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Restraint stress promotes nonalcoholic steatohepatitis by regulating the farnesoid X receptor/NLRP3 signaling pathway

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Abstract
Psychological stress promotes nonalcoholic steatohepatitis (NASH) development. However, the pathogenesis of psychological stress-induced NASH remains unclear. This study aimed to explore the underlying mechanism of restraint stress-induced NASH, which mimics psychological stress, and to discover potential NASH candidates. Methionine choline deficient diet- and high fat diet-induced hepatosteatotic mice are subjected to restraint stress to induce NASH. The mice are administrated Xiaoyaosan granules, NOD-like receptor family pyrin domain containing 3 (NLRP3) inhibitors, farnesoid X receptor (FXR) agonists, or macrophage scavengers. Pathological changes and NLRP3 signaling in the liver are determined. These results demonstrate that restraint stress promotes hepatic inflammation and fibrosis in hepatosteatotic mice. Restraint stress increases the expression of NLRP3, Caspase-1, Gasdermin D, interleukin-1β, cholesterol 7α-hydroxylase, and sterol 12α-hydroxylase and decreases the expression of FXR in NASH mice. Xiaoyaosan granules reverse hepatic inflammation and fibrosis and target FXR and NLRP3 signals. In addition, the inhibition of NLRP3 reduces the NLRP3 inflammasome and liver damage in mice with restraint stress-induced NASH. The elimination of macrophages and activation of FXR also attenuate inflammation and fibrosis by inhibiting NLRP3 signaling. However, the inhibition of NLRP3 or macrophage scavengers fail to affect the expression of FXR. In conclusion, restraint stress promotes NASH-related inflammation and fibrosis by regulating the FXR/NLRP3 signaling pathway. Xiaoyaosan granules, NLRP3 inhibitors, FXR agonists, and macrophage scavengers are potential candidates for the treatment of psychological stress-related NASH.

Keywords: psychological stress; liver fibrosis; macrophage; farnesoid X receptor; Xiaoyaosan granules

1 Introduction
Psychological stress promotes the progression of liver diseases[1]. Epidemiological studies have shown that nonalcoholic fatty liver disease (NAFLD), a highly prevalent liver disease, occurs or is
exacerbated in humans subjected to psychological stress[2, 3]. Severe psychological stresses such as depressive and anxious symptoms are accompanied by ballooning degeneration in patients with NAFLD[4]. In vivo experiments have shown that chronic psychological stress increases the incidence of steatohepatitis with lobular inflammation, ballooning degeneration[5], and mild fibrosis[6]. In mice with NASH, psychological stress aggravated depressive-like behaviors, inflammation, and oxidative liver injury[7]. Inflammation is a mechanism underlying psychological distress in patients with NAFLD.

Previous reports indicate that chronic restraint stress, a psychological stressor, is associated with increased adipocytokines, including interleukin (IL)-6, IL-1β, macrophage inflammatory protein-2, and monocyte chemoattractant protein-1[5]. Restraint stress inhibits liver injury repair in mesenchymal stem cells[8]. In the upstream pathways, restraint stress activates nuclear factor (NF)-κB and NOD-like receptor family pyrin domain containing 3 (NLRP3) in mice with NAFLD[9]. Furthermore, chronic social defeat stress increases susceptibility to colitis by promoting NLRP3 inflammasome production in macrophages[10]. The canonical pathway for NLRP3 activation involves two steps. One is to induce the nuclear transcription of NLRP3 by initiating NF-κB activity. The second mechanism involves activation of NLRP3 assembly by damage-associated molecular patterns. The oligomerization of NLRP3, apoptosis-associated speck-like protein containing a CARD (ASC), and cysteinyl aspartate specific proteinase-1 (Caspase-1) precursors leads to the activation of Caspase-1 and the release of IL-1β and IL-18[11]. NLRP3 knockout reverses NASH in high-cholesterol diet-fed mice owing to the lack of inflammasomes[12]. However, the association between NLRP3 inflammasome activation in hepatic macrophages and psychological stress-induced NASH requires further investigation.

Among the upstream regulators of NLRP3, farnesoid X receptor (FXR) relieves the development of NASH[13]. NLRP3 inflammasome activation is accompanied by the downregulation of FXR in patients with liver necrosis and liver injury in mice [14]. The NLRP3 inflammasome may be modulated by FXR in psychological stress-related NASH. Whether the FXR/NLRP3 signaling pathway is involved in psychological stress-induced NASH remains unclear. In addition, Xiaoyaosan granules, originating from the book Prescriptions of the Bureau of Taiping People’s Welfare Pharmacy in Song Dynasty of China, can ameliorate chronic restraint stress-induced depressive-like behavior[15]. Some reports indicate that Xiaoyaosan granules reduce liver fibrosis in rats[16]. Whether Xiaoyaosan granules are a potential candidate for psychological stress-related NASH also needs further investigation.
In this study, hepatosteatotic mice were subjected to restraint stress that mimics psychological stress to induce NASH. The underlying mechanism involved in psychological stress-induced NASH was explored. The effect of Xiaoyaosan granules on psychological stress-induced NASH was evaluated.

**2 Materials and Methods**

### 2.1 Animal experiments

Six-week-old specific pathogen-free (SPF) male C57BL/6 mice were divided into seven groups with six mice per group. Mice were fed a methionine choline-deficient diet (MCD) for eight weeks to induce hepatic steatosis[17]. Mice in the control group were fed a standard diet (3.4 kcal/g, 4% fat, etc.). The MCD-fed mice were subjected to restraint stress for 10 h (9:00-19:00) every day from the 3rd week to induce NASH for six weeks. The specific operation of restraint stress was provided below: A mouse was placed into a 50-mL centrifuge tube (CLS430829; Corning, Corning, USA) to limit the space for locomotor activity by making its head towards the bottom of the tube and its tail through the hole of the centrifuge tube cap. Each tube contained one hole at the bottom of the tube, one hole at the top of the tube and 9 holes in 3 walls of the tube to provide sufficient holes for the mouse to breathe. After restraint stress treatments were completed, the mouse was removed from the tube. The total restrained time was 420 h. The hepatosteatotic mice and restraint stress-treated hepatosteatotic mice were orally administered 0.51 g/kg of Xiaoyaosan granules, a clinically equivalent dosage in mice, daily for three weeks[18]. The details of Xiaoyaosan granules are shown in supplementary table 1. Xiaoyaosan granules contained 1.47 mg/g paeoniflorin, 0.27 mg/g ferulic acid and 0.02 mg/g liquiritin (Supplemental Figure S1). The mice with restraint stress-induced NASH received an intraperitoneal injection of 20 mg/kg MCC950 (Cat# S780907; Selleckchem, Houston, USA,) every other day for two weeks from the 7th week[19]. The mice with restraint stress-induced NASH were orally administered 30 mg/kg GW4064 (Selleckchem, Cat# S2782) every other day for two weeks from the 7th week[20].

Four-week-old male SPF ApoE\(^{-/-}\) mice were divided into six groups with six mice per group. Mice were fed a high-fat diet (HFD, 1.2% cholesterol, 4.5 kcal/g, 37% fat, etc.) for 20 weeks to induce hepatic steatosis[21]. Mice in the control group were fed a standard diet (3.4 kcal/g, 4% fat, etc.). The HFD-fed mice were subjected to restraint stress for 3 h (18:00-21:00) daily from the 9th week to induce NASH[22]. The total restrained time was 252 h. The mice with restraint stress-induced NASH were
orally administered 0.51 g/kg Xiaoyaosan granules daily for seven weeks. The mice with restraint stress-induced NASH were intraperitoneally administered clodronate liposomes (50 mg/kg, Yisheng Biotechnology Co., Ltd, Shanghai, China, 40337ES08/10)[23] twice at the 12th week and 16th week.

The mice with restraint stress-induced NASH received an intraperitoneal injection of lipopolysaccharide (LPS, Sigma, 15 μg/kg)[23] every three days for seven weeks. LPS was used as a positive control for NLRP3 activation to test whether restraint stress enhanced NLRP3 signaling via a canonical or noncanonical pathway because LPS induces NLRP3 inflammasome activation via the canonical NF-κB pathway[24] and the noncanonical Caspase-11 or Caspase-4 pathway[25]. The animals were sacrificed followed by anaesthetization with ethyl ether at the end of the experiment. Blood samples were collected via cardiac puncture. The liver tissues were obtained and stored at −80°C.

C57BL/6 mice were provided by Guangdong Provincial Medical Laboratory Animal Center (License No. SYXK (Yue) 2018-0002, Guangdong, China), and ApoE−/− mice were provided by Jicuiyaokang Biotechnology (License No. SYXK (Su) 2018-0008; Nanjing, China). As estrogen induces oxidative damage and promotes steatohepatitis, only male mice were used in this study[26]. All mice were maintained at 22 ± 2°C on a 12-h/12-h light/dark cycle and kept under SPF conditions. According to animal welfare, the feces of the restrained mice and centrifuge tube were cleaned, the hair of the restrained mice was dried, and the restrained mice were given food and water after the restraint stress treatment was completed. Each cage contained less than five mice with free water and food for feeding. All animal studies received prior approval by the Ethics Committee of Guangdong Pharmaceutical University. The ethical number of the certification is No. gdpulacspf2017379.

2.2 Plasma biochemical analysis

Plasma samples were obtained by centrifuging blood samples at 2683 g for 5 min. Plasma alanine transaminase (ALT) and aspartate transaminase (AST) activities were determined using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Plasma corticosterone contents were determined using a commercial kit (Meibiao Biotechnology, Yancheng, China). The analyses were carried out according to the manufacturer’s instructions.

2.3 Hepatic cholesterol analysis

The content of total cholesterol in the liver was determined using a cholesterol oxidase-peroxidase coupling method according to the manufacturer’s instructions for commercial kits from Nanjing Jiancheng Bioengineering Institute. The content of hepatic total cholesterol was quantified as mmol per
g of liver proteins.

2.4 Histological analysis

Liver tissues were fixed using 4% paraformaldehyde, embedded in paraffin, and then sliced into 3
μm sections. Liver sections were stained with hematoxylin & eosin (H&E) and Masson’s trichrome
(Masson) using commercial kits (Nanjing Jiancheng Bioengineering Institute).

2.5 Immunohistochemistry and immunofluorescence analyses

Liver tissue sections were heated at 60°C for 2 h and hydrated with gradient ethanol after
deparaffinization. The tissue sections were boiled with citrate buffer (pH 6.0) for 5 min to retrieve
antigens and then incubated with 3% hydrogen peroxide solution. Immunostaining assay was
performed using an immunohistochemistry SP-9000 kit (Zhongshan Golden Bridge Biotechnology,
Beijing, China) according to the manufacturer’s instructions. The sections were incubated with primary
antibodies against α-SMA (Cat# ab134175; Abcam, Boston, USA). Images were observed using an
automated digital slide scanner (Pannoramic MIDI; 3DHISTECH).

Frozen liver tissue sections were stained according to the instructions of TDT-mediated dUTP
Nick-End Labeling (TUNEL) kits (Beyotime, Shanghai, China) and observed by using an inverted
microscope (IX73; Olympus, Tokyo, Japan). In addition, primary antibodies against NLRP3 (Cat#
D4D8T; Cell Signaling Technology, Danvers, USA) and ASC (Cat# SC-514414; Santa Cruz, Dallas,
USA) were added to the liver tissue sections at 4°C. The sections were washed with PBS. Alexa Fluor
488-conjugated goat anti-rabbit antibody (1:1000; Abcam) and Alexa Fluor 555-conjugated goat
anti-mouse antibody (1:1000; Abcam) were dropped into the sections for 2 h at room temperature.
DAPI solution (1:1000) was added to the sections at 37°C for 10 min. Finally, anti-fluorescence
quenching sealing tablets were used for sealing. The samples were observed using a TCS SPE-II laser
confocal imaging system (Leica, Wetzlar, Germany). The negative control was performed using a
specific species of normal IgGs instead of the primary antibodies.

2.6 Western blot analysis

Total hepatic proteins were extracted using lysis buffer (Beyotime) containing a mixture of
phosphatase and protease inhibitors (MedChemExpress, Monmouth Junction, USA). Equal amounts of
the protein samples were separated by 8%-15% SDS-PAGE and transferred to polyvinylidene fluoride
membranes (Millipore Immobilon-P, USA). After incubation with 5% skimmed milk, the membranes
were incubated with primary antibodies against IL-1β (Cat# SC-52012; Santa Cruz), TNF-α (Cat#
SC-52746), Caspase-1 (Cat# SC-56036), cholesterol 7α-hydroxylase (CYP7A1, Cat# SC-518007), sterol 12α-hydroxylase (CYP8B1, Cat# SC-101387), β-actin (Cat# SC-47778), Gasdermin D (GSDMD, Cat# AF4012; Affinity Biosciences, Cincinnati, USA,), FXR (Cat# 25055-1-AP; Proteintech, Chicago, USA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Cat# FD0063; Fude Biological Technology, Hangzhou, China,), NLRP3, and ASC at 4 ℃ for 12 h. The membranes were then incubated with peroxidase (HRP)-conjugated anti-rabbit or anti-mouse IgG antibodies (Fude Biological Technology). The bands in membranes were visualized using an enhanced chemiluminescence kit (Fude Biological Technology) and detected using the Quick Chemi 5200 chemiluminescence imaging system (Monad Biotech).

2.7 Immunoprecipitation (IP) analysis

Liver lysate samples were incubated with Protein A/G beads at a ratio of 25:1 (Lysate volume: beads volume) by a four-dimensional rotary mixer for 30 min at 4℃. After centrifugation at 2683 g for 10 min, each supernatant was divided into three groups: the input group, IP group, and IgG group. The samples of the IP group were incubated with a primary antibody against NLRP3 (Cell Signaling Technology). Rabbit normal IgG was added to the samples of the IgG group. The mixture was incubated overnight at 4℃ by a four-dimensional rotary mixer for 12 h. Protein A/G beads were added to the samples at a ratio of 25:1 for 4 h. The supernatant was removed after centrifugation. The beads were washed with PBS five times and then lysed in ice-cold lysis buffer. Protein expression was determined as described in “Western blot analysis” section.

2.8 Reverse transcription (RT)-quantitative real-time polymerase chain reaction (qPCR) analysis

Total RNA in liver tissues was extracted using TRizol reagent (TaKaRa, Dalian, China) and reverse-transcribed to cDNA using a ReverTra Ace qPCR RT Master Mix kit (Toyobo, Osaka, Japan). The amplification of cDNA samples was performed on a CFX Connect™ Real-Time system (Bio-Rad, CA, USA) with THUNDERBIRD SYBR qPCR Mix solutions (Toyobo) and specific primers. Sequences of the forward and reverse primers are listed below: Shp (F: 5’-TATTCTGTATGCACTTCTGA-3’, R: 5’-AGGCTACTGTCTTGCTTAGG-3’) and 18S (F: 5’-ACGGCTACCACATCC-3’, R: 5’-CAGACTTGCCCTCCA-3’). Gene expression was analysed by using a delta cycle threshold method. Relative mRNA expression of each gene was calculated as fold changes to 18S, a reference gene.

2.9 Statistical analysis
Data were analysed and graphed using GraphPad Prism software 8.02 (GraphPad Software, San Diego, USA). The data are expressed as the mean ± SD. The normality of the distribution of the data was calculated by using the Shapiro-Wilk test. The statistical significance of the data was analysed by using one-way analysis of variance followed by a post hoc Newman-Keuls multiple comparison test or Tukey’s test. P< 0.05 was considered statistically significant.

3 Results

3.1 Restraint stress promoted hepatic inflammation and fibrosis in hepatosteatotic mice

In this study, MCD- and HFD-induced hepatosteatotic mice were subjected to restraint stress. The results showed that restraint stress increased the levels of corticosterone, a stress hormone, in hepatosteatotic mice (Figure 1A). An increase in inflammatory infiltration, nuclear pyknosis, necrosis, fibrosis, and α-SMA-positive expression was observed in restraint stress- and MCD-treated mice compared to MCD-fed mice (Figure 1B). In addition, restraint stress increased the levels of inflammatory infiltration, nuclear pyknosis, necrosis, fibrosis, and α-SMA-positive expression in HFD-fed mice (Figure 1C). Restraint stress enhanced the activities of plasma ALT and AST (Figure 1D) and increased hepatic total cholesterol content in hepatosteatotic mice (Figure 1E). The data above indicated that restraint stress promoted NASH-related inflammation and fibrosis. In addition, Xiaoyaosan granules were used to evaluate their effects on restraint stress-induced NASH. Levels of hepatic inflammation, fibrosis, ALT, AST, and total cholesterol in mice with restraint stress-induced NASH were reduced by treatment with Xiaoyaosan granules (Figure 1B-E). Thus, Xiaoyaosan granules ameliorated restraint stress-induced inflammation and fibrosis in NASH in hepatosteatotic mice.

3.2 Restraint stress regulated NLRP3 and FXR signaling in hepatosteatotic mice

Subsequently, inflammation- and cholesterol metabolism-related regulators were investigated. The results showed that restraint stress increased the expression of hepatic NLRP3, ASC, Caspase-1, precursor (Pro)-Caspase-1, GSDMDC1, GSDMD-N, IL-1β, and TNF-α in hepatosteatotic mice. Restraint stress failed to increase the NLRP3 signal induced by LPS. Few changes in pro-IL-1β expression were found in mice with restraint stress-induced NASH (Figure 2A-C). In addition, restraint stress inhibited the expression of FXR and increased the expression of CYP7A1 and CYP8B1 in hepatosteatotic mice (Figure 3A, and B). The data above indicated that alterations in NLRP3 and FXR signaling occurred in mice with restraint stress-induced NASH. Additionally, Xiaoyaosan granules reduced NLRP3 signaling and increased FXR signaling in hepatosteatotic mice (Figure 2 and 3),
indicating that Xiaoyaosan granules regulated FXR/NLRP3 signaling in mice with restraint stress-induced NASH.

3.3 Inhibition of NLRP3 by MCC950 reduced the NLRP3 inflammasome and liver damage in mice with restraint stress-induced NASH

Further investigations found that restraint stress promoted an interaction between hepatic NLRP3 and ASC in hepatosteatotic mice (Figure 4A). Colocalization of hepatic ASC and NLRP3 was increased in mice with restraint stress-induced NASH (Figure 4D). This demonstrated that restraint stress activated the NLRP3 inflammasome. Blockade of NLRP3 by MCC950 reduced the expression of hepatic NLRP3, Caspase-1, Pro-Caspase-1, IL-1β, and TNF-α in mice with restraint stress-induced NASH (Figure 4B, and C), indicating that the NLRP3 signal was blocked. Subsequently, MCC950 decreased the level of colocalization of ASC and NLRP3 (Figure 4D) and reduced the occurrence of inflammatory infiltration, nuclear pyknosis, necrosis, fibrosis, and α-SMA-positive expression in mice with restraint stress-induced NASH (Figure 4E and F). Increased programmed cell death in the liver occurs in mice with restraint stress-induced NASH. MCC950 rescued programmed cell death in mice with restraint stress-induced NASH (Figure 4G). Furthermore, comparable expression of hepatic FXR was observed in mice with restraint stress-induced NASH that received MCC950 (Figure 4H). Thus, the data confirmed that restraint stress promoted NASH-related inflammation and fibrosis via NLRP3 signaling. Additionally, NLRP3 failed to affect FXR expression in restraint stress-induced NASH.

3.4 Elimination of macrophages attenuates inflammation and NLRP3 signaling in mice with restraint stress-induced NASH

To explore the role of macrophages in restraint stress-induced NASH, clodronate liposomes were used to eliminate macrophages in the liver. The level of monocytes was significantly decreased in restraint stress-induced NASH mice that received clodronate liposomes (Figure 5A). Macrophage scavengers reduced fibrosis and α-SMA-positive expression in mice with restraint stress-induced NASH (Figure 5A). Elimination of macrophages by clodronate liposomes reversed the increased expression of NF-κB, NLRP3, Caspase-1, Pro-Caspase-1, GSDMD-N, IL-1β, and TNF-α in mice with restraint stress-induced NASH (Figure 5B, and C). However, comparable expression of hepatic FXR was observed in restraint stress-induced NASH mice treated with clodronate liposomes (Figure 5D). Thus, restraint stress activated the NLRP3 inflammasome in hepatic macrophages. Elimination of macrophages failed to affect hepatic FXR expression in restraint stress-induced NASH.
3.5 FXR activation by GW4064 attenuated NLRP3 signaling in mice with restraint stress-induced NASH

Further investigations showed that GW4064 enhanced the mRNA expression of Shp, a downstream regulator of FXR, in mice with restraint stress-induced NASH (Figure 6A). GW4064 repressed the expression of hepatic CYP7A1 and CYP8B1 in mice with restraint stress-induced NASH (Figure 6B). The results indicated that GW4064 activated FXR signaling. Activation of FXR by GW4064 reversed the increase in inflammatory infiltration, nuclear pyknosis, cell necrosis, fibrosis, and α-SMA-positive expression (Figure 6C) and reduced the expression of hepatic NLRP3, Caspase-1, and Pro-Caspase-1 (Figure 6D) in mice with restraint stress-induced NASH. The results indicated that restraint stress activated the NLRP3 inflammasome by attenuating FXR signaling in restraint stress-induced NASH.

4 Discussion

CRS has already been used to simulate psychological stress with anxiety-[27] and depression-like behaviors[28]. In the present study, 12 weeks of restraint stress increased the levels of plasma corticosterone, a stress hormone, in HFD-fed mice. In addition, six weeks of restraint stress reduced the ratio of sucrose preference and increased immobility time in the tail suspension test in mice (Supplementary figure 2). Long-term restraint stress treatment induces psychological stress-related behaviors. NASH demonstrates an accumulation of steatosis in the liver, and metabolic disorders increase the susceptibility to hepatocyte damage and lead to inflammation and fibrosis during the development of NAFLD[29]. Our data indicated that restraint stress aggravates hepatic inflammation and fibrosis in experimental NASH mice. Regarding hepatic steatosis, previous reports have shown that chronic restraint stress reduces the accumulation of hepatic fat[5, 30]. Collectively, these data suggest that restraint stress promotes NASH-related inflammation and fibrosis. Restraint stress activates the NF-κB/NLRP3/GSDMD signaling pathway and decreases the FXR/CYP7A1/CYP8B1 signaling pathway. These data indicate a link between restraint stress and FXR/NLRP3 signaling.

Further investigations focused on verifying the molecular mechanisms involving FXR and NLRP3 in restraint stress-induced NASH. The current data show that restraint stress promotes the assembly of the NLRP3 inflammasome and activates NLRP3 signaling in NASH mice. Blockade of NLRP3 signaling by MCC950[31] reduced programmed cell death in mice with restraint stress-induced NASH. The activation of the NLRP3 inflammasome increases the activity of Caspase-1 on the cleavage of
IL-1β and GSDMD[32]. As the characteristics of pyroptosis include cell death with excess secretion of IL-1β from the formation of GSDMD pores[33], the study indicated that restraint stress promotes NLRP3-mediated pyroptosis in NASH mice. Combined with the result that elimination of macrophages reversed the increase in the NLRP3 inflammasome, this study speculates that restraint stress activates the NLRP3 inflammasome and pyroptosis in hepatic macrophages. In addition, few changes in hepatic FXR expression were found after treatment with MCC950 and a macrophage scavenger, indicating that FXR might regulate the NLRP3 inflammasome in hepatic macrophages. The relationship between FXR and NLRP3 signaling in restraint stress-induced NASH is discussed below. First, previous studies indicate that crosstalk between FXR and NF-κB exists in the liver[34]. NF-κB is an upstream regulator of NLRP3 [35]. FXR can downregulate the transcriptional activity of NF-κB by interacting with NF-κB in the nucleus[36]. Combined with our results, FXR inhibits NLRP3 signaling by decreasing NF-κB transcriptional activity. Second, FXR negatively regulates the NLRP3 inflammasome through physical interactions with NLRP3 and Caspase-1[37]. Third, downregulation of FXR promotes cholesterol uptake by increasing the expression of CYP7A1 and CYP8B1 in restraint stress-induced NASH. FXR can improve bile acid metabolism by regulating CYP7A1 and CYP8B1[38]. The upregulation of CYP7A1 and CYP8B1 increases the synthesis of cholic acid, thereby increasing the absorption of cholesterol from the small intestine[39]. Cholesterol can induce liver fibrosis by activating HSCs[40] and activate the NLRP3 inflammasome to produce IL-1β[41]. Thus, FXR inhibited NLRP3 signaling by decreasing cholesterol accumulation. Fourth, inhibition of FXR can promote reactive oxygen species (ROS) production, followed by the activation of NLRP3 signaling[42]. Thus, another source of NLRP3 in restrained mice might be the stimulation of cholesterol and ROS via the blockade of FXR. Fifth, FXR attenuated CCAAT enhancer-binding protein homologous protein (CHOP)-dependent NLRP3 overexpression by inhibiting the ER stress-mediated activation of the protein kinase RNA-like ER kinase (PERK) [14]. Therefore, restraint stress activates the NLRP3 inflammasome in NASH mice by regulating FXR/NLRP3 signaling in five ways. Our data showed that Xiaoyaosan granules ameliorated inflammation and fibrosis in mice with restraint stress-induced NASH and reversed the changes in FXR and NLRP3 signals. Xiaoyaosan is an advantageous FXR activator for treating restraint stress-induced NASH. Obeticholic acid, an effective FXR agonist, has become a first-line clinical drug that has been shown to improve steatosis, hepatocyte ballooning, lobular inflammation and fibrosis[43]. However, side effects, including pruritus,
hepatobiliary toxicity, and increased low-density lipoprotein cholesterol levels, limit the application of FXR agonists[44]. In addition, high doses of obeticholic acid induce FXR-dependent liver injury [45]. Currently, few reports have shown that Xiaoyaosan granules induce side effects via FXR activation. It has been used to treat mental illnesses for centuries without obvious adverse reactions[46]. Previous reports have shown that Xiaoyaosan granules ameliorate glucocorticoid-induced hepatic steatosis by decreasing the translocation of GR into the nucleus[18]. Xiaoyaosan regulates glucocorticoid signaling in rats[47]. Thus, restraint stress-driven corticosterone may explain how restraint stress stimulates FXR/NLPR3 signaling. Previous reports have shown that chronic stress can activate the NLRP3 inflammasome by inducing glucocorticoid release and activating glucocorticoid receptors (GRs) to increase the nuclear transcription of NF-κB [48]. Additionally, GR interacts with FXR to inhibit its transcriptional activity[49]. The underlying mechanism of restraint stress-induced NASH may involve the FXR/NLRP3 signaling pathway, probably by activating GR.

In conclusion, the current study demonstrates that restraint stress promotes NASH-related inflammation and fibrosis by regulating the FXR/NLRP3 signaling pathway. The findings provide a theoretical basis for treatments including FXR agonists and Xiaoyaosan granules for psychological stress-related NASH.

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**Conflict of Interest**

The authors declare that they have no conflict of interest.
References


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502**Figure legends**

503**Figure 1. Effect of restraint stress on hepatic inflammation and fibrosis in hepatosteatotic mice**

504 HFD- and MCD-fed mice were subjected to restraint stress treatments. The restrained mice were administered Xiaoyaosan granules. (A) The level of plasma CORT was detected (n=6). Liver tissue sections in MCD- and HFD-fed mice (B,C) were analysed using H&E (bar=50 μm) and Masson's trichrome (bar=100 μm) staining methods, respectively. The liver tissue sections were incubated with α-SMA antibody using an immunohistochemical method (bar=100 μm). Blue arrows indicate
inflammatory cells. Yellow arrows indicate nuclear pyknosis. Red arrows indicate necrotic cells. Dark arrows indicate lipid droplets. Green arrows indicate collagen fibres. (D) The activities of plasma ALT and AST were determined \((n=6)\). (E) The level of hepatic total cholesterol was determined \((n=6)\). Data are expressed as the mean ± SD. \(^{*}P < 0.05\) versus CON group, \(^{**}P<0.01\) versus MCD group at, and \(*\) \(P < 0.05\) and \(^{**}P < 0.01\) versus MCD + RS group at. CON: control group; MCD: methionine choline deficient diet; HFD: high fat diet; RS: restraint stress; TC: total cholesterol; XYS: Xiaoyaosan granules.

**Figure 2. Effect of restraint stress on NLRP3 signaling in hepatosteatotic mice** The expression of hepatic NLRP3, ASC, Pro-Caspase-1, Caspase-1 (A), GSDMDC1, GSDMD-N (B), Pro-IL-1β, IL-1β, and TNF-α (C) was determined using Western blot analysis \((n=3)\). Expression of each protein was normalized to that of GAPDH or β-actin. The relative protein expression in different groups was normalized to that in the control group. Data are expressed as the mean ± SD. \(^{*}P<0.05\) and \(^{**}P<0.01\) versus HFD group, and \(*P<0.05\) and \(^{**}P<0.01\) versus HFD + RS group. CON: control group; HFD: high fat diet; RS: restraint stress; LPS: lipopolysaccharide; XYS: Xiaoyaosan granules.

**Figure 3. Effect of restraint stress on FXR signaling in hepatosteatotic mice** (A) The expression of hepatic FXR, CYP7A1, and CYP8B1 in MCD-fed mice was determined by Western blot analysis \((n=3)\). (B) The expression of hepatic FXR in HFD-fed mice was determined \((n=3)\). Expression of each protein was normalized to that of GAPDH. The relative protein expression in different groups was normalized to that in the control group. Data are expressed as the mean ± SD. \(^{**}P<0.05\) versus CON group, \(^{**}P<0.01\) versus MCD or HFD group, and \(*P<0.05\) versus MCD + RS or HFD + RS group. CON: control group; MCD: methionine choline deficient diet; HFD: high fat diet; RS: restraint stress; LPS: lipopolysaccharide; XYS: Xiaoyaosan granules.
Figure 4 Effect of MCC950 on the NLRP3 inflammasome and liver damage in mice with restraint stress-induced NASH. (A) The interaction between NLRP3 and ASC was detected using an immunoprecipitation method. The expression of hepatic NLRP3, Pro-Caspase-1, Caspase-1 (B), TNF-α, Pro-IL-1β and IL-1β (C) was measured by Western blot analysis (n=3). (D) The colocalization (orange) of ASC (green) and NLRP3 (red) was measured by an immunofluorescence double-staining method (bar=50 μm). H&E staining (E), Masson's trichrome staining, and immunohistochemical analyses (F) were conducted (bar=100 μm). Blue arrows indicate inflammatory cells. Yellow arrows indicate nuclear pyknosis. Red arrows indicate necrotic cells. Dark arrows indicate lipid droplets. α-SMA-positive expression is marked with brown. (G) TUNEL staining was conducted (bar=100 μm). (H) The expression of hepatic FXR was determined (n=3). Expression of each protein was normalized to that of GAPDH. The relative protein expression in different groups was normalized to that in the control group. Data are expressed as the mean ± SD. P<0.05 Versus CON group, #P<0.05 and ##P<0.01 versus MCD group, and **P<0.01 versus MCD + RS group. CON: control group; MCD: methionine choline deficient diet; RS: restraint stress; IB: immunoblotting; IP: immunoprecipitation.

Figure 5. Effect of clodronate liposomes on NLRP3 signaling in mice with restraint stress-induced NASH. (A) Monocyte density, collagen fibres, and α-SMA-positive expression were detected by H&E staining, Masson staining, and immunohistochemical analyses, respectively (bar=100 μm). Blue arrows indicate monocytes. Yellow arrows indicate collagen fibres. Dark arrows indicate positive expression of α-SMA. The levels of monocyte density, collagen fibres, and α-SMA-positive expression were calculated by ImageJ 1.52a software. The expression of hepatic NF-κB, NLRP3, Pro-Caspase-1, Caspase-1 (B), GSDMD-N, TNF-α, Pro-IL-1β, IL-1β (C), and FXR (D) was determined by Western blot analysis (n=3). Expression of each protein was normalized to that of GAPDH.

P<0.05 Versus CON group, #P<0.05 and ##P<0.01 versus MCD group, and **P<0.01 versus MCD + RS group. CON: control group; MCD: methionine choline deficient diet; RS: restraint stress; IB: immunoblotting; IP: immunoprecipitation.
GAPDH or β-actin. The relative protein expression in different groups was normalized to that in the control group. Data are expressed as the mean ± SD. $^{\text{a}}P<0.05$ versus CON group, $^{\text{b}}P<0.05$ and $^{\text{c}}P<0.01$ versus HFD group, and $^{\text{d}}P<0.05$ and $^{\text{e}}P<0.01$ versus HFD + RS group. CON: control group; HFD: high-fat diet; RS: restraint stress; CL: clodronate liposomes.

Figure 6. Effect of GW4064 on NLRP3 signaling in mice with restraint stress-induced NASH

(A) The mRNA expression of Shp was determined by RT‒PCR analysis ($n=4$). (B) The expression of CYP8B1 and CYP7A1 was detected by Western blot analysis ($n=3$). (C) H&E staining, Masson staining, and immunohistochemistry analyses were conducted (bar=100 μm). Blue arrows indicate inflammatory cells. Green arrows indicate collagen fibres. Yellow arrows indicate nuclear pyknosis. Red arrows indicate necrotic cells. Dark arrows indicate positive expression of α-SMA. (D) The expression of NLRP3, Pro-Caspase-1, and Caspase-1 was determined ($n=3$). Expression of each protein was normalized to that of GAPDH. The relative protein expression in different groups was normalized to that in the control group. Data are expressed as the mean ± SD. $^{\text{a}}P<0.05$ versus CON group, $^{\text{b}}P<0.05$ and $^{\text{c}}P<0.01$ versus MCD group, and $^{\text{d}}P<0.05$ and $^{\text{e}}P<0.01$ versus MCD + RS group. CON: control group; MCD: methionine choline deficient diet; RS: restraint stress.
Supplementary Materials

Preparation of samples of Xiaoyaosan granules

Xiaoyaosan granules are composed of eight single formula granules. The details of each granule are listed in supplementary Table 1. The granules including 0.525 g of *Bupleurum chinense* DC., 1.35 g of *Angelica sinensis* (Oliv.) Diels, 0.45 g of *Paeonia lactiflora* Pall., 1.35 g of *Atractylodes macrocephala* Koidz., 0.45 g of *Poria cocos* (Schw.) Wolf., 0.25 g of *Glycyrrhiza uralensis* Fisch. Ex DC., 0.25 g of *Mentha haplocalyx* Briq., and 0.125 g of *Zingiber officinale* Roscoe. were dissolved in 93 mL of distilled water. The solution was stored at 4°C. The formula granules are from Guangdong Yifang Pharmaceutical Co., Ltd (Guangdong, China). Plant name databases including “World Flora Online” (www.worldfloraonline.org) and MPNS (http://mpns.kew.org) were used to validate the scientific name of the plant.

High-performance liquid chromatography (HPLC) analysis

Xiaoyaosan granules (1.0 g) were extracted with methanol (25 mL) by ultrasonication at 50 kHz for 60 min. The supernatant fluid was obtained after centrifugation. Reference substances including paeoniflorin, ferulic acid, and liquiritin (Chengdu Chroma-Biotechnology Co., Ltd, Sichuan, China) were dissolved in methanol as standards. The samples solution were filtered using a 0.22 μm filter membranes. HPLC analysis was performed on Agilent 1260 Infinity II (Agilent Co., USA) using Welchrom C18 column (4.6 mm×250 mm, 5 μm). The mobile phases comprised eluent A (0.1% phosphoric acid) and eluent B (acetonitrile). The gradient flow was shown as follows: 0.00–45.00 min, 14% B; 45–50 min, 14%–90% B; 50–55 min, 90% B; 55–60 min, 90%–14% B; 60–65 min, 14% B. The analysis was performed at a flow rate of 1.0 mL/min with DAD detection at 230 nm. Each injection volume was 10 μL. The Agilent Openlab Control Panel software (Version 3.5) was used for data analysis.
### Supplementary Table S1. Details of Xiaoyaosan granules

<table>
<thead>
<tr>
<th>Plant name</th>
<th>Formula granules/Crude drug (g/g)</th>
<th>Product batch</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bupleurum chinense</em> DC.</td>
<td>18/60</td>
<td>0110711</td>
</tr>
<tr>
<td><em>Angelica sinensis</em> (Oliv.) Diels</td>
<td>20/60</td>
<td>0119281</td>
</tr>
<tr>
<td><em>Paeonia lactiflora</em> Pall.</td>
<td>20/60</td>
<td>0069401</td>
</tr>
<tr>
<td><em>Atractylodes macrocephala</em> Koidz.</td>
<td>20/60</td>
<td>9120621</td>
</tr>
<tr>
<td><em>Poria cocos</em> (Schw.) Wolf.</td>
<td>20/60</td>
<td>0092921</td>
</tr>
<tr>
<td><em>Glycyrrhiza uralensis</em> Fisch. Ex DC.</td>
<td>18/60</td>
<td>0099401</td>
</tr>
<tr>
<td><em>Mentha haplocalyx</em> Briq.</td>
<td>18/60</td>
<td>9081741</td>
</tr>
<tr>
<td><em>Zingiber officinale</em> Roscoe.</td>
<td>18/60</td>
<td>0040771</td>
</tr>
</tbody>
</table>
Supplementary Figure S1. The HPLC chromatogram demonstrated the content of main components in Xiaoyaosan granules. The main peaks were marked with paeoniflorin, ferulic acid, and liquiritin, respectively. Xiaoyaosan granules contained 1.47 mg/g of paeoniflorin and 0.27 mg/g of ferulic acid and 0.02 mg/g of liquiritin.
Supplementary Figure S2. Effect of restraint stress on psychological stress-related behaviors in mice. (A) The percentage of sucrose preference in restraint stress-treated mice was detected (n=6). (B) The immobility time in TST was detected (n=6). Data are expressed as mean ± SD. Versus CON group at $^{**}p < 0.01$. CON: Control group; RS: Restraint stress; TST: Tail suspension test.
Highlights

Psychological stress promotes the development of nonalcoholic steatohepatitis (NASH). The underlying mechanism of psychological stress-related NASH remains unclear. Our study shows that restraint stress, which mimics psychological stress, aggravates inflammation and fibrosis in NASH by regulating the FXR/NLRP3 signaling pathway.

1) Inhibition of NLRP3 by MCC950 reduces NLRP3 inflammasomes and liver damage in mice with restraint stress-induced NASH.

2) Elimination of macrophages attenuate inflammation and NLRP3 signaling.

3) FXR activation by GW4064 attenuates NLRP3 signaling.

4) Xiaoyaosan granules ameliorate restraint stress-induced NASH by regulating FXR/NLRP3 signaling pathway.