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Original Article

Ameliorative effects of glycine in an experimental nonalcoholic steatohepatitis and its correlation between TREM-1 and TREM-2

Zhang-Feng Dou[1], Ya-Rong Guo[1], Jin-Chun Liu[1], Ning Li[1], Bao Chai[3], Xiu-Qing Li[1], Rong Fu[2], Xiao-Juan Wang[4]

[1]Department of Gastroenterology, First hospital of Shanxi Medical University, Taiyuan, Shanxi Province, China; [2]The Shanxi Key Laboratory of Otorhinolaryngology Head and Neck Cancer, Taiyuan, Shanxi Province, China; [3]Department of Gastroenterology, Shanxi Academy of Medical Science, Shanxi DaYi Hospital, Taiyuan, Shanxi Province, China; [4]Department of Gastroenterology, Taiyuan Municipal People's Hospital, Taiyuan, Shanxi Province, China

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Abstract: Inflammation plays an important role in Nonalcoholic Steatohepatitis (NASH), triggering receptor expressed on myeloid cells-1 and 2 (TREM-1 and TREM-2) modulates inflammatory and innate immune, they have been investigated in various inflammatory diseases, but not in NASH. Meanwhile we added glycine in HFO (HFOG) to investigate if the liver pathologic relief is related with TREM-1 and TREM-2. Liver tissue staining and serum indexes showed that the NASH was successful from the 4th weekend and glycine can improved many features of NASH. Results from Q-PCR and ELISA study showed that compareaded with control, TREM-1 is upregulated and TREM-2 is downregulated respectively in 4 and 8-week NASH model (TREM-1: $p < 0.001$; TREM-2: $p < 0.001$).Compared with HFO group, HFOG group with an extra 5% Glycine into the diet of NASH, we found that all model liver pathologic and serum indexes ameliorated in this group. Furthermore, Results from Q-PCR and ELISA study showed that compareaded with HFO group, TREM-2 of this group is upregulated and TREM-1 is downregulated respectively from the 4th weekend, which is more significant at the 8th weekend (TREM-1: $p < 0.001$; TREM-2: $p = 0.048$). Pearson correlation showed that TREM-1 and TREM-2 were closely associated with serum ET, TNF-α, TLR-4 and PC III. Besides, using multiple-stepwise regression analysis, we found that the ameliorative effects of glycine in HFOG was mainly related to its counteraction of PC III, TREM-1 and upregulation of TREM-2. Furthermore, we detected the expression of TREM-1 and TREM-2 in gall stone patients without drinking excessively before undergoing cholecystectomy, and found that the rise of TREM-1 and reduction of TREM-2 was close associated with the severity of fatty liver. To conclude, our results support the concept that TREM-1 and TREM-2 were close strongly linked to NASH and NALFD. Glycine can relieve NASH by its anti-fibrosis effect, and this ameliorative effect is related to the expression change of TREM-1/2 to some extent.

Keywords: TREM-1/2, glycine, NASH

Introduction

The major feature in non-alcoholic steatohepatitis (NASH) is excessive fat in the liver, along with inflammation and damage of the liver tissue, which is different from simple fatty liver or nonalcoholic fatty liver disease (NAFLD) [1]. NASH can be lead to liver fibrosis, cirrhosis and even liver cancer [2]. Therefore, it is becoming more common to study mechanism and management of liver injury in NASH. Normally, unhealthy diet and lack of exercise, obesity, insulin resistance, type 2 diabetes, dyslipidemia and hypertension are consider to be risky factors. Age and gender are also associated with the development. However, in the Asia-Pacific area, NAFLD has been found in a high percentage of non-obese individuals [3]. Though NASH underlying cause is still not clear, it is certain that inflammatory reaction induces liver injure and damage in NASH [4].

Triggering receptor expressed on myeloid cells (TREM) is a classical inflammation-related immunoglobulin super family receptor highly expressed on the cell membrane of human and marine neutrophils, monocytes and macrophages. The immunoglobulin super family mem-

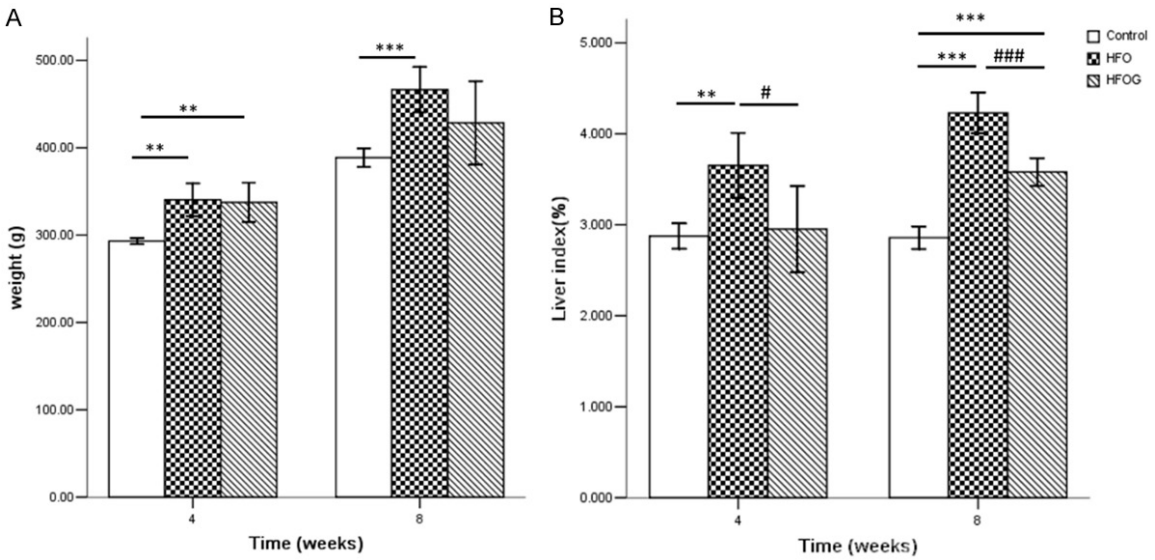

Figure 1. A. The mean body weight of each group at the 4th weekend and 8th weekend. B. The mean liver index of each group at the 4th weekend and 8th weekend. *VS Control, #VS HFOG, Data was analyzed by using one way analysis of variance (ANOVA) followed by Dunnett's post hoc test.

bers mainly include two activated receptors, TREM-1 and TREM-2. TREM-1 amplifies inflammation through promoting the secretion of proinflammatory factors, such as TNF-α, IFN-γ, IL-1β [5]. Previous studies have showed that TREM-1 is associated with disease activity in patients with IBD [6]. TREM-2 confers high risk for Alzheimer's disease and other neurodegenerative diseases, but its function remains contentious, some study shows that silencing of TREM 2 exacerbates neurodegenerative changes , others show that TREM 2 deficiency ameliorates pathology in AD [7, 8].

It has been known that endotoxin (ET) is related with NASH through Toll like receptor 4 (TLR4) and our previous and other studies have demonstrated that glycine alleviated the high-fat diet induced liver damage through inhibiting endotoxin [9-11]. However, the mechanism of the effect has not been fully elucidated. In this study, rat NASH model was established using the high-fat diet and intraperitoneal injection of oxytetracycline. The blood level of endotoxin and other factors of proinflammation were detected, and expression of TREM 1 and TREM 2 in liver was examined in NASH model. Through this study, we will research the effect of glycine on TREM 1 and TREM 2 in NASH. Moreover, utilizing correlation analysis and multiple-stepwise regression analysis, we will illustrate the relationship between ET, TNF-α, PC III, TLR-4, TREM-1/2 and glycine. Further, from the perspective of translational medicine, we detected the TREM expression in the liver specimen of some gall stone patients who were not drink excessively and underwent cholecystectomy, to study its relation with fatty liver, which is also our next research step.

Materials and methods

Animals

SD rats were maintained and housed at the animal facility center under specific pathogen-free conditions of Shanxi Medical University, Taiyuan, Shanxi, China. Male rats (6-7 weeks Age, weight 150-180 g) were selected for this study. All of the rats in the current study were approved by the Shanxi Medical University Animal Care and Use Committee.

NASH model preparation

Lettéron P [12] had reported that tetracycline can inhibit microsomal triglyceride transfer protein, which caused hepatic steatosis in mice. According to this result, making use of oxytetracycline and a high fat diet, Xing LX et al. [13] produced a NASH model in SD rats. Briefly, 54 rats with body weight range from 160 g to 180 g were purchased from Beijing Vital River Laboratory Animal Technology Co. and randomly divided into 6 groups of 9 animals each, which were 4-week and 8 week Control (chow), HFO (high-fat diet and oxytetracycline) and

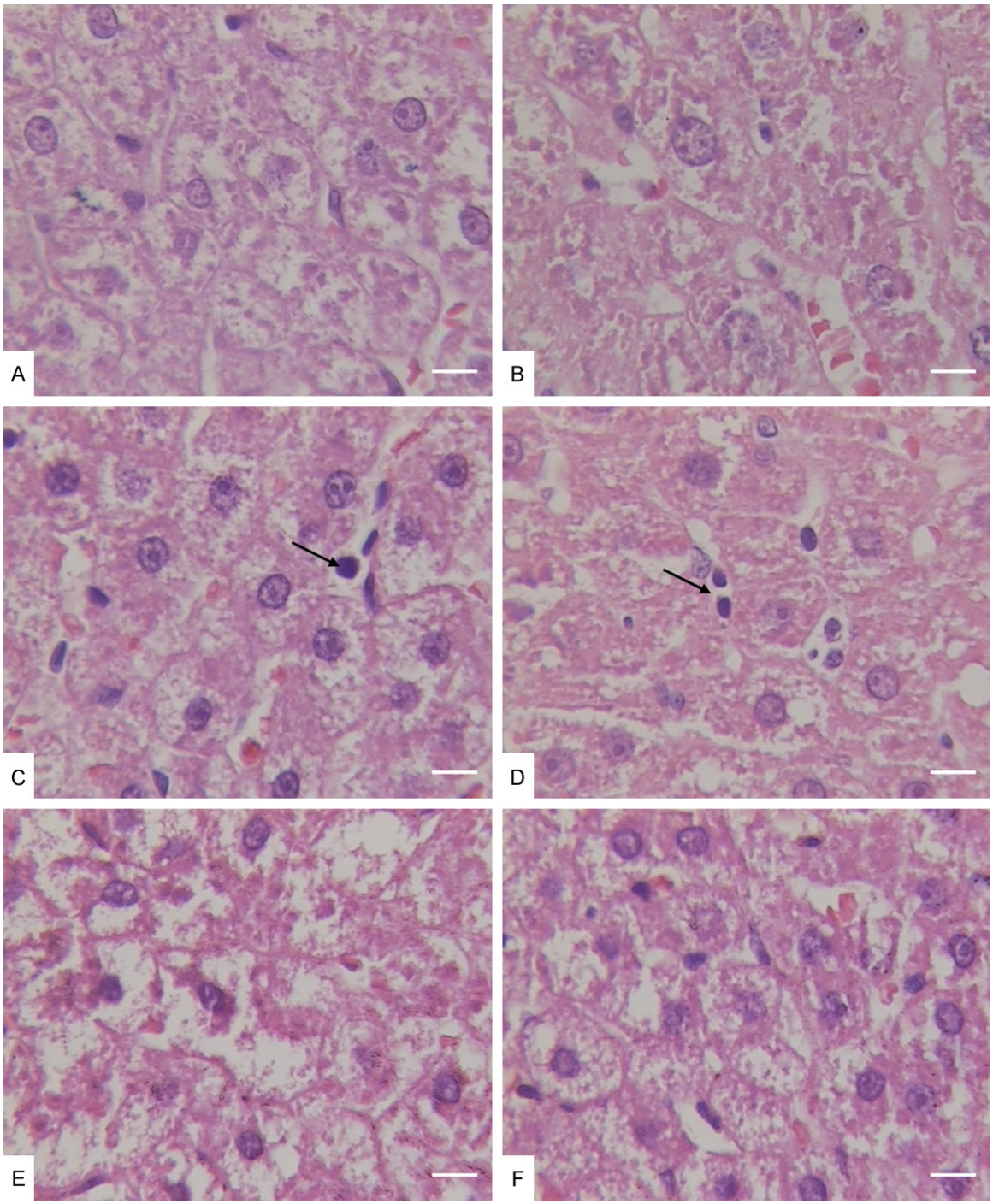

Figure 2. H&E staining of liver in each group at the 4th and 8th weekend (400X). A, B: Control group at the 4th and 8th weekend respectively, C: HFO group at the 4th weekend, D: HFOG group at the 4th weekend, E: HFO group at the 8th weekend, F: HFOG group at the 8th weekend. (Black arrow refers to inflammatory cells).

HFOG (high-fat diet, oxytetracycline and 5% glycine) groups. Briefly, NASH model was produced using a high-fat diet (0.5% sodium cholate, 2% cholesterol, 5% egg yolk powder, and 15% lard) and oxytetracycline (10 mg/100 g, peritoneal injection, once every 5 days). 5% glycine was added to the high-fat diet in HFOG groups. Rats were sacrificed at the 4th weekend and 8th weekend, respectively. On the condition of general anesthesia, blood samples were col-

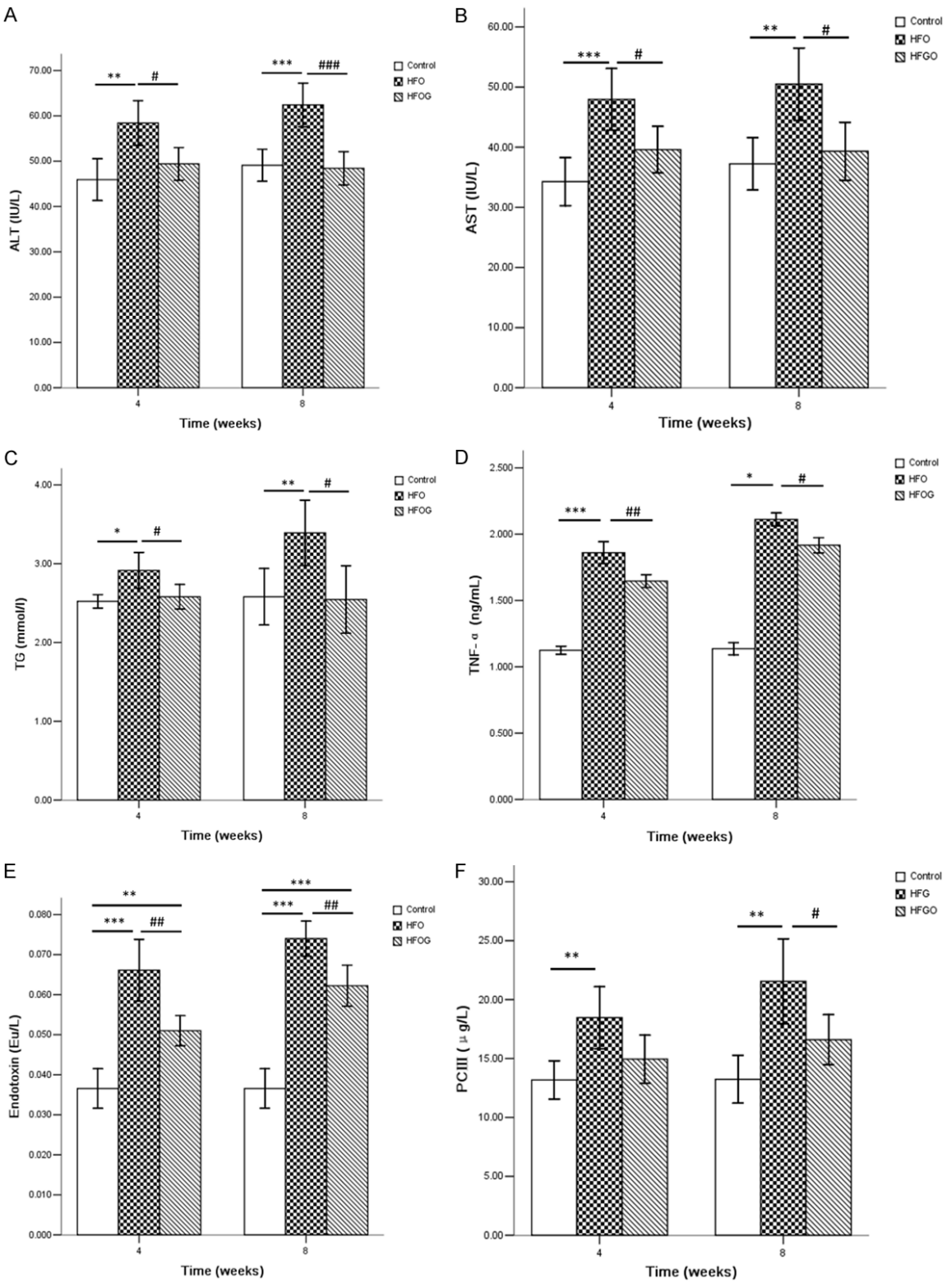

Figure 3. A-F. The mean Plasma ALT, AST, TG, TNF-α, ET, PC III content of each group at the 4th weekend and 8th weekend. *VS Control, #VS HFOG, Data was analyzed by using one way analysis of variance (ANOVA) followed by Dunnett's post hoc test.

lected from abdominal aortic to examine AST, ALT, PC III and TNF-α, and from portal vein to detect endotoxin. Spectrophotometry was used to measure levels of AST, ALT, and endotoxin

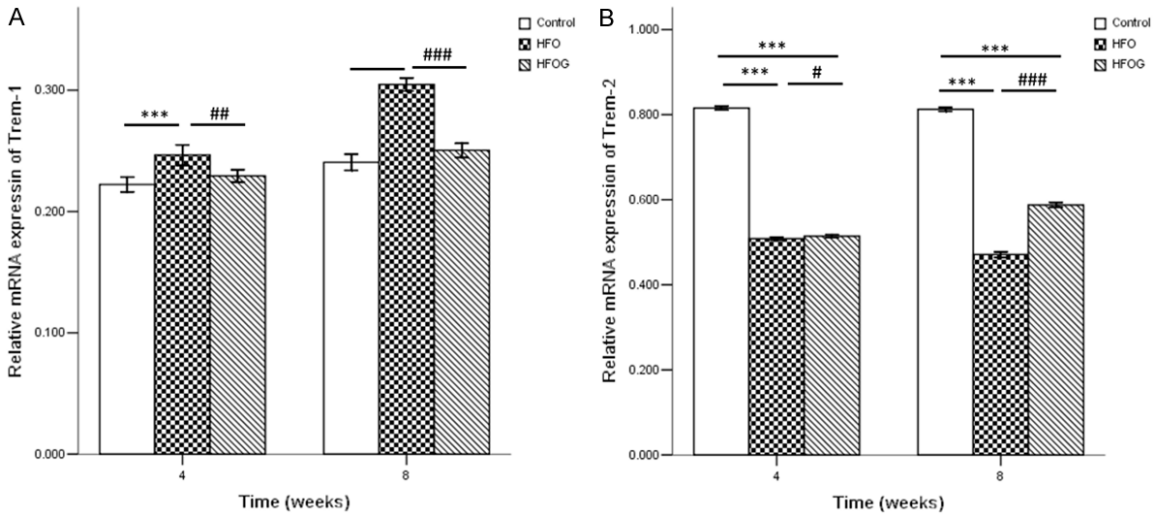

Figure 4. The relative mRNA level of Trem-1 and Trem-2 of each group at the 4th weekend and 8th weekend. *VS Control, #VS HFOG, Data was analyzed by using one way analysis of variance (ANOVA) followed by Dunnett's post hoc test.

while enzyme-linked immunosorbent assay (ELISA) was used to measure level of PC III and TNF-α. All reasonable efforts were made to minimize the animals' suffering.

Patients and samples

This study was approved by the Institutional Review Board of the First Hospital of Shanxi Medical University, 40 gall stone patients without drinking excessively before undergoing cholecystectomy, who met the inclusion criteria and signed informed consent were chose from 2009.11 to 2010.05. According to the extent of hepatocellular steatosis [14], we collected 13 patients' liver specimens whose hepatocellular steatosis <5%, 6 patients between 5% and 33%, 12 patients between 33% and 66%, and 9 patients steatosis >66%. Patients' blood samples were collected within 24 to 48 hours after admission. Before hemospasia, subjects should rest for 20 minutes, then we collected their fasting blood 3 ml from the morning and put them in heparin soldium-anticoagulant tubes, centrifugal separated and storaged in a -80°C refrigerator we had tested their blood lipid, blood glucose, ALT, AST in the blood plasma and calculated their BMI. During the cholecystectomy, we had got a piece of liver tissue for 0.5×0.5×0.3 cm in each subject, later we fixed them in a 10% formalin liquid , embedded with paraffin and sliced with 4 μm. This paraffin section were used for hematoxylin and eosin (HE) staining and Immunohistochemistry.

Immunohistochemistry

The liver specimens of gall stone patients were rinsed with PBS and prepared to detect the protein expression of TREM-1, 2 using immunohistochemistry. Briefly, formalin-fixed, paraffin-embedded tissue sections of 4 μM thickness were deparafinized and hydrated. Heat-induced epitope retrieval was performed using citrate buffer (pH=6) and a microwave histoprocessor. After antigen retrieval, tissue sections were incubated with 5% BSA for 20 minutes to block endogenous peroxidase activity. Adding first antibody for 1 to 2 hours in 37°C and then adding biotinylated secondary antibodies and StreptAvidin-Biotin-enzyme Complex-Alkaline phosphatase. Using an NBT/BCIP color detection system. Each patient we chose 4 specimens and each specimen with 4 views to calculate the mean integrate optical density (IOD) of TREM-1, 2 protein expression. The process of detecting TLR-4 in rats was much the same as above. All the immunochemistry results were measured using Image Pro Plus software.

Real-time PCR

The livers from rats were handled as above, using fluorescence quantitative RT-PC to determine the expression of TREM 1, 2. Total RNA extracted from collected liver tissue with TRIzol® Reagent (Ambion™.) was reverse transcribed to cDNA with reverse transcription kits (TIANGEN BIOTECH (BEIJING) CO.LTD.). Quantitative real-time polymerase chain reac-

Table 1A. The protein levels of TREM-1 in livers of different group

Time	Control (n=9)	HFO (n=9)	HFOG (n=9)
4th weekend	55.664±3.879	99.363±3.548[1]	88.559±3.760[2,3]
8th weekend	56.029±6.298	93.761±3.197[1]	84.634±5.660[2,3]

Note: [1,2]P<0.001, VS Control; [3]P=0.003, VS HFO.

Table 1B. The protein levels of TREM-2 in livers of different group

Time	Control (n=9)	HFO (n=9)	HFOG (n=9)
4th weekend	33.884±1.884	19.743±2.171[1]	22.691±2.679[2]
8th weekend	31.860±1.785	18.261±2.931[1]	22.978±3.842[2,3]

Note: [1,2]P<0.001, VS Control; [3]P=0.048, VS HFO

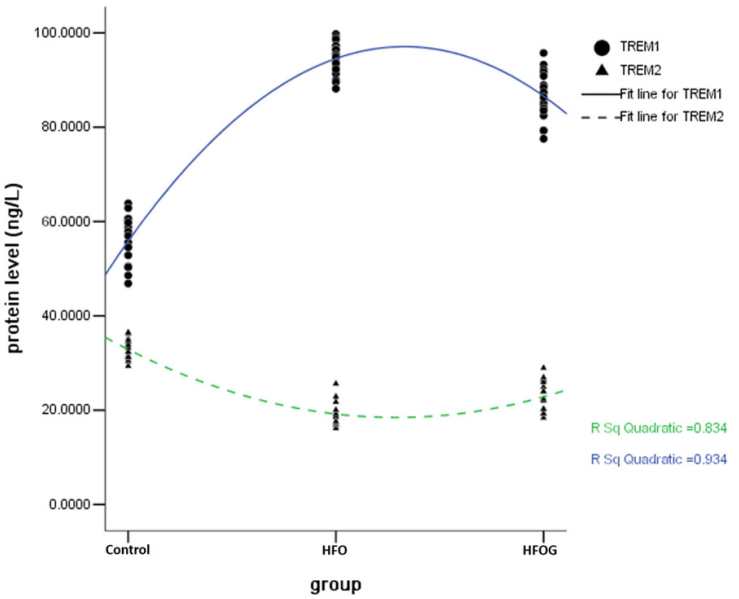

Figure 5. The scattergram of the protein level of TREM-1 and TREM-2 among groups.

tion was performed using fully automatic real-time fluorescence quantitative PCR. Sequences of the primer pairs used for this analysis were as follows: TREM 1 forward, 5'-GAAGTCCGTGTGGGGAAGTA-3' and reverse 5'-GGTTCTTCAGGTGTGTCCGT-3'; TREM 2 forward, 5'-CTTCTTACAGCCAGCATCC-3' and reverse 5'-TTCAGATCCTCACTGGACCC-3'; β-actin forward, 5'-GTCAGGTCATCACTATCGGCAAT-3 and reverse 5'-AGAGGTCTTTACGGATGTCAACGT-5'.

ELISA

Put 0.2 g rat liver tissue into tubes with 2 ml PBS (PH=7.4), fully grind, and stored overnight at -20°C. After two freeze-thaw cycles were performed to break the cell membranes, then centrifuged at 4°C, 5000 X g for 5 min, collect the supernatant. We use the Rat triggering receptor expressed on myeloid cells 1 (CSB-E09681r, Cusabio Biotech Co., Ltd.) and 2 (CSB-EL024405RA, Cusabio Biotech Co., Ltd.) Elisa kits, we chose 3 rats of the 4th weekend and 8th weekend from each group and draw standard curve following the instructions provided. other plasma indexes such as ET, TNF-α, PC III were detected following the instruction of the kits and the procedure was much similar as above.

Statistical analysis

The experimental data are expressed as mean ± SD. One-way ANOVA or T-test was used to determine whether sample mean values between groups were significantly different. The differences in values at $P < 0.05$ were statistically significant. Pearson's correlation and multiple-stepwise regression analysis were conducted at the levels of P=0.01. For categorical data, we adopted chi-square test. SPSS17.0 statistical software was used for all data analysis.

Results

High fat diet supplemented with oxytetracycline resulted in increased body weight and liver index at the 4th weekend and 8th weekend in HFO group, however, compared with HFO group, in HFOG group, rats' body weight and liver index decreased, especially at the 8th weekend

As **Figure 1** shows, rats fed a short-term 4-week HFD gained significantly more weight and higher liver index than chow-fed controls in HFO group (P=0.001), at 8th weekend, the tendency was much more significant (P<0.001). In HFOG group, rats' mean body weight was heavier than that of Control group at 4th weekend (P=0.006) but had no difference at 8th weekend (**Figure 1A**). The liver index of HFOG group was smaller than that of HFO group at the 4th

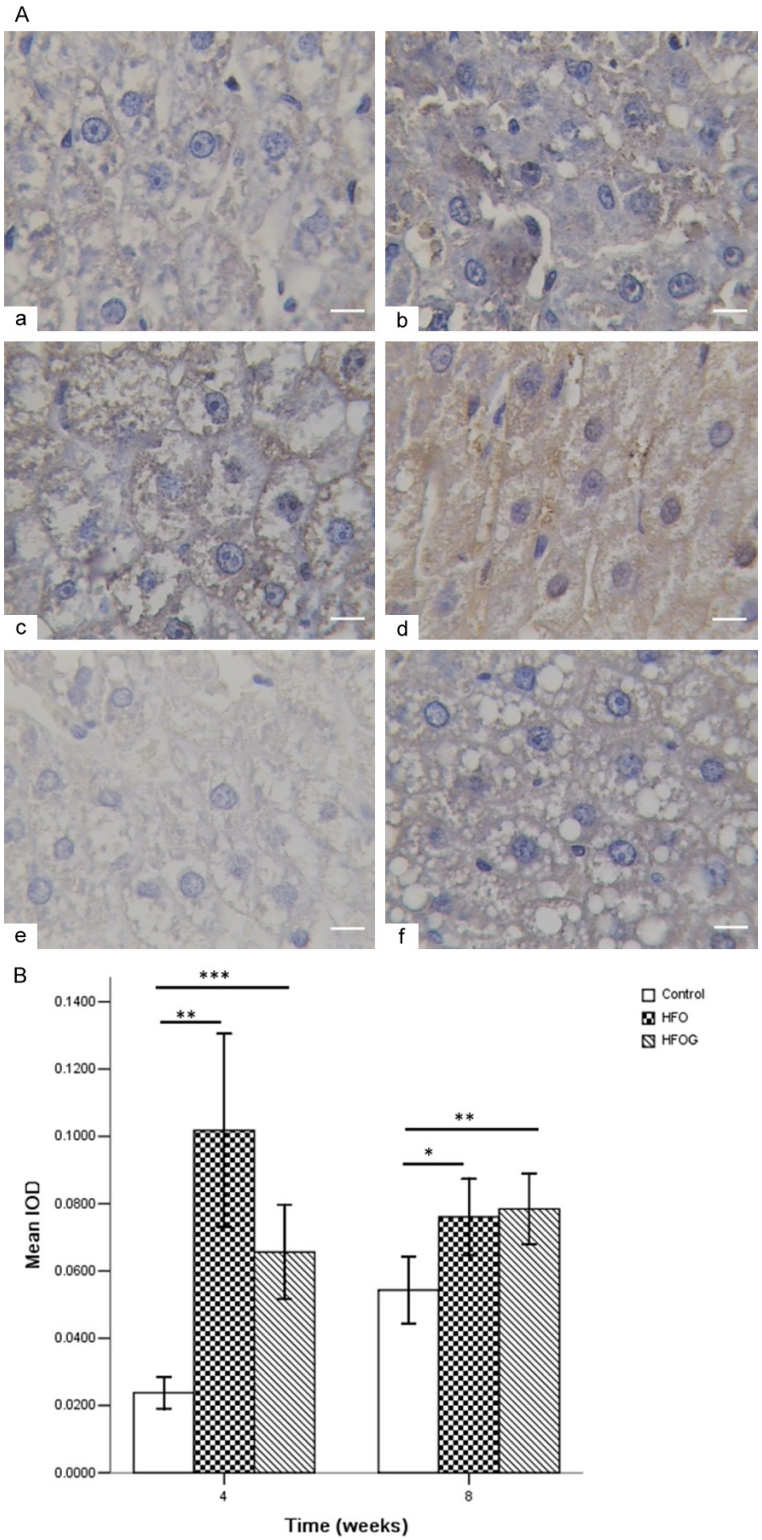

Figure 6. (A) Immunochemistry staining of TLR4 (400X) , in Control group at the 4th weekend (a) and the 8th weekend (b). Immunochemistry staining of TLR4 in HFO group at the 4th weekend (c) and the 8th weekend (d). Immunochemistry staining of TLR4 in HFOG group at the 4th weekend (e) and the 8th weekend (f). (B) The mean IOD of TLR-4 of each group at the 4th weekend and 8th weekend. *VS Control, Data was analyzed by using one way analysis of variance (ANOVA) followed by Dunnett's post hoc test.

weekend (P=0.047, **Figure 1B**). At the 8th weekend, the liver index of HFOG group was smaller than that of HFO group but greater than that of the Control group (P<0.001, **Figure 1B**).

High fat diet supplemented with oxytetracycline resulted in steatohepatitis in rats, however, compared with HFO group, in HFOG group, liver HE staining and the plasma indicators of rats reflected a lower levels of inflammation

We adopted H&E staining to assess liver steatosis and inflammation. In Control group, liver structure remained integrity and liver cells arranged compactly (**Figure 2A** and **2B**). In HFO group at the 4th weekend, we could see different degrees of hepatocyte steatosis, a small quantity of inflammatory cell infiltration and focal necrosis (**Figure 2C**). Compared with HFO group, the degree of hepatocyte steatosis and inflammatory cell infiltration is lessen and liver injury was alleviated in HFOG group at the 4th weekend (**Figure 2D**). The degree of hepatocyte steatosis and inflammatory cell infiltration is aggravated in HFO group at the 8th weekend (**Figure 2E**), however, at the same time, at the 8th weekend, the degree of hepatocyte steatosis and inflammatory cell infiltration is lessened In HFOG group (**Figure 2F**).

We further tested some plasma indicators of rats to evaluate liver steatosis and inflammation, including plasma ALT, AST, triglyceride (TG), TNF-α, endotoxin (ET) and type 3 precollagen (PC III) at the 4th and 8th weekend, representative figures were shown in **Figure 3**. Plasma ALT was the most sen-

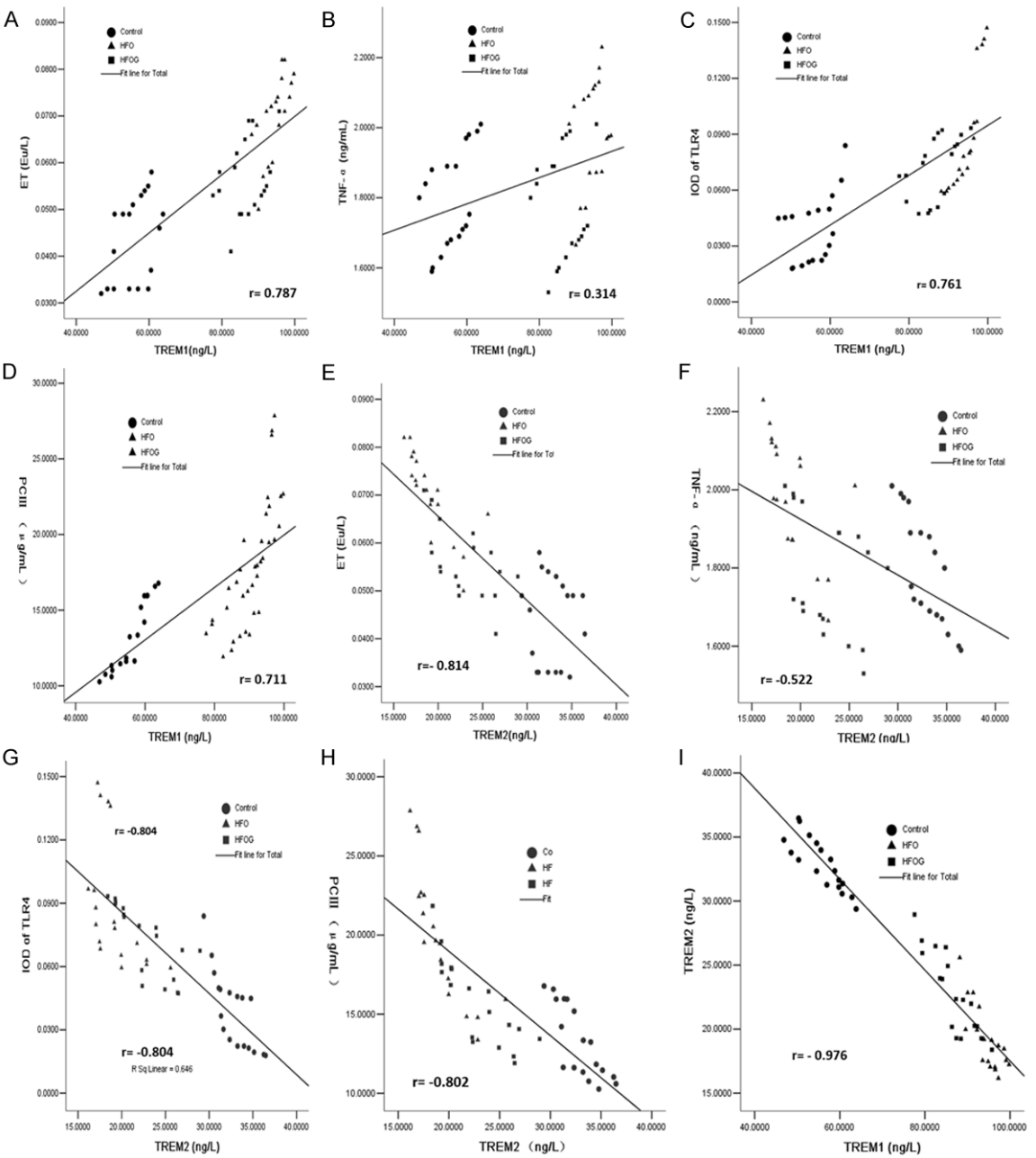

Figure 7. The correlation between ET and TREM-1 (A), TNF-α and TREM-1 (B), TLR-4 and TREM-1 (C), PC III and TREM-1 (D), ET and TREM-2 (E), TNF-α and TREM-2 (F), TLR-4 and TREM-2 (G), PC III and TREM-2 (H), TREM-1 and TREM-2 (I).

sitive indicator of liver injury, in HFO group were higher than that of Control group and HFOG from the 4th weekend (VS Control P=0.002, VS HFOG P=0.012), at 8th weekend, the trend is much more significant (both P<0.001) (**Figure 3A**). The average mean of AST was also the maximal from the 4th weekend (VS Control P<0.001, VS HFOG P=0.027) (**Figure 3B**). As an indicator of hepatic lipid, the average means of TG in HFO was also the highest between the 3 groups at the 4th (VS Control P=0.031, VS HFOG P=0.011) and 8th (VS Control P=0.006, VS HFOG P=0.014) weekend (**Figure 3C**). TNF-á was a factor of earlier period of inflammation, it rise the most obviously from the 4th weekend (VS Control P<0.001, VS HFOG P=0.002) (**Figure 3D**). ET was related with NASH, it had rise in both HFO (P<0.001) and HFOG (P=0.002) group from the 4th weekend, however, compared with HFO group, ET in HFOG was in a

Table 2A. Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	144.808	2.476		58.478	.000
	TREM-2	-2.636	0.096	-0.967	-27.418	.000
2	(Constant)	184.650	7.585		24.344	.000
	TREM-2	-2.894	0.091	-1.062	-31.958	.000
	TNF-α	-18.027	3.312	-0.181	-5.433	.000

a. Dependent Variable: TREM-1.

Table 2B. Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	52.992	1.046		50.639	.000
	TREM-1	-0.355	0.013	-0.967	-27.418	.000
2	(Constant)	63.651	1.777		35.827	.000
	TREM-1	-0.329	0.010	-0.897	-31.958	.000
	TNF-α	-6.842	1.027	-0.187	-6.663	.000
3	(Constant)	60.690	2.153		28.184	.000
	TREM-1	-0.308	0.014	-0.839	-22.550	.000
	TNF-α	-4.654	1.383	-0.127	-3.365	.001
	PC III	-0.170	0.075	-0.113	-2.261	.028

a. Dependent Variable: TREM-2.

Table 2C. Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	0.163	0.010		15.986	.000
	TREM-2	-0.004	0.000	-0.804	-9.739	.000
2	(Constant)	0.104	0.030		3.424	.001
	TREM-2	-0.003	0.001	-0.582	-4.344	.000
	PC III	0.002	0.001	0.277	2.067	.044

a. Dependent Variable: TLR-4.

lower level from the 4th weekend (P=0.005). PC III is a indicator of hepatic fibrosis, it had rise from the 4th weekend in HFO group (P=0.005), at the 8th weekend, the trend is much more significant (VS Control P=0.001, VS HFOG P= 0.0049).

mRNA expression of TREM-1 and TREM-2 in the liver tissue of the 3 groups

mRNA expression of TREM 1 and TREM 2 in liver tissues were quantitated using real-time PCR. As showed in **Figure 4A**, in HFO group, levels of *Trem 1* were upregulated at the 4th weekend (VS Control P<0.001, VS HFOG P= 0.005), at the 8th weekend, it became more significant (both P<0.001). Both levels of *Trem 2* were downregulated in HFO and HFOG group from the 4th weekend (both P<0.001), but HFO declined even more (P=0.048). At the 8th weekend, compared with HFO, levels of *Trem 2 had got an obvious rise* (P<0.001) (**Figure 4B**).

Protein levels of TREM-1 and TREM-2 in the liver tissue of the 3 groups

We adopted ELISA to detect the protein levels of TREM-1 and TREM-2. As we saw from **Table 1A**, the protein level of TREM-1 upregulated in HFO and HFOG group (both P<0.001), but compared to HFO, TREM-1 in HFOG had a smaller extent (P=0.003). This trend extended to the 8th weekend. On the contrary, in **Table 1B**, the protein level of TREM-2 downregulated in HFO and HFOG group at the 4th weekend (both P<0.001). This trend extended to the 8th weekend, however, compared with HFO, the protein level of TREM-2 in HFOG was a little higher than that in HFO (P=0.048). In **Figure 5**, Compared to HFO group, we can see clearly that Glycine in HFOG group can downregulate TREM-1 and upregulate TREM-2 at the same time.

Protein levels of Toll-like receptor 4 (TLR4) in the liver tissue of the 3 groups

It had been reported that the TLR4 expression was upregulated in a large number of NASH patients [11], what's more, TREM-1 had been characterized as an amplifier of TLR4 signaling. So we further quantitated TLR4 using immunohistochemistry to investigate its relationship with TREM-1 and TREM-2 in the 3 groups. As shown in **Figure 6**, the protein level of TLR-4 upregulated in HFO (P=0.001) and HFOG group (P<0.001) at the 4th weekend, and this trend extended to the 8th weekend (VS Control P1= 0.013, P2=0.004).

Table 3. The detection of liver ALT, AST, glucose, blood lipids and BMI in gallstones patients

	n	Rise in the number of ALT (Likelihood Ratio)	Rise in the number of AST (Likelihood Ratio)	Rise in the number of TC (Likelihood Ratio)	Rise in the number of TG (Likelihood Ratio)	Rise in the number of glucose (Likelihood Ratio)	Rise in the number of BMI (Likelihood Ratio)
Patients with fatty liver*	27	18 (66.7)*	16 (59.3) *	5 (18.5)	14 (51.9)*	20 (74.1)*	22 (81.5)*
Patients without fatty liver	13	2 (15.4)	3 (23.1)	1 (7.69)	2 (15.4)	4 (30.8)	5 (38.5)
Pearson Chi-square		9.231	4.067	Fisher's Exact Test	4.862	6.857	Fisher's Exact Test
P		0.002	0.032	0.351	0.027	0.009	0.011

Note: *P<0.05, VS Patients without fatty liver.

Correlation analysis of TREM 1 and TREM 2 with ET, TNF-α, PC III and TLR4 in control, HFO and HFOG group

Correlation analysis at the 0.01 level demonstrated that the expression of TREM 1 was positively correlated with the content levels of ET, TNF-α , TLR4 and PC III in the 3 groups (**Figure 7A-D**). However, the TREM 2 expression was negatively correlated with the levels of ET, TNF-α, TLR4 and PC III expression in the 3 groups (**Figure 7E-H**). Besides, there was a significantly negative correlation between TREM-1 and TREM-2 (**Figure 7I**). Using multiple-stepwise regression analysis, we found that PC III, TREM-1 were the top two predictors of ET; PC III, TREM-1 and TREM-2 were the top three predictors of TNF-α; TREM-2 and PC III were the top two predictors of TLR4; ET, TNF-α and TLR4 were the top three predictors of PC III.

Multiple-stepwise regression analysis of TREM-1, TREM-2 and TLR-4

Using Multiple-stepwise regression analysis, make the expression of TREM-1, TREM-2 and TLR-4 as dependent variable respectively, and the rest factors as independent variable. Results showed that TNF-α and TREM-2 were the top two predictors of TREM-1 (**Table 2A**); TREM-1, TNF-α and PC III were the top three predictors of TREM-2 (**Table 2B**); TREM-1, TREM-2 and PC III were the top two predictors of TLR-4 (**Table 2C**).

Protein levels of TREM-1 and TREM-2 in liver specimens of gall stone patients without drinking excessively before undergoing cholecystectomy

As we see in **Table 3**, in patients with fatty liver, the ratio of rise in ALT, AST, TG, blood glucose and BMI was greater than that in patients without fatty liver (P< 0.05). However, the total cholesterol (TC) had no difference in two groups. The mean IOD value of TREM-1 in patients without fatty liver was lower than that in patients who had developed fatty liver (P<0.001), on the contrary, The mean IOD value of TREM-2 in patients without fatty liver was higher than that in patients who had developed fatty liver (P=0.005) (**Figure 8**). Furthermore, the mean IOD of TREM-1 in patients with fatty liver were increased with the severity of fatty liver. Instead, the mean IOD of TREM-2 in patients with fatty liver were descended with the severity of fatty liver.

Discussion

Oxytetracycline is one kind of tetracycline medicaments, our results in HFO group showed that the method of injecting oxytetracycline intraperitoneally and supplementing with fat-rich food was effective and economical to establish NASH model in rats. Glycine has been demonstrated beneficial for hepatitis for many years. The mechanism is that glycine can prevent the LPS-induced elevation of intracellular Ca^{2+} concentration in Kupffer cells, thereby minimizing LPS receptor signaling and cytokine production. What's more, glycine can antagonize apoptosis of sinusoidal endothelial cells (SECs), which is one of the initial events in the development of liver injury [15]. The results of HFOG group showed that glycine was also beneficial for NASH, which was essentially a sort of inflammatory diseases.

Endotoxin is a major constituent of the outer cell membrane of Gram-negative bacteria. In NASH, endotoxin not only directly damages the liver, but also combines with lipopolysaccharide-binding protein (LBP) to form complex transporter. The latter activates the liver Kupffer cell and neutrophils to produce a series of inflammatory mediators, such as TNF-α and so on, inducing the damage of the liver [16]. Furthermore, the degree of fat accumulation in the liver was positively correlated with TNF-α level [17]. Tanwar S et al. reported that terminal

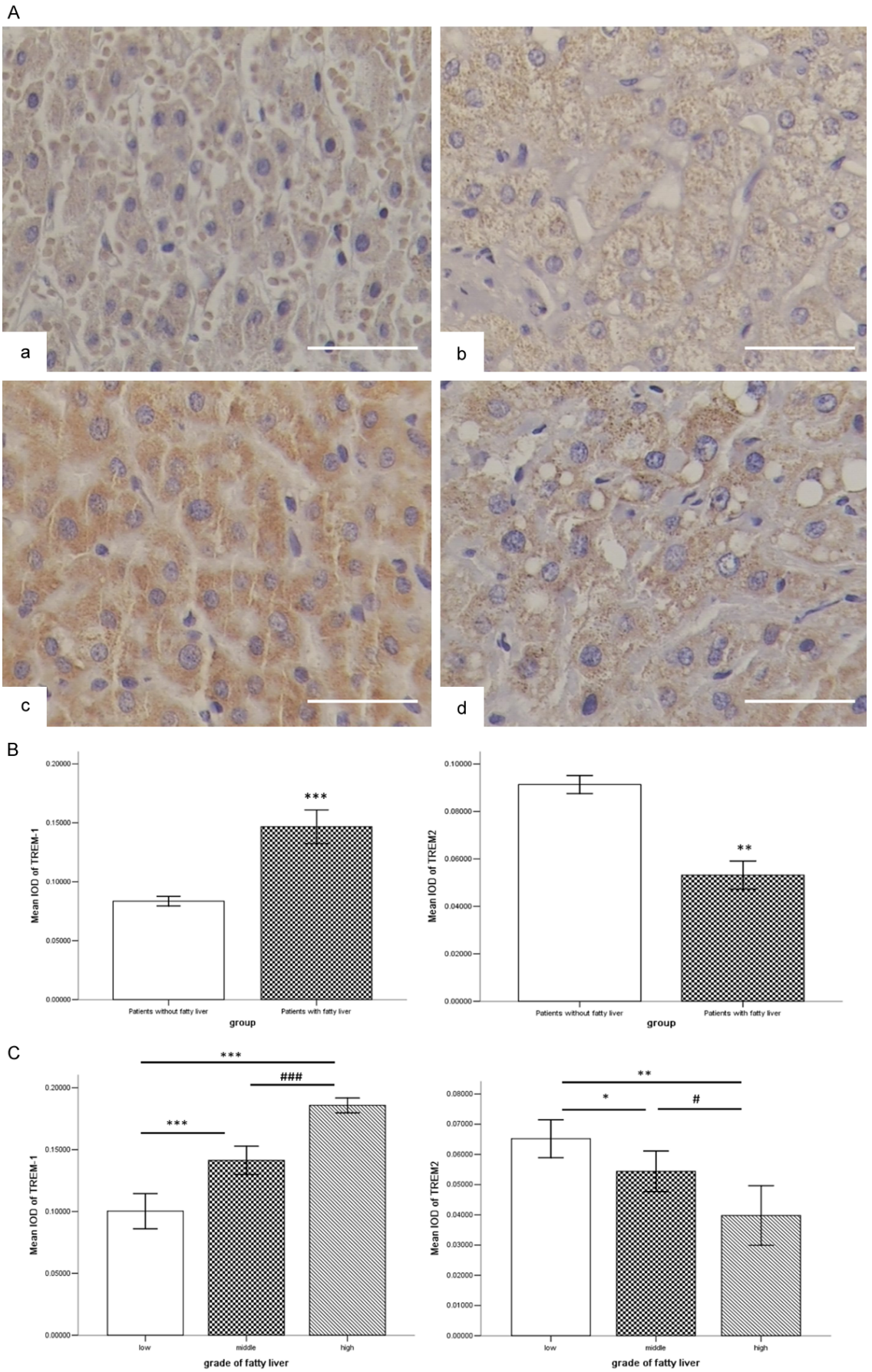
A
a
b
c
d
B
Mean IOD of TREM-1
0.20000
0.15000
0.10000
0.05000
0.00000
***
Patients without fatty liver
Patients with fatty liver
group
Mean IOD of TREM2
0.10000
0.08000
0.06000
0.04000
0.02000
0.00000
**
Patients without fatty liver
Patients with fatty liver
group
C
***
###
***
Mean IOD of TREM-1
low
middle
high
grade of fatty liver
**
*
#
Mean IOD of TREM2
0.08000
0.07000
0.06000
0.05000
0.04000
0.03000
0.02000
0.01000
0.00000
low
middle
high
grade of fatty liver

Figure 8. A. Immunochemistry staining of TREM in *gall stone patients without drinking excessively before undergoing cholecystectomy* (250X). a. TREM-1 in patients without fatty liver. b. TREM-2 in patients without fatty liver. c. TREM-1 in patients with fatty liver. d. TREM-2 in patients with fatty liver. B. The mean IOD of TREM-1 and TREM-2 between patients without fatty liver and with fatty liver. Data was analyzed by using T-test. C. The mean IOD of TREM-1 and TREM-2 among patients with different degree of fatty liver. *VS low grade , #VS middle grade, Data was analyzed by using one way analysis of variance (ANOVA) followed by Dunnett's post hoc test.

peptide of PC III as a biomarker for clinical utility in detecting the minority of patients with NAFLD who have NASH or advanced fibrosis related to NASH [18]. Our study showed that in HFO group, the plasma levels of portal vein endotoxin, aorta abdominalis TNF-α and PC III were significantly increased. Multiple-stepwise regression analysis showed that PC III, TREM-1 were the main factors of promoting NASH, but TREM-2 was the main factor of restraining NASH. In this entire indicators, PC III was the leading factor of mirroring NASH, which was in line with the study of Tanwar S et al. Besides, TREM 1, which was a secondary factor in downstream signaling events ,was reported to be an amplifier of inflammation by TLR2 and TLR4 signaling [19, 20], and Chen et al. reported that both endotoxin and TNF-α unregulated TREM-1 expression and suppressed TREM 2 expression in hepatic macrophages and endothelia cells [21]. Further more, Gao X et al. reported that Silencing of TREM-2 enhances the inflammatory responses of alveolar macrophages to LPS, which means TREM-2 played an important role of antagonizing LPS and TREM-1. In our study, from the multiple-stepwise regression analysis, if TREM-1 to be a secondary amplifier of downstream inflammation by TLR4 signaling, we can probably speculate that TREM-2 can be an negative factor which makes up the negative feedback of regulating TLR-4 signaling pathway. The possible mechanism here maybe that small intestinal bacterial overgrowth (SIBO) produce ET (LPS) [22], which combine TLR-4, through the MyD88-independent pathway [19] and caused the expression of TREM-1 and other transcription factors, finally leaded to the augmentative inflammation in NASH. During this cascade reaction, TREM-2 was activated, maybe by combining DAP12 and activating PI3K and ERK signaling pathway [23], which restrain the TLR-4 signaling pathway and combat the damaging inflammation.

In this study, Using glycine in HFOG can prevented the increase in ALT, AST, TNF-α, ET, PC III and TREM-1 in high-fat diet rats, and maintained a normal level of TREM-2 expression in the high-fat diet rats, which suggesting that glycine may target many of inflammatory factors to prevent and treat the liver injury in NASH rats. Therefore, glycine may inhibit ET and TNF-α production leading to suppression of TREM 1 expression and upregulation of TREM 2 expression resulting in protecting the liver cells in NASH. TREM-1 and TREM-2 in the liver tissue of 3 groups showed negative correlation, that's another evidence that TREM-1 and TREM-2 may be antagonistic each other.

In gall stone patients without drinking excessively before undergoing cholecystectomy, comparing to those who without fatty liver, patients with fatty liver shows higher expression of TREM-1 and lower TREM-2 in their liver specimen. What's more, the expression of TREM-1 was increased with the severity of fatty liver. To the contrary, the expression of TREM-2 declined with the severity of fatty liver. This suggested that TREM-1/2 was related to the fatty content of liver to some extent, The TREM-1/2 may develop to be an indicator of evaluating NAFLD.

In summary, by feeding high fat diet and peritoneal injection with oxytetracycline (HFO group), we demonstrated that this method was economical and convenient. By adding glycine into the high fat diet (HFOG group), we validated that glycine can relieve NASH. Through comparison the expression of TREM-1 and TREM-2 in control, HFO and HFOG, we investigated the role of TREM-1 TREM-2 and their correlations with ET, TNF-α, PC III in the process of forming NASH and its possible mechanism through TLR-4. Finally, we illustrated the relationship between NASH, glycine and TREM-1 and TREM-2. In conclusion, our data provide new insight into the mechanism responsible for the development of NASH. Therefore, targeting TREM-1 and TREM-2 could be a promising therapeutic strategy in NASH.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jin-Chun Liu, Department of Gastroenterology, First Hospital of Shanxi Medical University, Jiefang Road South 85th, Taiyuan, Shanxi Province, China. Tel: +86 351 4639384; Fax: +86 351 4639384; E-mail: zxr610624@163.com

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