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Original Article

Anti-inflammation effect via TLR4-mediated MyD88-dependent and -independent signalling pathways in non-alcoholic fatty liver disease rats: Chinese herb formula

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Abstract: Non-alcoholic fatty liver disease (NAFLD) is a global health problem that has been the most common cause of chronic liver disease. No promising treatment has been recommended. Toll-like receptor 4 (TLR4) is the essential factor in pathogenesis and closely linked to non-alcoholic steatohepatitis. In this study, we expected to explore the potential effect of Hongqi Jiangzhi Formula (HJF) in alleviating inflammation via TLR4-mediated MyD88-dependent and -independent signalling pathways in NAFLD rat liver tissue. In total, 32 rats were randomized into 4 groups: Normal, Model, Probiotic and HJF groups, except the normal group, which was fed a chow diet, while the other three groups were all fed a high fat diet (HFD). The probiotic and HJF group were additionally fed probiotic and Hongqi Jiangzhi Formula, respectively. The course of treatment was 16 weeks. Basic characteristics of the rats were measured before sacrifice. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol (TC), triglyceride (TG), fasting glucose (GLU) and insulin in the aorta serum was measured, and the HOMA-IR (homeostasis model of assessment for insulin resistance index) was calculated. Tumour necrosis factor-alpha in the serum was also measured by means of an enzyme-linked immunosorbent assay. The liver tissues were stained with Haematoxylin-Eosin. Protein expression of MyD88-dependent and -independent signalling pathways, including TLR4, TIRAP, MyD88, p-NF-κB, NF-κB p65, TRAM, pIRF3 and IRF3, were further detected by Western blotting. Compared with the model group, HJF could reduce the level of the body weight, ALT, HOMA-IR index, insulin, AST, TG and TNF-α significantly. Regarding the proteins in TRL4 downstream pathways, HJF could down-regulate the overexpression of TLR4, TIRAP, MyD88, p-NF-κB and TRAM, TRIF, and pIRF3. In conclusion, HFD can activate TLR4 MyD88-dependent and -independent pathways, which might be related to the inflammatory production of NF-κB. HJF could effectively improve lipid accumulation in the high-fat rat liver and could down-regulate the expression of NF-κB through TLR4 downstream signalling pathways.

Keywords: Non-alcoholic fatty liver disease, TLR4, NF-κB, Chinese medicine formula

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a global health problem that has been the most common cause of chronic liver disease [1]. Non-alcoholic steatohepatitis (NASH)-a serious form of NAFLD-is predicted to become the leading cause of liver transplantation in the USA by the year 2020 [2]. At present, it is increasingly clear that NAFLD not only affect the liver but also increase the risk of developing extra-hepatic diseases, including diabetes, cardiovascular disease and chronic kidney disease [3]. To date, NAFLD is not only limited to developed countries, but has gradually expanded to Asia [4]. As reported, the prevalence in China climbed to 24.8% for males and 13.1% for females by 2014 [5]. In addition, the harm expanded to children, as 9.03% of children were already suspected to suffer from NAFLD [6]. Although the morbidity is continuously rising, the mechanism of pathogenesis remains unclear [7]. The

most widely accepted theory lies in the "two-hit theory"[8].Theessentialprocessisthatfataccumulation in hepatocytes results from a series of inflammation and fibrosis [9]. Recent studies mostly concentrate on the gut microbiota, which is believed to be intimately associated with the severity of NAFLD [10]. Furthermore, changes in the gut microbiota would strengthen intestinal permeability, resulting in the accumulation of bacterial metabolites, such as endotoxin lipopolysaccharide (LPS) in the liver [11]. A human liver biopsy study also reported when the LPS and total free fatty acid levels increased in the plasma of NASH patients, while the Toll-like receptor 4 (TLR4) mRNA levels would increase in succession [12]. Therefore, probiotics are considered to be another therapeutic strategy to cure NAFLD [13]. An evidence-based case report also showed that probiotics could effectively alleviate the serum ALT and inflammation response, but substantial evidence on lowering the liver lipids is still needed [14]. Since there is no promising cure for patients with NAFLD, current treatment mostly focuses on lifestyle changes and inflammatory responses [15, 16]. Toll-like receptor sensors are important for the innate immune system, which is responsible for mounting a protective immune response [17]. This family receptor, recognising a broad range of pathogens, recruits a specific set of adaptor molecules that harbour the Toll/interleukin-1 receptor domain, such as MyD88 (Myeloid Differentiation Factor 88) and TRIF, and initiates downstream signalling, resulting in the secretion of inflammatory cytokines [18]. TLR4 for LPS and components of the TLR4 signalling pathway are widely distributed in the liver [12]. Increasing reports support that TLR4 is an essential factor in the pathogenesis of NAFLD and links to NASH [12].

TLR4 engages two distinct adapter proteins: MyD88 (dependent), which triggers inflammatory responses, and TRIF (MyD88-independent), which activates the production of type I Interferon (IFN), as well as pro-inflammatory cytokines [31, 32]. TLR4, once stimulated, can induce NF-κB up-regulation [19]. NF-κB is regarded as the central mediator in the inflammatory process and participates in innate immunity [20]. In addition, this protein's activation occurs along with the recruitment of inflammatory cells and pro-inflammatory mediators, such as tumour necrosis factor-alpha (TNF-α) [21]. Therefore, NF-κB is also taken as an important reference for evaluating anti-inflammatory treatment [22]. Chinese medicine formula-Hongqi Jiangzhi Formula (HJF) has been placed into practical use and has achieved satisfying effects. Therefore, supposing that this formula's pharmacological mechanism is derived from anti-inflammation, we launched this study to explore the possible mechanism and how it alleviates liver inflammation via TLR4-mediated MyD88-dependent and -independent signalling pathways.

Materials and methods

Animals

Thirty-two Sprague Dawley male rats, sanitary degree, weighing (200 ± 20) g, were bought from the Experimental Animal Science Center of Guangzhou University of Chinese Medicine (China, Animal License Key No. 440058000-03578; License No. SCXK (Yue) 2008-0020). This animal experiment was approved by the laboratory animal ethics committee of Jinan University. The rats were separately housed in the Institute of Laboratory Animal Science, Jinan University. The rats were housed under controlled temperature conditions of (24 ± 2)°C and humidity of (60% ± 10%) in a 12-12 h light dark cycle (lights on from 8:00 am to 8:00 pm) with free access to diet and water.

Animal grouping, modelling and drugs

We arranged a 1-week acclamation period before experimental drug treatment. Thirty-two rats were randomized into four groups: normal group, model group, probiotic group and HJF group (n=8). Except the normal group, which was fed a normal chow diet, the other three groups were all fed high fat diets (HFD), which was composed of 83% regular chow, 10% axungia porci, 1.5% cholesterol, 5% sucrose and 0.5% bile salt. The HJF group and probiotic group were additionally fed with HJF decoction (19.05 g/kg/d) and compound probiotic (more than 600 billion CFU/100 g) at 0.6 g/kg/d, respectively. HJF consist of 7 traditional Chinese medicines (**Table 1**): Astragali Radix (Huangqi) 15 g, Red yeast rice (Hongqu) 12 g, Nelumbinis Folium (Heye) 10 g, Curcumae Longae Rhizoma (Jianghuang) 6 g, Lych Fructus (Gouqi)

Table 1. Constitute of HJF and its components

English name	Chinese name	Source	Active ingredient	Effect
Astragali Radix	Huangqi	Neimenggu province, China	Astragalus polysaccharides	Improving insulin sensitivity and anti-diabetic
Red yeast rice	Hongqu	Zhejiang province, China	Monascus purpureus	Lowering serum lipid
Nelumbinis Folium	Heye	Guangdong province, China	Lotus alkaloid	Regulating blood lipid and controlling body mass
Curcumae Longae Rhizoma	Jianghuang	Fujian province, China	Curcumin	Anti-cancer
Lych Fructus	Gouqi	Ningxia province, China	Lycium barbarum polysaccharides	Anti-oxidation
Magnoliae Officinals Cortex	Houpo	Shanxi province, China	Magnolol	Anti-oxidation and suppress liver cancer cell proliferation
Artemisiae Scopariae Herba	Yinchen	Fujian province, China	Artemisia capillaris	Anti hepatotoxic and anti-oxidation

10 g, Magnoliae Officinals Cortex (Houpo) 6 g, Artemisiae Scopariae Herba (Yinchen) 10 g. Red Yeast Rice was purchased from Jun Tong Pharmaceutical Co., Ltd., China (batch number: 160302) and ground into powder before use. The other Chinese medicines were purchased from Jiangyin Tian Jiang Pharmaceutical Co., Ltd., China (batch number: 1601058). The formula granules were mixed together, dissolved in distilled water and later preserved in a -4°C refrigerator. Compound probiotic (Purchased from Professor Heping Zhang, College of Life Science, Inner Mongolia Agricultural University, China) contains 9 probiotic strains, including 6 strains of Lactobacillus (Lactobacillus casei Zhang, Lactobacillus plantarum HM-P8, Lactobacillus paracasei HM-P9, Lactobacillus rhlaricus HM-R1, Lactobacillus acidophilus HM-A2, Lactobacillus bularicus HM-B1), 3 strains of Bifidobacterium (Bifidobacterium lactis HM-V9, Bifidobacterium longum HM-A1, Bifidobacterium longum HM-L), and 15 g/100 g Galactooligosaccharides (GOS). The other two groups were fed with the same dose of distilled water. The course of treatment was 16 weeks.

Basic characteristic in rats

Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured in conscious rats by the standard tail-cuff method (IITC Life Science, USA). Lee's index, a standard used to evaluate the obesity level, was calculated as follows: weight (g)^(1/3) * 1000/nasoanal length (cm) [23].

Histopathological examination of liver

After treatment by macroscopic observation on general changes of rat livers in the model group, the livers were paraffin embedded and H&E staining, and steatosis of the liver was observed under the light microscope.

Biochemical test in abdominal aorta serum

After rats were anaesthetized by injection of 3% pentobarbital (2 mL/kg body weight), the livers were taken out quickly. Liver tissues were placed into isopropanol. Homogenates were manufactured using a Tissue Lyser-II Homogenizer (Qiagen, Germany), centrifuged at 8000 × g for 10 min at 4°C, and then the clear supernatants were collected. The liver tissues were used to detect the total cholesterol (TC) and triglyceride (TG) with an automatic biochemical analyser (Hitachi, Japan). A blood sample (5 mL) was drawn from the abdominal aorta into a tube and centrifuged at 3000 × g for 10 min at 4°C. The supernatant was later used to measure the contents of TC, TG, alanine aminotransferase (ALT), aspartate aminotransferase (AST), fasting plasma glucose (GLU) and insulin in the serum by an automatic biochemical analyser. The homeostasis model of assessment for insulin resistance index (HOMA-IR) was = GLU × fasting insulin/22.5. Tumour necrosis factor-alpha (TNF-α) in the serum was also measured using an enzyme-linked immunosorbent assay (ELISA) kit (Invitrogen Corporation, USA).

Western blotting

Western blotting was used to determine the proteins of MyD88-dependent pathways: TLR4, TIRAP, MyD88, p-NF-κB, NF-κB p65, and MyD-88-independent pathways: TRAM, TRIF, pIRF3 and IRF3. β-actin was used as the internal control. In brief, a Teflon homogenizer was used to thaw and homogenize the frozen sample at 4°C in 50 mM Tris-HCL (pH 7.6). Proteins were extracted by RIPA Lysis Buffer (Beyotime Institute of Biotechnology, China) with a protease inhibitor cocktail (Sigma, USA). These proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis with 10% polyacrylamide gels. Next, the proteins were transferred to a polyvinylidene fluoride membrane (Millipore, USA) and blocked with 5% skim milk in Tris buffered saline Tween for 1 h. After incubation with primary antibody: anti-TLR4 (Novus Biologicals, USA), anti-TIRAP (Abcam, UK), anti-MyD88 (Cell Signaling Technology, USA), anti-p-NF-κB (Cell Signaling Technology, USA), anti-NF-κB p65 (Cell Signaling Technology, USA), anti-TRAM (Santa Cruz Biotechnology, USA), anti-TRIF (Novus Biologicals, USA), anti-p-IRF3 (Cell Signaling Technology, USA) and anti IRF3 (Cell Signaling Technology, USA) overnight at 4°C, horseradish peroxidase labelled second antibody (KangChen Bio-tech Inc., China) was added and continually incubated for 1 h at 37°C. Afterwards, we used TBST to wash the membrane 10 min 4 times.

Additionally, we put the hybrid film on a transparent plastic plate and dried the film.

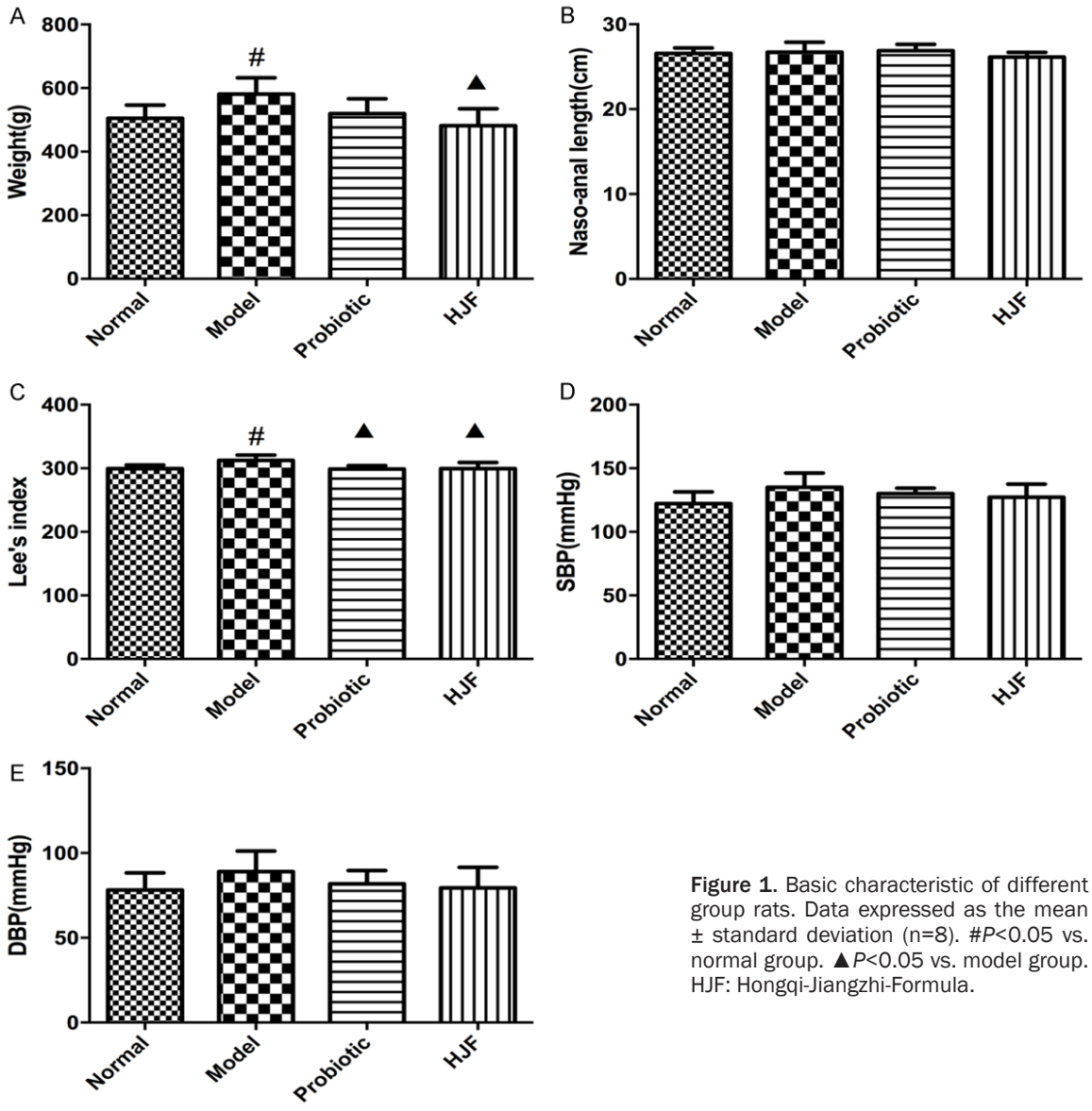

Figure 1. Basic characteristic of different group rats. Data expressed as the mean ± standard deviation (n=8). #*P*<0.05 vs. normal group. ▲*P*<0.05 vs. model group. HJF: Hongqi-Jiangzhi-Formula.

We prepared a clean pipette on the surface of the membrane and let the reaction last for 5 min. Finally, to clean up the solution on the surface of the substrate and placed it into the X-ray film. The films were scanned and analysed by a gel image processing system.

Statistical analysis

Data were represented in terms of the mean ± standard deviation ($\bar{x} \pm S$). SPSS (Statistical Product and Service Solutions) 22.0 was used to calculate the statistical information. Differences between the groups were analysed with One-way Anova followed by a post hoc Bonferroni or Tamhane T2 test (according to homogeneity of variance). *P*<0.05 were considered to be statistically significant.

Results

Basic characteristics in rats

Basic characteristics, including length, weight, lee's index and blood pressure, were illustrated in **Figure 1**. After the HFD, the level of the weight and Lee's index in the model group were markedly higher than the normal group (P<0.05). Compared with the model group, probiotic treatment decreased Lee's index significantly (*P*<0.05), while HJF could reduce the level of Lee's index and weight (*P*<0.05).

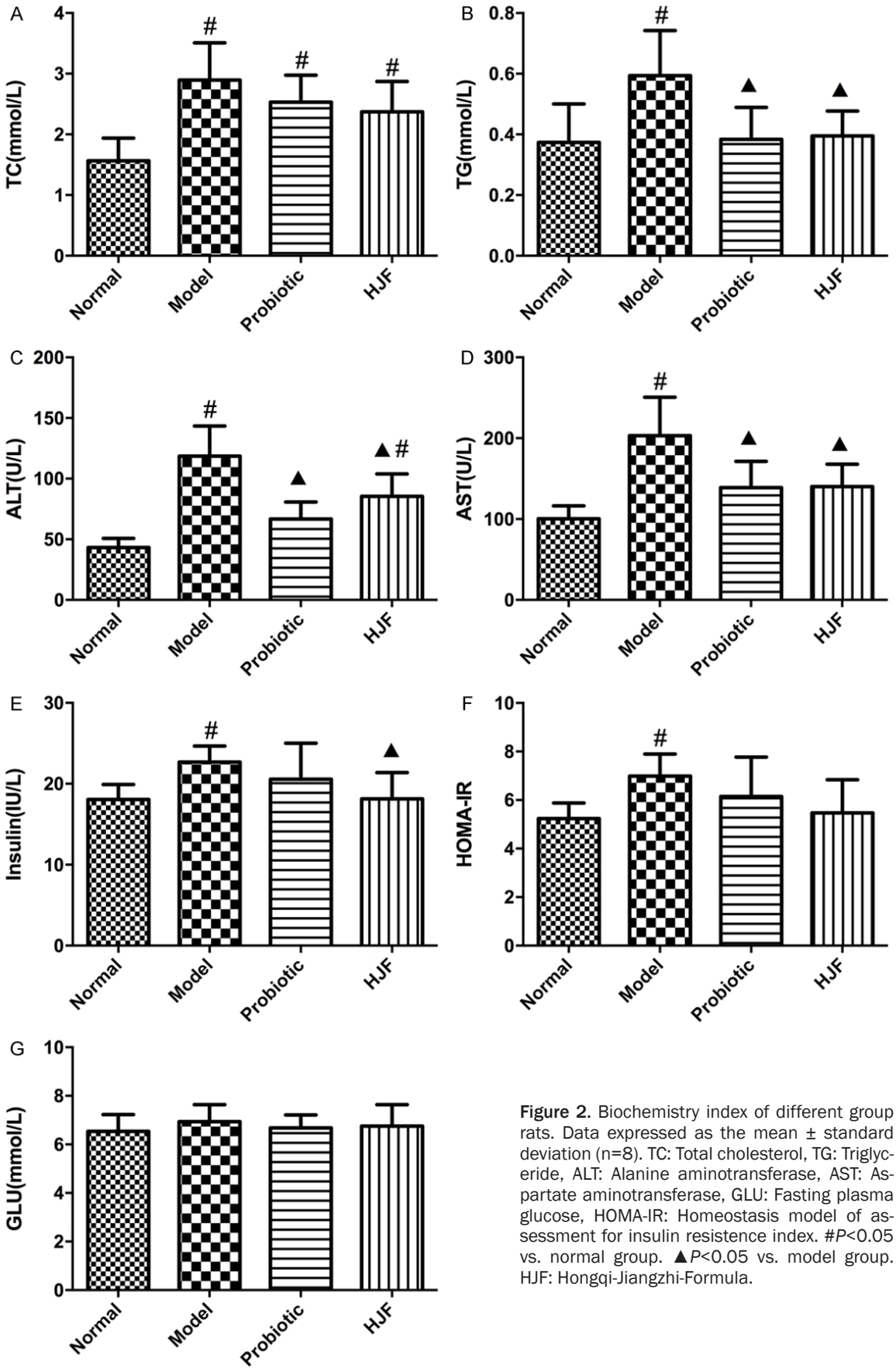

Figure 2. Biochemistry index of different group rats. Data expressed as the mean ± standard deviation (n=8). TC: Total cholesterol, TG: Triglyceride, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, GLU: Fasting plasma glucose, HOMA-IR: Homeostasis model of assessment for insulin resistence index. #P<0.05 vs. normal group. ▲P<0.05 vs. model group. HJF: Hongqi-Jiangzhi-Formula.

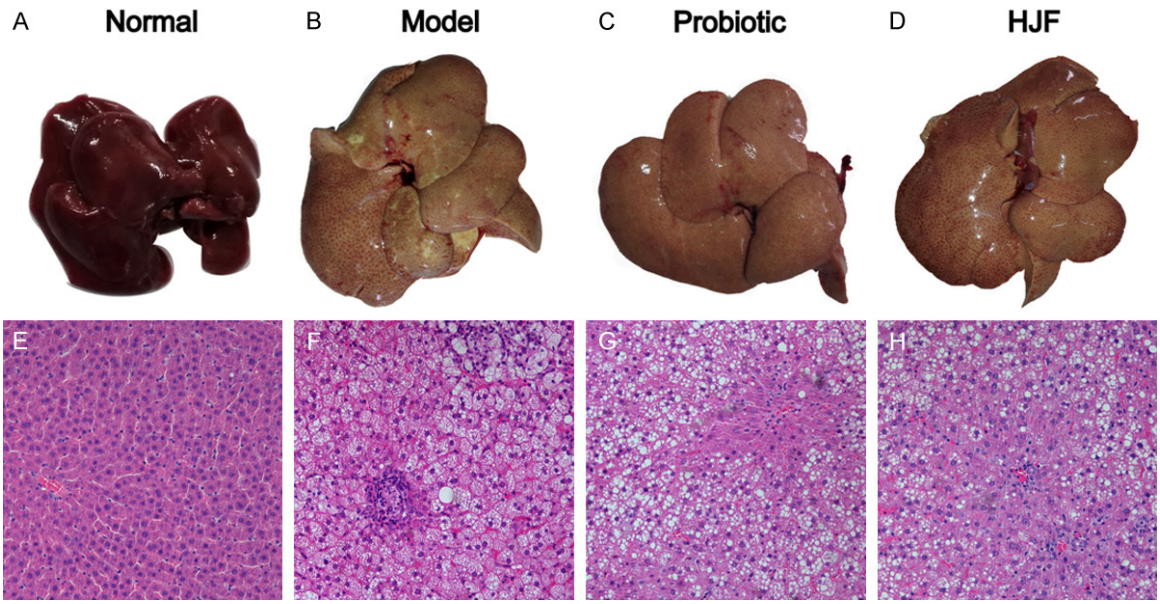

Figure 3. Observation and histological changes of liver tissue in different groups. HE staining of the hepatic tissue (magnification × 200), HJF: Hongqi-Jiangzh-Formula.

Serum biochemistry indexes in rats

Shown in **Figure 2**, compared with the normal group, the serum TC, TG, ALT, AST, insulin index and HOMA-IR markedly increased in the model group. Probiotic treatment significantly decreased TG, ALT and AST ($P<0.05$). However, the level of TG, ALT, AST and the insulin index were reduced remarkably by HJF treatment ($P<0.05$).

Observation and HE morphology of liver tissue in NAFLD rats

From **Figure 3A**, **3B**, we could see that the colour, as well as the size, of the liver in the model group was extinguished from normal group. The high-fat-induced liver was dark-stained and swollen with tense diolame and poor elasticity. We could also observe that the surface was cover by yellow lipid droplets, resulting from a number of bleeding spots. The conditions of liver characteristics in the probiotic and HJF group were better compared to the model group, with its coloure being approximately next to the normal group (**Figure 3C**, **3D**). Total lipid levels were measured by H&E staining. As shown in **Figure 3E-H**, the liver in the model group can been distinguished from the normal group by its obscure structure, irregular size, accumulation of lipid drops, as well as hepatocellular ballooning. Compared with the model group, the two treatment groups, probiotic and HJF, can differ by the alleviation of their lobular inflammation, fewer lipid drops and improved ballooning.

Proteins in MyD88-dependent pathways and MyD88-independent pathways via TLR4

As shown in **Figure 4**, except NF-κB p65, expression levels of the protein in the MyD88-dependent pathway in the model group were all higher compared with the normal group down-regulation ($P<0.05$). Alternatively, probiotics inhibited the expression of TLR4, TIRAP, MyD88, and p-NF-κB ($P<0.05$). The HJF group also resulted in the down-regulation of TLR4, TIRAP, MyD88, and p-NF-κB ($P<0.05$).

The result of **Figure 5** indicated the expressions of TRAM, TRIF and pIRF3 in the MyD88-independent pathway in the model group, where all of the groups were up-regulated compared with the normal group ($P<0.05$). Probiotic and HJF treatment could absolutely down-regulate the expression of TRAM, TRIF and pIRF3 ($P<0.05$).

Pro-inflammatory adipokines in serum

The results of TNF-α in the serum was performed in **Figure 6**. Similarly, the level in the model group was higher than the normal group ($P<0.05$). In contrast, the level of TNF-α in the HJF group declined and was notably distinguished from the model group ($P<0.05$).

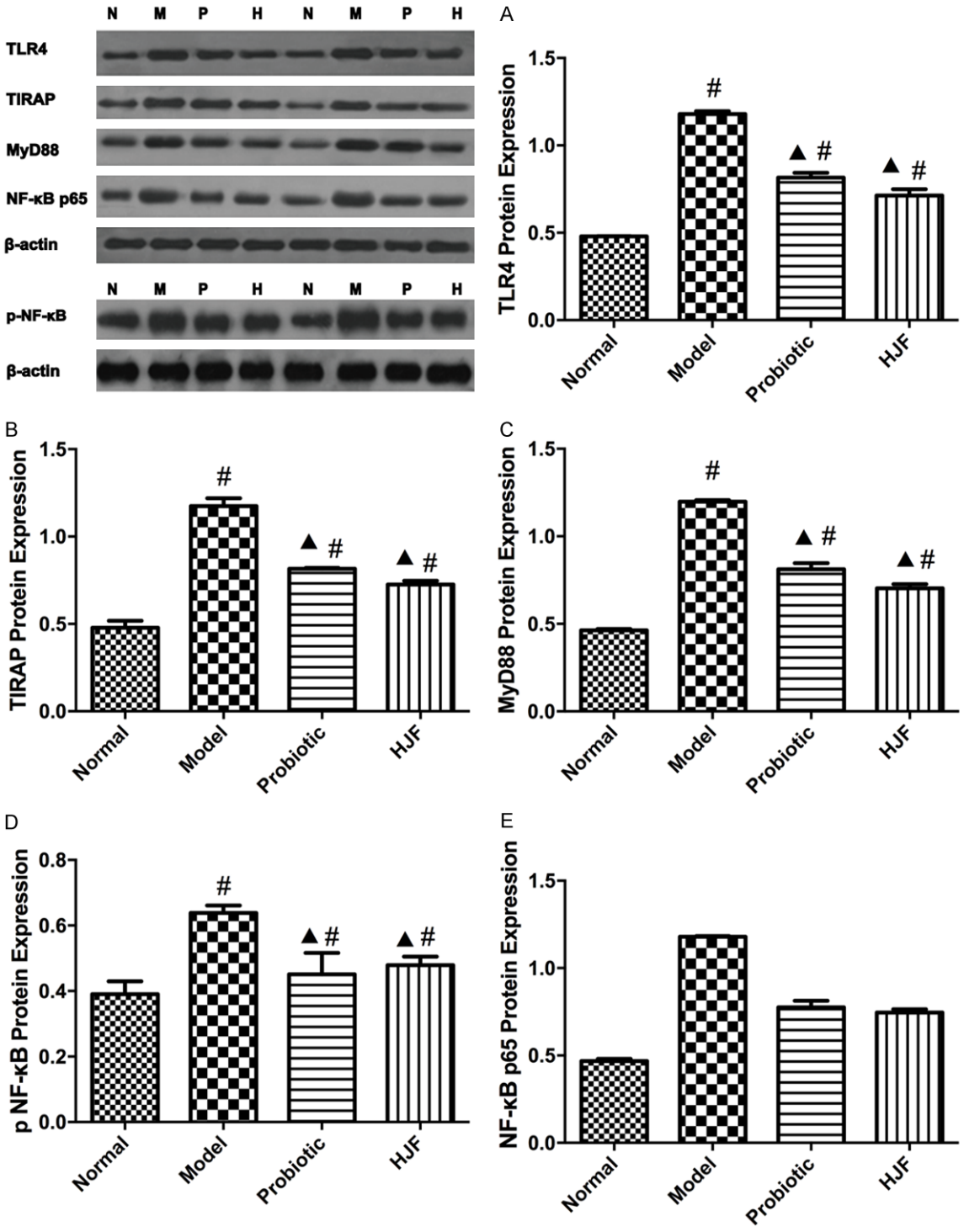

Figure 4. Western blotting of proteins expression in MyD88 dependent pathway. Data expressed as the mean ± standard deviation (n=5). #P<0.05 vs. normal group. ▲P<0.05 vs. model group. N: Normal group; M: Model group; P: Probiotic group; H: Hongqi-Jiangzh-Formula.

Discussion

Currently, with the global increase in obesity and diabetes, NAFLD is becoming the most common liver disease in Europe and the US and the main cause of liver transplantation due to end-stage liver disease and hepatocellular carcinoma [24]. NAFLD includes a series of abnormalities from hepatic steatosis to steatohepatitis. Accumulating evidence implies that

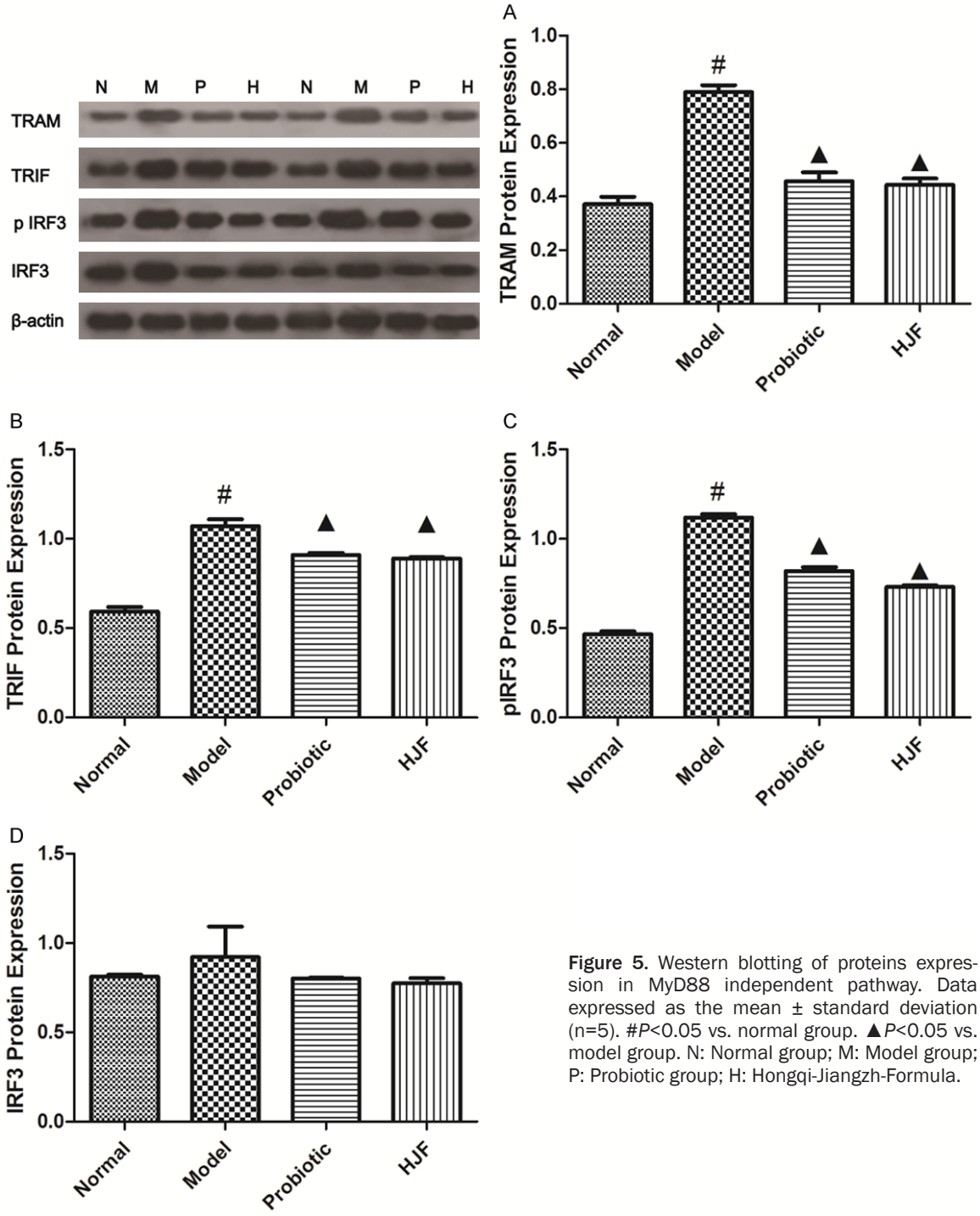

Figure 5. Western blotting of proteins expression in MyD88 independent pathway. Data expressed as the mean ± standard deviation (n=5). #P<0.05 vs. normal group. ▲P<0.05 vs. model group. N: Normal group; M: Model group; P: Probiotic group; H: Hongqi-Jiangzh-Formula.

NAFLD, is usually linked with lipid metabolic disorders and inflammatory reactions, which especially worsened in the NASH stage [21]. NAFLD models induced by HFD are suggested to be consistent with not only the characteristics of human disease but also the signs of oxidative stress, increased sensitivity to endotoxin and alterations in TLR4 signalling in the liver [22]. ALT and AST are the basic transaminases that reflect the conditions of liver injury. When damages, such as inflammations, occur in the liver. ALT and AST would be released to the serum and consequently lead to the increase of transaminases [25]. As mentioned above, excessive lipid accumulation is taken as the first “hit” for the initiation of secondary inflam-

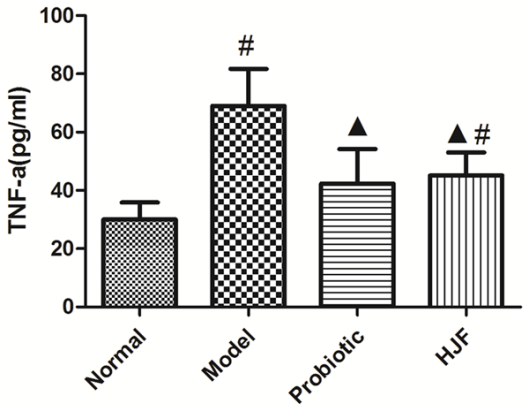

Figure 6. Expression of TNF-α in serum. Data expressed as the mean ± standard deviation (n=8). #P<0.05 vs. normal group. ▲P<0.05 vs. model group. HJF: Hongqi-Jiangzh-Formula; TNF-α: Tumor necrosis factor-alpha.

mation, for elevated TG was a strong marker of NAFLD [26]. A systemic review stated that there was a strong association of hyperlipidaemia with inflammatory status in experimental models while lacking clinical evidence [27]. In obese individuals, insulin and the HOMA-IR index are also tools to evaluate insulin resistance and inflammation [28]. Thus, even if levels of serum AST, ALT, TC, TG and insulin, as well as the HOMA-IR index, cannot be an independent marker for fat accumulation, as well as inflammation factors in the liver, it could help to understand the condition of the livers. Our experiment showed that the level of serum TC, TG, ALT, AST and insulin decreased apparently after HJF treatment. Additionally, histopathology is the gold standard for the diagnosis of NAFLD and prognosis [29]. The morphological and biological results demonstrated that HFD can model the fatty rat liver successfully [30]. According to the HE results, the degree of hepatocyte steatosis was considerably more severe in the model group. The condition of hepatocyte steatosis in HJF was significantly alleviated, indicating that excessive fat distribution was improved in the HJF group.

To explore how HJF prevents inflammation through the TLR4 pathways for NAFLD, we selected several proteins and the serum TNF-α, which were intimately related to the overexpression of NF-κB. It is clear that the activation of TLR4 might accelerate the oxidation of fatty acids and worsen NAFLD afterwards [31, 32]. Compared with the normal group, our present study clearly displayed that the rats in the model group were exposed to more severe inflammation due to the induction of NF-κB for the overexpression of proteins in the TLR4 pathways. In the liver, TLR4 is an essential initiator of the inflammatory reaction. The activation of TLR4 could release a series of pro-inflammatory responses, consequently resulting in NF-κB activation [33]. NF-κB, a critical master regulator of inflammation that can be activated in the rodent liver by models of HFD [34], is also a transcription factor of eukaryotic cells. The inhibition of TLR4/NF-κB is typically intended to inhibit inflammation [35]. Furthermore, MyD88 is not only an indispensable adaptor molecule for most TLRs but also induces inflammatory cytokines through NF-κB [36]. On one hand, MyD88's dysregulation could activates innate B cell activity, resulting in of liver fibrosis [37]. On the other hand, the MyD88-dependent signalling pathway is also the initiator and processor of HFD-induced systemic inflammation and metabolic inflammatory disease [38]. The MyD88-independent pathway it is an essential pathway that is functionally responsible for inhibiting viral infections through the activation of type I IFN and other IFN-inducible genes [39, 40]. The DNA-binding activity of NF-κB was clearly induced by multiple type 1 IFNs and was promoted by IFNs in rat cells [41].

Shown in the study, HFD would activate TLR4-related inflammatory reactions. As illustrated in the figure, probiotics and HJF could alleviate its inflammatory response to a certain extent. In our previous study, we have reported that HJF could significantly alleviate the HFD-induced endotoxaemia and influence specific gut bacteria in NAFLD rats [11]. In this study, we supposed that that HJF could also reduce the inflammatory response via TLR4 downstream pathways. In addition, the data demostrated that whether using MyD88-dependent or -independent pathways, expression mediated via TLR4 in the HJF group is clearly lower than in the model group, suggesting that HJF could effectively down-regulate the expressions of the TLR4/MyD88-dependent and -independent pathways. Furthermore, we observed that significance also occurred in the phosphorylation of NF-κB p65, which is closely related to the activation of NF-κB. The crucial contribution of NF-κB phosphorylation is directly controlling

NF-κB transactivation [44]. Our results indicate that the activation of TLR4/NF-κB signalling pathways would finally result in the release of pro-inflammatory cytokines, such as TNF-α [42].

HJF is a compound Chinese formula, which consists of natural herb medicines, all of which are prescribed according to the characteristics of disease. The Chinese medicine formula exerts its pharmacological effects through a multi-systemic mechanism. In Chinese medicine, NAFLD is a live qi stasis category syndrome [45]. The primary therapeutic principle for NAFLD is soothing the liver. HJF has the ability to soothe the liver, eliminating dampness and heat and promoting blood circulation by removing blood stasis. Previous studies proved that the classical formula, Shenling Baizhu San and Chaihu Shugan San, can effectively relieve NAFLD via the LXRα/FAS signalling pathway, as well as regulating SREBP-1c mRNA and protein [46, 47]. HJF is the enhanced and improved version. HJF is composed of different effective herbs, all of which are naturally made and demonstrated to be clinically effective. Astragalus membranaceusis is one of the most important herbs in HJF. This herb's extraction has led to prevention and treatment of NAFLD in rats induced by HFD, improving the ability of oxidation resistance [48]. In a recent study, the active ingredients of astragalus polysaccharides tend to activate of TLR4-mediated MyD88-dependent signalling pathway for the modulation of the immunity of the host organism [49]. Red yeast rice is a traditional Chinese food that is anticipated to alleviate metabolic syndrome by its active monascin and ankaflavin [50]. Although information on NAFLD is currently limited, studies in vivo and in vitro have shown them to be effective [51]. Many studies may argue that rice yeast rice is limited to monacolin K. However, the formular's ability in anti-obesity-related inflammation, insulin resistance, and NAFLD is reported to be irrespective of the monacolin K levels [52]. The Chinese formula is a type of constitutional medicine. Cooperation and communication of international medical societies have become increasingly frequent in the 21st century [53]. We hope that based on clinical practice, our Chinese formula would be an alternative option for patients when no promising cure is available.

In conclusion, our study demonstrated that HFD can activate TLR4-MyD88-dependent and -independent pathways, which might be related to the activation of NF-κB and the release of TNF-α. Our HJF could effectively improve lipid accumulation in HFD rat livers and down-regulate the expression of NF-κB through TLR4 downstream signnalling pathways. Our experimental evidence supports the use of HJF as a therapeutic treatment for NAFLD clinical use.

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Disclosure of conflict of interest

None.

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