

## Effect of Exogenous Fibroblast Growth Factor-21 on Inflammation in a High-fat Diet and Cholesterol Mice Model

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### Abstract

Fibroblast Growth Factor 21 (FGF21) is a novel metabolic regulator involved in lipid utilization, and it can involve in the regulation of lipid metabolism. To determine the physiological function of FGF21 in relation to a high-fat diet (HFD) and high cholesterol, the effect of FGF21 on immune response indicators and hematological parameters was investigated. In the experiment, a total of 24 female mice were selected, 12 of which were fed with HFDs (group 1) and the other were fed with normal diet (group 2). After 30 days, the mice of two groups were respectively divided into two groups on average: 1. HFDs mice were used as model group; 2 normal diet mice were used as control group; 3. HFDs mice injection were used as model + FGF21 group; 4. normal diet injection were used as control + FGF21 group. Mice were sacrificed at 3-day intervals and the livers were isolated and analyzed. Blood was collected and analyzed for CD3 and CD19 by flow cytometry and IL-4, IL-6 and TNF- $\alpha$  by ELISA. Serum TG, TC, NEFA, LDL-c, and HDL-c levels were measured by automatic biochemical analyzer. Although CD3 and CD19 varied, the difference was not significant, and the levels of IL-4, IL-6, TNF- $\alpha$ , TC, TG LDL-c, and NEFA in the model exogenous injection FGF21 group were lower than in the model group ( $P < 0.05$ ). The present results indicate that exogenous FGF21 regulates IL-4, IL-6 and TNF- $\alpha$  and protects the liver from inflammation damage induced by high dietary fat and cholesterol.

**Keywords:** Exogenous FGF21, High-fat diets, Cholesterol, Immunological factor

## Yüksek Yağlı Diyet ve Kolesterolle Beslenen Farelerde Eksojen Fibroblast Büyüme Faktörü-21'in Yangı Üzerine Etkisi

### Özet

Fibroblast Büyüme Faktörü 21 (FGF21) yağların kullanımında görev alan metabolik bir düzenleyicidir ve yağ metabolizmasında rol oynar. Yüksek yağlı ve kolesterollü diyet ile FGF21'in fizyolojik fonksiyonu arasındaki ilişkiyi araştırmak amacıyla FGF21'in bağışıklık cevap indikatörleri ve kan parametreleri üzerine etkisi incelendi. Çalışmada toplam 24 dişi fare kullanıldı ve bunların 12'si yüksek yağlı diyet (Grup 1) diğer 12'si ise normal diyet (Grup 2) ile beslendi. Otuz gün sonunda iki gruptaki fareler tekrar ikiye gruba bölündü; 1: Yüksek yağlı diyet ile beslenen fareler "model grup" olarak kullanıldı; 2: Normal diyet fareler "kontrol grubu" olarak kullanıldı; 3: Yüksek yağlı diyet ile beslenen ve FGF21 enjekte edilen fareler "model + FGF21 grubu" olarak kullanıldı; 4: normal ile beslenen ve FGF21 enjekte edilen fareler "kontrol + FGF21 grubu" olarak kullanıldı. Fareler 3 gün aralıklarla öldürüldü ve karaciğer dokuları incelendi. Kan toplanarak CD3 ve CD19 akışkan sitometresi ve IL-4, IL-6 ve TNF- $\alpha$  ise ELISA ile analiz edildi. Serum TG, TC, NEFA, LDL-c ve HDL-c seviyeleri otomatik biyokimyasal analizleyici ile ölçüldü. CD3 ve CD19 değişimle birlikte değişim istatistiksel bakımdan anlamlı bulunmadı ve model eksojen enjeksiyon FGF21 grubunda IL-4, IL-6, TNF- $\alpha$ , TC, TG LDL-c ve NEFA seviyeleri model grubundan daha düşük olarak tespit edildi ( $P < 0.05$ ). Mevcut çalışmanın sonuçları eksojen FGF21'in IL-4, IL-6 ve TNF- $\alpha$  ekspresyonlarını düzenlediğini ve yüksek diyetel yağ ve kolesterol ile oluşturulan karaciğer yangısal hasarına karşı organı koruduğunu göstermektedir.

**Anahtar sözcükler:** Eksojen FGF21, Yüksek yağlı diyet, Kolesterol, İmmunolojik faktör

## INTRODUCTION

High-fat diets (HFDs) can increase the risk of many metabolic

diseases, such as insulin resistance, hypertension, hyperlipemia, and liver disease<sup>[1]</sup>. HFDs is also closely related to metabolic inflammation, especially in the liver<sup>[2,3]</sup>. Liver



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is an important organ that regulates the energy homeostasis of the body [4]. Liver steatosis is not only confined to liver damage, but also closely associated with disorders of glucolipid metabolism [5].

A HFDs causes resistance of skeletal muscle glucose transport to insulin and contractions. HFDs-fed mice show glucose intolerance and decreased insulin sensitivity, accompanying by impaired insulin signaling. A HFDs can affect immunity functions and energy metabolism in the body. HFDs cause chronic inflammation, potentially damaging the liver's innate immune system. The abnormalities of the hepatic innate immune system (macrophages, neutrophils, and natural killer T cells) lead to the increased production of inflammatory cytokines, which contribute to the chronic inflammatory state of liver injury [6]. Inflammatory stress is closely related to metabolic disease and insulin resistance, implying that chronic inflammation accelerates the deterioration of B cell function. There are a number of ways to regulate the energy disorders caused by HFDs, such as reducing FFA production, inhibiting TNF- $\alpha$  production and its activity, inhibiting or inhibiting oxidative stress, triglyceride (TG) synthesis, and hepatic stellate cell activation. However, fibroblast growth factor (FGF21) have a variety of adverse effects or contraindications and there is still no consensus regarding the most effective drug therapy for liver steatosis. Therefore, new agents such as active endogenous molecules with high efficacy and minimal side effects are eagerly needed for the treatment of liver steatosis. FGF21 is a hepatic protein that plays a critical role in metabolism, stimulating fatty acid oxidation and liver uptake of fat [7]. Systemic metabolize of obese rodents adjusted by FGF21 leads to improved glucose homeostasis and weight loss. The concentration of immunity factors reduced and resulted in blood glucose and lipid levels decreased in the fatty rats by administrating FGF21, it proved that FGF21 can improve the metabolic disorders [8,9]. FGF21 changes the degree of inflammation of the liver caused by high fat, indirectly affecting the immune level changes. FGF21 plays an important role in anti-inflammatory responses induced by HFDs in mice. However, the underlying anti-inflammatory mechanism remains to be elucidated. In this study, we injected recombinant FGF21 into mice to evaluate the effect of exogenous FGF21 on the immunocytokines in mice fed a HFDs to determine the effect on inflammation.

## MATERIAL and METHODS

### Ethics

The animal study was approved by the Institutional Animal Care and Use Committee (IACUC). All mice experimental procedures were performed in accordance with the regulations for the Administration of Affairs Concerning Experimental Animals approved by the school

Council of Heilongjiang Bayi Agricultural University of China daqing.

### Animals

Twenty-four female 4 weeks old KM mice (15-20 g) were purchased from Yongquan Animal Experiment Center, Heilongjiang, China. Twelve mice were fed a HFDs (feed formula: 10% load, 2% bilineurine, 5% albumen powder, 0.3% pig bile salt, and 82.7% basal feed), and the other twelve mice were fed a normal diet; mice were provided free access to the diet food and drinking water. After 30 days, the animals were divided into four groups: 1. HFDs mice below refer to 'model group' (n=6, intraperitoneal injection of 0.9% saline); 2. normal diet mice below refer to 'control group' (n=6, intraperitoneal injection of 0.9% saline); 3. HFDs mice exogenous injection FGF21 with 200 mg/kg/day FGF21 below refer to 'model + FGF21 group' (n=6, intraperitoneal injection); and 4. normal diet exogenous injection with 200 mg/kg/day FGF21 below refer to 'control + FGF21 group' (n=6, intraperitoneal injection). Mice were sacrificed at 3 and 6 days after injection. The experiment mice were lightly anesthetized and blood was collected from the retro-orbital plexus. The whole blood sample was then centrifuged at 2500 g for 10 min to yield the serum fraction and frozen at  $-20^{\circ}\text{C}$  until it was needed for subsequent biochemical analyses. Blood was collected at 3 and 6 days, and after blood collection, the mice were sacrificed, and the liver was removed and fixed in buffered formalin for 24 h and embedded in paraffin wax for histological analyses.

### Histological Analyses

The liver tissues were fixed in 10% neutral buffered formaldehyde, embedded in paraffin, and sectioned (4  $\mu\text{m}$ ). The sections were stained with hematoxylin and eosin and observed under a microscope (Nikon, Tokyo, Japan).

### Flow Cytometry

Whole blood was collected in anticoagulant tubes and the separation of lymphocytes from peripheral blood lymphocytes was performed (Solarbio, Beijing, China). Monoclonal antibodies against CD3 (SC-20047FITC, PC3/188A, Santa Cruz Biotechnology Inc) and CD19 (SC-8499PE, R20, Santa Cruz Biotechnology Inc) were used. The presence of immune T and B leukomonocytes in serum was analyzed by flow cytometry. For each analysis, 10,000 events were recorded [10].

### ELISA Assay

Serum IL-4 and IL-6 levels were measured using a commercial ELISA kit (BOSTER, Wuhan, China) according to the manufacturer's guidelines. The optical density was determined at 450 nm using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). The calibration curves

were constructed by plotting the net average absorbance of the standards on the Y-axis and the concentrations on the X-axis using the logit-log function to linearize and draw the best fitting curve.

### Biochemical Index Examination

Serum total cholesterol TC, TG, and NEFA were measured by personnel at the University Hospital of Jilin according to routine clinical chemistry methods (Siemens, Erlangen, Germany).

### Statistical Analysis

Data were pooled as mean values  $\pm$  standard errors. The ANOVA corrected using the Bonferroni test was used to determine differences between groups comparing the internal variability of groups with the variability among all experimental groups.  $P < 0.05$  was considered statistically significant. Data were randomly collected and the analyses were performed using IBM SPSS Statistics 22.0 software (SPSS, Chicago, IL, USA).

## RESULTS

To study the potential anti-obesity effect of exogenous recombinant FGF21, a HFDs model was successfully established. We first injected the FGF21 protein to HFDs

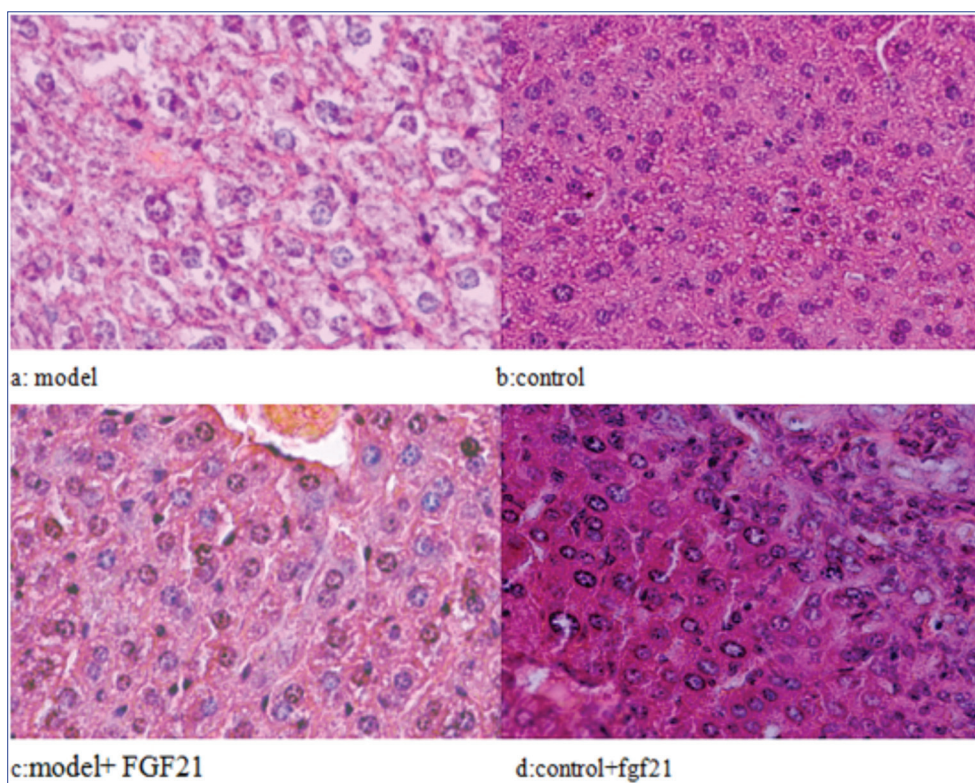
mice after 30 days days via intraperitoneal injection. The daily doses were 200 mg/kg of FGF21.

### Histological Analysis of Liver

Weight gain in HFDs feeding results from the accumulation of adipose tissue. Therefore we performed histological analyses of the liver. The livers of mice were collected after 6 days and analyzed. The number of vacuoles was lower in the model + FGF21 group than in the model group, and vacuole size decreased slightly (Fig. 1c). The normal liver structure of the control group showed normal-sized liver cells, and the liver cord, nuclear circle, and cytoplasm were normal, neatly arranged and located in the central cells (Fig. 1b). The degree of steatosis in the control + FGF21 group has no significant difference compared with the control group. These findings indicated that HFDs feeding resulted in significant hepatic steatosis and liver injury in mice. Based on the results of the NAFLD (nonalcoholic fatty liver disease) diagnostic gold standard, namely the histological analysis, these findings indicated that exogenous injection FGF21 had a significant hepatoprotective effect on HFDs-induced steatosis.

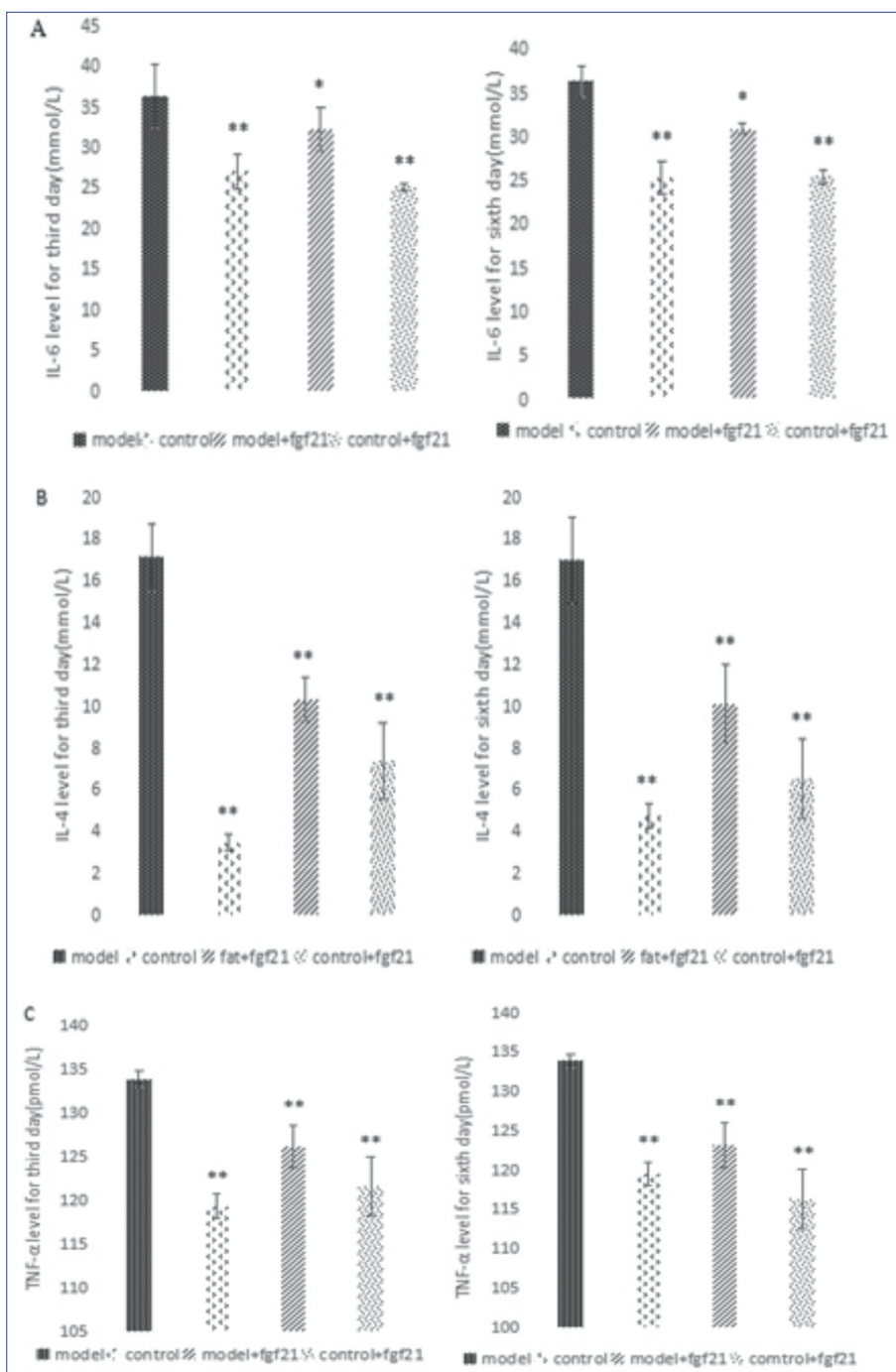
### Changes of Cytokines and Biochemical Indices

Next, we investigated how FGF21 suppresses hepatic inflammation. FGF21 affected slightly increased cytokine-



**Fig 1.** A HFDs caused liver pathological changes in exogenous injection FGF21 HFDs animals. Representative photomicrographs of liver sections stained with hematoxylin and eosin (200 $\times$ ). Notable differences in liver morphology are observed. After consuming a HFDs for 30 days, exogenous injection FGF21 HFDs mice (b) had reduce vacuolar changes compared with obese mice (a)





**Fig 2.** FGF21 reverses diet-induced obesity as indicated by immune factors and biochemical indicators. HDFs mice were subcutaneously injected with FGF21. The levels of IL-4 and IL-6 were measured at 3 and 6 days after exogenous injection FGF21. Data represent the mean ± SEM (three animals per group). All statistical comparisons were performed by one-way ANOVA followed by a multiple-comparison test. \*P<0.05, \*\*P<0.01 compared with the model group. A, The changes of serum IL-6 at 3 and 6 days. B, The changes of serum IL-4 at 3 and 6 days. C, The changes of serum TNF-α at 3 and 6 days

induced (i.e. TNF-α) inflammatory factor expression (Fig. 2C). The changes of IL-4, IL-6, and TNF-α in each group are shown in Fig. 2. IL-6, IL-4, and TNF-α levels were significantly higher in the model group than in the model + FGF21 group at 3 and 6 days (P<0.01) (Fig. 2A,B). There were no significant difference in IL-4, IL-6, TNF-α between the control group and the exogenous injection group.

TG levels were measured as the main NAFLD markers biochemical indicator. On days 3 and 6, TG was significantly lower in the model exogenous injection group, and on day 3, the model group and the control group were very significantly different (P<0.01). On days 3 and 6, LDL-c,

TC, and NEFA had significantly different between model group and model exogenous injection FGF21 group. HDL-c was followed exogenous injection change. The above results indicate that FGF21 can significantly improve Lipid metabolism in KM HDFs mice.

After 3 days of exogenous injection, blood samples were collected for assessment of serum TC, TG, NEFA, LDL-C, and HDL-C levels using an automatic biochemical analyzer (Table 1).

After 6 days of exogenous injection, blood samples were collected for assessment of serum TC, TG, NEFA, LDL-C and

**Table 1.** Effect of FGF21 on the changes in serum lipid parameters at the third day

Group	TG/mmol·L <sup>-1</sup> (mean±SEM)	TC/mmol·L <sup>-1</sup> (mean±SEM)	NEFA/mmol·L <sup>-1</sup> (mean±SEM)	LDL-c/mmol·L <sup>-1</sup> (mean±SEM)	HDL-c/mmol·L <sup>-1</sup> (mean±SEM)
Model (n=3)	0.720±0.072	2.00±0.057	0.440±0.023	0.407±0.012	0.593±0.024
Control (n=3)	0.413±0.073**	1.20±0.050**	0.260±0.031**	0.237±0.027**	0.447±0.053
Model + FGF21 group (n=3)	0.567±0.024	1.67±0.037*	0.340±0.012*	0.297±0.019*	0.807±0.066*
Control + FGF21 group (n=3)	0.537±0.038*	1.23±0.043**	0.233±0.037**	0.287±0.024*	0.693±0.103

\* P<0.05, \*\* P<0.01 vs. the model group

**Table 2.** Effect of FGF21 on the changes in serum lipid parameters at the sixth day

Group	TG/mmol·L <sup>-1</sup> (mean±SEM)	TC/mmol·L <sup>-1</sup> (mean±SEM)	NEFA/mmol·L <sup>-1</sup> (mean±SEM)	LDL-c/mmol·L <sup>-1</sup> (mean±SEM)	HDL-c/mmol·L <sup>-1</sup> (mean±SEM)
Model (n=3)	0.673±0.077	1.99±0.068	0.426±0.017	0.400±0.023	0.587±0.035
Control (n=3)	0.521±0.029**	1.06±0.094**	0.220±0.020**	0.247±0.029**	0.467±0.038
Model + FGF21 group (n=3)	0.454±0.024*	1.57±0.037**	0.353±0.017*	0.300±0.002**	0.793±0.096*
Control + FGF21 group (n=3)	0.423±0.052**	1.26±0.219**	0.243±0.015**	0.220±0.058**	0.774±0.070*

\* P<0.05, \*\* P<0.01 vs. the model group

HDL-C levels using an automatic biochemical analyzer (Table 2).

#### Different Exogenous Injection FGF21 Days Contast

Compared with the index of model + FGF21 and control + FGF21 on the third day, the index on the sixth day has no significant differences in IL-4, IL-6, TNF- $\alpha$ , TC, NEFA, LDL-c and HDL-c (Fig. 3). But the trend of model exogenous injection group to the normal development in IL-4, IL-6, TNF- $\alpha$ , TC, NEFA, LDL-c and HDL-c.

In the model exogenous injection groups, the third day was compared TG, TC, NEFA, LDL-c and HDL-c with the sixth day (Table 3).

In the control exogenous injection groups, the third day was compared was compared TG, TC, NEFA, LDL-c and HDL-c with the sixth day (Table 4).

#### Immune Cells

Immune cells from blood of 30-days HDFs mice for 6 days were analyzed by flow cytometry. T lymphocytes (CD3 cells) and B lymphocytes (CD19 cells) showed no marked differences between fat exogenous injection and HDFs mice, and no differences were observed between the control and exogenous injection groups (Fig. 4). In the HDFs mice, T (CD3) and B (CD19) lymphocyte numbers showed a similar profile in response to the nutritional challenge, i.e. HDFs does not cause a strong inflammatory response. However, CD3/CD19 values decreased significantly.

## DISCUSSION

HFDs can cause chronic inflammation and potentially damage the liver's innate immune system in long - term

edible. The liver is an important organ that plays a critical role in the innate immune system [11,12]. The inflammatory response is a complex reaction of the immune system. Activation of lymphocytes plays a pivotal role in the development and progression of inflammation. T and B lymphocytes play a major role in cellular and humoral immunity, in which CD3 cells represent the total number of T lymphocytes and CD19 is an important marker of B lymphocytes.

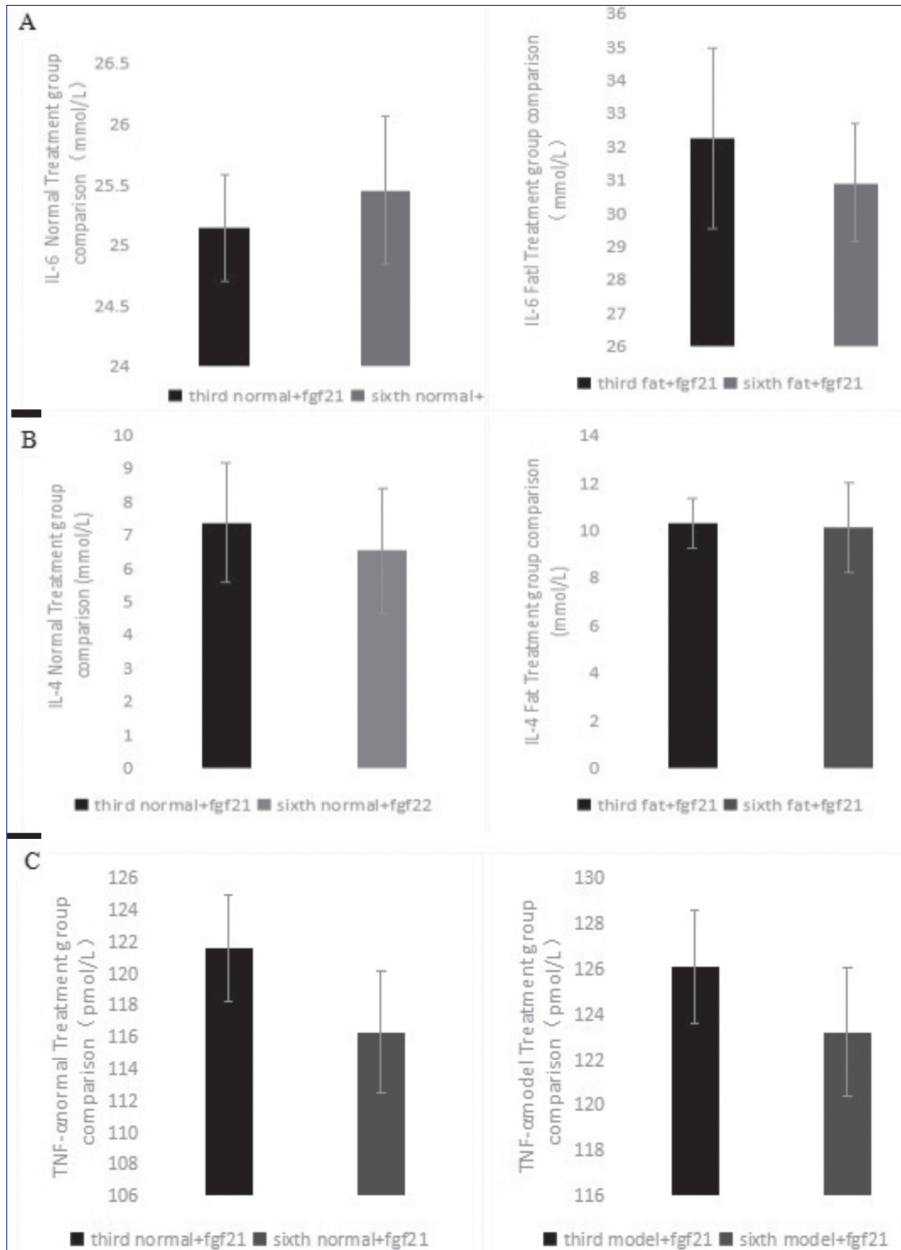
In our study, we found that FGF21 could improve lymphocytes to reduce inflammation. we investigated the effect of FGF21 on HFDs mice CD3 and CD19. The results showed that blood CD19 was increased in mice fed a HFDs. As the severity of NAFLD increased, CD19 tended to increase, indicating that humoral immune enhancement, which may be involved in the pathogenesis of NAFLD, may be caused by the production of excessive immunoglobulin. The increased percentage of CD3/CD19 cells may be the result of a decrease in the absolute number of other lymphocyte subsets.

In this study, we investigated the mechanism underlying the effect of FGF21 on inflammatory factors in mice fed a HFDs. The results showed that FGF21 effectively reduced TC and NEFA levels and alleviated hepatic damage. The lack of IL-6 expression was associated with exacerbated steatosis in HFD conditions. In our study, The level of serum IL-6 was higher in the model group than that in the control group. An important source of IL-6 in obesity is the expanding visceral adipose tissue mass. Regarding the hepatic lipid metabolism, there is evidence that IL-6 affects the opposing fatty acid pathways: degradation and Synthesis. The HFD-derived effects were aggravated in IL-6-deficient mice: higher cholesterol levels and decreased TG levels in serum. In the context of HFD-induced obesity, the

**Table 3.** Exogenous FGF21 between model groups in serum lipid parameters at the third day and sixth day

Group	TG/mmol·L <sup>-1</sup> (mean±SEM)	TC/mmol·L <sup>-1</sup> (mean±SEM)	NEFA/mmol·L <sup>-1</sup> (mean±SEM)	LDL-c/mmol·L <sup>-1</sup> (mean±SEM)	HDL-c/mmol·L <sup>-1</sup> (mean±SEM)
Model + FGF21 group at third day (n=3)	0.567±0.024	1.67±0.037	0.340±0.012	0.297±0.019	0.807±0.066
Model + FGF21 group at sixth day (n=3)	0.454±0.024	1.57±0.037	0.353±0.017	0.300±0.002	0.793±0.096

\*P<0.05, \*\*P<0.01 vs. the model+FGF21 group



**Fig 3.** In the control exogenous injection and model exogenous injection groups, the third day was compared IL-4, IL-6 and TNF-α with the sixth day. Data represent means±SEM (three animals per group). All statistical comparisons were performed by one-way ANOVA followed by a multiple-comparison test. A, Comparison of the effects of exogenous injection FGF21 on IL-6 at 3 and 6 days. B, Comparison of the effects of exogenous injection on IL-4 at 3 and 6 days. C, Comparison of the effects of exogenous injection FGF21 on TNF-α at 3 and 6 days

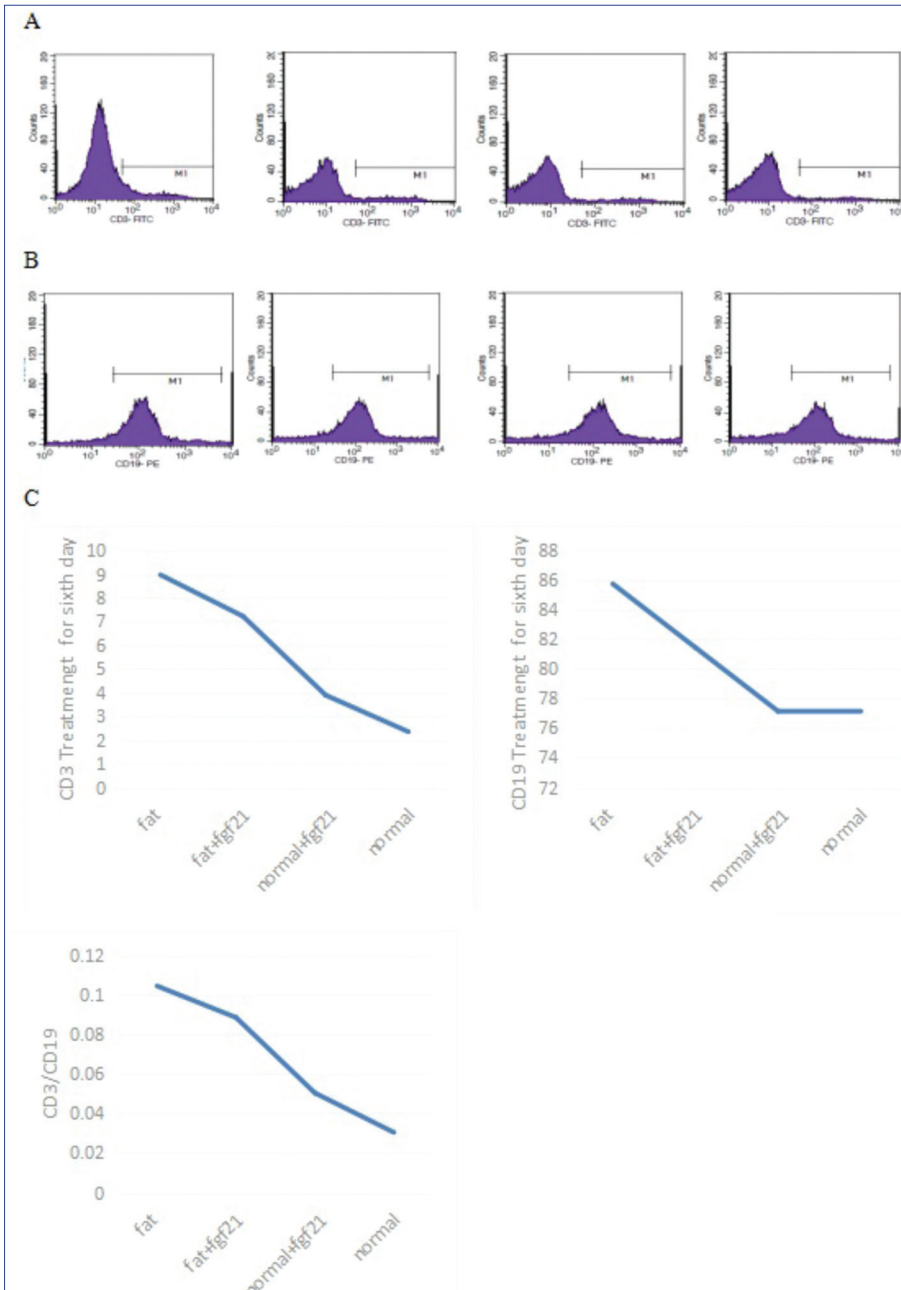
administration of rIL-6 might contribute to the aggravation of fatty liver disease through increasing lipogenesis [13]. IL-6 is considered as an anti-inflammatory cytokine through its inhibitory effects on TNF-α [14,15]. FGF21 can effectively change the production of IL-6, thereby reducing the production of TNF-α. In previous studies, the functions of IL-4 were primarily investigated in the immune system, where it exerts pleiotropic

effects ranging from Th2 differentiation of helper T cells, activation of B cells to release IgE, and stimulation of alternative macrophage activation [16,17]. IL-4 is usually higher in the early stages of inflammation. Because we established a HDFs model, IL-4 values may be high in the model group, however, the exogenous injection in the same period and the relative reduction in IL-4 may indicate a therapeutic effect. Elevated IL-4 levels in

**Table 4.** Exogenous FGF21 between control groups in serum lipid parameters at the third day and sixth day

Group	TG/mmol-L <sup>-1</sup> (mean±SEM)	TC/mmol-L <sup>-1</sup> (mean±SEM)	NEFA/mmol-L <sup>-1</sup> (mean±SEM)	LDL-c/mmol-L <sup>-1</sup> (mean±SEM)	HDL-c/mmol-L <sup>-1</sup> (mean±SEM)
Control + FGF21 group at third day (n=3)	0.537±0.038	1.23±0.043	0.233±0.037	0.287±0.024	0.693±0.103
Control + FGF21 group at sixth day (n=3)	0.423±0.052	1.26±0.219	0.243±0.015	0.220±0.058	0.774±0.070

\* P<0.05, \*\* P<0.01 vs. the model + FGF21 group

**Fig 4.** CD3 and CD19 were detected by flow cytometry. A, CD3 cell numbers by flow cytometry. B, CD19 cell numbers by flow cytometry. C, CD3, CD19 and CD3/CD19 linear analysis

the control + FGF21 group compared with that in the control group may be due to exogenous injection.

Our result indicated that the model + FGF21 group improved after exogenous injection on days 3 and 6. However, there were no significant differences between days 3 and 6 in the exogenous injection group, which may

be due to the fact that the injection cycle was too short. The effects of FGF21 on decreasing body fat in model mice may have been mediated by FGF21-induced suppression of hepatic carbohydrate oxidation may occur via PDK4-mediated suppression of PDC activity [18]. A HFDs induced hepatic steatosis in mice, leading to increased TG and NEFA. This resulted in NAFLD leading to inflammatory

changes in the liver. IL-4, IL-6, and TNF- $\alpha$  were increased by different degrees. FGF21 effectively relieved hepatic fat deformation, inhibited the synthesis of NEFA and TG and reduced the inflammation caused by changes in the inflammatory factors IL-4, IL-6, and TNF- $\alpha$ , and thus alleviated inflammation. Induced by HFDs.

### COMPETING INTERESTS

The authors declare that they have no competing interests.

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