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Changes in cytokines and signaling pathways in different stages of hepatic fibrosis in rats

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Abstract

Background & Purpose: Liver fibrosis is a disease that seriously threatens people's health, and its etiopathogenesis has not been described clearly. Experimental Approach: Female rats were subjected to common bile duct ligation (BDL) for one month, and male rats were treated with thioacetamide for 23 weeks. The expression of cytokines, signal pathways, histopathology in liver and biochemical indexes in serum were detected. Key Results: The levels of transaminases in serum and hydroxyproline and α-smooth muscle actin in the liver were remarkably increased in both models, although the degree of liver fibrosis was more severe in thioacetamide rats than in BDL rats. However, the levels of IL-1α, IL-4, IL-10, TNFα, MCP-1, PDGF-BB, p-Akt and p-STAT5 decreased, and the levels of IL-18, TGFβ1 and p-p70s6k increased in the livers of BDL rats, while the levels of IL-1α, IL-4, IL-10, IL-1β, IL-6 and IL-12p70, TNFα, MCP-1, PDGF-BB, p-CREB, p-JNK, p-NFκB, p-Akt, p-p70s6k, p-STAT3 and p-STAT5 decreased, and the levels of IL-18 and TGF-β1 increased in the livers in thioacetamide rats. Conclusion and Implications: These data suggest that TGFβ1 and IL-18 may be the main fibrogenic factors at different stages of liver fibrosis and that the levels of inflammation-associated cytokines and signaling pathway components decrease as the severity of hepatic fibrosis progresses. Therefore, it may be better to apply anti-inflammatory drugs in the early stage or use these drugs which facilitating more inflammatory cells or cytokines into liver tissue at the end stage of hepatic fibrosis.

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Data availability:

The data is available on request from the authors.

Competing interests:

The authors declare no competing interests.

Authors contributions:

All authors contributed to the manuscript. Q.Pi, X.Hua and L.Wang performed the experiments, analyzed data and wrote the manuscript. They contribute the same. D.Fu and L.Zhang performed the experiments and graphed the data. Z. Tan and W.Tan contributed to the design of the experiments and supervised the project. All authors read and approved the final version of the manuscript.

Ethics approval:

All animal experiments were performed in accordance with the guidelines of the Ethical Committee for the Care and Welfare of Laboratory Animals from the Sichuan province government.

Abbreviations: BDL, bile duct ligation; α-SMA, α-smooth muscle actin; TAA, thioacetamide; HBV, hepatitis B virus; HCV, hepatitis C virus; NASH, nonalcoholic steatohepatitis; HSCs, hepatic stellate cells; NK, natural killer; TNFα, tumor necrosis factor α· ILs, interleukins; TGF-β, transforming growth factor β· ALT, alanine aminotransferase; AST, aspartate amino transferase; ALP, alkaline phosphatase; TBIL, total bilirubin; DBIL, direct bilirubin; H&E, hematoxylin and eosin; SABC, streptavidin-biotin complex; DAB, 3,3'-diaminobenzidine; PDGF, platelet-derived growth factor; IFN, interferon; TMB, Tetramethylbenzidine; MCP-1, monocyte chemoattractant protein-1; MIP, macrophage inflammatory protein; MFI, median fluorescent intensity; EMCs, extracellular matrix components; FN, fibronectin; LN, laminin; NLRP, NOD-like receptor protein;

Bullet point summary:

What is already known:

Inflammatory factors increase in early stage of liver fibrosis.

What this study adds:

Most inflammatory cytokines decrease in advanced hepatic cirrhosis.

Clinical significance:

According to the different degree of liver fibrosis, change the treatment strategy.

Background & Purpose:

Liver fibrosis is a disease that seriously threatens people's health, and its etiopathogenesis has not been described clearly.

Experimental Approach:

Female rats were subjected to common bile duct ligation (BDL) for one month, and male rats were treated with thioacetamide for 23 weeks. The expression of cytokines, signal pathways, histopathology in liver and biochemical indexes in serum were detected.

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Key Results:

The levels of transaminases in serum and hydroxyproline and α-smooth muscle actin in the liver were remarkably increased in both models, although the degree of liver fibrosis was more severe in thioacetamide rats than in BDL rats. However, the levels of IL-1α, IL-4, IL-10, TNFα, MCP-1, PDGF-BB, p-Akt and p-STAT5 decreased, and the levels of IL-18, TGFβ1 and p-p70s6k increased in the livers of BDL rats, while the levels of IL-1α, IL-4, IL-10, IL-1β, IL-6 and IL-12p70, TNFα, MCP-1, PDGF-BB, p-CREB, p-JNK, p-NFκB, p-Akt, p-p70s6k, p-STAT3 and p-STAT5 decreased, and the levels of IL-18 and TGF-β1 increased in the livers in thioacetamide rats.

Conclusion and Implications:

These data suggest that TGFβ_1 and IL-18 may be the main fibrogenic factors at different stages of liver fibrosis and that the levels of inflammation-associated cytokines and signaling pathway components decrease as the severity of hepatic fibrosis progresses. Therefore, it may be better to apply anti-inflammatory drugs in the early stage or use these drugs which facilitating more inflammatory cells or cytokines into liver tissue at the end stage of hepatic fibrosis.

Key words : liver fibrosis; cytokines; common bile duct ligation (BDL); thioacetamide (TAA); signal pathway;

Introduction

Liver fibrosis is a wound healing response to various types of injury, and it can progress into liver cirrhosis and even to hepatocellular carcinoma (HCC). Alcohol abuse, hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, and nonalcoholic steatohepatitis (NASH) are the main causes of liver fibrosis. In the European Union, 0.1% of the population is affected by cirrhosis, which leads to approximately 170,000 deaths each year.(1) In China, the morbidity rate of HBV was 9.75% in 1992, and approximately 300,000 patients died of HBV-related diseases (2). Unfortunately, there are currently no effective drugs for liver fibrosis, especially liver cirrhosis.

The etiopathogenesis of liver fibrosis is very complex. Many cells, including hepatic stellate cells (HSCs), fibroblasts, bone marrow-derived myofibroblasts, hepatocytes, Kupffer cells, and natural killer (NK) cells, are involved in this pathological processs(3-7) . These cells interact with each other through cytokines, resulting in the accumulation of extracellular matrix, as it is formed faster than it is degraded, and the gradual formation of fibrotic tissue that can further develop into cirrhosis. In particular, tumor necrosis factor α (TNFα), interleukins (ILs), and transforming growth factor β (TGF-β) are involved in the formation and development of liver fibrosis(8-11). However, whether these cytokines exhibit obvious changes with the development of liver fibrosis or whether those changes differ depending on the fibrosis stimulus is not fully understood.

Biliary cirrhosis is caused by biliary obstruction and cholestasis (12). Cholestasis leads to the sedimentation of bilirubin, bile salts, bile acids, cholesterol and other substances in the liver. Long-term cholestasis causes liver fibrosis and even cirrhosis(13). Bile duct ligation (BDL) is a common way to establish an animal model of cholestatic liver fibrosis (12). The animal model can simulate human liver fibrosis caused by long-term cholestasis, with good repeatability and a high success rate.

Thioacetamide (TAA) has hepatotoxicity and leads to the destruction of liver cells (14). Long-term and low-dose exposure to TAA may promote gradual hepatocyte necrosis, which is similar to the pathological process of human liver fibrosis, meaning that it is a good model for chronic liver injury and liver fibrosis(15).

This paper focuses on comparing the early stage of liver fibrosis or cirrhosis (caused by choledochal ligation for 1 month) with the late stage of cirrhosis (treated with TAA for 3 months) and exploring the similarities and differences in the main cytokines and signaling pathways in female and male rats to identify the common factors promoting liver fibrosis and find new targets or target groups for the treatment of liver fibrosis and cirrhosis.

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Materials and Methods

Animals

Female and male SD rats (body weight 200-250 g) were supplied by the Center of Experimental Animals of Sichuan Academy of Chinese Medicine Sciences (eligibility certification no. SCXK (chuan) 2013-19). The rats were maintained under standard conditions with a 12 h:12 h light–dark cycle in a temperature- and humidity-controlled environment. The rats were provided food and water *ad libudum.* All animal experiments were performed in accordance with the guidelines of the Ethical Committee for the Care and Welfare of Laboratory Animals from the Sichuan province government.

Induction of liver fibrosis by BDL

BDL operation was performed under sterile conditions. 20 female SD rats were randomly into control and BDL groups, rats were anesthetized by intraperitoneal injection of pentobarbital 50mg/kg, the abdomen was opened by a median incision, and the common bile duct was double-ligated and cut between the ligatures. Sham-operated control animals underwent similar manipulation without bile duct ligation. On the 29^{th} day, the animals were sacrificed, and sampling was performed to detect related indicators.

Induction of liver fibrosis with TAA

20 male SD rats were randomly into control and TAA treated groups. The model animals were fed 0.035% TAA solution for 23 weeks, and the controls were fed pure water. Twenty-four hours after the last administration, the animals were sacrificed, and sampling was performed to detect related indicators.

Serum biochemistry assays

Blood samples were taken from the abdominal aorta of anesthetized animals, and the serum levels of alanine aminotransferase (ALT), aspartate amino transferase (AST), alkaline phosphatase (ALP), total bilirubin (TBIL), and direct bilirubin (DBIL)(Sichuan Maker Biotechnology Co. Ltd, Chengdu, China) were measured using a standard automatic biochemical analyzer (HITACHI 7020).

Measurement of hepatic hydroxyproline content

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Liver tissue (60 mg) was mixed with 1.0mL of 6 N NaOH and incubated at 100°C for 20 min with frequent vigorous shaking. After cooling, 1.0 mL of 3N HCl was added to the solution, the pH was regulated to 6.0˜6.8, and then 10 mL of water was added and mixed. Then, 3.0 mL of the suspension was added to 20˜30 mg active carbon, and the sample was mixed and centrifuged at 3500 rpm for 10 min. The supernatant was taken to measure the hydroxyproline contents using a colorimetric hydroxyproline test kit according to the manufacturer's instructions (Institute of Jiancheng Bioengineering, Nanjing, China). The hydroxyproline content was expressed as micrograms per gram of liver weight (μg/g liver).

Hepatic histological and pathological

assay

The wet weight of the liver or spleen was measured and then divided by the body weight to obtain the liver or spleen index. Then, the liver tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Tissue sections (4-μm thick) were prepared and placed on glass slides. They were then stained with hematoxylin and eosin (H&E) or Masson's trichrome stain following standard protocols. A pathologist who was blinded to the experimental groups scored liver fibrosis under a microscope according to the METAVIR scoring system (F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis with rare septa; F3, numerous septa without cirrhosis; F4, liver cirrhosis) (16).

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Tissue sections (4-μm thick) were prepared and placed on glass slides. Endogenous enzymes were inactivated, and antigen retrieval was performed using heat (in 0.01 M citrate salt buffer, pH 6.0, heating to 100°C two times for 10 mins). Then, the sections were incubated with 5% BSA at room temperature for 20 min, followed by the addition of anti-α-SMA (1:40) antibody (Santa Cruz Biotechnology, Inc., CA, USA) at 2–8°C overnight. They were then incubated with biotinylated goat anti-rabbit IgG (1:100) (Wuhan Boster, Wuhan, Hubei, China) at 37°C for 30 min. Next, streptavidin-biotin complex (SABC) detection reagent (1:100) (Wuhan Boster, Wuhan, Hubei, China) was added dropwise, and the samples were incubated for 30 min before 3,3'-diaminobenzidine (DAB) reagent (Wuhan Boster, Wuhan, Hubei, China) was added. After washing with distilled water, the sections were stained with hematoxylin, and a pathologist who was blinded to the treatment groups counted the number of positively stained cells under a microscope.

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Extraction of protein

Segments (100 mg) of frozen livers were ground up at 0°C, and then 500 μl diluted cell signaling lysis buffer supplemented with 5 μl protease inhibitor cocktail set III (Millipore Corporation, USA) was added. The mixture was vortexed for 3 cycles of 20 min on/3 min off. The suspended liver tissues were sheared with an ultrasonic cell disruptor and centrifuged at 4°C (14000 rpm for 20 min). The supernatant was stored at -80°C until measurement of the levels of cytokines and phosphorylation of the key protein in the signal pathways by ELISA or MILLIPLEX®MAP assay.

Protein assay

The BCA Protein Assay Kit (Thermo Scientific Pierce Protein Research Products, USA) is a comprehensive and robust protein quantification assay based on the colorimetric detection of copper ions that are chelated with proteins. The protein content in the liver samples was measured according to the manufacturer's instructions.

Enzyme-linked immunosorbent assay (ELISA)

The contents of platelet-derived growth factor (PDGF)-BB, interferon (IFN)-γ and TGF-β1 (R&D Systems Inc., Minneapolis, USA) in the protein extracts were measured by ELISA according to the manufacturer's instructions**.** Briefly, the protein extracts were lysed with 100 mM Tris (pH: 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, and 0.5% sodium deoxycholate. Then, 50 μL of standard, control, or sample was added to each well of the microplate, which was precoated with capture antibody; A second HRP-labeled antibody was added and bound to the antigen-antibody complex.Unbound detection antibody was washed away. Tetramethylbenzidine (TMB) substrate solution was added to the wells, and a blue color developed in proportion to the amount of analyte present in the sample. Color development was stopped, turning the color in the wells yellow. The absorbance of the color at 450 nm was measured. The contents of PDGF-BB, IFN-γ and TGFβ_1 were calculated by means of standard curves.

Multiplex bead assay for detecting cytokines and phosphorylated proteins of the signaling pathway

Multiplex bead assays were used to quantify the amounts of IL-10, IL-13, IL-12p70, IL-4, IL-6, IL-12, IL-18, IL-1α, IL-1β, monocyte chemoattractant protein-1 (MCP-1/CCL2), macrophage inflammatory protein (MIP)-1α, MIP-2, and TNFα (the MILLIPLEX MAP Rat Cytokine/Chemokine Magnetic Bead Panel; Cat # RECYTMAG-65K-12, Billerica, MA, USA) and P-CREB, P-JNK, P-NF-ϰB, P-P38MAPK, P-ERK1/2, P-Akt, P-p70S6K, P-STAT3 and P-STAT5 (MILLIPLEX® MAP 9-Plex Multi-Pathway Signaling Magnetic Bead Kit for Phosphoprotein, 96-well; Cat # 48-680MAG, Billerica, MA, USA) in liver protein extracts. All assays were performed and analyzed according to the manufacturer's instructions. The plate was analyzed using a Luminex 200 analyzer and xPONENT (Billerica, MA, USA) software, and the median fluorescent intensity (MFI) data were saved and analyzed using a five-parameter logistic or spline curve-fitting method to calculate the analytical concentrations in the samples.

Statistical analysis

All the data are expressed as the mean+-SD. The differences in the biomarkers between groups were analyzed using one-way analysis of variance test, and then the individual differences between groups were evaluated using Dunnett's test. The differences in histopathology between groups were analyzed using the Kruskal-Wallis test and Dunn's multiple comparison test.

Results

TAA and BDL caused significant liver dysfunction in rats and led to obvious liver fibrosis

Elevated serum levels of ALT and AST are indicators of liver injury(17). ALP levels are related to chronic liver injury(18). The serum levels of ALT, AST and ALP and the liver weights of normal female rats were significantly lower than those of male rats. The levels of ALT, AST, and ALP and the liver weights or liver

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indexes were significantly increased by BDL for 1 month and were much higher than those in rats treated with TAA for 23 weeks. These data suggest that BDL for 1 month induced an obvious inflammatory reaction in the livers of rats that was more potent than that of TAA (Figure 1).

Fibrosis is the excessive accumulation of collagen and other extracellular matrix components (EMCs) such as fibronectin (FN) and laminin (LN), due to chronic injury (19-21). Hydroxyproline is a characteristic component of collagen, and the content of hydroxyproline is related to the level of tissue fibrosis(22). As shown in Table 1, hydroxyproline in the livers of female rats was significantly higher than that in those of male rats. In addition, 1 month after BDL, the increase in hydroxyproline was significantly higher in female rats than in male rats treated with TAA for 23 weeks but BDL for 1 month showed lower pathological scoring, suggesting that the hydroxyproline level in liver was not completely proportional to the degree of fibrosis. The mediastinal form and connective tissue hyperplasia in the liver were more obvious in TAA rats than in BDL rats (Table 1, Figure 2), and α-SMA, the marker of HSC activation, was upregulated in the livers of BDL and TAA rats (Figure 3).

The effect of BDL on the levels of ILs in female rats is different from the effect of TAA in male rats

ILs include many cytokines that play an important role in liver injury or fibrosis. Many cells can synthesize or secrete ILs. Many cells can synthesis or secretion of ILs. As Figure 4 shows, the levels of interleukins, especially IL-4, IL-10, IL-13, IL-18 and L-12p70, in liver tissue of female rats were higher than those in the liver tissue of male rats. After BDL or TAA treatment, IL-1α, IL- 4 and IL-10 decreased, but IL-18 increased; BDL for a month upregulated IL-1β in the livers of female rats but had no significant effect on IL-6 and IL-12p70, while TAA decreased IL-1 β, IL-6 and IL-12p70 levels in the livers of male rats (Figure 4).

ΒΔΛ σιγνιφιςαντλψ ινςρεασες ΜΙΠ-1α βυτ δεςρεασες ΤΝΦα εξπρεσσιον, ωηιλε ΤΑΑ μαρκεδλψ δεςρεασες ΤΝΦα ανδ ΜΆΠ-1 εξπρεσσιον ιν τηε λιερς οφ ρατς

Macrophages are key regulators of fibrosis development(23). The liver contains macrophages including Kupffer cells and macrophages filtered from the blood. When activated, they can produce and release many proinflammatory cytokines, such as TNFα, MIP-2, MIP-1α and MCP-1. These cytokines may attract other proinflammatory cells and recruit macrophages themselves to sites of inflammation. As Table 4 shows, the TNFα, MIP-2 and MCP-1 levels were significantly higher in the livers of control female rats than in the livers of male rats. BDL or TAA treatment significantly reduced TNFα and MCP-1 levels and slightly increased MIP-2 levels; BDL significantly increased the level of MIP-1 α in female rats, but TAA had no obvious effect on MIP-1α in male rats (Figure 5).

ΒΔΛ σιγνιφιςαντλψ ινςρεασες ΤΓΦβ1 εξπρεσσιον ιν φεμαλε ρατς

PDGF-BB possesses mitogenic(24), differentiation-promoting (25), chemotactic(26) and angiogenic(27) properties, and is produced by osteoblasts, platelets and monocytes/macrophages, PDGF-BB and TGF-β1both activate the HSCs and promote liver fibrosis(28). TGF-β1is ubiquitously present in both normal cells and transformed cells and almost all cells have receptors for it(22). IFN-γ plays critical roles in promoting pathologic inflammatory processes(29). In Table 5, the levels of PDGF-BB, IFN-γ and TGF-β 1 in the liver of female and male rats showed no significant difference; BDL or TAA slightly decreased PDGF-BB and increased in TGF-β 1 but had no obvious effect on IFN-γ. The increase in TGF-β 1 in livers of female rats with BDL was significantly greater than that with TAA treatment (Figure 6).

BDL and TAA have similar inhibitory effects on signaling pathway components except p-p70S6K in the livers of rats

Many signaling pathway components, such as CREB, c-JNK, NF-ϰB, P38MAPK, ERK1/2, Akt, p70S6K, STAT3 and STAT5, are involved in the occurrence, development and dissolution of liver fibrosis(30-37). Activation of the signaling pathways occurs via phosphorylation of these key proteins, so the levels of phosphorylated protein represent the level of signaling pathway activation.

As shown as Table 6, p-CREB, p-JNK, p-NF-ϰB, p-P38MAPK, p-ERK1/2, p-Akt, p-p70s6k, p-STAT3 and p-STAT5 expression was significantly higher in the livers of female rats than those of male rats; BDL significantly decreased p-NFϰB p-P38MAPK, p-Akt, p-STAT5 expression and increased p-p70s6k expression in the livers of female rats. TAA significantly decreased the levels of p-CREB, p-JNK, p-NFϰB, p-Akt, p-p70s6k, p-STAT3 and p-STAT5 in male rats but had no significant effect on the levels of p-P38MAPK and p-ERK1/2 (Figure7).

Discussion

The TAA- and BDL-induced models of liver fibrosis in rats are good models for exploring the pathogenesis of liver fibrosis and evaluating the action of drugs for liver fibrosis and cirrhosis. Here, we found that BDL for one month significantly increased the ALT, AST, ALP, and Hyp levels, liver indexes and pathological score of liver fibrosis in female rats. TAA treatment for 23 weeks has similar effects on the male rats. However, the level of Hyp in the livers of BDL-treated rats was higher than that in those of TAA-treated rats, but the pathological score of liver fibrosis was much lower than that in TAA-treated rats, which suggested that the composition of liver fibrosis in BDL rats was different from that in TAA-treated rats, which may be due to the difference in sex.

Interleukins (IL) including a lot of cytokines which play an important role in liver injury or fibrosis.IL-1α and IL -6 correlates with severity of liver diseases (38, 39) while IL-4 levels can be used to predict advanced fibrosis in chronic hepatitis C (40). IL-1β, Il-6, IL-9, IL-10, IL-13, IL-18 and L-12 are higher in patients with cirrhosis (41-47), However, we found that with BDL or TAA treatment, IL-1α, IL- 4, IL-10, TNFα, MCP-1 and PDGF-BB decreased, but IL-18 and TGF-β 1 increased; BDL for a month upregulated IL-1β and MIP-1α in the livers of female rats, while TAA decreased IL-1β, IL-6 and IL-12p70 in the livers of male rats.

BDL significantly decreased p-NFϰB, p-P38MAPK, p-Akt, and p-STAT5 and increased p-p70s6k in the livers of female rats. TAA significantly decreased the levels of p-CREB, p-JNK, p-NFϰB, p-Akt, p-p70s6k, p-STAT3 and p-STAT 5 in male rats but had no significant effect on the levels of p-P38MAPK and p-ERK1/2.

IL-1 refers to two similar cytokines (IL-1α and IL-1β) that bind to the same receptor and play an important role in acute and chronic inflammation. IL-1β is produced by monocytes, macrophages and neutrophils; it also recruits and activates these cells and induces local inflammation. In addition, IL-1β can activate HSCs, which contributes to fibrosis. When IL-1β levels are elevated, depletion of IL-1R1 ameliorates this fibrotic phenotype in mouse models of liver fibrosis, and the absence of IL-1 signaling attenuates thioacetamide-induced liver fibrogenesis in rats. IL-1 may regulate fibrosis and tissue remodeling by modulating the expression of matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases. IL-18 is constitutively expressed in myeloid cells and epithelial cells; it signals via a heteromeric receptor, increases the production of nitric oxide (NO) and chemokines, and increases cell adhesion molecules for leukocyte trafficking. The maturation and secretion of IL-1β and IL-18 are mediated by inflammatory caspases within inflammasomes containing the NOD-like receptor (NLR) protein (NLRP) 3. Large amounts of IL-1β are produced by Kupffer cells, which also express most NLRs. Inflammasome signaling often induces pyroptosis that allows the passive release of alarmins, including IL-1α. Inflammasomes may regulate liver fibrosis directly by mediating inflammasome expression in HSCs or indirectly by HSC activation via Kupffer cell-derived IL-1β and IL-18. In addition, depletion of Nlrp3 significantly reduced the expression of TGF-β1 and collagen-1α1 in carbon tetrachloride-induced or TAA-induced liver fibrosis mouse models.

p-p70s6k is the active form of p70s6k, which plays an important role in protein synthesis and cell cycle control. p70s6k is activated by growth factors and hormones through the phosphatidylinositol 3-kinase (PI3K)-dependent signaling pathway. IL-1β increases the p-p70s6k protein level in a time-dependent manner(48) .

Overall, BDL for one month increased the degree of liver fibrosis by augmenting the production of TGFβ1, induced the activation of HSCs by increasing the production of IL-1β and IL-18 to enhance inflammasome function and accelerated the formation of the collagenous connective tissue via the p70s6k signaling pathway.

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However, a key function of IL-18 is that it cooperates with IL-12 to induce IFN-γ production from T helper cells and NK cells, leading to NK cell activation, but BDL slightly decreased the levels of IL-12p70 and IFN-γ.

However, the level of MIP-1α markedly increased in the livers of BDL rats. MIP-1α is also known as CCL3 and is strongly expressed on T cells, macrophages, neutrophils, endothelial cells and HSCs. It may recruit several cell types, such as T cells, neutrophils and eosinophils, to the site of inflammation by interacting with its receptors CCR1 and CCR5. HSCs also express CCR5, which is the target of CCL3 in the liver. CCR1 and CCR5 deficiency can alleviate liver fibrosis from chronic carbon tetrachloride treatment or BDL.

CREB, JNK, NFκB, P38MAPK, ERK1/2, Akt, STAT3 and STAT5 are related to liver injury or fibrosis. Unexpectedly, most of the ILs and phosphorylated signaling pathway proteins we assessed were decreased by BDL for one month or by TAA treatment for 23 weeks. Under a microscope, we found that liver tissue slices from TAA-treated male rats exhibited a high degree of hyperplasia of connective tissue and scar tissue formation, and this effect was more serious than that of BDL female rats. There was no inflammation or necrotic tissue, which may be the cause of the decrease in inflammation-related signaling pathways in the two liver fibrosis groups, especially in TAA-treated rats.

Conclusion

The levels of most inflammation-associated cytokines and signaling pathway components in the livers of normal young female rats are higher than those in the livers of male rats, and liver fibrosis induced by BDL in female rats or TAA in male rats is analogous; however, due to the different degrees of fibrosis, there are some variations in the levels of inflammation-associated cytokines and signaling pathway components. As the severity of hepatic fibrosis increases, the scar form and connective tissue hyperplasia become more obvious, and the levels of inflammation-associated cytokines and signaling pathways decrease, with some levels falling even lower than those in normal controls. These data suggest that the therapeutic strategies for liver fibrosis must change along with the state of liver fibrosis: anti-inflammatory drugs should be used at the early stage to prevent liver fibrosis progress, and improved liver microcirculation drugs should be used at the end stage of liver fibrosis (liver cirrhosis); this strategy may facilitate the recruitment of more inflammatory cells or cytokines into liver tissue activating these enzymes such as matris metalloproteinases to eliminate the scar structure and reverse liver cirrhosis.

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Table 1. Effects of BDL or TAA on the liver fibrosis in rats

Groups	N	Hydroxyproline (mg·g)	Degree of liver fibrosis 0	Degree of liver fibrosis 1	Degree of liver fibrosis 2	Degree o 3
Female	10	276.1±17.4	10	0	0	0
DBL	10	496.3±79.8**	0	1	7	0
Male	10	228.84±21.80	10	0	0	0
TAA	9	376.57±160.27*	0	0	2	3

Compared with the same sex controls, $^{*}p<0.05$, ** $p<0.01$, *** $p<0.001$

Hydroxyproline in the livers of female rats was significantly higher than that in those of male rats. And liver hydroxyproline level in BDL female rats was significantly higher but the degree of fibrosis is much less than the male rats treated with TAA.

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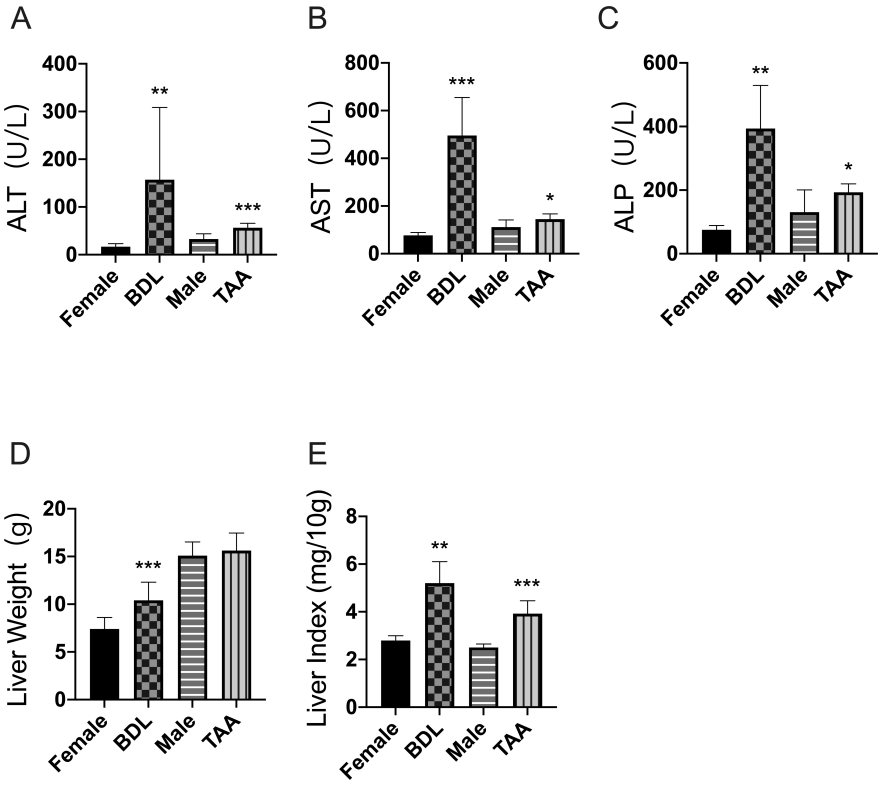

Figure 1. Effects of BDL or TAA on the liver functions in rats.

(A) ALT, (B) AST and (C) ALP in Serum and (E) liver index significantly increased by BDL or TAA. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ compared with the same sex control group.

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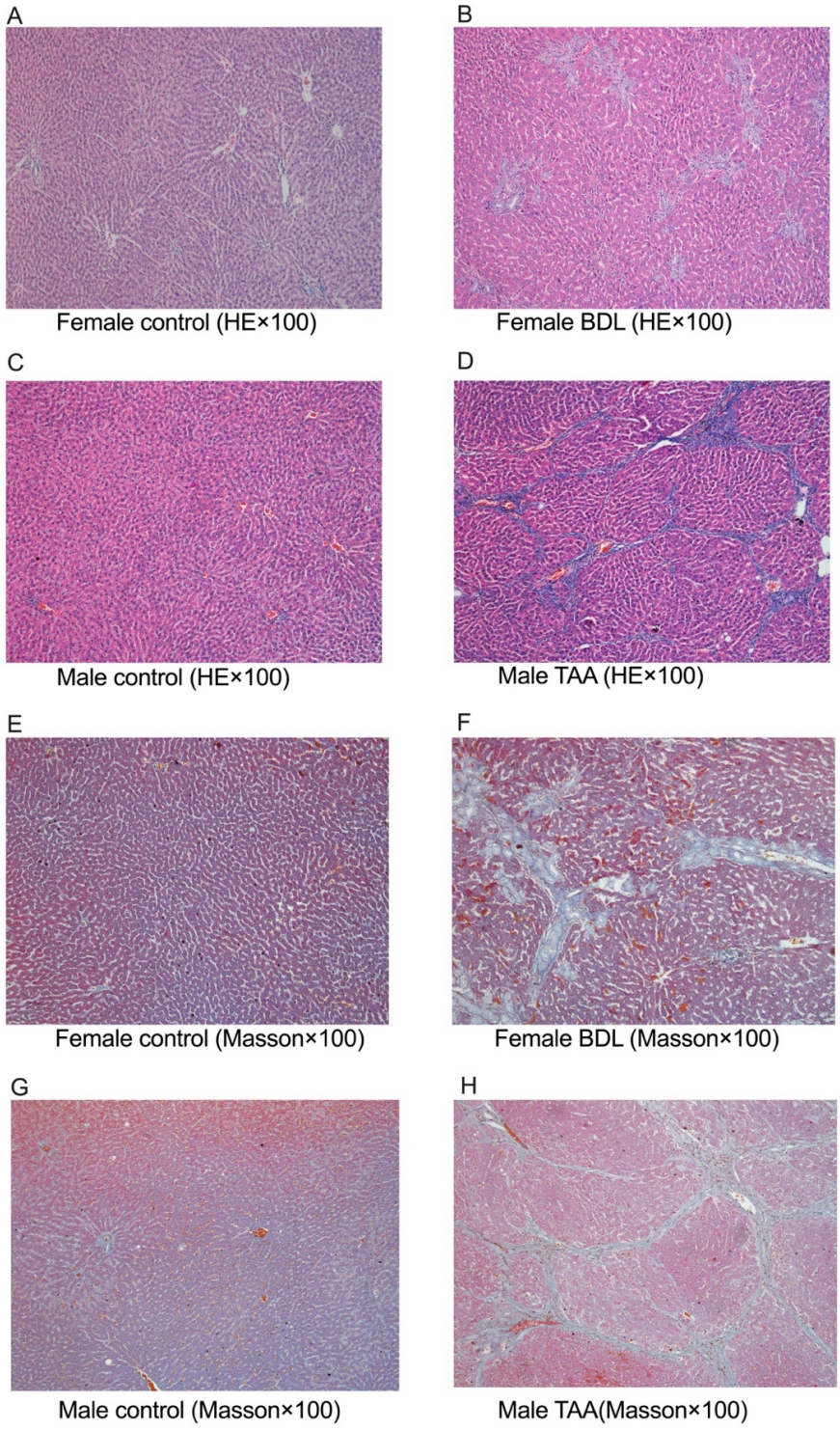
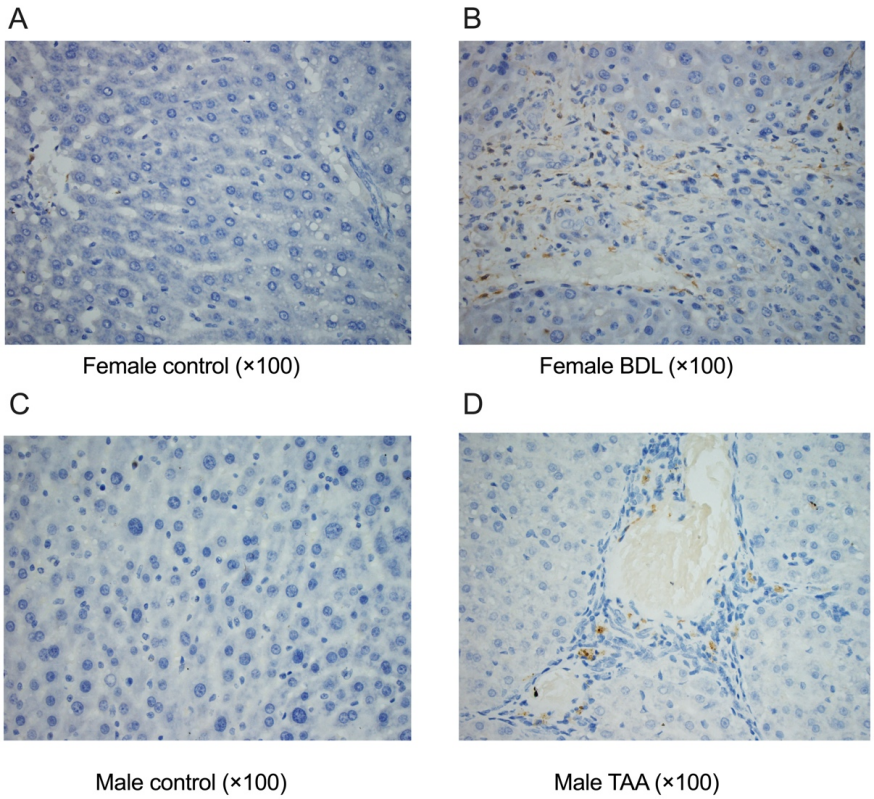


Figure 2. Effects of BDL or TAA on histopathology of liver in rats.

A, C and E, G are normal pictures of hepatic tissue stained by H&E or masson under microscope, while B, D, F, H are liver fibrosis induced by BDL or TAA, however, the mediastinal form in the liver in TAA rats

are more seriousness than that in BDL rats.



Φιγυρε 3. Εφφεςτς οφ ΒΔΛ ορ ΤΑΑ ον τηε εξπρεσσιον οφ α-ΣΜΑ ιν τηε λιερ.

Brown color indicates positive. (A) negligible positive immunostaining in female normal rat livers; (B) strong α-SMA expression in liver of BDL rats; (C) negligible positive immunostaining in male normal rat livers; (D) α-SMA expression markedly increased in TAA rat livers. (α-SMA immunohistochemistry, haematoxylin counterstain, ×100).

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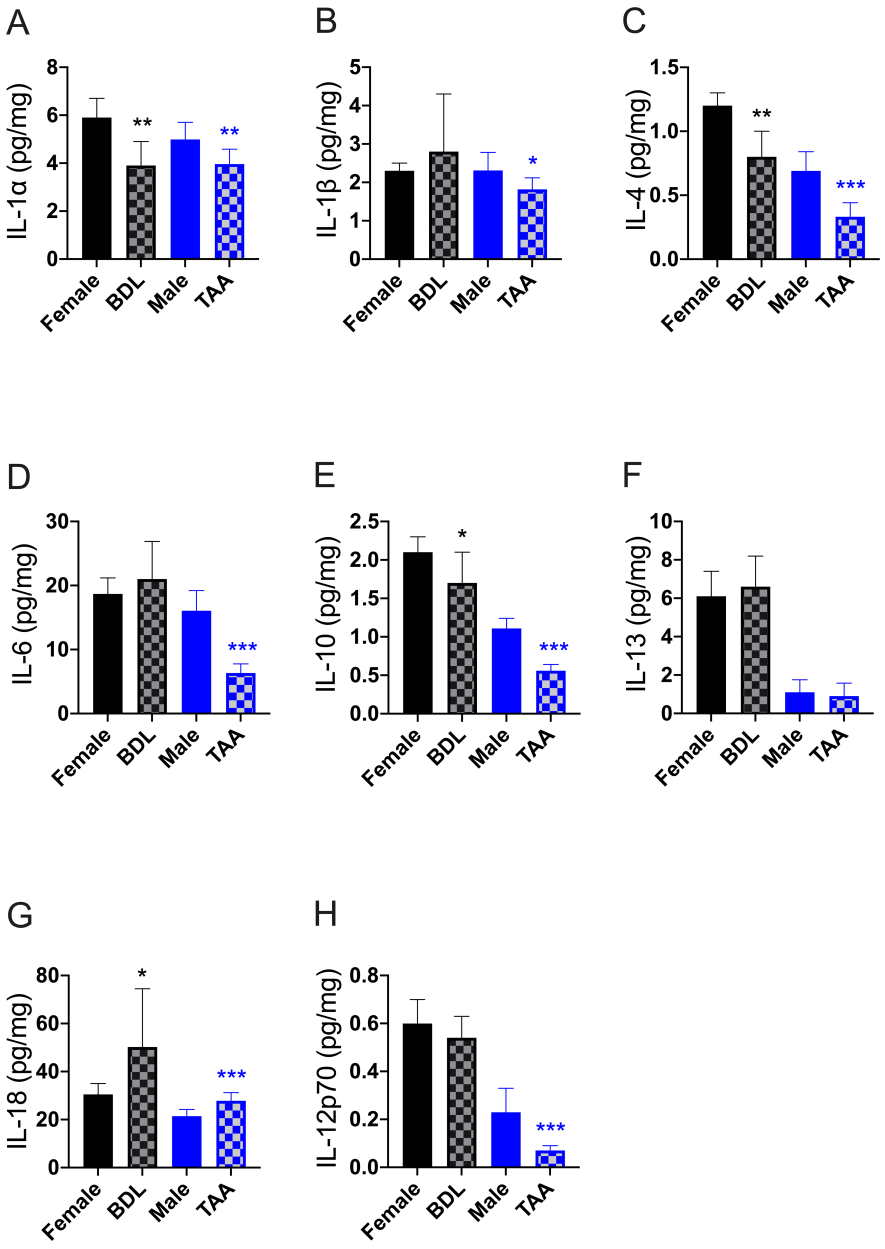

Figure 4. Effects of BDL or TAA on the levels of ILs in livers in rats

BDL significantly decrease the levels of (A) IL-1α, (C) IL-4, (E) IL-10 in livers in female rats, while TAA decreased the levels of (A) IL-1α, (B) IL-1β, (C) IL-4, (D) IL-6, (E) IL-10, and (H) IL-12p70 in livers in male rats, however, BDL or TAA both significantly increased the levels of (G) IL-18 in livers in female or male rats. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ compared with the same sex control group.

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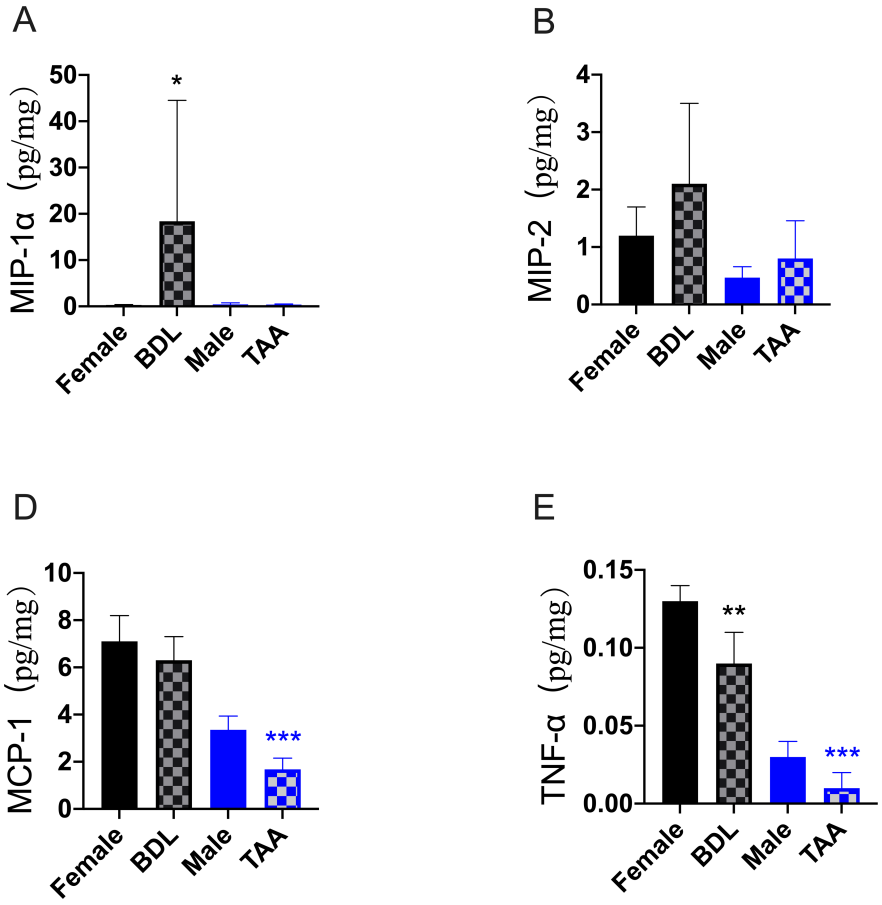
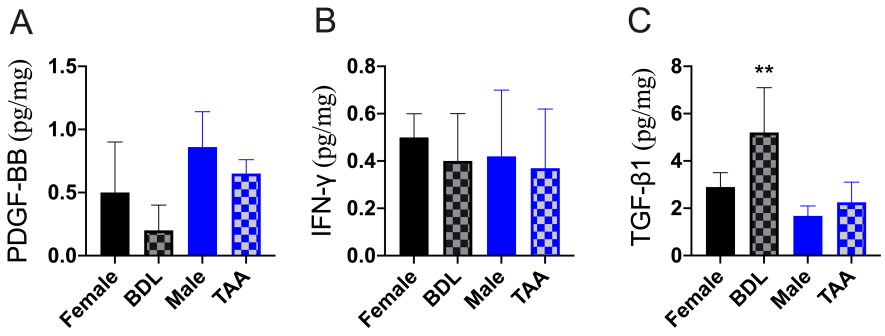


Φιγυρε 5. Εφφεστς οφ ΒΔΛ ορ ΤΑΑ ον τηε λεελς οφ ΜΙΠ-1α, ΜΙΠ-2, Μ˚Π-1ανδ ΤΝΦ-α ιν λιερς ιν ρατς.

BDL markedly increased the levels of (A) MIP-1α and decreased the level of TNFα in liver in female rats, while TAA decreased the levels of MCP-1 and TNFαin liver in male rats. * p<0.05, ** p <0.01 and *** p <0.001 compared with the same sex control group.



Φιγυρε 6. Εφφεστς οφ ΒΔΛ ορ ΤΑΑ ον τηε λεελς οφ ΠΔΓΦ-ΒΒ, ΙΦΝ-γ ανδ ΤΓΦ-β1 ιν λιερς ιν ρατς.

BDL markedly increased the level of (C) TGFβ1 in liver in female rats. But has no effects on the levels

of (A) PDGF-BB and (B) IFN-γ,while TAA has no the levels of (A) PDGF-BB, (B) IFN-γor TGF-β1. * p<0.05, ** p <0.01 and *** p <0.001 compared with the same sex control group.

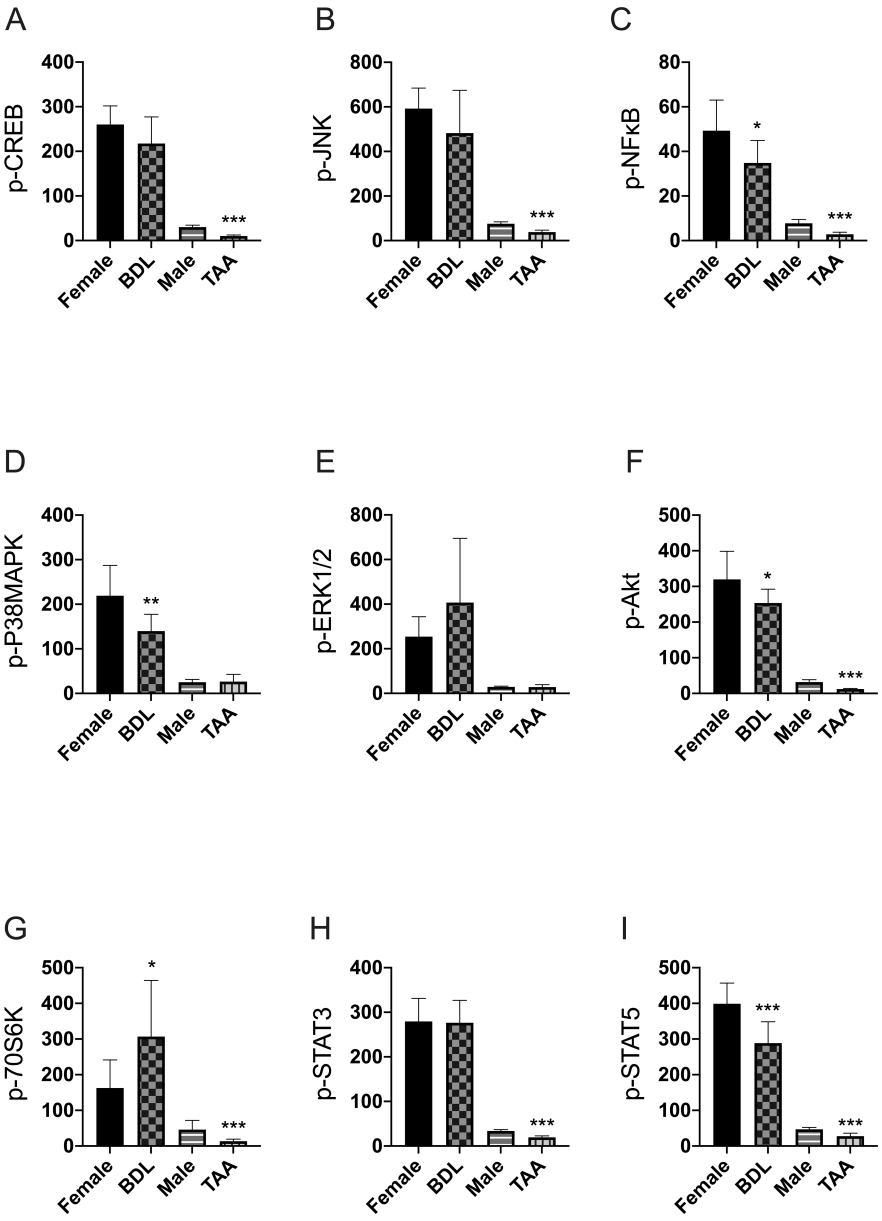


Figure 7. Effects of BDL or TAA on the phosphorylated protein levels of signaling pathway-related peotein in liver in rats.

BDL markedly decreased the level of (C) p-NFκB, (D)p-P38MAPK, (F) p-Akt, (I) p-STAT5and increased (G) p-70S6K in liver in female rats, and TAA markedly decreased the level of (A) p-CREB, (B) p-JNK, (C)p-NFκB, (F) p-Akt, (G) p-p70S6K, (H) p-STAT3 and (I) p-STAT5 in liver in male rats. * p<0.05, ** p <0.01 and *** p <0.001 compared with the same sex control group.

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