

Original Article

Study on the phenotype changes of kupffer cells in non-alcoholic fatty liver

Yang Li¹, Haitao Jiang², Jianchun Xian¹, Wei Zhang³, Li Xiao¹, Jianhe Gan⁴

Departments of ¹Liver Diseases, ²Articular surgery, ³Infectious Diseases, Taizhou People's Hospital, Taizhou 225300, China; ⁴Department of Infectious Diseases, The First Affiliated Hospital of Soochow University, Suzhou 215006, China

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Abstract: Objective: To establish the model of non-alcoholic fatty liver (NAFL) in mice and isolate morphologically sound and biologically active kupffer cells (KCs) based on the improved hepatic perfusion digestion method, so as to study the function of phenotypic changes of kupffer cells in NAFL. Methods: C57BL/6J male mice were classified into the control group and the high-fat-diet group. The mice were observed after being fed for 12 weeks. Their serum hepatic indexes were detected, and fixed hepatic tissues were made into pathological sections. Some liver tissues were taken to detect the serum concentration of ALT, AST, TC, TG according to the serum ALT, AST, TC, TG assay kit instructions. Mice KCs were isolated by improved perfusion digestion method, and the isolated KCs were identified by ED1 immunocytochemical staining. The ratio changes of the types of cells (CD11b⁺ and CD68⁺) in mice with non-alcoholic fatty liver were detected by means of the flow cytometer. The expression level of TNF- α , CD-14, IL-6 and TLR-4 protein of primary KCs in the mice with non-alcoholic fatty liver disease were detected by the Western blot. Results: The serum concentration of TG, AST, TC, TG in mice in the high-fat-diet group were significantly higher than that of the control group ($P < 0.05$). Compared with the control group, the proportion level of the two types of (CD11b⁺CD68⁺ and CD11b⁺CD68⁻) cells in the the process of establishing non-alcoholic fatty liver model significantly changed, and the ratio of CD11b⁺CD68⁻ cells increased. The Western blot assay results showed that compared with the control group, the expression level of TNF- α , CD-14, IL-6 and TLR-4 protein in primary KCs of the mice in the high-fat-diet group were significantly increased ($P < 0.05$). Conclusion: Kupffer cells (KCs) transferred to CD68⁺CD11b⁺ in the occurrence and development of NAFL, which may promote the further progression of NAFL by elevating the pro-inflammatory cytokines.

Keywords: Non-alcoholic fatty liver, kupffer cells, mice, inflammatory cytokines

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a clinical syndrome with histological changes of liver similar to alcoholic hepatitis in patients without history of alcoholism [1]. The lesion of the liver lobule is shown in fatty change of hepatocyte and accumulation of fat. In terms of lesion, the disease can be classified into three sustained stages of simple fatty liver (sFL), non-alcoholic steatohepatitis (NASH), and non-alcohol fatty liver cirrhosis (NAFLC), associated with genetic-environment-metabolism stresses [2]. NAFLD can further develop into the advanced-staged liver diseases, such as hepatocellular carcinoma and hepatic failure. Its damages to the body are not only involved in the lesion liver,

but also correlated to other diseases, such as obesity, hyperlipidemia, type 2 diabetes mellitus, hypertension, and coronary heart disease. Currently, the pathogenesis of NAFLD is yet to be elucidated.

In recent years, with further exploration of the functions of macrophages in lipid homeostasis and in the immune system, the functions of Kupffer cells (KCs) in the occurrence and development of NAFLD has gradually become a hot spot. KCs are mononuclear macrophages located in the hepatic sinusoids, which can migrate between the vessels and sinusoids and establish the relationship between them. In a pathological state, activated KCs can secrete a variety of inflammatory mediators such as pro-

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teolytic enzymes, chemotaxines, and cytokines (CK). These inflammatory mediators play important roles in liver injury [4]. With the action of different cytokines secreted by CD4⁺ T cells, KCs activate toward two directions. Therefore, KCs can be divided into two phenotypes: histologically intrinsic KCs (CD68⁺, M2 type) and inflammatory infiltration type (CD11b⁺, M1 type), both of which are involved in regulating inflammation. The main function of CD11b⁺ is to secrete a variety of cytokines, while CD68⁺ is to induce repairing and phagocytosis. In view of the various functions of KCs in the development of diseases, the transformation of KCs phenotypes may affect the occurrence and development of NAFLD, but which is yet to be elucidated. This study is designed to establish a model of mice with non-alcoholic fatty liver fed with high-fat-diet, to further study the relationship between the morphology and functions of the Kupffer cells as well as the correlation among fatty changes, inflammation, and necrosis so as to identify the links of KCs to the occurrence and development of NAFLD.

Materials and methods

Materials and reagents

Cholesterol was purchased from Sijiqing Biological Technology Co Ltd. in Hangzhou; Polyvinylidenedifluoride (PVDF) was purchased from Millipore company (USA). Percoll (cell isolation liquid) was purchased from the R&D company (USA). DMEM culture medium was purchased from Gibco company (USA). TNF- α , CD-14, IL-6 and TLR-4 antibodies were purchased from R&D company (USA).

Establishment of the animal model

After adaptively fed for a week, twenty SPF C57BL/6J wild male mice aged six weeks (purchased from the Slack experimental animal Co. Ltd.) were classified into two groups: the control group fed with normal diet and the high-fat-diet group fed with high fat diet (standard forage with lard, cholesterol, egg yolk powder, milk powder and sodium cholate). After the model was established, every week the weight of the mice was measured, observing whether there were any changes in the mice's habits of appetite and behavior. 12 weeks later, the mice were killed for tissue specimens, of which the left hepatic lobe was removed and placed in

10% neutral formalin for pathological section making, and the rest liver tissues were cryopreserved at -80°C. At the same time, the serum concentrations (ALT, AST, TC, TG) of the mice in the high-fat-diet group were detected by the serum (ALT, AST, TC, TG) assay kits.

The mice liver pathological examination

The paraffin section of mice liver by HE staining was observed to verify the pathological changes, fatty change, inflammatory activity and fibrosis of hepatic tissues. The steps of HE staining were as follows: the tissue specimens were baked in the oven at 60°C for 6 h after conventional procedure of dehydration, paraffin embedding, and slicing. After that, the paraffin section was dewaxed in the xylene (I) for 15 min, and in the xylene (II) for 15 min; treated with gradient hydration in 100% ethanol (I) for 10 min, in 100% ethanol (II) for 10 min, 90% ethanol (I) for 10 min, 90% ethanol (II) for 10 min, 80% ethanol for 10 min, and washed more than 10 min. The nucleus was treated with hematoxylin staining for 5 min, bathed for 5 s in the clean water, differentiated in hydrochloric acid alcohol for 2-3 s, washed for 3 times, and bathed for more than 15 min in clean water to become blue again. Cytosol was strained in eosin for 2-3 min, washed for 3 times, bathed in 80% ethanol for 1 min, in 100% ethanol for 2 min and in xylene for 5 min; and the sections were sealed by neutral gum. In terms of the severity of fatty changes under optical microscope, semi quantitative is mild "+" (hepatic cells with fatty changes accounting for hepatic lobule <1/3), moderate "++" (hepatic cells fatty changes accounting for the liver lobule 1/3-2/3), and severe "+++" (hepatic cells fatty changes accounting for hepatic lobule >2/3).

Isolation, culture, identification, morphology and function detection of kupffer cells

Kupffer cells of the liver in mice were isolated by the improved perfusion digestion method (steps: after the mice were anaesthetized by chloral hydrate, the abdominal cavities were cut and open to expose the liver, and the portal vein isolated into which the needle was injected; then the inferior vena cava was cut off and the liver was perfused with perfusion fluid. When the color of the liver turned into pale without blood, the liver was perfused by collagenase solution instead of the perfusion fluid,

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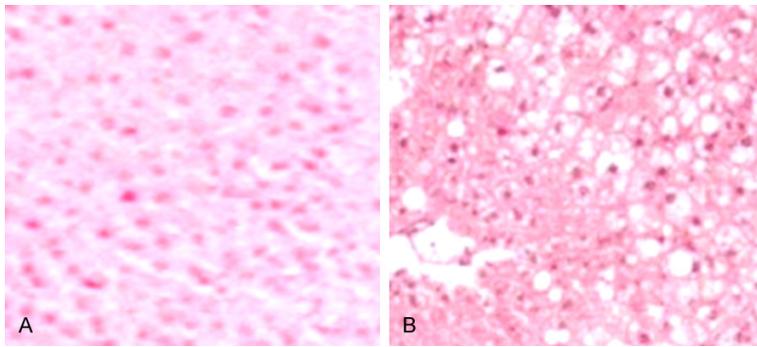


Figure 1. HE staining of the hepatic tissues of the mice in the high-fat-diet group. A: The normal control group, B: The high-fat-diet group.

and the inferior vena cava was clamped at the same time. The liver was isolated after complete digestion, which was then torn into pieces by the tweezers and filtered through a strainer, with cell suspension stored for future utilization) and Percoll density gradient centrifugation. With the cell concentration regulated to $5 \times 10^6/\text{ml}$, the KCs were cultured in the 35 mm culture dish in the conditions of 5% CO_2 and 37°C for 4 h till the cells were adherent, and then cultured in 10% FBS DMEM medium. The culture solution was replaced once a day, and images were collected by taking photos. The KCs were cultured for another 48 h and the ratios of $\text{CD11b}^+\text{CD68}^-$ and $\text{CD11b}^+\text{CD68}^+$ cells (KCs cells) were detected by flow cytometry.

Mouse hepatic tissues by Western blot

KCs cells were extracted from the livers of mice in the control group and in the high-fat-diet model group by the improved perfusion digestion method. Next, protein was extracted from the KCs cells, and the protein content of the tissues was determined by the BCA method. Equal amounts of samples were mixed with $5 \times$ sample buffer, boiled for 5 minutes, and then cooled quickly in the ice bath. The protein was isolated in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). By the electrical transfer method the isolated protein was transferred to PVDF membrane which was immersed in the sealing solution at room temperature for 1 h. Then PVDF membrane was incubated in the primary antibody at 4°C for overnight. After being rinsed for 3 times, the PVDF membrane was placed into the secondary antibody and incubated at room temperature for another 1 h, and then into witch appro-

priate amount of ECL color reagents were added after being rinsed for 3 times. The PVDF membrane was placed into the gel image analysis system for imaging in the chemical exposure mode. Finally, the integrated optical density value (IOD value) of the target band was determined by the Bio-Rad image software.

Statistical methods

The data were statistically analyzed by the SPSS 18.0 software. The measurement data were expressed as mean \pm standard deviation, and the compared results between the two groups were detected by t test; while the count data were presented by the rate, and the compared results between the two groups were detected by the chi square test. Only when $P < 0.05$, the differences were statistically significant.

Results

Pathological changes of hepatic tissues

During the model establishment, the two mice groups were sound in growth, but the mice in the high-fat diet group significantly increased in weight ($P < 0.05$). HE staining results showed in the control group, clear structure in the hepatic lobules and portal areas, neatly-arranged hepatic cords and normal hepatic sinusoids; while in the high-fat-diet group, hepatocytes showed as fatty change, severe fatty liver, Grade 4 pathological changes; without necrosis of hepatocytes, without inflammatory cells infiltrated in portal area nor without fibrous tissue hyperplasia in portal tracts, as shown in **Figure 1**.

Detection of serum lipids and hepatic function

The results of mice serum indexes detection showed that in the fasting state, the serum Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Total cholesterol (TC) and Triglyceride (TG) in the high-fat-diet group were significantly higher than those in the normal group ($P < 0.05$), as shown in **Table 1** and **Figures 2** and **3**.

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Table 1. Comparison between the two groups in body weight, liver index, serum lipids, serum amino-transferase level and HAI ($\bar{x} \pm S$)

Groups	Weight (g)	Liver index	AST (U/L)	ALT (U/L)	TG (mmol/L)	TC (mmol/L)	Knodel HAI
Control group	21.3±1.5	2.5±0.5	110.5±15.5	62.3±4.5	0.75±0.15	2.34±0.15	0
High-fat-diet group	41.5±5.5*	4.1±0.7*	434.1±21.6*	164.2±10.5*	1.75±0.25*	6.75±2.15*	2.50±1.50*
T	4.73	5.08	8.87	4.00	6.14	5.43	26.78
P	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05

Notes: *compared with control group, P<0.05.

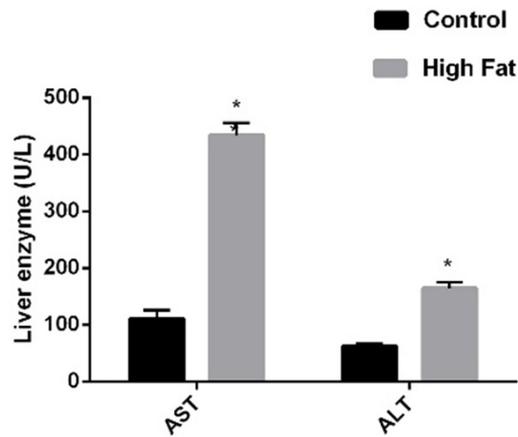


Figure 2. Comparison between the two groups in serum aminotransferase level. *P<0.05.

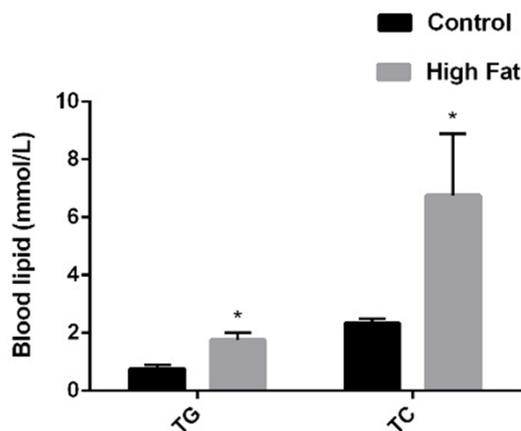


Figure 3. Comparison between the two groups in serum lipid level. *P<0.05.

Purity assessment of isolated primary KCs and primary culture morphology

Under the light microscope, the KCs were observed to be small, round in shape, and equal-sized; and after adhered, and the boundary of the cells are unclear due to membrane

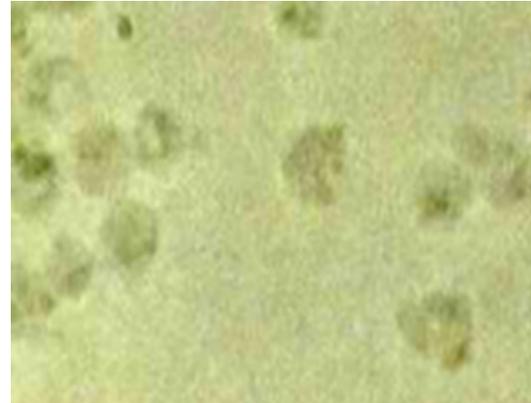


Figure 4. KCs in vitro observed under the optical microscope.

extending; the fully-extended cells are shown to be morphologically polygonal or stellate, as shown in **Figure 4**. KCs cells were successfully identified by the ED1 immunocytochemistry staining method, as shown in **Figure 5**. Flow cytometry results showed that, in the collected cell populations of the high-fat-diet group and the control group, the proportion of CD45⁺ and F4/80⁺ (KCs markers) accounted for about 27% in hepatic non-parenchymal cells. And F4/80⁺ purity analysis results showed that the purity of the cells in the two groups was more than 95%. The results showed that the purity of the isolated primary cells could meet the requirements of the future experiments.

Flow cytometry detection of two cell types (CD11b⁺CD68⁻ and CD11b⁺CD68⁺)

CD11b⁺CD68⁺ cells accounted for 91.2% in all the macrophages in the normal group; while in the high-fat-diet group, the percentage of double-positive cells was 51.8%, and the percentage of CD11b⁺CD68⁻ cells was 47.1%. The results showed that the ratios of the two types of cells CD11b⁺CD68⁻ and CD11b⁺CD68⁺ changed

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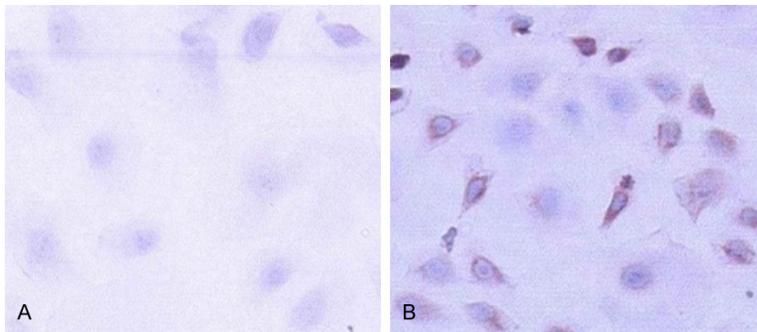


Figure 5. ED1 immunocytochemistry staining. A: The normal control group, B: The high-fat-diet group.

Table 2. IOD values of TNF- α , CD-14, IL-6, and TLR-4 in KCs in the mice liver of the high-fat-diet mice group

	Control group	High-fat-diet group	T value	P value
TNF- α	7355.45 \pm 134.75	12456.45 \pm 256.48	8.49	<0.05
CD-14	8245.45 \pm 138.39	11349.34 \pm 238.45	7.16	<0.05
IL-6	7734.00 \pm 122.35	12618.11 \pm 198.32	6.29	<0.05
TLR-4	8345.13 \pm 134.35	9235.55 \pm 245.13	5.56	<0.05

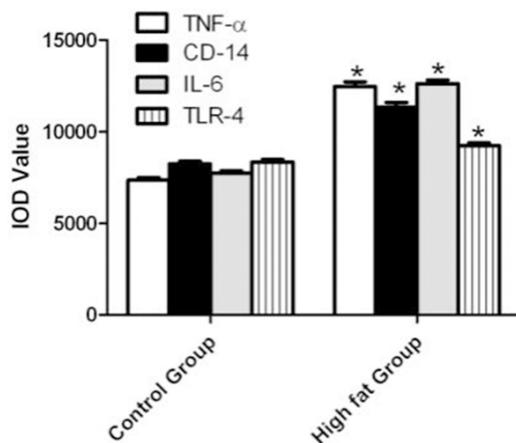


Figure 6. IOD values of TNF- α , CD-14, IL-6, and TLR-4 in the KCs cells in the liver of two groups of mice. *P<0.05.

significantly in the process of establishing the non-alcoholic fatty liver model. There is possibility that the increased CD11b⁺CD68⁺ cells in the high-fat-diet group may involve in the pathogenesis of inflammation, contributing to the progression of inflammation.

Results of the Western blot assay

Compared with the control group, the IOD values of TNF- α , CD-14, IL-6, and TLR-4 in KCs in

the mice liver of the high-fat-diet group were significantly elevated, and the difference was statistically significant (P<0.05), as show in **Table 2**; **Figures 6** and **7**.

Discussion

In this study, C57BL/6J mice were fed with high fat diet for 12 weeks, and the pathological sections of the non-alcoholic fatty liver demonstrated that the mice model of NAFLD was successfully established. The serum levels of AST, ALT, TC and TG in the high-fat-diet model group were higher than those in the control group. The occurrence and development of NFLD in mice were similar to the nonalcoholic fatty liver disease in patients

with obesity and/or hyperlipidemia induced by human liverlesion, providing a good animal model for the further study of the pathogenesis of human NAFLD [6, 7].

The results of this experiment showed that: Kupffer cells in the nonalcoholic fatty livermodel established based on the high fat diet of mice can be successfully isolated by the improved two-stepped collagenase perfusion method and Percoll density gradient centrifugation. In this study, the morphology of primary KCs was isolated under the light microscopy, and the cells were identified by means of the ED1 immunocytochemical staining. Morphological observation showed that: the Kupffer cells in the mice liver of the high-fat-diet group were uneven in volume, of thick somas, prone to deformation, extended cell pseudopodia and easy to make adherent growth. By contrast, the Kupffer cells in mice liver in the normal group were morphologically even, and of thin somas and round shape. These are major differences between the Kupffer cells in the normal mice and the ones fed by the high fat diet [8-10]. Some studies have indicated that there are certain relationships between the aberrant morphology of Kupffer cells and occurrence and development of fatty liver. The results of this study show that it is the high fat diet induces

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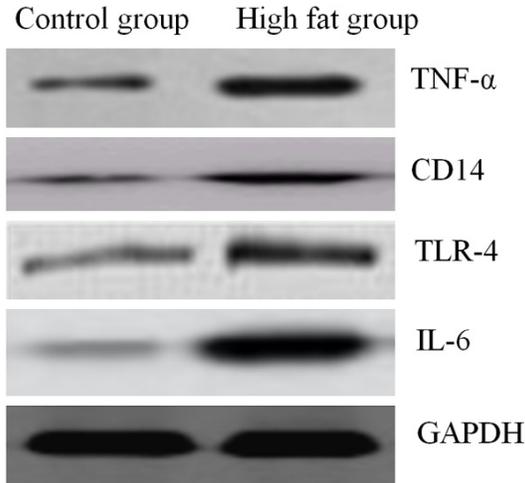


Figure 7. TNF- α , CD-14, IL-6, and TLR-4 in the KCs in liver of mice identified by the Western Blot assay.

the elevation in the levels of plasma triglyceride and cholesterol.

The development of KCs is derived from bone marrow, which plays a critical role in inflammation and defense reaction. Different functional phenotypes can be obtained in response to the environment in the tissues. It is reported that in the pathological state and in the normal state, the proportion functions of the CD11b⁺ and CD68⁺ in KCs were not exactly the same [13]. In this study, the KCs were extracted by the flow cytometry with the improved perfusion digestion method. In the non-alcoholic fatty liver model, the ratios of the two types of CD11b⁺ and CD68⁺ cells in the high-fat-diet group were considerably different from those in the control group, which is consistent with the reported literature [14]. The flow cytometry in the two groups demonstrated that in control group, CD11b⁺CD68⁺ (hepatic macrophages) accounted for a larger proportion of 91.2%, while in model of NAFLD, CD11b⁺CD68⁺ decreased to 51.8% but CD11b⁺CD68⁻ dramatically increased, so the majority of the CD11b⁺CD68⁻ cells are deemed to be infiltrated monocytes.

The further findings of this study showed that Kupffer cells (KCs) in the mice liver in the high-fat-diet group secreted pro-inflammatory cytokines, such as TNF- α , CD-14, IL-6 and TLR-4, the levels of which are elevated significantly. This maybe positively correlated to the elevation of CD11b⁺CD68⁻ cells in experimental mice with NAFLD. CD68⁺ cells may be in apoptosis

after repairing and phagocytosis, or during the development of the diseases, the increased secretion of pro-inflammatory cytokines may mediated infiltration of peripheral blood mononuclear cells (PBMC). There was no time for them to transform into macrophages, so they were only expressed as CD11b⁺CD68⁻ cells. In recent years, the functions of the KCs in the non-alcoholic fatty liver have been mainly identified by the spontaneously formed mechanism of leptin-deficient mice and the mice studies on leptin resistance [15]: KCs have no inductive synthase on their surface. Abnormally active KCs can increase inductive synthases expressed on the surface and catalyze a large quantity of leptin, which not only increases fatty deposition in hepatocytes but combines with oxygen free radicals to produce nitride cytotoxicity which mediates hepatocyte injury and circulatory failure [16-18]. Additionally, hepatic tumor necrosis factors secreted by KCs have the following functions: infiltration of granulocytes and T-lymphocyte cells, stimulating the expression of adhesion molecules on the surface of endothelial cells, inducing and deteriorating inflammation, thus causing hepatocyte necrosis directly or indirectly [19]. Besides, tumor necrosis factors secreted by KCs can stimulate serine phosphorylation of insulin substrate, promoting tyrosine kinase (TK) inhibitors on insulin receptor substrates (IRSs) and inducing insulin resistance (IR) [20]. It has been reported that liver damage can be induced as follows: LPS was combined with CD14, one of endotoxin receptors on KCs' plasma membrane. NF- κ B was activated via transduction of intracellular signaling, initiating transcription and expression of pro-inflammatory gene TNF- α to induce liver injury. Toll-like receptors expressed by KCs, such as TLR-4 stimulated by oxidized products, fat in the diet and the lysates from damaged hepatocytes and start to promote inflammation reaction.

In conclusion, the mice fed with high-fat diets existed dysfunction of hepatocyte. Phenotype changes of KCs may be correlated to the level of histological damages in the hepatic tissues of mice with NAFLD fed by high-fat diet, which indicates that abnormality in morphology and function of KCs may play a vital role in pathogenesis of NAFLD induced by human obesity and/or hyperlipidemia. However, the specific mechanisms are yet to be further studied. With

our increasing understanding of the biological function of KCs, KCs are expected to be preferable targeted cells for future studies on the treatment of non-alcoholic fatty liver.

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Disclosure of conflict of interest

None.

Address correspondence to: Haitao Jiang, Department of Articular Surgery, Taizhou People's Hospital, No. 399 Hailing South Road, Taizhou 225300, China. Tel: +86-0523-86361437; Fax: +86-0523-86361437; E-mail: TZ635582246@163.com

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