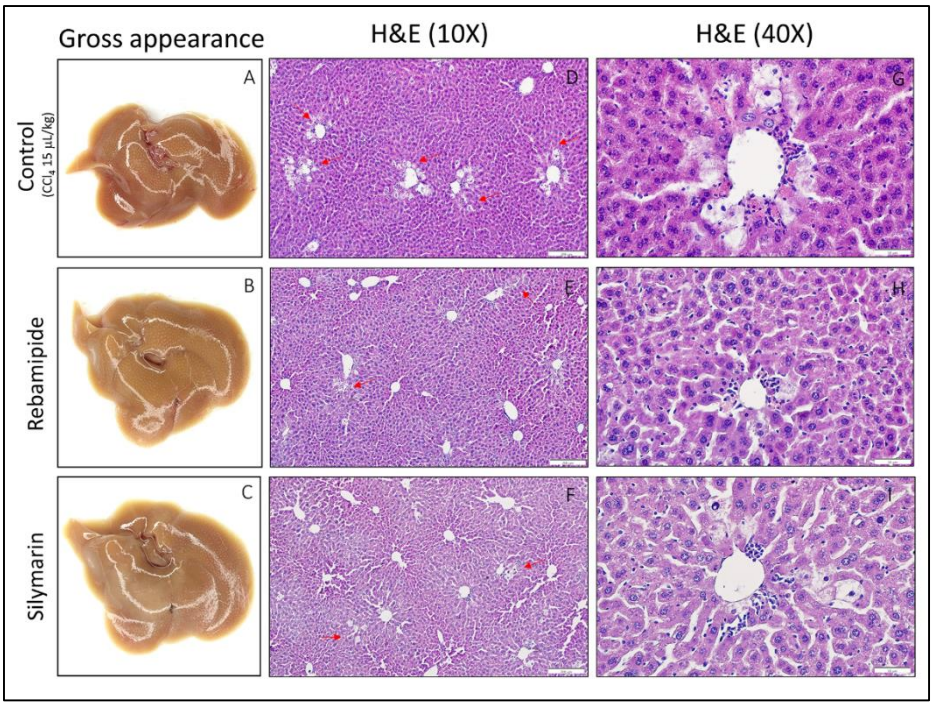

Figure 4. Representative gross appearances (A-C), hematoxylin/eosin (H&E) staining at 10X (D-F, scaled bar = 200 µM), and at 40X (G-I, scaled bar = 50 µM) in the liver of ICR outbred mice administered rebamipide (300 mg/kg) or silymarin (100 mg/kg) to protect against 15 µL/kg CCl_4-induced liver injury. A, D, G = control; B, E, H = rebamipide; C, F, I = silymarin. Arrows (↑) indicate hepatocyte vacuolation/ballooning.

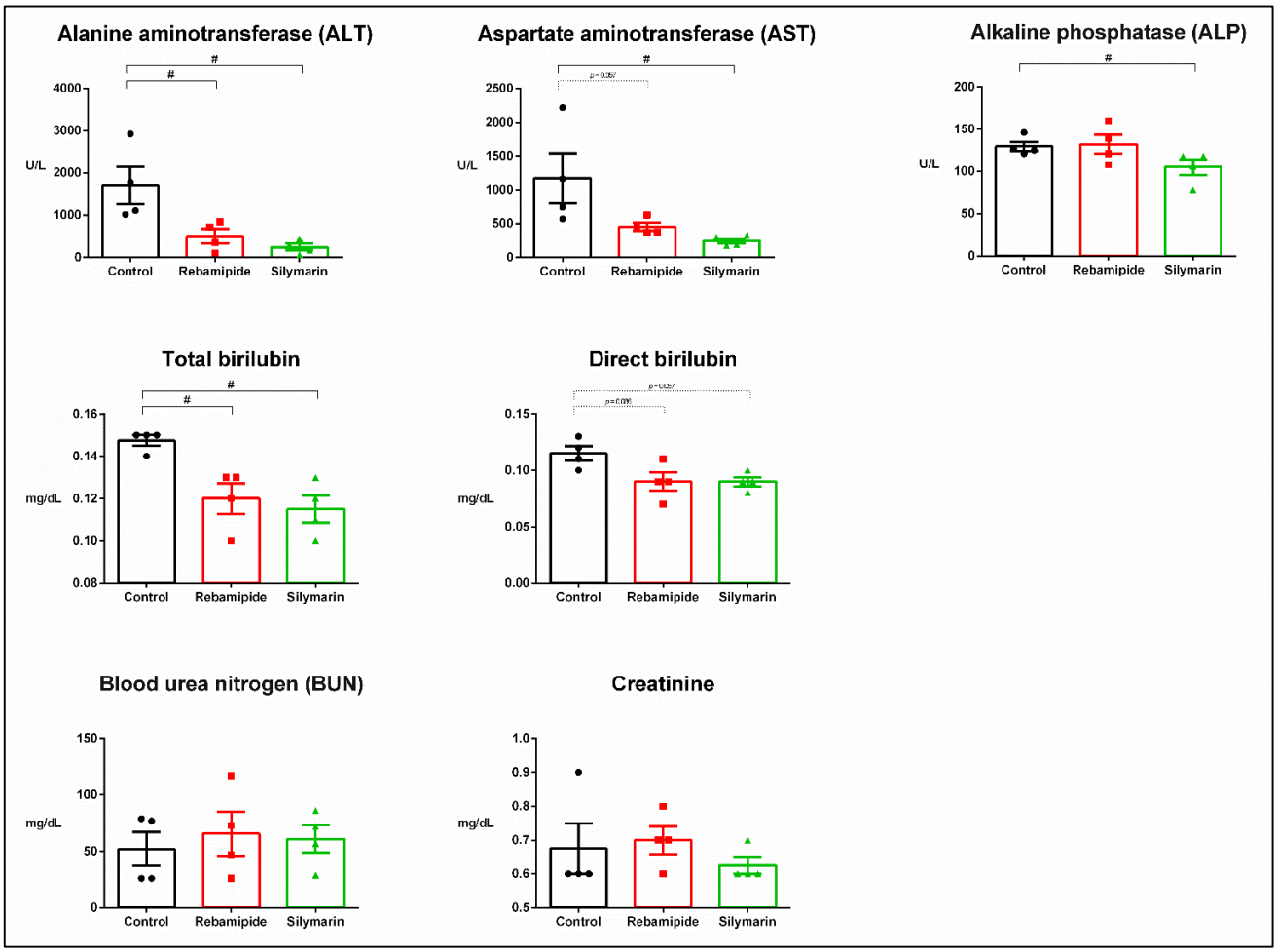

Figure 5. Serum biomarkers in ICR outbred mice administered rebamipide (300 mg/kg) or silymarin (100 mg/kg) to protect against 15 µL/kg CCl_4-induced liver injury. Scatter plot represents individual values. Bar graphs and corresponding error bars indicate means and SEM among the same treatment, respectively (n=4). # indicates $p<0.05$ when compared to control. U = units.

hepatocytes could be noticeably seen in control. However, infiltrated inflammatory cells were observed in the control and drug treatments.

In accordance with liver histology, rebamipide and silymarin apparently reduced serum levels of ALT, AST, and total bilirubin when compared to those of control (Figure 5). Even though, the decrease of AST in mice received rebamipide was not statistically significant. Silymarin also slightly decreased ALP. However, it should be reminded that the levels of bilirubin and ALP were unaltered in response to 15 μL/kg of CCl_4 (Figure 3). Although the level of direct bilirubin in the serum of mice received both drugs seemed to be lower than that of control, the differences were not statistically significant. Conversely, serum BUN and creatinine of mice were unchanged.

3.3. Effects of reference drugs against high dosage of CCl_4 (150 μL/kg)

Against 150 μL/kg of CCl_4, the highest tested dosage, rebamipide and silymarin were unable to protect liver from injury (Figure 6). As seen in the control, nodule formation and hemorrhagic area could be similarly observed in all treatments. Moreover, H&E staining showed that the livers of mice received drugs comprised of disseminated necrotic regions as observed in the control.

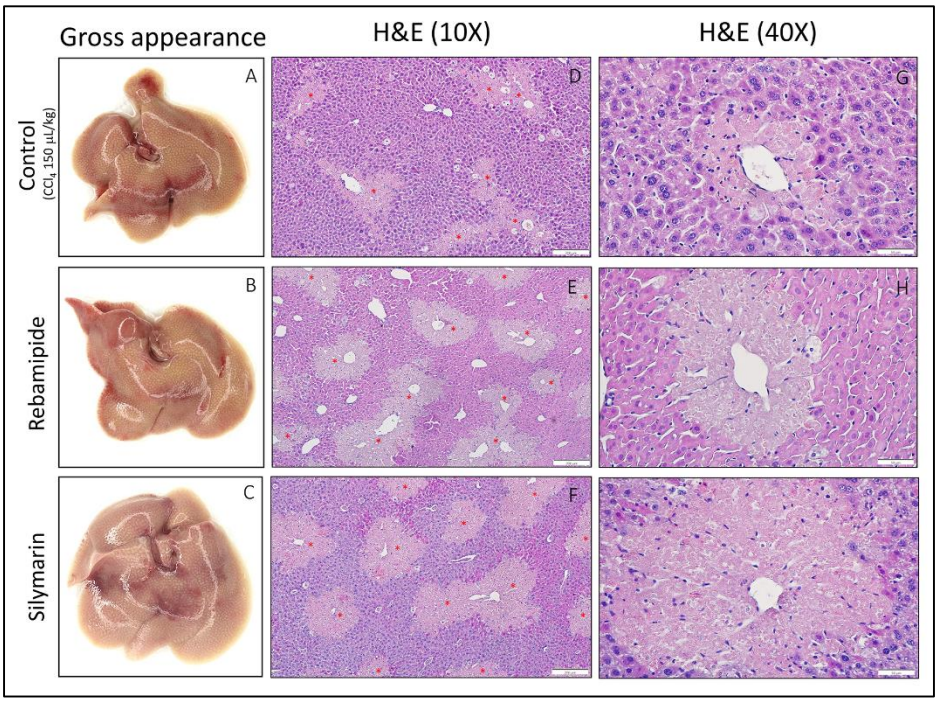

Figure 6. Representative gross appearances (A-C), hematoxylin/eosin (H&E) staining at 10X (D-F, scaled bar = 200 μM), and at 40X (G-I, scaled bar = 50 μM) in the liver of ICR outbred mice administered rebamipide (300 mg/kg) or silymarin (100 mg/kg) to protect against 150 μL/kg CCl_4-induced liver injury. A, D, G = control; B, E, H = rebamipide; C, F, I = silymarin. Asterisks (*) indicate necrotic region.

Correlate with the liver histology, all serum biomarkers of mice received rebamipide or silymarin appeared to be comparable to those found in the control (Figure 7). Furthermore, we found that the average levels of AST in mice received drugs were significantly higher than that of control.

4. DISCUSSION

A mouse model of CCl_4-induced acute liver damage is often used to simulate liver injury in human due to its potency and reproducibility[1]. Nevertheless, the dosage of CCl_4 for the induction of liver injury were diverse, even with the same tested species. In a study showing hepatoprotective effects of blue honeysuckle in ICR mice with a similar age and sex as used in our study, 500 μL/kg of CCl_4 was selected to induce liver damage, and this study revealed that the 500 μL/kg dosage increased the average levels of ALT and AST to be approximately 2-3 folds when compared to intact control[5]. In contrast, our study showed that 15 μL/kg of CCl_4, approximately 30-fold relatively lower amount, was sufficient to induce liver damage as seen via histological analysis and increased liver enzymes to be more than 50- and 16-folds for ALT and AST, respectively, when compared to control. Our preliminary found that the high dosage of CCl_4 at 500 μL/kg also caused watery diarrhea, a common systemic effect of CCl_4 intoxication as observed by wet and brownish bottom in mice[12]. However, the diarrhea was unnecessary for this model. In addition, the histological liver damages and increased biomarkers in mice received 500 μL/kg were comparable to those

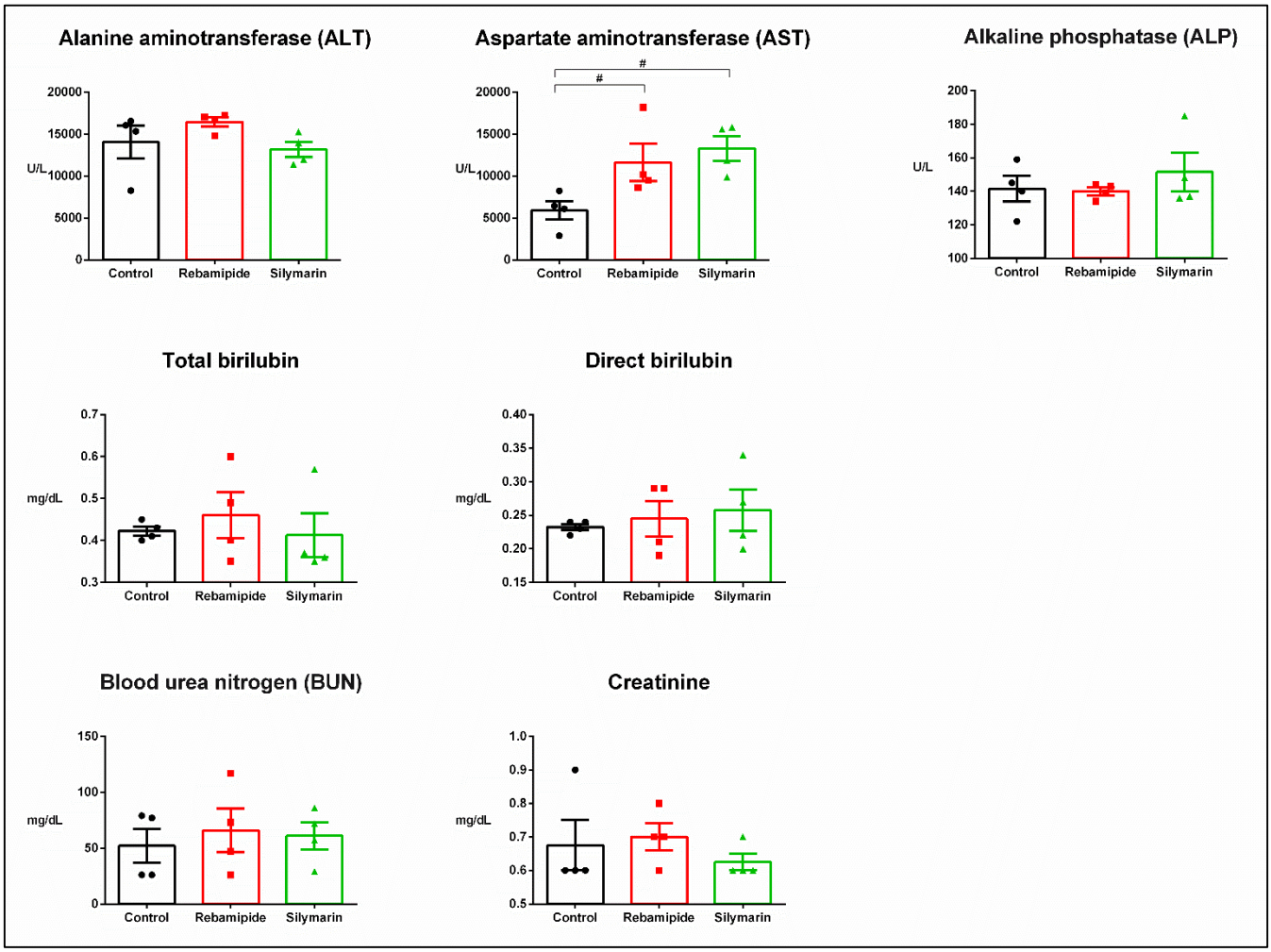

Figure 7. Serum biochemical measurements of ICR outbred mice administered rebamipide (300 mg/kg) or silymarin (100 mg/kg) to protect against 150 μL/kg CCl_4-induced liver injury. Scatter plot represents individual values. Bar graphs and corresponding error bars indicate means and SEM among the same treatment, respectively (n=4). # indicates $p<0.05$ when compared to control. U = units.

received 150 μL/kg of CCl_4. Furthermore, we found that silymarin could not demonstrate its hepatoprotective potency against 500 μL/kg of CCl_4. Therefore, 500 μL/kg of CCl_4 was considered to be an excessive induction of liver damage for our ICR outbred mice. Even though we were unable to compare the background of mice in this previous study to the outbred mice in our experiment, a myriad of factor, such as genetic origin, breeding procedure, and housing environment, would possibly be the reason for the difference in response against CCl_4 and drugs. Interestingly, the diversity of response against CCl_4 in other mouse strains or even among substrains was reported[13]. Therefore, to initiate an experiment, the dosage optimization of CCl_4 is primarily recommended.

Most importantly, the hepatoprotective effects of rebamipide and silymarin could be observed only at 15 μL/kg, but not at 150 μL/kg of CCl_4. This finding emphasized the necessity in the dosage selection of CCl_4 to evaluate hepatoprotective effects of putative agents. For silymarin, we found that at 100 mg/kg, which was used as a hepatoprotective reference in many studies[5,9], elicited protective effect against 15 μL/kg of CCl_4. Therefore, this dosage was primarily chosen to indicate the least sufficient amount of CCl_4 for optimizing the protocol. However, silymarin at other dosages might be appropriate in different conditions. In our preliminary study, 100 mg/kg of rebamipide was selected due to this dosage demonstrated hepatoprotective effects in a rat model of circulatory shock induced by bacterial endotoxin[14]. Nonetheless, we found that the 100 mg/kg of rebamipide could not protect the liver against 15 μL/kg of CCl_4. Thus, rebamipide at 300 mg/kg was evaluated and revealed its hepatoprotective effects. Besides the previous study against endotoxin, rebamipide also demonstrated its protective effect in ischemia/reperfusion liver injury[15]. However, in-depth beneficial mechanism against acute exposure of CCl_4 remains to be further elucidated. Focusing on the protocol, our finding implied that the amount of both hepatoprotective agents and CCl_4 must be optimal. Besides dosages, duration of drug treatment should be considered. In general, 3-7 days of pretreatment are preferentially used, depending on pharmacokinetic and pharmacodynamic properties of individual drug.

Biomarkers which highly correlated with the acute

hepatic damages were ALT and AST, whereas ALP, total bilirubin, and direct bilirubin appeared to be less dependent. In contrast to ALT and AST which are abundant in hepatocytes, ALP is mostly found in bile canaliculi and it is commonly used to signify biliary disorders[16]. According to our results, the levels of ALP appeared to be unchanged reflecting that the exposure of CCl_4 at the tested dosage might not directly injure cells in biliary tract. In addition, this finding implicated that, when compared to ALT and AST, serum ALP appeared to be less sensitive in response to the effect of CCl_4. BUN and creatinine, indicators for signifying kidney function, were quantified to compare their sensitivity with liver-related biomarkers, and we found that they were not significantly altered in response to CCl_4, especially in the lower dosages. Interestingly, since BUN is used to indicate serum urea, the end product of ammonia detoxification in the liver, this biomarker would possibly reflect liver function. Also, serum level of BUN could be increased in certain liver disorders, such as non-alcoholic fatty liver disease (NAFLD)[17]. Therefore, the increment of BUN in mice received 75 and 150 μL/kg CCl_4 might be associated with the liver injury. Nevertheless, the alteration of these biomarkers may be dissimilar in other species.

It is worthwhile to discuss why the average levels of AST in mice received drugs was significantly higher than that of control. In fact, the maximal individual value of AST was approximately 18,000 U/L. This level of AST was greatly less than the highest level observed in mice received 150 μL/kg of CCl_4 in the experiment to demonstrate dosage-dependent effects. Therefore, rebamipide, and also silymarin, might not affect liver damage in this condition. The observed statistical significance would possibly be due to the inadvertently difference in drug response between control and treatments of outbred mice.

Besides the dosages of CCl_4 and biomarkers for evaluating liver injury, the time point for evaluating drug effects is essential. As demonstrated in a previous study, at 24-hours after the mice received CCl_4 was the time that serum AST and ALT were maximally elevated. In addition, this critical time was the same period showing the highest damaged hepatic histology[4]. Finally, the route of CCl_4 administration is another factor to be considered. Besides intraperitoneal injection as conducted in our study, CCl_4 may be administered via oral or inhalation route[1]. Nonetheless, for researchers with skillful laboratory technique, intraperitoneal injection might be the most precise and simplest procedure for delivering the definite amount of CCl_4 to the rodents.

5. CONCLUSION

This study demonstrated how to perform an experiment to select the optimal dosage of CCl_4 for testing the hepatoprotective effect of putative agents in ICR outbred mice, and our procedure could be applied for initiating the experiment in other settings. Silymarin could be used as a reference drug in the experiment. In addition, together with histological evaluation, we showed that ALT and AST could be used as a reliable biomarker for assessing hepatoprotective effects of drug in response to acute CCl_4 exposure. However, the dosage of silymarin and the sensitivity of liver-related biomarkers in other studies might be unequalled to our experiment.

6. ACKNOWLEDGEMENT

We would like to thank Thai Otsuka Pharmaceutical for providing rebamipide.

Conflict of interest
No conflict of interest.

Funding
This research project was supported by Mahidol University.

Ethics approval
This study was approved by the Animal Ethical Committee of the Faculty of Pharmacy, Mahidol University (PYR002/2019 and PYR001/2020).

Article info:
Received June 8, 2022
Received in revised form August 11, 2022
Accepted August 22, 2022

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