Rosmarinic acid protects mice from Concanavalin A-induced hepatic injury through AMPK signaling

Yangyang Wang\textsuperscript{a}, Jie Meng\textsuperscript{a}, Lu Men\textsuperscript{a}, Boran An\textsuperscript{a}, Xiaoxu Jin\textsuperscript{b}, Wenjuan He\textsuperscript{c}, Sucai Lu\textsuperscript{a}, Na Li\textsuperscript{d,*}

\textsuperscript{a} Department of Gastroenterology and Hepatology, Affiliated Hospital of Hebei University; Baoding, Hebei Province, 071000, China;
\textsuperscript{b} Department of Gastroenterology and Hepatology, Hebei Medical University No.2 Hospital; Shijiazhuang, Hebei Province, 050000, China;
\textsuperscript{c} Internal Medicine Department, Yi Country Hospital; Baoding, Hebei Province, 071000, China; and
\textsuperscript{d} Department of Physiology, Hebei University College of Medicine; Baoding, Hebei Province, 071000, China.

Corresponding author: Na Li, Department of Physiology, Hebei University College of Medicine; Baoding, Hebei Province, 071000, China. E-mail: lina001823@126.com.
Summary

Rosmarinic acid (RA) is extensively utilized in herbal medicine in China. The adenosine 5'-monophosphate-activated protein kinase (AMPK) signaling can be activated by RA and inhibited by the synthetic, reversible AMP-competitive inhibitor, Compound C (CC). The objective of this study was to investigate the role of AMPK signaling involving the protective effects of RA on concanavalin A (Con A)-induced (AIH) in mice. BALB/c mice were treated with RA, with or without CC, followed by the pretreatment with concanavalin A (Con A). Analysis of serum aminotransferases and cytokines were conducted and liver tissue histology was performed to evaluate hepatic injury. Cytokine levels in serum and hepatic tissue were respectively measured by enzyme-linked immunoassay (ELISA) and used quantitative polymerase chain reaction (qPCR). Levels of phosphorylated acetyl coenzyme-A carboxylase in the liver, representing AMPK activation, were detected by Western blotting. Compared with the Con A group, serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in RA group (100 and 150 mg/kg/day) were significantly reduced. RA also reduced hepatocyte swelling, cell death, and infiltration of leukocytes in the liver of Con A-treated mice. Serum levels of cytokines, such as interferon-γ (IFN-γ), interleukin-2 (IL-2) and interferon-1 (IL-1β), were reduced by RA pretreatment, while the levels of serum interferon-10 (IL-10), an anti-inflammatory cytokine, was elevated. These protective effects were reversed by treatment with CC. RA treatment reduced the hepatic damage via the activation of AMPK in the mice of Con A-induced. So RA acts as a potential part in the therapy of autoimmune hepatitis.

Keyword: Rosmarinic acid; autoimmune hepatitis; adenosine 5'-monophosphate protein kinase; Concanavalin A.
Introduction:
Liver disease is a type of damage to or disease of the liver. Parasites and viruses can infect the liver, causing inflammation that reduces liver function. The most common types of liver disease include hepatitis B, alcoholic liver disease, fatty liver disease, and autoimmune hepatitis (AIH).

AIH is a severe liver disease that arises in genetically predisposed male and female individuals worldwide. It is characterized by a hepatocellular pattern of elevated levels of liver enzymes, positive tests for non-disease-specific antinuclear antibodies (ANA) and/or smooth muscle antibodies (SMA), which are the histological hallmark of AIH interface hepatitis on liver biopsy, and an optimal response to steroids in the majority of patients. If AIH is remained untreated, it may lead to end-stage liver failure. Standard treatment for AIH is based on steroids and azathioprine, and may result in disease remission in 80-90% of patients. To our knowledge, steroids and azathioprine have been reported with various side-effects. Thus, finding an optimal therapeutic approach for AIH with few adverse reactions is highly vital.

Rosmarinic acid (RA) is the ester of caffeic acid and 3,4-dihydroxyphenyl lactic acid that is widely identified in different plant species and is extensively utilized in herbal medicine in China. It has been shown to possess a wide range of biological activities including antioxidant, anti-inflammatory, anticancer, antimicrobial, neuroprotective, and cardioprotective and radioprotective effects. A number of scholars reviewed the hepatoprotective effects of RA. Besides, its protective role against lipopolysaccharide (LPS)-induced liver injury has been reported. The protective mechanism of RA on liver of mice was confirmed by the effects of anti-tumor necrosis factor (TNF) and superoxide dismutase (SOD) via reducing the serum level of aspartate aminotransferase (AST).

Dracocephalum heterophyllum (D. heterophyllum) is a traditional Tibetan medicine that possesses various pharmacological effects involved in anti-inflammatory, antibacterial activities. A study reported that RA is one of the main compounds of D. heterophyllum. Although RA can alleviate liver injury induced by inflammation, immune unbalance, and oxidative
stress, to date, no study has reported its capability to relieve AIH.

Neutrophil infiltration, hepatocyte apoptosis, and immune imbalance are the main mechanisms of AIH. concanavalin A (Con A) animal model, which is a typical T cell and macrophage-dependent model, mimics the mechanisms and characteristics of clinical AIH.⁶)

In AIH research, a mouse model of Con A-induced liver injury is highly similar to the pathogenesis and pathological changes of patients.⁷) As a classical immune disease, rheumatoid arthritis is treated by steroids and azathioprine. Besides, it has been shown to suppress synovitis in a murine collagen-induced arthritis (CIA) model.⁸)

Rheumatoid arthritis develops when the immune system produces antibodies that attach to the linings of joints. Immune system cells then attack the joints, causing inflammation, swelling, and pain.⁹)

The adenosine 5'-monophosphate-activated protein kinase (AMPK) is a highly preserved sensor of cellular energy status, and appears to exist in essentially all eukaryotes as heterotrimeric complexes composed of a catalytic α subunit and regulatory β and γ subunits.¹⁰) Adiponectin, statins, berberine, and metformin were used as a positive control to verify the activation of AMPK in cellular models.¹¹,¹²)

Intracellular deficiency in ATP activates AMPK, which, in turn, promotes catabolic processes and inhibits anabolic processes by phosphorylation of multiple substrates, including acetyl-coenzyme A (CoA) carboxylase (ACC) and hydroxymethylglutaryl-CoA (HMG-CoA) reductase. A study reported that RA activates AMPK to inhibit metastasis of colorectal cancer.¹³) Another research demonstrated that RA increases skeletal muscle cell glucose uptake and activates AMPK.¹⁴)

The objective of this study was to investigate the effects of RA on Con A-induced liver injury in mice via AMPK signaling.

Material and Methods:

Antibodies and reagents

RA, Con A, and CC were purchased from Sigma-Aldrich (St. Louis, MO, USA).
Primary mouse monoclonal antibodies against ACCα and ACCβ, and anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). RA was dissolved in 5% dimethyl sulfoxide (DMSO).

**Animals**

Adult male BALB/c mice (age, 6–8 weeks old; body weight, 19±1 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China), and kept at 25 °C under a 12-h light/dark cycle (09:00 a.m.-09:00 p.m.) with free access to food and water. All experimental procedures were conducted in accordance with the 8th edition of the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH, Bethesda, MD, USA). The animal experimental protocol was approved by the Institutional Animal Care and Use Committee of Hebei University (Baoding, China).

First, to study the effects of RA on hepatic injury, mice were assigned to the groups of control, Con A, Con A+RA (50, 100, and 150 mg/kg) and RA alone (see Fig. 1A). Next, to investigate whether RA activates AMPK, mice were divided into control and different RA groups (50, 100 and 150 mg/kg) (see Fig. 4A). Besides, to assess the role of AMPK activation in RA effects, mice were divided into 4 groups, including control, Con A, RA and RA+CC groups (see Fig. 4D).

**Analysis of aminotransferases and cytokines**

Herein, 12 h after injection of murine intravenously with Con A (20 mg/kg), blood samples were collected from the murine heart under anesthesia, and we centrifugated the blood samples at 1000×g for 10 min to acquire serum samples. Serum levels of AST and alanine aminotransferase (ALT) were measured using a fully automated biochemical analyzer (7170; Hitachi, Tokyo, Japan). Levels of IFN-γ, IL-2, IL-1β, and IL-10 were detected by ELISA kits, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

**Histopathological evaluation**

A portion of mouse hepatic tissue was harvested 12 h after intravenously
administration of Con A, fixed in 4% paraformaldehyde for 24 h, and embedded into paraffin. Sections (3-μm thick) were cut onto glass slides and stored at room temperature. The paraffin-embedded tissues were then stained with hematoxylin and eosin (H&E) for histopathological evaluation.

**RNA extraction and quantitative polymerase chain reaction (qPCR)**

After 12 h of Con A injection (20 mg/kg), a 2 mg sample of hepatic tissue was collected. Total RNA was acquired from hepatic tissue of mice using RNA-TriQuick reagent (Solarbio, Beijing, China). After quantification, RNA was reversely transcribed to complementary DNA (cDNA) using a first-strand synthesis kit (Sigma-Aldrich, St. Louis, MO, USA). Amplification was performed in a total volume of 20 µl comprising the cDNA template, primers, 2×Taq PCR Master Mix (Solarbio, Beijing, China).

The standard PCR conditions were as follows: at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and at 60 °C for 34 s. The samples were run on an MX3000P qPCR detection system (Stratagene, San Diego, CA, USA). The amounts of target genes were detected and normalized to the corresponding β-actin results. The primer sequences used for qPCR are presented in Table 1.

**Western blot analysis**

The liver tissue samples of mice were lysed in lysis buffer consisting of 100 mM Tris-HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml pepstatin A, 20 mM Na3P2O7, and 2 mM Na3VO4. Total protein was extracted and measured using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of protein (20-40 µg) were resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinyl difluoride (PVDF) membranes. Membranes were blocked in 5% (w/v) dried milk powder in Tris-buffered saline with Tween 20 (TBST) for at least 2 h at room temperature, and then incubated overnight with particular primary antibody. The PVDF membranes were washed with TBST for three times, and then, incubated with appropriate
HRP-conjugated secondary antibodies. The protein bands were visualized by enhanced chemiluminescence using an enhanced chemiluminescence (ECL) Western Blotting Detection kit (Sigma-Aldrich, St. Louis, MO, USA). Eventually, the images were observed by autoradiography.

**Statistical Analysis**

The data were expressed as mean ± standard deviation (SD). Groups were compared by One-way analysis of variance (ANOVA) and Dunnett’s multiple comparison tests using GraphPad Prism 5.01 (GraphPad Software Inc., San Diego, CA, USA). P<0.05 was considered statistically significant.

**Result**

**RA alleviated Con A-induced liver injury**

Mice received intraperitoneal injection of solvent or RA (50, 100, and 150 mg/kg/day) for 7 consecutive days. Mice were given a single intravenous injection in tail with Con A (20 mg/kg) 12 h before to be sacrificed. As RA alone group, RA (150 mg/kg/day) was intravenously injected into mice.

To assess the effects of RA, we detected the serum levels of ALT and AST in mice. We found that those levels were noticeably increased 12 h after Con A injection compared with control group (P < 0.01; Fig. 1B and 1C). Compared with the Con A group, the serum levels of ALT and AST in the medium- and high-dose RA-treated group showed an obvious decreasing trend, which was statistically significant (P <0.01). However, there was no significant difference between the two groups (Fig. 1B and 1C) (P > 0.05). However, the serum levels of ALT and AST in low-dose RA-treated group were reduced compared with Con A group (P > 0.05). Besides, no statistical difference was noted between control group and RA alone group (150 mg/kg) (P > 0.05; Fig. 1B and 1C). The above-mentioned results indicated that RA had an influence on Con A-induced liver injury in mice according to reduction of the serum levels of ALT and AST.
As illustrated in Fig. 1D, liver of mice was stained with H&E to assess histopathological changes. We noted that histology of liver tissue was normal in both control group and RA alone group. In contrast to control group, the liver tissue in Con A group showed severe pathological changes. However, the liver tissue in medium- and high-dose RA-treated groups (100 and 150 mg/kg) was dramatically improved compared with that in the Con A group. Thus, it can be concluded that administration of RA reduced the cell swelling, necrosis, and monocytes infiltration.

**Effects of RA on pro- and anti-inflammatory cytokines in Con A-induced liver injury in mice**

Con A-induced liver injury was found to be associated with the release of cytokines, as indicated by ELISA findings. It was unveiled that compared with the control group, the serum levels of IFN-γ, IL-1β, IL-2, and IL-10 were remarkably elevated 12 h after Con A injection (Fig. 2, P < 0.01). The serum levels of IFN-γ, IL-1β, and IL-2 were notably decreased after the administration of RA (100 and 150 mg/kg) in Con A -induced liver injury in mice (Fig. 2A-2C, P < 0.01), while the serum level of IL-10 was increased (Fig. 2D, P < 0.01). However, the serum levels of cytokines in low-dose RA- treated group (50 mg/kg) were not significantly different compared with control group (P>0.05). No significant differences were found in the serum levels of IFN-γ, IL-1β, and IL-2 between the high-dose RA-treated group (150 mg/kg) and the control group (Fig. 2A–2C, P>0.05), while the serum level of IL-10 was noticeably increased (Fig. 2D, P < 0.01).

We additionally studied the mRNA levels of IL-1β, IL-2, IFN-γ, and IL-10 by qPCR. Total RNA was extracted from liver tissue of mice using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then, DNA was synthesized with RNA as the template using a cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA). The results showed the mRNA levels of IL-1β, IL-2, and IFN-γ in the Con A group were significantly higher than those in the control group (Fig. 3A-3C, P < 0.01). The mRNA levels of IL-1β, IL-2, and IFN-γ in the middle- and high-dose RA-treated
groups (100 and 150 mg/kg) were lower than those in the Con A group (Fig. 3A-3C, P < 0.01). There was no significant difference in the mRNA levels of pre-inflammatory cytokines between the low-dose RA-treated group (50 mg/kg) and the control group (Fig. 3A-3D, P > 0.05). However, mRNA level of IL-10 was did not change in both Con A group and RA-based-treated groups (Fig. 3D, P > 0.05). It was disclosed that RA influenced Con A-induced liver injury in mice via inhibiting the inflammation cytokines (IL-1β, IL-2, and IFN-γ).

The activation of AMPK signaling in mouse liver was induced by RA

The mice were intraperitoneally injected with 50, 100, and 150 mg/kg RA for 7 consecutive days. It is well-known that ACC is a downstream target of AMPK. Using Western blotting, we noted that RA promoted phosphorylation of ACC, which reflects activation of AMPK signaling. As displayed in Fig. 4B and 4C, treatment with RA (100 and 150 mg/kg) increased the ratio of phosphorylated ACC/total ACC (P<0.01), which indicated that RA activated AMPK. There was no statistical difference in AMPK activation between two different doses (100 and 150 mg/kg) of RA treatment (P>0.05).

A specific inhibitor of AMPK, CC (20 mg/kg), was injected into the mice with RA for 7 consecutive days prior to administration of Con A. We found that RA noticeably increased the ratio of phosphorylated ACC/total ACC compared with Con A administration, as shown in Fig. 4E and 4F, which revealed that RA-activated AMPK was at least partially blocked by CC (P < 0.05).

CC blocked the protective effect of RA on liver injury in mice

In the present study, RA (100 mg/kg) with or without CC (20 mg/kg) was administrated into mice for 7 consecutive days. Then, mice were injected in tail vein with Con A 12 h before they were sacrificed. The results uncovered that only RA treatment noticeably reduced the serum levels of ALT and AST in Con A group compared with the control group (Fig. 5A and 5B, P < 0.01).

After H&E staining, histopathological changes in liver tissues of mice were observed
(Fig. 5C). The liver necrosis and mononuclear cell infiltration in hepatic lobule were remarkable in the combined administration of RA plus CC in comparison with RA alone treatment. Thus, AMPK might be activated at least partly in mice treated with RA, and showed to have an influence on protective role of RA in liver injury.

**Alteration in cytokine secretion after treatment with CC plus RA**

RA (100 mg/kg), with or without CC (20 mg/kg), was injected into mice for 7 consecutive days, followed by injection of Con A. Herein, 12 h after Con A injection, blood and liver samples were collected. Besides, serum levels of cytokines were measured by ELISA. Compared with the RA-treated group, the serum levels of IL-1β, IL-2, and IFN-γ were markedly increased in the RA plus CC-treated group (Fig. 6A-6C, P < 0.01), while the serum level of IL-10 was declined (Fig. 6D, P < 0.01). The mRNA levels of cytokines in the liver tissue, detected by qPCR, showed that compared with the RA-treated group, the mRNA levels of IL-1β, IL-2, and IFN-γ in the RA plus CC-treated group were significantly elevated (Fig. 7A-7C, P < 0.01). The results also indicated that the mRNA level of IL-10 was not significantly changed after treatment with RA + CC (Fig. 7D, P > 0.05).

**Discussion:**

AIH is a serious autoimmune liver disease that is characterized by a progressive destruction of the liver parenchyma and the development of chronic fibrosis. The early treatment of AIH is highly vital, otherwise, it may lead to the fibrosis and cirrhosis of liver. At the end-stage of AIH, patients and their family members may suffer from higher degree of pain and experience greater economic loss. AIH responds favorably to steroids and pharmacologic immunosuppression. Although they may cause remission of the disease, their side effects are obvious to AIH patients, including peptic ulcer, diabetes mellitus, osteoporosis, and hypertension. As a result, the therapy of AIH has markedly gained scholars’ attention.

RA is a caffeic acid ester and a naturally-occurring phenolic compound, and it is widely used due to its antiviral, antibacterial, anticancer, antioxidant, antiaging,
antidiabetic, cardioprotective, hepatoprotective, nephroprotective, antidepressant, antiallergic, and anti-inflammatory effects. RA has nutritional and pharmaceutical properties, thus, it can be reliably utilized as a new target to treat AIH.

Previous researches reported that liver injury in rats induced by alcohol and acetaminophen could be alleviated by pretreatment with RA. In the present study, an animal model of immune-mediated liver injury was established by tail vein injection of Con A, which could imitate the pathogenesis of AIH in human. Previous studies have shown that Th1 and Th2 cells play a role in the mechanisms of immune-mediated liver injury. Besides, it was pointed out that imbalance of Th17/Treg cells is involved in the progression of nonalcoholic fatty liver disease in mice. Con A is a well-known T cell mitogen that can activate the immune system, recruit lymphocytes, and elicit cytokine production. Additionally, the present study demonstrated that activation of AMPK provides significant protection against Con A-induced liver injury, which may justify why RA can relieve the serum levels of ALT and AST, as well as the levels of pro-inflammatory cytokines in mice.

In the present study, we demonstrated that RA notably reduced the serum levels of ALT and AST in mice. Additionally, the protective role of RA was suppressed when CC was injected into mice.

A previous research pointed out that ethyl acetate extract has the highest antioxidant activities, followed by petroleum ether extract. Besides, in that research, 9 compounds could be isolated from petroleum ether and ethyl acetate extracts, including four triterpenes: oleanolic acid, ursolic acid, pomolic acid, and 2α-hydroxyl ursolic acid, three flavonoids: apigenin-7-O-rutinoside, luteolin, and diosmetin, as well as two phenolic acids: RA and methyl rosmarinate. Another study showed that RA could protect C57BL/6 mice from LPS/D-GalN-induced acute liver injury. A number of scholars demonstrated that RA suppressed synovitis in a murine collagen-induced arthritis model, and this effect may be beneficial for treatment of rheumatoid arthritis in clinical settings. Moreover, a previous research pointed out that RA could inhibit T-cell antigen receptor (TCR)-induced IL-2 expression and subsequent T-cell
Thus, it can be concluded that RA plays a significant role in the autoimmune diseases and liver injury. Because a limited number of studies concentrated on the effects of RA on Con A-induced liver injury, we, in the current research, attempted to investigate the effects of RA on Con A-induced liver injury in mice via activation of AMPK signaling.

In inflammation process, pro- and anti-inflammatory cytokines play a pivotal role. The intravenous injection of the Con A is a widely used model for acute immune-mediated hepatitis in mice. In contrast to several other models for acute hepatic damage, Con A-induced liver injury is primarily driven by the activation and recruitment of T cells to the liver. In another immune-mediated liver injury mice model that was presented for treatment of human immunodeficiency virus (HIV), a remarkable elevation was found in the levels of IL-2 and IFN-γ, while a reduction was noted in the levels of IL-10 and IL-6. Nuclear factor-κB (NF-κB) may interfere with the injury process in the liver by regulating the inflammatory cytokines IL-1β and TNF-α. In an immune-mediated liver injury model developed by administration of pseudomonas aeruginosa exotoxin A, the serum levels of pro-inflammatory cytokines, including TNF-α, IL-2, and IL-6 were markedly increased. To our knowledge, various production of pro-inflammatory cytokines including IL-2 and IFN-γ was the symbol of hepatitis, and the concentrations of IL-2 and IFN-γ were found extremely higher by the administration of Con A. In addition, our previous study demonstrated that serum level of IL-10 was decreased, whereas it was elevated by berberine treatment. Therefore, it can be concluded that the level of inflammatory cytokines may reflect the severity of the hepatic injury in the Con A-mediated mice model. Luan et al. pointed out that the production of NOD-like receptor protein 3 (NLRP3) inflammasome-dependent IL-1β is vital in the pathogenesis of Con A-induced liver injury in mice.

The pro-inflammatory cytokines (e.g., IL-1 and IL-6) play a major role in the pathogenesis of AIH. Abe et al. analyzed and compared the composition of the oral microbiota of 56 patients with autoimmune liver disease (AILD) and 15 healthy
controls (HCs) to evaluate its association with salivary immunological biomarkers and gut microbiota. Their findings showed elevated levels of inflammatory cytokines (IL-1β, IFN-γ, TNF-α, IL-8) and immunoglobulin A in the saliva of patients with AILD.\textsuperscript{32} In patients with AIH, the levels of TNF-α and IL-1β in peripheral blood are significantly higher than those in patients with non-alcoholic fatty liver disease (NAFLD).\textsuperscript{33} Thus, assessment of changes in level of inflammatory cytokines in a Con A animal model is advantageous to the therapy of AIH.

The results of the present study indicated that RA reduced the levels of pro-inflammatory cytokines (IL-1β, IL-2, IFN-γ), whereas increased the level of anti-inflammatory cytokines, IL-10, in the peripheral blood. Thus, RA plays a protective role in the immune-mediated liver injury by Con A in mice. Our results suggested that RA can inhibit the inflammatory reaction of liver of mice and protect liver via decreasing the serum levels of pro-inflammatory cytokines (IL-1β, IL-2, IFN-γ) and increasing the serum levels of anti-inflammatory cytokine (IL-10) in vitro. The serum level of IL-10 in our model inversely changed with the serum levels of IL-1β, IL-2, and IFN-γ, while the serum level of IL-10 didn’t change in Sass et al.’s model.\textsuperscript{34} A number of scholars demonstrated that regulation of the levels of pro-inflammatory cytokines (IL-17F, IL-21, IL-23, IL-10, and IL-6) in AIH patients is associated with the progression of AIH.\textsuperscript{35}

Several drugs showed to have a protective role in acute Con A-induced liver injury in animal model through modulating the inflammation.\textsuperscript{12, 36-38} Ma et al. found that the serum level of IL-10 was negatively correlated with the serum levels of IgG and IgM in patients with AIH. The present research demonstrated that RA could increase the serum level of IL-10, thus, it can be a potential therapeutics candidate for AIH patients in the future studies.

AMPK is a highly conserved master regulator of metabolism, which restores energy balance during metabolic stress both at the cellular and physiological levels.\textsuperscript{39} AMPK was found to have roles in lipid metabolism, anti-inflammation\textsuperscript{40}, anti-cancer,\textsuperscript{41} etc. AMPK signaling is related to autoimmune diseases, such as experimental autoimmune
uveitis, autoimmune rheumatic diseases, autoimmune insulitis, etc. To our knowledge, AMPK activity can inhibit inflammatory responses in diverse types of cells and tissues. Hence, AMPK serves as a key component in the immunodeficiency disorders.

A variety of natural resources and synthetic molecules can enhance AMPK activation. The anti-diabetic effects of the black ginseng extract in type 2 diabetes were investigated by activating AMPK in the liver, AICAR, an agonist of AMPK, can alleviate liver cirrhosis in bile duct ligation (BDL) model rats.

CC can reserve the inhibitory effect of metformin on the NLRP3 inflammasome by activating AMPK signaling in diabetic cardiomyopathy. We, in the current research, observed that CC restrained the protective effect of RA on the liver of mice that received Con A by inhibiting AMPK signaling. CC was injected into rats to verify the close relationship between AMPK expression and inflammatory response in brain. It decreased serum levels of IL-1, TNF-α, and intercellular adhesion molecule 1.

AIH is a rare autoimmune disorder causing chronic liver inflammation. AIH is considered to be associated with the CD4+ helper T cells, mediating the release of inflammatory cytokines. Con A-induced hepatitis is a well-established T-cell-mediated murine model that mimics human AIH. In the Con A model, the activated T-cells and macrophages were observed to infiltrate into the tissue of liver, inducing inflammatory cytokines to secret into the peripheral blood. The immune-mediated inflammation leads to the severe damage in liver, and even progresses to cirrhosis or hepatocellular carcinoma. However, the main treatment for AIH is steroids and immunosuppressive medicine, imposing some side-effects on patients. The findings of the present research demonstrated that RA has a protective influence on liver damage in mice that received Con A by decreasing the serum levels of pro-inflammatory cytokines and AMPK activation. It presented a new insight for the treatment of AIH.

**Conclusion:**

RA pretreatment protected the mice liver from the injury induced by Con A
mimicking AIH. The activation of AMPK signaling plays a vital role in the protection. Our study may recommend a new potential therapy for AIH and further research will be focused on the mechanism of RA in the treatment of AIH.

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**CONFLICT OF INTEREST :**

The authors declare no conflict of interest.
REFERENCE:

1) Sciveres M, Nastasio S, Maggiore G. Novel diagnostic and therapeutic strategies in juvenile autoimmune hepatitis. Front Pediatr, 7, 382 (2019).

2) Terziroli Beretta-Piccoli B, Mieli-Vergani G, Vergani D. Autoimmune hepatitis: Standard treatment and systematic review of alternative treatments. World J Gastroenterol, 23, 6030-6048 (2017).

3) Elufioye TO, Habtemariam S. Hepatoprotective effects of rosmarinic acid: Insight into its mechanisms of action. Biomed Pharmacother, 112, 108600 (2019).

4) Osakabe N, Yasuda A, Natsume M, Sanbongi C, Kato Y, Osawa T, Yoshikawa T. Rosmarinic acid, a major polyphenolic component of Perilla frutescens, reduces lipopolysaccharide (LPS)-induced liver injury in D-galactosamine (D-GalN)-sensitized mice. Free Radic Biol Med, 33, 798-806 (2002).

5) Shi QQ, Dang J, Wen HX, Yuan X, Tao YD, Wang QL. Anti-hepatitis, antioxidant activities and bioactive compounds of dracocephalum heterophyllum extracts. Bot Stud, 57, 16 (2016).

6) Ye T, Wang T, Yang X, Fan X, Wen M, Shen Y, Xi X, Men R, Yang L. Comparison of concanavalin a-induced murine autoimmune hepatitis models. Cell Physiol Biochem, 46, 1241-1251 (2018).

7) Wang HX, Liu M, Weng SY, Li JJ, Xie C, He HL, Guan W, Yuan YS, Gao J. Immune mechanisms of concanavalin A model of autoimmune hepatitis. World J Gastroenterol, 18, 119-125 (2012).

8) Youn J, Lee KH, Won J, Huh SJ, Yun HS, Cho WG, Paik DJ. Beneficial effects of rosmarinic acid on suppression of collagen induced arthritis. J Rheumatol, 30, 1203-1207 (2003).

9) Li Z, Feng H, Wang Y, Shen B, Tian Y, Wu L, Zhang Q, Jin M, Liu G. Rosmarinic acid protects mice from lipopolysaccharide/ dgalactosamine-induced acute liver injury by inhibiting MAPKs/NF-κB and activating Nrf2 /HO-1 signaling pathways. Int Immunopharmacol, 67, 465-472 (2019).

10) Marin-Aguilar F, Pavillard LE, Giampieri F, Bullón P, Cordero MD. Adenosine
monophosphate (AMP)-activated protein kinase: a New target for nutraceutical compounds. Int J Mol Sci, 18, 288 (2017).

11) Li J, Zhong L, Wang F, Zhu H. Dissecting the role of AMP-activated protein kinase in human diseases. Acta Pharm Sin B, 7, 249-259 (2017).

12) Wang YY, Zhou L, Li YN, Guo LP, Zhou Z, Xie HR, Hou YJ, Wang BM. The effects of berberine on concanavalin A-induced autoimmune hepatitis (AIH) in mice and the adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK) pathway. Medical Science Monitor, 28, 6150-6161 (2017).

13) Han YH, Kee JY, Hong SH. Rosmarinic acid activates AMPK to inhibit metastasis of colorectal cancer. Front Pharmacol, 9, 68 (2018).

14) Vlavcheski F, Naimi M, Murphy B, Hudlicky T, Tsiani E. Rosmarinic acid, a rosemary extract polyphenol, increases skeletal muscle cell glucose uptake and activates AMPK. Molecules, 22, 1669 (2017).

15) Mieli-Vergani G, Vergani D, Czaja AJ, Manns MP, Krawitt EL, Vierling JM, Lohse AW, Montano-Loza AJ. Autoimmune hepatitis. Nat Rev Dis Primers, 4, 18017 (2018).

16) Pape S, Schramm C, Gevers TJ. Clinical management of autoimmune hepatitis. United European Gastroenterol J, 7, 1156-1163. (2019).

17) Oğuz A, Böyük A, Ekinci A, Alabalik U, Türkoğlu A, Tuncer MC, Ekingen A, Deveci E, Gültürk B, Aday U. Investigation of antioxidant effects of rosmarinic acid on liver, lung and kidney in rats: a biochemical and histopathological study. Folia Morphol (Warsz), 79, 288-295 (2020).

18) Lin SY, Wang YY, Chen WY, Liao SL, Chou ST, Yang CP, Chen CJ. Hepatoprotective activities of rosmarinic acid against extrahepatic cholestasis in Rats. Food Chem Toxicol, 108, 214-223 (2017).

19) Hasanein P, Seifi R. Beneficial effects of rosmarinic acid against alcohol-induced hepatotoxicity in rats. Can J Physiol Pharmacol, 96, 32-37 (2018).

20) Hasanein P, Sharifi M. Effects of rosmarinic acid on acetaminophen-induced hepatotoxicity in male Wistar rats. Pharm Biol, 55, 1809-1816 (2017).
21) Tian X, Liu Y, Liu X, Gao S, Sun X. Glycyrrhizic acid ammonium salt alleviates concanavalin A-induced immunological liver injury in mice through the regulation of the balance of immune cells and the inhibition of hepatocyte apoptosis. Biomed Pharmacother, 120, 109481 (2019).

22) Herkel J. Regulatory T cells in hepatic immune tolerance and autoimmune liver diseases. Dig Dis, 33, 70-74 (2015).

23) Park SH, Oh HS, Kang MA, Cho H, Prasad JB, Won J, Lee KH. The structure-activity relationship of the series of non-peptide small antagonists for p56lck SH2 domain. Bioorg Med Chem, 15, 3938-3950 (2007).

24) Wu W, Lv L, Shi D, Ye J, Fang D, Guo F, Li Y, He X, Li L. Protective effect of akkermansia muciniphila against immune-mediated liver injury in a mouse model. Front Microbiol, 8, 1804 (2017).

25) Zhang H, Bai Y, Gao M, Zhang JF, Dong GJ, Yan FL, Ma Q, Fu XQ, Zhang QQ, Li CX, Shi H, Ning ZC, Dai J, Li ZH, Ming JK, Xue QJ, Si CP, Xiong HB. Hepatoprotective effect of capsaicin against concanavalin A-induced hepatic injury via inhibiting oxidative stress and inflammation. Am J Transl Res, 11, 3029-3038 (2019).

26) Bekker Z, Walubo A, du Plessis JB. The role of the immune system in nevirapine-induced subclinical liver injury of a rat model. ISRN Pharm, 2012, 932542 (2012).

27) Lin Q, Kang XL, Li XF, Wang T, Liu FT, Jia JX, Jin ZQ, Xue YZ. NF-κB-mediated regulation of rat CYP2E1 by two independent signaling pathways. PLoS One, 14, e0225531 (2019).

28) Chiu CC, Wang YC, Huang WC, Chen YH, Hung SW, Huang YT, Chuang HL, Chang YC. Differences in genetic background contribute to pseudomonas exotoxin A-induced hepatotoxicity in rats. Toxins (Basel), 9, 224 (2017).

29) Zhao XH, Ding S, Geng C, Man Z, Pan MZ, Sun LL, Hu BJ, Wang H. Anti-CD200 attenuates concanavalin A induced hepatitis via modulating the imbalance of CD4+ T lymphocyte differentiation in mice. Am J Transl Res, 10,
4202-4209 (2018).
30) Luan JY, Zhang XY, Wang SF, Li YB, Fan jj, Chen W, Zai WJ, Wang SJ, Wang YC, Chen MK, Meng GG, Ju DW. NOD-like receptor protein 3 inflammasome-dependent IL-1β accelerated con A-induced hepatitis. Front Immunol, 9, 758 (2018).
31) Yousefi A, Najafi M, Motamed F, Mahmoudi E, Bidoki AZ, Sadr M, Rahmani F, Farhmand F, Khodadad A, Fallahi G, Rezaei N. Association of interleukin-6 and interleukin-1 family gene polymorphisms in autoimmune hepatitis. Ann Hepatol, 17, 1021-1025 (2018).
32) Abe K, Takahashi A, Fujita M, Imaizumi H, Hayashi M, Okai K, Ohira H. Dysbiosis of oral microbiota and its association with salivary immunological biomarkers in autoimmune liver disease. PLoS One, 13, e0198757 (2018).
33) Guo LP, Zhou L, Li HX, Zhang J, Wang BM. The study of liver macrophages polarization in patients with autoimmune hepatitis. Zhonghua Nei Ke Za Zhi, 56, 763-765 (2017).
34) Sass G, Heinlein S, Agli A, Bang R, Schümann J, Tiegs G. Cytokine expression in three mouse models of experimental hepatitis. Cytokine, 19, 115-120 (2002).
35) Thomas-Dupont P, Remes-Troche JM, Izaguirre-Hernández IY, Sánchez-Vargas LA, Maldonado-Rentería Mde J, Hernández-Flores KG, Torre A, Bravo-Sarmiento E, Vivanco-Cid H. Elevated circulating levels of IL-21 and IL-22 define a cytokine signature profile in type 2 autoimmune hepatitis patients. Ann Hepatol, 15, 550-558 (2016).
36) Zhao X, Liu M, Li J, Yin S, Wu Y, Wang A. Antimalarial agent artesunate protects Concanavalin A-induced autoimmune hepatitis in mice by inhibiting inflammatory responses. Chem Biol Interact, 274, 116-123 (2017).
37) Fei M, Xie Q, Zou Y, He R, Zhang Y, Wang J, Bo L, Li J, Deng X. Alpha-lipoic acid protects mice against concanavalin A-induced hepatitis by modulating cytokine secretion and reducing reactive oxygen species generation. Int Immunopharmacol, 35, 53-60 (2016).
38) Wang L, Du H, Liu Y, Wang L, Ma X, Zhang W. Chinese medicine bu xu hua yu recipe for the regulation of treg/th17 ratio imbalance in autoimmune hepatitis. Evid Based Complement Alternat Med, 2015, 461294 (2015).

39) Ren Y, Shen HM. Critical role of AMPK in redox regulation under glucose starvation. Redox Biol, 25, 101154 (2019).

40) Shen BY, Zhao CX, Wang Y, Peng Y, Cheng JQ, Li Z, Wu L, Jin MY, Feng HH. Aucubin inhibited lipid accumulation and oxidative stress via Nrf2/HO-1 and AMPK signaling pathways. J Cell Mol Med, 23, 4063-4075 (2019).

41) Jiang SP, Shi FL, Lin H, Ying Y, Luo LY, Huang DQ, Luo ZJ. Inonotus obliquus polysaccharides induces apoptosis of lung cancer cells and alters energy metabolism via the LKB1/AMPK axis. Int J Biol Macromol, 151, 1277-1286 (2019).

42) Niu T, Cheng L, Wang HY, Zhu SP, Yang XL, Liu K, Jin HY, Xu X. KS23, A novel peptide derived from adiponectin, inhibits retinal inflammation and downregulates the proportions of Th1 and Th17 cells during experimental autoimmune uveitis. J Neuroinflammation, 16, 278 (2019).

43) Liu E, Perl A. Pathogenesis and treatment of autoimmune rheumatic diseases. Curr Opin Rheumatol, 31, 307-315 (2019).

44) Duan W, Ding YC, Yu XF, Ma DX, Yang B, Li Y, Huang L, Chen ZH, Zheng JM, Yang C. Metformin mitigates autoimmune insulitis by inhibiting Th1 and Th17 responses while promoting Treg production. Am J Transl Res, 11, 2393-2402 (2019).

45) Noor HB, Mou NA, Salem L, Shimul MFA, Biswas S, Akther R, Khan S, Raihan S, Mohib MM, Sagor MAT. Anti-inflammatory property of AMP-activated protein kinase.Antiinflamm Antiallergy Agents Med Chem, 19, 2-41 (2019).

46) Kang OH, Mi-Yae Shon MY, Kong R, Seo YS, Zhou T, Kim DY, Kim YS, Kwon DY. Anti-diabetic effect of black ginseng extract by augmentation of AMPK protein activity and upregulation of GLUT2 and GLUT4 Expression in Db/Db Mice. BMC Complement Altern Med, 17,341 (2017).
47) Hu LS, Su L, Dong ZX, Wu YH, Lv Y, George J, Wang JH. AMPK agonist AICAR ameliorates portal hypertension and liver cirrhosis via NO pathway in the BDL rat model. J Mol Med (Berl), 97, 423-434 (2019).

48) Yang F, Qin Y, Wang YQ, Meng SY, Xian HM, Che H, Lv J, Li Y, Yu YH, Bai YL, Wang LH. Metformin inhibits the NLRP3 inflammasome via AMPK/ Mtor dependente effects in diabetic cardiomyopathy. Int J Biol Sci, 15, 1010-1019 (2019).

49) Yang L, Gong NR, Zhang Q, Ma YB, Zhou H. Apparent correlations between AMPK expression and brain inflammatory response and neurological function factors in rats with chronic renal failure. J Mol Neurosci, 68, 204-213 (2019).

50) Matsuoka N, Kozuru H, Koga T, Abiru S, Yamasaki K, Komori A, Fujita Y, Tenmoku J, Asano T, Sato S, Suzuki E, Furuya M, Kobayashi H, Watanabe H, Naganuma A, Yoshizawa K, Shimada M, Ario K, Yamashita H, Kohno H, Kaneyoshi T, Nakamura M, Furukawa H, Takahashi A, Kawakami A, Ohira H, Yatsuhashi H, Migita K. Galectin-9 in autoimmune hepatitis: correlation between serum levels of galectin-9 and M2BPGi in patients with autoimmune hepatitis. Medicine (Baltimore), 98, e16924 (2019).
Table 1: The sequence of primers used in this study

| Gene     | Forward(5’-3’)          | Reverse(5’-3’)         |
|----------|-------------------------|-------------------------|
| IL-1β    | actcattgtggctgtggaga    | ttgttcatctcggagcttgt    |
| IL-2     | agcagctgttgatggaccta    | aatccagagaacatgccgacag  |
| IL-10    | ggtgagaagctgaagaccct    | tgtctaggtcctggagtcca    |
| IFN-γ    | ttcttcagcaacacagcaagcc  | actctttttcagttctgta     |
| β-actin  | gtgggaatgggctgaagga     | tctcttttcacggttgtgcc    |
Fig. 1. RA reduced Con A-induced AIH and liver injury in mice

After intraperitoneal injection of solvent or RA (50, 100, 150 mg/kg) into mice once daily for 7 consecutive days, they were intravenously injected with Con A (20 mg/kg).
Besides, 12 h after Con A injection, the serum samples of liver were collected. Fig. 1B and 1C show the serum levels of ALT and AST. Fig. 1D illustrates results of histopathological evaluation in treatment group and control group according to hematoxylin and eosin (H&E) staining (magnification, ×200). The grouping information of mice was shown in Fig. 1A. Data were expressed as mean ± standard deviation (SD). (n = 6, **P < 0.01 vs. control group, ##P < 0.01 vs. Con A group). The black arrows indicate liver necrosis in mice (original magnification ×200).

(Color figure can be accessed in the online version.)
Fig. 2. RA affects the serum levels of pro- and anti-inflammatory cytokines in Con A-induced liver injury in mice.

The mice were treated with intraperitoneal injection of solvent or RA (50, 100, and 150 mg/kg) for 7 consecutive days. Then, they were injected intravenously with Con A (20 mg/kg), and 12 h after injection, serum samples were used to detect the levels of IL-1β (A), IL-2 (B), IFN-γ (C), and IL-10 (D) by ELISA (n = 6). **P < 0.01 vs. the control group, ##P < 0.01 vs. the Con A group. The grouping information of mice was shown in Fig. 1A.
Fig. 3: Effects of RA on the levels of cytokines in Con A-induced liver injury in mice.

After mice were intraperitoneally injected with solvent or RA (50, 100, and 150 mg/kg) for seven consecutive days, they were intravenously injected with 20 mg/kg Con A. After 12 h of Con A injection, the mRNA levels of IL-1β (A), IL-2 (B), IFN-γ (C), and IL-10 (D) in hepatic tissue were detected by qPCR ($n = 6$, **$P < 0.01$ vs. the control group, ##$P < 0.01$ vs. the Con A group. The grouping information of mice was shown in Fig. 1A.
## Mice Administration

| Group   | Treatment       | Day0  |
|---------|-----------------|-------|
| Ctrl    | 5%DMSO 0.4ml    | sacrifice |
| RA_{50} | RA 50mg/kg      |       |
| RA_{100}| RA 100mg/kg     |       |
| RA_{150}| RA 150mg/kg     |       |

### Figure B

- P-ACC
- t-ACC
- GAPDH

### Figure C

Bar graph showing the ratio of pACC/ACC for different groups. Ctrl, RA_{50}, RA_{100}, RA_{150}.
Fig. 4.-RA activated AMPK in liver tissue of mice.

Fig. 4B and C: Mice were treated with RA (50, 100, and 150 mg/kg) for 7 consecutive days. After 12 h of intraperitoneal injection with RA, we detected the phosphorylation of ACC by Western blot analysis. The grouping information of mice was shown in Fig. 4A.

Fig. 4E and 4F: Con A-induced liver injury in mice treated with RA (100 mg/kg) with or without CC (20 mg/kg) for 7 consecutive days. After 12 h of Con A injection into tail vein, phosphorylation levels of ACC were detected by Western blot analysis. The
grouping information of mice was shown in Fig. 4D.

The protein bands were visualized by enhanced chemiluminescence using an enhanced chemiluminescence (ECL) Western Blotting Detection kit and normalized to β-actin (Fig. 4C and F) (n = 6. **P < 0.01 vs. control group, #P < 0.05 vs. Con A group, §P < 0.05 vs. Con A + RA group).
Fig. 5. The changes in serum levels of cytokines in RA-treated mice after administration of CC.

Con A-induced liver injury in mice was treated with intraperitoneal injection of RA (100 mg/kg) with or without CC (20 mg/kg) for 7 consecutive days. After 12 h of Con A injection, the serum and liver tissues were collected immediately. The serum levels of ALT (A) and AST (B) in mice were detected by ELISA. The liver samples were stained with hematoxylin and eosin (H&E) to show the histopathological changes (Fig. 5C, magnification, ×200). The grouping information of mice was shown in Fig. 4D. Data were expressed as mean ± standard deviation (SD) (n = 6, **P < 0.01 vs. control group, ##P < 0.01 vs. Con A group, §§P < 0.01 vs. Con A + RA group).

(Color figure can be accessed in the online version.)
Con A-induced liver injury in mice was treated with 100 mg/kg RA and with or without 20 mg/kg CC for 7 consecutive days. After 12 h of Con A injection, the serum levels of IL-1β (A), IL-2 (B), IFN-γ (C) and IL-10 (D) were detected by ELISA (n = 6, **P < 0.01 vs. control group, ##P < 0.01 vs. Con A group, §§P < 0.01 vs. Con A + RA group). The grouping information of mice was shown in Fig. 4D.
Fig. 7. Effects of CC on the levels of cytokines in the liver of RA-treated mice.
Con A was injected via tail vein into mice that were treated with 100 mg/kg RA with or without 20 mg/kg CC for 7 consecutive days. After 12 h of Con A injection, the mRNA levels of IL-1β (A), IL-2 (B), IFN-γ (C), and IL-10 (D) in liver tissue were detected by qPCR (n = 6, **P < 0.01 vs. control group, ##P < 0.01 vs. Con A group, §§P < 0.01 vs. Con A + RA group). The grouping information of mice was shown in Fig. 4D.