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Hepatoprotective Effect of Silymarin Peptide on Carbon Tetrachloride-Induced Acute Liver Injury in Mice

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Running title: Role of silymarin peptide in CCL4-induced ALI

Objective: Liver diseases and injuries are significant global health concerns; in particular, acute liver injury (ALI) is a prominent cause of liver diseases and is associated with high morbidity and mortality. The application of natural products in preventing and treating liver diseases is considerable. Silymarin and silymarin peptides derive from the Milk thistle (Silybum marianum). Still, they differ in their composition and effects: Silymarin is a complex mixture of flavonoids, primarily made up of silybinin, silybinin, and silychristin. Silymarin is well-known for its antioxidant, anti-inflammatory, and hepatoprotective properties. It is often a dietary supplement to support liver health. Silymarin Peptide refers to specific peptides derived from silymarin. These peptides have more enhanced bioavailability and activity of effect compared to the whole silymarin compound. Therefore, this study aimed to investigate the hepatoprotective effects of silymarin peptide on acute liver injury (ALI) in mice induced by carbon tetrachloride (CCl4) compared with Silymarin. **Methods:** Forty-eight male C57BL/6J mice were randomly divided into six groups (n=8 per group): Control group: regular saline+olive oil, Negative control group: 10% CCl4 solution (10 μl/g), Treatment group 1: CCl4+50 mg/kg silymarin peptide, Treatment group 2: CCl4+100 mg/kg silymarin peptide, Treatment group 3: CCl4+200 mg/kg silymarin peptide, and Positive control group: CCl4+100 mg/kg silymarin. The treatment of silymarin was used as a positive control. At the end of the experiments, mice were euthanized, and the blood and liver samples were collected. **Results:** The results showed that silymarin peptide ameliorated the histopathological damage of liver tissues caused by CCl4 and decreased the CCl4-induced serum AST and ALT levels, among which the 200 mg/kg dose demonstrated the most notable protective effect. Additionally, silymarin peptide showed no significant influence on CCL2 levels but markedly reduced TNF-α and CXCL5 levels, with the most apparent impact at 100 mg/kg. Finally, the terminal deoxynucleotidyl transferase-mediated dUTP-nick end labeling (TUNEL) staining indicated that the 200 mg/kg dose of silymarin peptide restrained CCl4-induced hepatocytic apoptosis. **Conclusion:** Silymarin peptide alleviated CCl4-induced ALI in mice by inhibiting inflammatory cytokines release and decreasing hepatocyte apoptosis.

Keywords: Acute liver injury, Carbon tetrachloride (CCl4), Silymarin, Apoptosis, Hepatoprotective.

Introduction

Liver diseases and injuries are significant global health concerns; in particular, acute liver injury

(ALI) is a prominent cause of liver diseases and is associated with high morbidity and mortality.[1,2] Currently, the approved pharmaceutical interventions for liver injury frequently exhibit many side effects and limited efficacy.[2] Therefore, safer and more effective hepatoprotective drugs remain an unaddressed medical need. The application of natural products in preventing and treating liver diseases is considerable.[3] Silymarin is a polyphenolic component extracted from the fruits and seeds of the Milk thistle family.[4] The silymarin extract contains flavonolignans, flavonoids, fatty acids, and polyphenolic compounds, which possess a range of pharmacological effects, including hepatoprotective, anti-inflammatory, antioxidant, and anti-fibrosis effects.[5-7] Previous studies have shown that silymarin protects carbon tetrachloride (CCl4)-induced liver fibrosis, lipopolysaccharide (LPS)-induced liver injury, and drug-induced liver injury.[8-9] CCl4 is commonly used as an inducing agent in animal models of ALI, which is employed to explore potential therapeutic strategies due to its similarity to ALI in humans.[10-11] Based on this evidence, this study investigated the hepatoprotective effects of silymarin peptide on CCl4-induced ALI in mice. Here, we found that silymarin peptide ameliorated liver histopathological damage, decreased serum AST and ALT levels, reduced TNF-α and CXCL5 levels, and restrained hepatocytic apoptosis in CCl4-induced mice. These findings suggest a previously unidentified effect of silymarin peptide on CCl4-induced ALI, which may provide novel strategies for treating ALI.

Material and Methods

Animal Model and Drug Given.

Eight-week-old male C57BL/6J mice were purchased from SLAC Laboratory Animal Co.Ltd. (Shanghai, China). Mice were raised in a specific pathogen-free (SPF) environment at 22°C, 50–60% humidity, and a 12-hour light and dark cycle. The experiment was conducted one week after the mice had adapted to the new environment. The Institutional Animal Use and Care Committee of Inner Mongolia Medical University approved this study. Forty-eight mice were randomly divided into six groups (n=8 per group): Control group: standard saline+olive oil, Negative control group: 10% CCl4 solution (10 µl/g), Treatment group 1: CCl4+50 mg/kg silymarin peptide, Treatment group 2: CCl4+100 mg/kg silymarin peptide, Treatment group 3: CCl4+200 mg/kg silymarin peptide, and Positive control group: CCl4+100 mg/kg silymarin. CCl4 was diluted with olive oil. For the control group, mice received normal saline daily through gavage for 14 consecutive days and received olive oil through intraperitoneal injection on day 8. For the CCl4 group, mice were given normal saline daily through gavage for 14 consecutive days and received 10% CCl4 solution (10 µl/g) through intraperitoneal injection on day 8. For CCl4+50/100/200 mg/kg silymarin peptide, mice were given 50/100/200 mg/kg silymarin peptide daily through gavage for 14 consecutive days and then received 10% CCl4 solution (10 µl/g) through intraperitoneal injection on day 8. For the CCl4+100 mg/kg silymarin group, mice were given 100 mg/kg silymarin daily through gavage for 14 consecutive days and then received 10% CCl4 solution (10 µl/g) through intraperitoneal injection on day 8. Mice in the control group and CCl4 group received an equal volume of normal saline to silymarin peptide treatment groups, and mice in the control group received an equal volume of normal saline to the CCl4 group. At the end of the experiment, blood samples were collected, and the mice were sacrificed.

Blood and Tissue Sample Collection

The orbital blood collection was performed after modeling, kept at room temperature for 30 minutes, and centrifuged at 3000 rpm for 15 minutes. The upper supernatants were obtained, transferred into a new tube, and stored at −80°C. Liver tissues were collected, frozen in liquid nitrogen for proteins and RNA extraction, or fixed a patch with 4% paraformaldehyde for histological staining.

Hematoxylin and Eosin (H&E) Staining of Liver Tissues

The liver tissue was fixed with 4% paraformaldehyde overnight. The fixed samples were embedded in paraffin and sectioned into slices with 3-4 µm thickness, which were then dehydrated with different concentrations of ethanol and xylol, followed by staining with 5% hematoxylin solution for 10 minutes. After washing, the sections were incubated in 0.1% HCl-ethanol for 30 s and counterstained with eosin solution for 2 minutes. The liver lesions were observed under a fluorescence microscope (IX-51, Olympus), and Suzuki's pathological score was used as a semi-quantitative method to evaluate necrosis, congestion, and vacuole-like changes. Suzuki's pathologic score standard was as follows: 0: no necrosis, congestion, or vacuole-like change in hepatocytes; 1: mild congestion and acceptable vacuole-like changes (<10%), occasionally single cell necrosis;

2: mild congestion and cell vacuolation (11%-30%), mild tissue necrosis (< 30%); 3: moderate and severe congestion, cellular vacuolation, tissue necrosis (31%-60%).

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) detection assay

To evaluate liver injury, the levels of AST and ALT in the serum of mice were detected. According to the manufacturer's guidelines, a Hitachi 7600 automatic biochemical analyzer was employed to detect the serum ALT and AST levels in mice.

Enzyme-linked immunosorbent assay (ELISA)

The levels of serum TNF-α, CCL2, and CXCL5 were detected using mouse TNF-α (Solarbio, Beijing, China), mouse CCL2 (Solarbio), and mouse CXCL5 (Solarbio) ELISA kits according to the manufacturer's instructions.

TUNEL Staining

Hepatocyte apoptosis in liver tissues was assessed by performing the terminal deoxynucleotidyl transferase-mediated dUTP-nick end labeling (TUNEL) assay. The staining was constructed on paraffin-embedded liver sections using the TUNEL apoptosis assay kit (Sangon Biotech, Shanghai, China), following the manufacturer's instructions. The nuclei were stained with a DAPI solution (Servicebio, Wuhan, China). Images were captured using fluorescence microscopy, and the positive-staining cells were counted using Image J software.

Statistical Analysis

Data were expressed as mean ± SD and statistically analyzed using SPSS 19.0 software. The differences between groups were analyzed by one-way ANOVA followed by Tukey's post hoc test. $P<0.05$ was considered a significant difference.

Results

Silymarin peptide ameliorated CCl4-induced acute liver injury in mice.

To investigate the protective effects of silymarin peptide on CCl4-induced ALI, the liver histopathology was evaluated by H&E staining. As shown in Fig 1 A, the hepatocytes in the control group exhibited a well-organized, compact, and radial arrangement. In the CCl4 model group, a significant presence of hepatocyte disordered arrangement, ballooning, and abundant inflammatory cell infiltration was observed. In silymarin peptide-pretreatment groups, the impairment of hepatocytes was ameliorated at specified concentrations, with the 200 mg/kg dose demonstrating the most notable protective effect. Similarly, the liver injury score was significantly higher in the CCl4 model group compared to the control group. At the same time, the

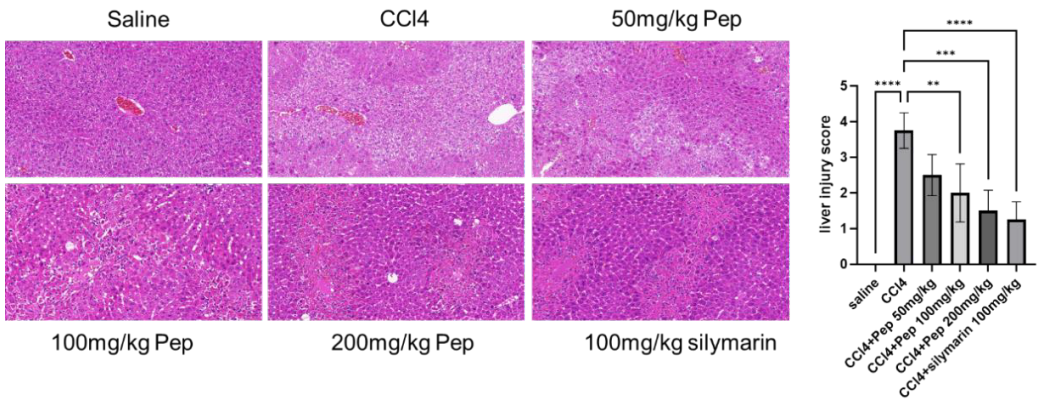

Figure 1. Silymarin peptide ameliorated CCl4-induced acute liver injury in mice.

*(A) Representative histopathological images of H&E staining on liver tissues of mice. (B) The histological scores for liver lesions. n = 8. *P < 0.05, ***P < 0.001, and ****P < 0.0001 vs. control group or CCl4 group.*

different doses of silymarin peptide treatment reduced the liver injury score, and the reduction was more significant at 200 mg/kg dose (Fig1B).

Silymarin Peptide Decreased Serum AST and ALT Levels in CCl4-Induced Mice.

As shown in Fig 2A and B, the serum AST and ALT levels in the CCl4 model group were significantly higher than in the control group. Compared with the CCl4 model group, silymarin peptide pretreatment markedly reduced serum ALT and AST levels, with

the 200 mg/kg dose showing a more distinct effect.

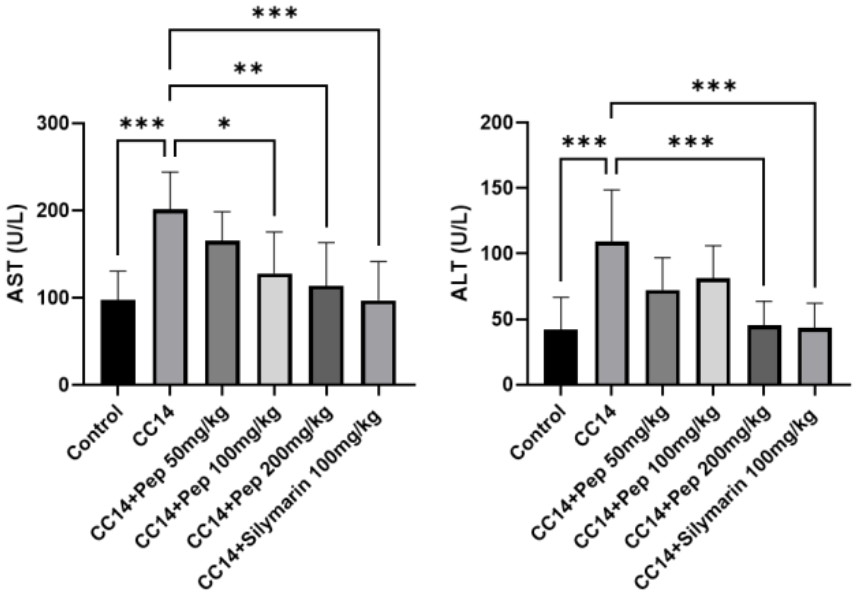

Figure 2. Silymarin peptide decreased serum AST and ALT levels in CCl4-induced mice. *(A, B)*
*Serum AST and ALT levels in mice were detected. n = 8. *P < 0.05 and **P < 0.001 vs. control group or CCl4 group.*

Silymarin Peptide Reduced the Release of Inflammatory Cytokines in CCl4-Induced Mice.

The effect of silymarin peptide on the production of inflammatory cytokines was studied after the CCl4 administration. ELISA results revealed that the CCl4-treated group showed a marked increase of TNF-α, CCL2, and CXCL5 levels in mouse serum compared to the control mice. The silymarin peptide treatment at 50 mg/kg had no significant impact on TNF-α, CCL2, and CXCL5 levels in the serum of CCl4-induced mice, while the 100 or 200 mg/kg dose of silymarin peptide distinctly reduced TNF-α and CXCL5 levels. Meanwhile, the silymarin peptide at 50, 100, and 200 mg/kg doses did not influence the CCL2 level in the serum of CCl4-induced mice (Fig 3A-C).

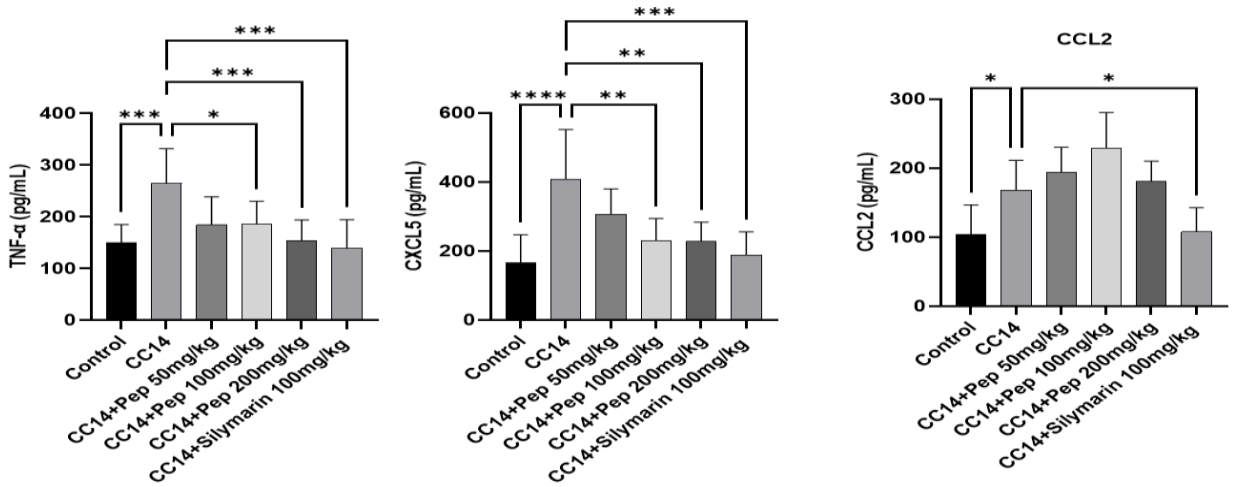

Figure 3. Silymarin peptide inhibited the release of inflammatory cytokines in CCl4-induced mice.

Silymarin Peptide Decreased Hepatocyte Apoptosis in CCl4-Induced Mice.

TUNEL staining was carried out to evaluate the impact of silymarin peptide on hepatocyte apoptosis. CCl4 administration markedly increased TUNEL-positive cells, which was attenuated by a 200 mg/kg dose of silymarin peptide treatment. Notably, the 50 and 100 mg/kg doses of silymarin peptide showed little effect on hepatocyte apoptosis in CCl4-induced mice (Fig 4).

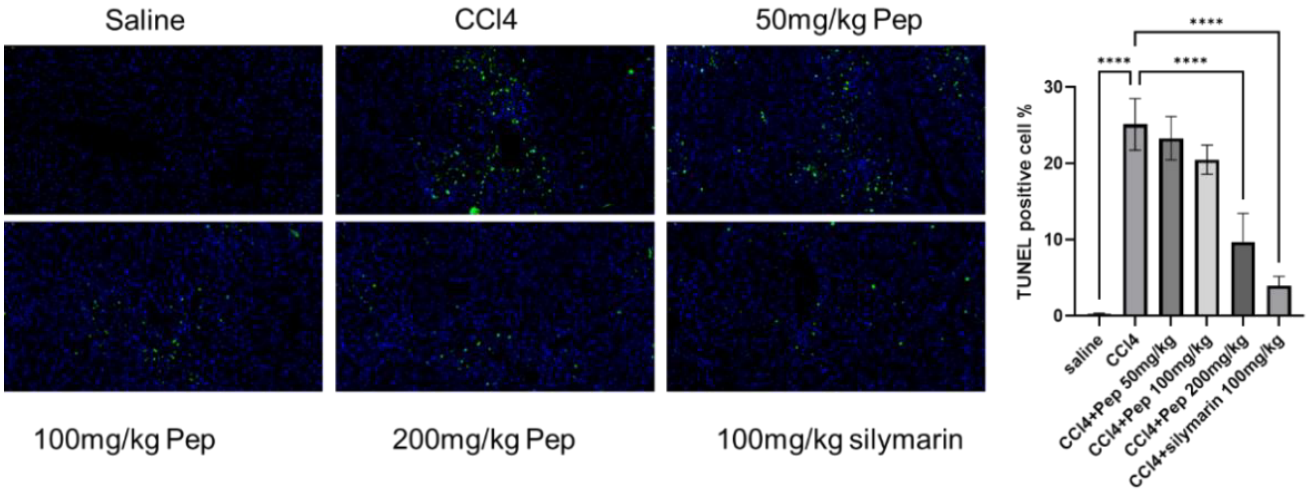

Figure 4. Silymarin peptide reduced hepatocyte apoptosis in CCl4-induced mice.
*TUNEL staining was performed on the liver tissues of mice, and the positive cells were counted and statistically analyzed. n = 8. ***P < 0.001 and ****P < 0.0001 vs. control group or CCl4 group*

Discussion

Silymarin has been reported to have a positive effect as a support for treating various liver diseases.[4] This study identified the role of silymarin peptide in CCl4-indued ALI. It was found that silymarin peptide alleviated histopathological damage of liver tissues, reduced serum AST and ALT levels, decreased inflammatory cytokines levels, and inhibited hepatocyte apoptosis in CCl4-induced mice. The present study discovered a previously unidentified role of silymarin peptide in CCl4-indued ALI. Inflammatory response is one of the crucial pathological mechanisms of CCl4-induced liver injury.[12] During ALI, various inflammatory mediators in liver tissues are released from inflammatory cells, further escalating liver damage.[13-14] In CCl4-induced mice, we found increased liver injury markers such as serum ALT and AST and prominent histopathological damage, including disordered hepatocyte arrangement, hepatocyte balloon formation, and extensive inflammatory cell infiltration. The inflammatory mediators TNF-α, CCL2, and CXCL5 were markedly elevated in mouse serum after CCl4 induction. Significantly, the increased liver injury markers, histopathological damage, and inflammatory mediators (except CCL2) were abrogated by silymarin peptide treatment. These data suggested that silymarin peptide ameliorated CCl4-induced ALI and inhibited inflammatory response. The CCL2 and CXCL5 are also the chemokines that affect the recruitment and infiltration of inflammatory cells.[15] In this study, the CCl4-induced up-regulation of CXCL5 and CCL2 levels may be responsible for the massive recruitment of inflammatory cells in liver tissues, as observed. The reduced recruitment and infiltration of inflammatory cells caused by silymarin peptide may be related to the decrease of CXCL5 level. However, the reason why silymarin peptide does not affect serum CCL2 levels is unclear and needs further investigation.

Hepatocyte apoptosis is the primary pathophysiological process for ALI, which can trigger intracellular or extracellular signals.[16] Numerous studies have demonstrated that apoptosis plays an indispensable role in ALI. For example, Chen. et al, revealed that inhibition of inflammation and apoptosis could attenuate LPS-induced ALI.[17] Dai. et al, confirmed that suppressing inflammation and apoptosis ameliorated CCl4-induced ALI in mice.[18]

Jia. et al, validated that inhibiting oxidative stress and apoptosis alleviated CCl4-induced ALI in mice.[19] In the present study, hepatocyte apoptosis was assessed by performing a TUNEL

assay. Consequently, CCl4 induction led to a marked increase in the number of TUNEL-positive cells in CCl4-challenged mice, and the silymarin peptide treatment profoundly reduced the number of TUNEL-positive cells, indicating that silymarin peptide repressed hepatocyte apoptosis in CCl4-induced ALI. Early studies demonstrated that liver injury would trigger apoptosis, amplifying the pro-inflammatory response, which is consistent with our results.[20] In addition, researchers reported that oxidative stress and mitochondrial dysfunction played a significant role in ALI, involving the activation of inflammatory cells and apoptosis of hepatocytes, thus affecting the pathological progression of ALI.[21-22] This study will focus on this direction to further explore the possible mechanism of silymarin peptide in regulating ALI. Our data indicate that silymarin peptide has a protective effect on CCl4-induced ALI, closely related to inhibiting inflammatory response and hepatocytic apoptosis. Considering that the present study is still in the primary stage, the next step is to investigate the potential mechanism by which silymarin peptide regulates CCl4-induced ALI.

Conclusion

Silymarin peptide alleviated CCl4-induced ALI in mice by inhibiting inflammatory cytokines release and decreasing hepatocyte apoptosis.

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Conflict of Interest

We declare no conflict of interest.

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