

Investigating the Anti-inflammatory Effects of Rutin in Carbon Tetrachloride-induced Hepatotoxicity: Role of TLR4/MyD88/NFκB Signaling Pathway Modulation

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Abstract

Objectives: Rutin protects and stabilizes hepatocytes and is used to treat hepatitis, liver cirrhosis, and other diseases. However, there is little evidence to suggest that Rutin is associated with CCl₄-induced hepatotoxicity. In this study, we investigated the effects of Rutin on CCl₄-induced liver inflammation and discussed *in vivo* and *in vitro* mechanisms.

Materials and Methods: CCl₄ was used to induce liver inflammation, and Rutin was used for intervention in mice. Then the body weight, liver weight, and liver inflammation of all mice were measured. Western blotting and RT-PCR were used to assess TLR4/MyD88/NFκB inflammation signaling pathways and inflammatory gene expression. Finally, TLR4 was activated in primary hepatocytes to verify the above signal pathways.

Findings: CCl₄ mice developed liver function damage and liver inflammation. Further investigations showed that the inhibitory effects of Si on inflammation were mediated by TLR4/MyD88/NFκB inflammation signaling pathways inhibition. Primary hepatocyte studies also confirmed the participation of these signaling pathways and proteins.

Significance: Rutin improved CCl₄-induced liver inflammation. Such mechanisms may be related to TLR4/MyD88/NFκB inflammation signaling pathways.

Keywords

Rutin, CCl₄, hepatotoxicity, inflammation, TLR4

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Introduction

The liver is the largest organ in the human body, with multiple functions, including material catabolism, biotransformation, and endocrine secretion, and it is vulnerable to various pathogenic factors and stimuli *in vivo* and abroad, leading to traumatic hepatitis (Trefts et al., 2017). Drugs, poisons, viruses, alcohol, fatty liver, and autoimmune diseases can all damage the liver (Jetter & Kullak-Ublick, 2020; Koppe, 2014; Lin et al., 2020;). If it progresses to cirrhosis or liver cancer, treatment is extremely difficult and mortality is extremely high (Chung et al., 2018). CCl₄, an organic compound, is mainly used as an excellent organic solvent, dry cleaning agent, fire extinguishing agent, refrigerant, extract of spices, pesticide, and chemical raw materials (Meaden et al., 2020). However, CCl₄ has been reported to have strong toxicity, which is recognized as a liver poison and a classic reagent for establishing liver injury models in basic research

(Pessayre et al., 1982; Zhao et al., 2019). CCl₄ caused liver damage in mice mainly by destroying the liver cells of mice, which led to the ALT and AST in the liver cells entering the blood through the liver cell membrane, leading to the increase of transaminase activity in the blood (Tuominen et al., 2021;

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Zhao et al., 2019). The relevant mechanism is mainly that CCl_4 is metabolized into trichloromethyl radical by cytochrome P450 superfamily monooxygenase in the liver (Jeong et al., 2002). Subsequently, this free radical reacts with nucleic acids, proteins, and lipids, thereby damaging key cellular processes and inducing systemic liver injury characterized by liver inflammation, fibrosis, cirrhosis, and liver cancer (Lee et al., 2021).

Rutin, also known as vitamin P, is a natural flavonoid glycoside, which widely exists in plants and has anti-inflammatory, antioxidant, anti-allergic, anti-virus, and other effects (Chua, 2013). Rutin is a strong antioxidant that can scavenge free radicals, inhibit biofilm peroxidation, and inhibit the occurrence of inflammatory reaction and plays an important role in the body (Chua, 2013; Ghorbani, 2017). Inflammation is an immune defense reaction of living tissues with the vascular system against various inflammatory factors and the damage caused by them, and also a defense adaptive response of the body to eliminate various inflammatory factors and promote the repair of damage (Medzhitov, 2008; Muvhulawa et al., 2022). However, its occurrence process is often accompanied by blood circulation disorders, the release of inflammatory mediators, tissue degeneration, and necrosis, which will cause damage to the body to a certain extent (Arulselvan et al., 2016; Medzhitov, 2008). Research shows that Rutin can regulate the cytokines and inflammatory mediators in LPS-induced RAW264.7 cells by mediating TLR4-MyD88-TRAF6-NF- κ B inflammatory signal pathway (Tian et al., 2021). In the study of liver diseases, it is also found that Rutin can effectively prevent and treat liver damage caused by doxorubicin, sorafenib, diabetes, and ischemia-reperfusion, and the main mechanism is that Rutin has strong anti-inflammatory and antioxidant capacity (Ahmed et al., 2022; Liang et al., 2018; Zhou et al., 2021). However, there is still no report on the liver inflammation caused by Rutin treatment of CCl_4 .

Therefore, the purpose of this study was to determine whether Rutin contributes to the anti-inflammatory effect in the CCl_4 -induced liver inflammation model in mice and determine its action target, so as to provide a strong experimental basis and theoretical basis for the clinical application of Rutin.

Materials and Methods

Experimental Animals

CCl_4 was provided by Sigma-Aldrich (St. Louis, MO, USA), and Rutin was purchased from Med Chem Express. Male C57BL/6 mice (18–22 g, 4–6 weeks) were purchased from the Nanjing Junke Bioengineering Co., Ltd (Nanjing, China). Mice were induced through intraperitoneal

injections of CCl_4 (50%, 1% mL/100 g) weekly (Wu et al., 2019) and intragastric administration of Rutin every day (100 mg/1000 g) for 4 weeks (Ma et al., 2017). The mice are specifically divided into the following groups: (1) Control group (CON, intraperitoneal injection + gavage of an equal amount of physiological saline); (2) Rutin group (Rutin, intraperitoneal injection of an equal amount of physiological saline + gavage an equal amount of Rutin); (3) CCl_4 group (CCl_4 , intraperitoneal injection of an equal amount of CCl_4 + gavage an equal amount of physiological saline); and (4) Rutin + CCl_4 group (Rutin + CCl_4 , intraperitoneal injection of an equal amount of CCl_4 + gavage an equal amount of Rutin). All the animals were in a standard laboratory (12-h light/dark cycle) and had free access to drinking water. Body weight was measured every week.

Tissue and Blood Sampling Collection

The mice were euthanized. The blood and liver samples were removed. The serum was obtained immediately (2,000 rpm, 30 min). The liver was partially immersed in 4% paraformaldehyde and partially frozen in -80°C . Notably, 4% paraformaldehyde was purchased from Biyuntian Biotechnology Co., Ltd (Shanghai, China).

Histopathological Examinations

Liver samples in 4% paraformaldehyde were dehydrated in graded alcohol and embedded in paraffin. The liver sample was cut into 5- μm -thick sections. The sections were stained with H&E for histological examination. The specimens were placed on normal slides and examined under an optical microscope. H&E Staining Kit (C0105M) was purchased from Shanghai Biyuntian Biotechnology Co., Ltd (Shanghai, China).

Biochemical Studies

The ALP, ALT, AST, GGT, IL-1 β , IL-6, IL-10, and LDH assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). According to the protocols of the kits, the contents of ALP, ALT, AST, GGT, IL-1 β , IL-6, IL-10, and LDH in serum were measured in the spectrophotometer.

Cell

Primary Hepatocytes Extraction

Male mice (4 weeks) were purchased from the Nanjing Junke Bioengineering Co., Ltd. (Nanjing, China). Primary hepatocytes were obtained from mouse liver by perfusion, digestion (collagenase IV), filtration, centrifugation, and

inoculation. Collagenase IV was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Hepatocytes Viability

First, CCl₄ is divided into concentration gradients of 0%, 0.25%, 0.5%, 1%, and 2%. After determining the optimal cell treatment concentration through the CCK-8 kit, Rutin was divided into 25, 50, 100, and 200 μ M according to concentration gradients.

CCK-8 was purchased from Shanghai Biyuntian Biotechnology Co., Ltd (Shanghai, China). According to the CCK-8 kit protocol, the reagent was added to each group of hepatocytes, the cells incubated at 37°C for 1.5 h, and finally, the cell viability was measured in the spectrophotometer. The hepatocyte viability of the CON group was defined as 100%.

Cell Grouping

Primary hepatocytes were divided into six groups. All groups of liver cells were cultured using DMEM medium +10% PBS. Rutin is dissolved in DMSO solution in a ratio of 1 (μ mol): 5000 (μ L). The specific cell grouping is as follows: (1) CON group: DMSO; (2) CCl₄ group: 0.5% CCl₄; (3) Rutin + CCl₄: 100 μ M Rutin +0.5% CCl₄; (4) Lv-EGFP:Lv-EGFP-Control; (5) Lv-EGFP-TLR4: Lv-EGFP-TLR4; and (6) Lv-EGFP-TLR4 + Rutin: Lv-EGFP-TLR4 + 100 μ M Rutin.

Western Blot

Liver tissue and primary hepatocytes were homogenized in standard protein extraction buffer at 4°C. The tissue homogenate was centrifuged at 12,000 rpm at 4°C for 15 min. The supernatant was collected for Western blot analysis. The total protein concentration was quantified by Bradford analysis (Bio-Rad, California, USA). Samples containing the same amount of protein were boiled in SDS sample buffer at 95°C for 10 min. Subsequently, protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to PVDF membrane (Bio-Rad), sealed with 5% skimmed milk at room temperature for 1 h, and incubated with primary antibody at 4°C overnight. The membrane was then washed and incubated with secondary antibodies. A chemiluminescence detection system (Millipore Corp., Billerica, MA, USA) was used to detect protein expression. All secondary antibodies were purchased from Wuhan Proteintech Biotechnology Co., Ltd. (Wuhan, China).

PCR

Using TRIzol reagent (Invitrogen, Shanghai, China), we isolated RNA from liver tissue according to the instructions. The gene expression level was quantified by qRT PCR.

Table 1. Sequences of the Primers Used in the Real-Time PCR Measurement.

Gene	Sense	Sequence (5' to 3')
MyD88	FWD	GGCTGCTCTCAACATGCGA
	REV	CTGTGTCCGCACGTTCAAGA
MCP-1	FWD	GCTGACCCCAAGAAGGAATG
	REV	GTGCTTGAGGTGGTTGTGGA
TNF α	FWD	CCGATGGGTTGTACCTTGTC
	REV	GGGCTGGGTAGAGAATGGAT
IL-1 β	FWD	CCAGCTTCAAATCTCACAGCAG
	REV	CTTCTTTGGGTATTGCTTGGGATC
IL-6	FWD	TCCTACCCCAATTTCCAATGC
	REV	CATAACGCACTAGGTTTGCCG
NF- κ B	FWD	GCGAGAGAAGCACAGATACCA
	REV	GGTCAGCCTCATAGTAGCCA
β -actin	FWD	CCACTGCCGCATCCTCTTCCTC
	REV	TCCTGCTTGCTGATCCACATCT

Abbreviations: MyD88: myeloid differentiation primary response 88; IL, interleukin; MCP-1, monocyte chemotactic protein-1; TNF α , tumor necrosis factor- α ; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells.

Adopt^{2- $\Delta\Delta$ CT} calculation is used to calculate the relative number of tested genes. GAPDH was used as an internal control. The primer sequences of these genes are shown in Table 1.

Statistical Analysis

The normal distribution and homogeneity of variance of the data were detected using one-way or two-way (Figure 1) ANOVA, and Tukey's multiple comparison *post hoc* test was used to analyze the significant differences between the groups. $p \leq 0.05$ was considered statistically significant.

Result

Rutin and CCl₄ Affect the Body and Liver Weight of Mice

As shown in Figure 1, the body weight of mice in CCl₄ group decreased significantly from the 14th day, while Rutin effectively prevented the weight loss from the 21st day.

As shown in Table 2, at the end of the model establishment, there was no significant difference in the liver weight of mice in each group, but the body weight of mice in the CCl₄ group decreased significantly, and Rutin effectively reversed this change. At the same time, the liver/body weight was significantly increased in the CCl₄ group, and Rutin effectively reversed this change.

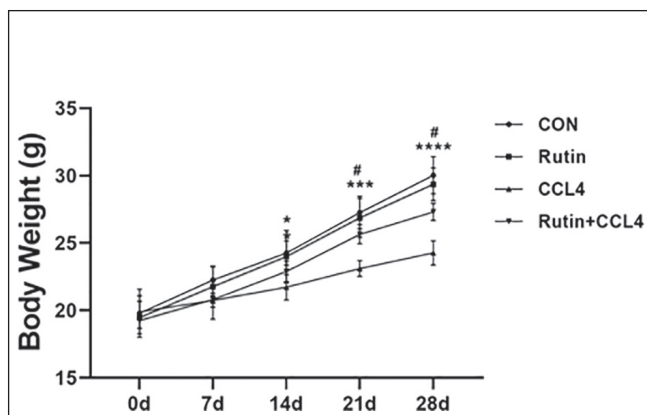


Figure 1. Effects of Rutin and CCl₄ on Body Weight in Mice (n = 3). Body Weight Changes of Mice During 28 days of Model Establishment.

Note: **p* < 0.05, ****p* < 0.001, *****p* < 0.0001 vs. CON, #*p* < 0.05 vs. CCl₄.

Abbreviations: CON, control; CCl₄, carbon tetrachloride.

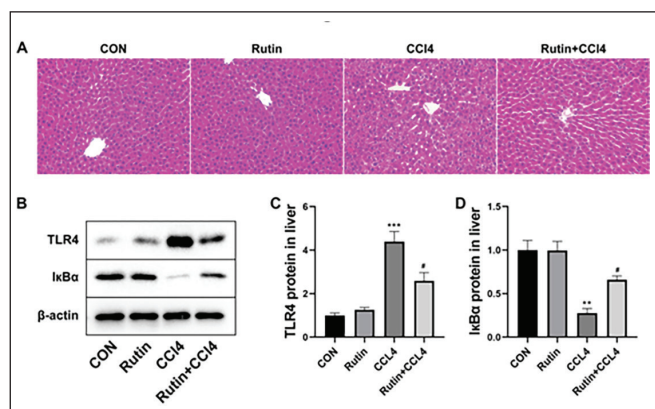


Figure 2. Effects of Rutin and CCl₄ on Liver Morphology and Protein Expression in Mice (n = 3). The Changes of Liver Morphology (A), Liver TLR4 and IκBα Protein Expression (B), After 6 Weeks of Model Establishment. Semi-quantitative Analysis of TLR4 (C) and IκBα (D) Protein Expression.

Note: ***p* < 0.01, ****p* < 0.001 vs. CON, #*p* < 0.05 vs. CCl₄.

Abbreviations: CON, control; CCl₄, carbon tetrachloride; TLR4, toll-like receptor 4; I-κB: NF-κB inhibitor protein.

Rutin and CCl₄ Affect the Liver Function of Mice

As shown in Table 2, CCl₄ successfully induced liver function damage in mice, specifically manifested as follows: blood concentrations of ALP, ALT, AST, and GGT increased significantly. However, the above abnormalities in the blood of the Rutin group mice were significantly reversed.

As shown in Figure 2A, CCl₄ causes damage to hepatocytes, which is manifested by abnormal nuclear morphology, multinucleated cells, unclear or even disappeared cell membrane boundaries, and abnormal cytoplasm.

Rutin and CCl₄ Affect the TLR4-induced Inflammation Signaling Pathway in Mice Liver

As shown in Figure 2B, TLR4 was significantly downregulated and IκBα was significantly upregulated in the liver of the CCl₄ group mice compared with the CON group. However, Rutin significantly reversed the above changes. Figures 2C and D provide more intuitive data for the above results.

As shown in Table 3, the content of inflammatory factors in serum was significantly changed. Specifically, IL-1β, IL-6, and LDH were significantly increased, while IL-10 was significantly decreased in the serum of CCl₄ mice. However, Rutin finally reversed the above changes.

As shown in Table 4, the expression of inflammatory genes in mice liver was significantly changed. Specifically, MyD88, TNF-α, IL-1β, IL-6, NF-κB, MCP-1, and VCAM-1 genes were significantly increased. However, Rutin finally reversed the above changes.

Rutin and CCl₄ Affect the Cell Viability in Primary Hepatocytes

As shown in Figure 3A, when the concentration of CCl₄ is 0.25% and 0.5%, the primary hepatocytes have a certain degree of cell viability decline, whereas, when the concentration of CCl₄ is 1% and 2%, the primary hepatocytes have a large number of deaths.

As shown in Figure 3B, under the condition that the concentration of CCl₄ is 0.5%, when the concentration of

Table 2. Rutin Inhibited Liver Function Deterioration Caused by CCl₄ (n = 3).

	CON	Rutin	CCl ₄	Rutin + CCl ₄
Liver weight (g)	0.90 ± 0.06	0.87 ± 0.06	0.84 ± 0.02	0.84 ± 0.04
Body weight (g)	30.03 ± 1.13	29.36 ± 0.99	24.27 ± 0.73****	27.31 ± 0.52#
Liver/Body weight (%)	2.98 ± 0.10	2.97 ± 0.13	3.45 ± 0.10**	3.09 ± 0.09#
ALP (U/L)	231.77 ± 53.42	205.35 ± 53.87	1,101.91 ± 217.94***	570.36 ± 96.36#
ALT (U/L)	8.20 ± 1.20	8.49 ± 1.38	44.82 ± 6.22***	22.41 ± 5.69##
AST (U/L)	88.84 ± 11.12	101.09 ± 27.87	685.75 ± 85.36****	229.04 ± 53.86###
GGT (U/L)	0.86 ± 0.43	1.01 ± 0.27	8.69 ± 1.24****	4.56 ± 1.05###

Rutin has no significant effect on liver weight, but it reduces the increase of liver/body weight caused by CCl₄. Rutin increases body weight due to CCl₄. In addition, Rutin reduces the elevation of ALP, ALT, AST, and GGT caused by CCl₄.

Note: ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001 vs. CON, #*p* < 0.05, ##*p* < 0.01, ###*p* < 0.001 vs. CCl₄.

Abbreviations: CON, control; CCl₄, carbon tetrachloride; GGT, γ-glutamyl transferase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

Table 3. Rutin Inhibited the Abnormality of Serum Inflammatory Factors Caused by CCl₄ (n = 3).

	CON	Rutin	CCl ₄	Rutin + CCl ₄
IL-1 β (mmol/L)	11.79 $\pm$ 3.06	12.49 $\pm$ 1.80	34.45 $\pm$ 6.57**	21.64 $\pm$ 2.52 [#]
IL-6 (mmol/L)	5.69 $\pm$ 2.08	6.05 $\pm$ 1.61	17.64 $\pm$ 1.91***	12.80 $\pm$ 2.16 [#]
IL-10 (mmol/L)	38.30 $\pm$ 5.09	41.64 $\pm$ 5.71	9.95 $\pm$ 2.20***	24.39 $\pm$ 3.86 [#]
LDH (U/L)	124.54 $\pm$ 22.83	117.37 $\pm$ 20.61	565.81 $\pm$ 60.18***	296.16 $\pm$ 75.75 ^{###}

Rutin reduced the increase of serum inflammatory factors IL-1 β , IL-6, and LDH and increased the decrease of IL-10 caused by CCl₄.

Note: **p < 0.01, ***p < 0.001 vs. CON, [#]p < 0.05, ^{###}p < 0.01 vs. CCl₄.

Abbreviations: CON, control; CCl₄, carbon tetrachloride; IL, interleukin; LDH, lactate dehydrogenase.

Table 4. Effect of Rutin on Liver Inflammation-related Genes (n = 3).

	CON	Rutin	CCl ₄	Rutin + CCl ₄
MyD88	1.00 $\pm$ 0.21	0.91 $\pm$ 0.10	3.42 $\pm$ 0.93**	1.74 $\pm$ 0.31 [#]
TNF- α	1.00 $\pm$ 0.11	0.90 $\pm$ 0.09	3.97 $\pm$ 0.46****	2.04 $\pm$ 0.35 ^{###}
IL-1 β	1.00 $\pm$ 0.12	0.99 $\pm$ 0.18	4.05 $\pm$ 0.59***	2.57 $\pm$ 0.49 [#]
IL-6	1.00 $\pm$ 0.10	1.05 $\pm$ 0.16	3.29 $\pm$ 0.49***	1.81 $\pm$ 0.31 ^{###}
NF- κ B	1.00 $\pm$ 0.10	1.08 $\pm$ 0.20	3.97 $\pm$ 0.65***	2.14 $\pm$ 0.30 ^{###}
MCP-1	1.00 $\pm$ 0.08	1.13 $\pm$ 0.14	3.75 $\pm$ 0.44****	2.17 $\pm$ 0.30 ^{###}
VCAM-1	1.00 $\pm$ 0.15	1.14 $\pm$ 0.12	3.48 $\pm$ 0.48***	1.97 $\pm$ 0.29 ^{###}

Rutin reduced the increase of expression of MyD88, TNF- α , IL-1 β , IL-6, NF- κ B, MCP-1, and VCAM-1 in the liver inflammation gene induced by CCl₄.

Note: **p < 0.01, ***p < 0.001, ****p < 0.0001 vs. CON, [#]p < 0.05, ^{###}p < 0.01, ^{####}p < 0.001 vs. CCl₄.

Abbreviations: MyD88: myeloid differentiation primary response 88; CON, control; CCl₄, carbon tetrachloride; IL, interleukin; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; VCAM-1, vascular cell adhesion molecule-1.

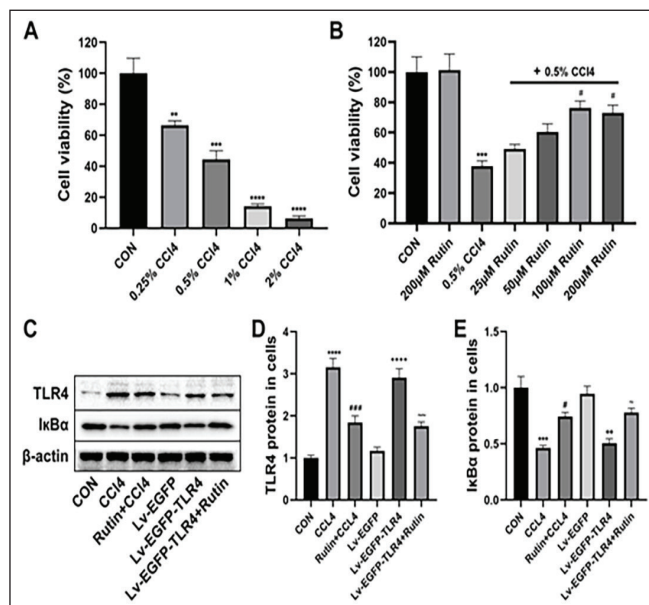


Figure 3. Effects of Rutin and CCl₄ on Protein Expression in Hepatocytes (n = 3). Screening of Treatment Concentration of CCl₄ (A) and Rutin (B) in Primary Hepatocytes. The Changes of Hepatocytes TLR4 and IκBα Protein Expression (C). Semi-quantitative Analysis of TLR4 (D) and IκBα (E) Protein Expression in Hepatocytes.

Note: ***p < 0.001, ****p < 0.0001 vs. CON, [#]p < 0.05, ^{###}p < 0.001 vs. CCl₄, ⁺⁺p < 0.01, ⁺⁺⁺⁺p < 0.0001 vs. Lv-EGFP, [~]p < 0.05, [~]p < 0.01 vs. Lv-EGFP+TLR4.

Abbreviations: CON, control; CCl₄, carbon tetrachloride; TLR4, toll-like receptor 4; I- κ B, NF- κ B inhibitor protein; Lv, lentiviral vector; EGFP, enhanced green fluorescent protein.

Rutin is 25 and 50 μ M, it does not significantly reverse the decline of primary hepatocyte viability caused by CCl₄, but when the concentration of Rutin is 100 and 200 μ M, the primary hepatocyte viability is significantly reversed, the difference is statistically significant.

Rutin and CCl₄ Affect the TLR4-induced Inflammation Signaling Pathway in Primary Hepatocytes

As shown in Figure 3C, TLR4 was significantly downregulated and IκBα was significantly upregulated in the hepatocytes of the CCl₄ and Lv-EGFP-TLR4 groups compared with the CON and Lv-EGFP groups. However, Rutin significantly reversed the above changes. Figures 3D and E provide more intuitive data for the above results.

As shown in Table 5, the expression of inflammatory genes in hepatocytes was significantly changed. Specifically, MyD88, TNF- α , IL-1 β , IL-6, NF- κ B, and VCAM-1 genes were significantly increased in the hepatocytes of the CCl₄ and Lv-EGFP-TLR4 groups compared with the CON and Lv-EGFP groups. However, Rutin finally reversed the above changes.

Discussion

Liver injury refers to liver cell injury and liver function change caused by various reasons (Trefts et al., 2017). The long-term existence of liver injury inducement such as external force damage, lipid metabolism damage, and drug

Table 5. Effect of Rutin on Primary Hepatocytes Inflammation Related Genes ($n = 3$).

	CON	CCl ₄	Rutin + CCl ₄	Lv-EGFP	Lv-EGFP-TLR4	Lv-EGFP-TLR4 + Rutin
MyD88	1.00 ± 0.12	4.57 ± 1.00 ^{***}	1.92 ± 0.33 ^{###}	0.87 ± 0.11	5.04 ± 0.68 ⁺⁺⁺	2.56 ± 0.33 [~]
TNF- α	1.00 ± 0.15	4.24 ± 0.95 ^{**}	2.07 ± 0.26 [#]	0.92 ± 0.10	5.40 ± 1.08 ⁺⁺⁺	2.56 ± 0.47 [~]
IL-1 β	1.00 ± 0.15	3.23 ± 0.38 ^{***}	1.84 ± 0.31 [#]	1.11 ± 0.15	3.59 ± 0.49 ⁺⁺⁺⁺	2.04 ± 0.36 [~]
IL-6	1.00 ± 0.12	3.24 ± 0.44 ^{****}	1.84 ± 0.25 ^{###}	0.99 ± 0.16	3.63 ± 0.32 ⁺⁺⁺⁺	2.05 ± 0.27 [~]
NF- κ B	1.00 ± 0.16	4.50 ± 0.79 ^{***}	2.33 ± 0.17 ^{###}	0.90 ± 0.13	5.38 ± 0.65 ⁺⁺⁺⁺	2.93 ± 0.23 [~]
VCAM-I	1.00 ± 0.14	3.63 ± 0.37 ^{***}	1.80 ± 0.28 ^{###}	0.99 ± 0.20	3.92 ± 0.70 ⁺⁺⁺⁺	2.26 ± 0.22 [~]

Rutin reduced the increase of expression of MyD88, TNF- α , IL-1 β , IL-6, NF- κ B, and VCAM-I in the liver inflammation gene induced by CCl₄ or Lv-EGFP-TLR4.

Note: ^{**} $p < 0.01$, ^{***} $p < 0.001$, ^{****} $p < 0.0001$ vs. CON, [#] $p < 0.05$, ^{###} $p < 0.01$, ^{####} $p < 0.001$ vs. CCl₄, ⁺⁺⁺ $p < 0.001$, ⁺⁺⁺⁺ $p < 0.0001$ vs. Lv-EGFP, [~] $p < 0.01$ vs. Lv-EGFP-TLR4.

Abbreviations: CON, control; CCl₄, carbon tetrachloride; TLR4, toll-like receptor 4; Lv, lentiviral vector; EGFP, enhanced green fluorescent protein; MyD88: myeloid differentiation primary response 88; IL, interleukin; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; VCAM-I, vascular cell adhesion molecule-I.

and poison damage will lead to irreversible pathological changes in liver tissue (Ginès et al., 2021). CCl₄ is mainly inhaled by the respiratory tract and has serious damage to the liver, kidney, and central system, among which the liver is the most (Zhao et al., 2019). In hepatocytes, CCl₄ can be converted into free radicals *in vivo*, which can disrupt the metabolism of lipids on the hepatocyte membrane and cause hepatocyte necrosis (Unsal et al., 2021). The toxicity of CCl₄ is mainly manifested by the increase of liver-specific enzyme activity, the decrease of pseudocholinesterase activity, fat accumulation, the significant increase of serum ALT, AST, and other markers, and the decrease of liver function (Lee et al., 2021; Zhang et al., 2021). In this study, we successfully induced liver injury in mice by CCl₄, which was specifically manifested as weight loss and liver/body weight increase, abnormal liver tissue morphology, abnormal serum liver function indicators such as ALP, ALT, AST, and GGT, abnormal liver TLR4/MyD88/NF- κ B signal pathway, and liver inflammation outbreak.

As a monomer of traditional Chinese medicine, Rutin has anti-inflammatory, anti-microbial, anti-oxidative stress, and other pharmacological effects, which can significantly protect multiple organs from damage (Chua, 2013; Ghorbani, 2017). One of the practical findings in this study is that Rutin can effectively alleviate the liver damage caused by CCl₄ and play an anti-inflammatory role by regulating the TLR4/MyD88/NF- κ B signal pathway. Serum levels of ALP, ALT, AST, and GGT are indicators to evaluate liver function and reflect the state of liver tissue cells (Albhaisi & Qayyum, 2022; Faris et al., 2021). Rutin effectively reverses the increase of their levels caused by CCl₄, which is consistent with the findings in research on the treatment of liver damage caused by doxorubicin with Rutin (Ahmed et al., 2022). Serum levels of IL-6, IL-1, LDH, and IL-10 are representative

indicators reflecting the inflammatory state of the body (Libby, 2021; Ridker, 2016; Saraiva et al., 2020). IL-6, IL-1, and LDH promote inflammation, while IL-10 plays an anti-inflammatory role (Libby, 2021; Ridker, 2016; Saraiva et al., 2020). The results showed that Rutin reversed the increase of serum ALP, ALT, AST, GGT, IL-6, IL-1, and LDH levels and the decrease of IL-10 levels in mice caused by CCl₄. There are various opinions on how Rutin exerts liver protection through anti-inflammatory effects. Rutin can alleviate liver inflammation caused by doxorubicin by regulating NRF2, regulate autophagy by regulating the BANCER/miRNA-590-5P/OLR1 axis, and promote liver cell proliferation by promoting the insulin IRS-2/PI3K/Akt/GSK-3 β signaling pathway (Ahmed et al., 2022; Liang et al., 2018; Zhou et al., 2021). In summary, Rutin may exert anti-inflammatory effects in liver injury through multiple pathways, but the TLR4/MyD88/NF- κ B signal pathway may play an extremely important role in CCl₄-induced liver injury.

TLR4/MyD88/NF- κ B signal pathway is a signal transduction pathway closely related to cellular inflammation (Li et al., 2020). Recent studies have shown that the abnormal activation of TLR4/MyD88/NF- κ B signaling pathway is closely related to the pathogenesis and pathological progress of liver injury (Tian et al., 2020; Yao et al., 2015). TLR4 is a pattern recognition receptor that plays an important role in innate immunity and is usually overexpressed in inflammatory cells (Tian et al., 2020; Vijay, 2018; Zamyatina & Heine, 2020). After TLR4 is stimulated by various reasons, it recruits its downstream MyD88 and other related cytokines, triggering the signal cascade, leading to the activation of downstream NF- κ B, and ultimately leading to the occurrence of inflammatory reaction (Liu et al., 2014; Sun et al., 2021). Specifically, after TLR4 is activated, MyD88 protein is recruited to a specific binding site. Through phosphorylation

reaction, IRAK and TRAF6 are combined to form a complex (Li et al., 2019; Roy et al., 2016). Finally, the downstream NF- κ B inhibitor protein kinase is phosphorylated by the complex, making the I- κ B ubiquitinated, resulting in the dissociation of NF- κ B from the NF- κ B/I- κ B complex and living, translocation from the cytoplasm to the nucleus, and plays its pro-inflammatory activity (He et al., 2016). In this study, Rutin reduced the expression of TLR4 in experimental animals, increased the expression of I- κ B, and decreased the gene expression level of MyD88 and NF- κ B, revealing the role of TLR4/MyD88/NF- κ B signaling pathway in the treatment of CCl₄-induced liver inflammation by Rutin.

Currently, there are few clinical applications of Rutin, but there are also some preclinical studies (Choi et al., 2016; Hashizume & Tandia, 2020; Ragheb et al., 2020). For example, when cream containing or without Rutin is applied to human skin, it is found that Rutin increases the mRNA expression of type I collagen by clearing ROS, reduces the expression of Messenger RNA of matrix metalloproteinase 1, increases skin elasticity, reduces the length, area, and number of wrinkles, and delays skin aging (Choi et al., 2016). The combination of Rutin and vitamin C also strengthened the effects of blood glucose control, insulin resistance, lipid status, and oxidative stress markers in type 2 diabetes patients (Ragheb et al., 2020). In addition, the enzyme-modified form of Rutin, water-soluble α -glycosylated Rutin, can be applied to obese individuals to improve overall health by reducing abdominal visceral fat (Hashizume & Tandia, 2020). These all provide a reference value for the clinical application of Rutin, and in future clinical research on the treatment of liver diseases with Rutin, the following methods can also be used, but not limited to: (1) adding traditional drug compatibility, (2) combining with clinical medication, and (3) preparing new Rutin formulations. In summary, the application of Rutin in the clinical treatment of liver diseases is still in its early stages, and further research is needed to determine the safe dosage, specific mechanism of action, and therapeutic effect of Rutin in the human body. This is the current flaw and the direction of future work.

Conclusion

Our research shows that Rutin may be a natural inhibitor of TLR4, which can inhibit liver inflammation by regulating TLR4 and finally reverse CCl₄-induced liver injury, highlighting its important potential mechanism. More importantly, it provides a more theoretical basis and clinical guidance for Rutin to regulate liver inflammation.

Abbreviations

CCl₄: Carbon tetrachloride; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: γ -glutamyl transferase; H&E: Hematoxylin & eosin; IL: Interleukin;

CCK-8: Cell counting kit-8; CON: Control; Lv: Lentiviral vector; EGFP: Enhanced green fluorescent protein; TLR4: Toll-like receptor 4; MyD88: Myeloid differentiation primary response 88; NF- κ B: Nuclear factor kappa-light-chain-enhancer of activated B cells; I- κ B: NF- κ B inhibitor protein; VCAM-1: Vascular cell adhesion molecule-1; LDH: Lactate dehydrogenase.

Authors Contributions

QH and KZ are responsible for the conception and design of the research, the key revision of the knowledge content, and the approval of the final manuscript version to be published and agree to be responsible for all aspects of the work. HH has made substantial contributions to data acquisition, analysis, and interpretation. QH is responsible for the drafting of the manuscript. QH and KZ confirm the authenticity of all original data.

Declaration of Conflicting of Interests

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Statement of Informed Consent and Ethical Approval

All experimental procedures in this study follow the guidelines of the Research Ethics Committee of Hubei University of Medicine.

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