

• BASIC RESEARCH •

Effects of emodin on treating murine nonalcoholic fatty liver induced by high caloric laboratory chaw

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Abstract

AIM: To investigate the effects of emodin on the treatment of non-alcoholic fatty liver in rats induced by high caloric laboratory chaw.

METHODS: Non-alcoholic fatty liver model was successfully established by feeding with high caloric laboratory chaw for 12 wk. Then the model rats were randomly divided into 3 groups, namely model control group, emodin group and dietary treatment group. The rats in emodin group were given emodin at dose of 40 mg/(kg·d) while animals in other groups were given distilled water of the same volume. The rats in model control group were fed with high caloric laboratory chaw while animals in other groups were fed with normal diet. Four weeks later, liver index (liver/body weight ratio), serum activities of liver-associated enzymes, blood lipid, fasting blood glucose, fasting plasma insulin, HOMA insulin resistance index (HOMA-IR), hepatic triglyceride content and histology features of all groups were assayed. The expression of hepatic peroxisomal proliferator activated receptor (PPAR) gamma was determined by RT-PCR.

RESULTS: The body weight, liver index, serum activities of alanine aminotransferase (ALT), blood lipid, hepatic triglyceride content of model control group were significantly elevated, with moderate to severe hepatocyte steatosis. The expression of hepatic PPAR gamma mRNA was obviously reduced in model control group. Compared with model control group, the body weight, liver index, serum activities of ALT, blood lipids and hepatic triglyceride of emodin group significantly decreased and hepatic histology display was also greatly improved. Meanwhile, the expression of hepatic PPAR gamma mRNA was elevated. However, high serum activities of ALT and hyperlipidemia

were persisted in dietary treatment group although liver index was decreased and liver histology was somewhat improved.

CONCLUSION: It is suggested that emodin might be effective in the treatment of non-alcoholic fatty liver in rats. Its therapeutic mechanism could be associated with increasing the expression of hepatic PPAR gamma mRNA.

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Key words: Emodin; Nonalcoholic fatty liver disease

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INTRODUCTION

Fatty liver is increasingly recognized as a major health problem in China. It is believed that sustained liver injury may partially lead to progressive liver fibrosis and cirrhosis, possibly up to one-third of those with non-alcoholic steatohepatitis (NASH). NASH may be a primary cause of cryptogenic cirrhosis^[1-4]. Controlling NASH would be expected to reduce the risk of developing degenerative liver diseases such as liver fibrosis or cirrhosis. Currently less medical therapy is available for patients with non-alcoholic fatty liver disease (NAFLD). Our previous research indicated that emodin might be effective to prevent and treat murine alcoholic fatty liver^[5]. Because the pathophysiological and pathological features of non-alcoholic fatty liver resemble those of alcohol-induced liver injury in certain extent^[6,7], we established the murine model with NASH to explore the possible therapeutic effects of emodin on nonalcoholic fatty liver.

MATERIALS AND METHODS

Animals

Forty male Sprague-Dawley rats weighing 140-160 g obtained from Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China) were used in the present study. The rats were housed in plastic cages and exposed to a 12/12 h controlled light cycle. The rats were given free access to food and water.

Reagents

Cholesterol was purchased from Liwei Biochemical Company (Zhengzhou, China). Lard oil was prepared in our laboratory. Sodium Tauroglycocholate was from Luye Company (Huainan, China). Ninety-nine percent emodin from Juzhan Biochemical Company (Shenzhen, China) was diluted by 0.5% Sodium Carboxymethyl Cellulose (CMC) and the end concentration were 2, 4 and 8 mg/mL, respectively. Alanine aminotransferase (ALT), aspartic aminotransferase (AST), serum total cholesterol (TC) and triglyceride (TG) assay kits were purchased from Dong-Ou Biological Engineering Co. Ltd (Zhejiang, China). Serum levels of insulin were determined by radioimmunoassay (kit purchased from Northern Biot Co., China). The reagents for reverse transcription and polymerase chain reaction (RT-PCR) were obtained from Promega Co. USA. DEPC, TRIzol reagents, primers for amplifying PPAR- γ and β -actin mRNA were synthesized by Shanghai Biology Engineering Co. Ltd. PPAR γ : (+)5'AGG AGC AGA GCA AAG AGG TG-3', (-) 5'ACC GTT TAT TTC CCA TCT3'. β -actin: (+) 5'CCT AGG CCA ACC GTG AAA AG3', (-) 5'TCT TCA TGG TGC TAG GAG CCA3'.

Animal model and experimental protocol

After fed with standard rat diet for one week, Sprague-Dawley rats were randomly divided into model group ($n = 30$) and normal control group ($n = 10$). Non-alcoholic fatty liver model was established by feeding with high caloric laboratory chaw (2% cholesterol, 10% lard oil, 0.5% sodium tauroglycocholate and 87.5% standard forage) for 12 wk. Then 6 rats from model group and 2 rats from normal control group were killed. All the murine liver tissue slides stained with HE in model group showed moderate to severe macrovesicular steatosis by optical microscopy. Then 24 rats fed with high caloric laboratory chaw were further randomly divided into 3 groups. The rats in model control group were fed with high caloric laboratory chaw continuously. The rats in dietary treatment group were fed with normal forage. The rats in emodin group were orally administered with emodin 40 mg/(kg·d) and they were also fed with normal forage. The animals in both model control group and normal control group were given distilled water in same volume by lavage. Oral administration of drugs were performed between 8:30 and 9:30 am everyday. All the animals were killed at the end of the 4th wk. The animals were weighed before experiment and one-day prior to sacrifice. Blood samples were obtained by aorta abdominalis puncture at the time of sacrifice and the specimen serum was kept storing at -20 °C until being put forward to analysis. Meanwhile, murine liver samples were rapidly excised, weighed and frozen in fluid nitrogen or fixed in 4% buffered formaldehyde solution until using.

Blood biochemical analysis

Serum biochemical parameters such as ALT, AST, TC, TG, fasting blood glucose (FBG) were automatically analyzed with biochemistry analyzer. Serum levels of insulin (FINS) were determined by radioimmunoassays in the Department of Nuclear Medicine in Tongji Hospital. HOMA-insulin resistance (IR) index was calculated by the method as follows: (HOMA-IR) = FBG×FINS/22.5^[8]

Hepatic triglycerides and histopathological examination

A section of liver was taken for quantitative assessment of triglycerides content by using solvent extraction method. Hepatic tissue slides were prepared and stained with hematoxylin and eosin (HE) for routine histopathological examination.

Total RNA isolation

Total RNA was extracted from 100 mg liver tissue using TRIzol reagent. To check the purity of RNA, it took 2 μ L RNA sample dissolved in the solution (1:50) to measure A_{260} and A_{280} by UV spectrophotometer. The ratio of A_{260} to A_{280} was determined as 1.8-2.0, which may reflect the high quality of extracted RNA. Further 3 μ L RNA sample was put into 1.1% denaturing agarose gel with ethidium bromide for electrophoresis.

RT-PCR Four microgram of total RNA was conducted into a reverse transcription reaction to synthesize cDNA. The following reagents were added to a 100 μ L polypropylene microcentrifuge tube sequentially: (1) 10×Taq buffer 2.5 μ L; (2) 10×dNTP 0.4 μ L; (3) 2.0 U/ μ L TaqE 1 μ L; (4) 2 pairs primer mixture (β -actin primer was used for internal control) 1 μ L; (5) sample 2 μ L; and (6) water up to 25 μ L. PCR for amplifying PPAR- γ mRNA was carried out in a programmable thermal controller. During each PPAR- γ PCR cycle, the samples were firstly heated to denature template complexes at 94 °C (180 s initially and 60 s during all subsequent cycles), then cooled down to 60 °C to anneal template and primers (60 s) and, in the end, heated up to 72 °C to extend the newly synthesized strand (50 s). The final 72 °C incubation after 34 cycles was extended for additional 10 min to maximize strand completion. During each β -actin PCR cycle, the samples were heated to denature template complexes at 94 °C (240 s initially and 60 s during all subsequent cycles), then cooled down to 54 °C to anneal template and primers (60 s) and then heated up to 72 °C to prolong the new strand (60 s). The final 72 °C incubation after 30 cycles was extended for additional 10 min to completely prolong PCR product. After PCR processing, the samples were rapidly cooled down to 4 °C and stored at -20 °C for later analysis. After amplification, 6 μ L of each PCR reaction mixture was electrophoresed through 2.0% agarose gel with ethidium bromide (0.1%). The gel was photographed over UV light at the same exposure and development time for all gels photographed. The bands on the film were scanned by densitometry for quantitation. The PCR products were electrophoresed in the same gel and ratios for PPAR- γ / β -actin were determined to eliminate film-to-film variance.

Statistical analysis

Data were expressed as mean \pm SD. The Student's *t*-test was used to test individual differences. $P < 0.05$ was considered to be statistically significant.

RESULTS

Body and liver weight changes

During the experimental period, the body weight of the rats fed on high-fat chaw was increased quickly. At the end

of the experiment, the body weight and liver index of rats in model control group were significantly increased compared with those in normal group ($P<0.05$ and $P<0.01$, respectively). Compared with model control group, the body weight and liver index in emodin group were significantly decreased ($P<0.05$ and $P<0.01$, respectively). The liver index in dietary group was significantly decreased ($P<0.01$) but there was no significant difference between the body weights in model control group and dietary group (Figure 1).

Hepatic enzyme activity, blood lipids levels and hepatic TG contents

At the end of 16 wk, serum ALT levels were significantly elevated in model control group compared with those in normal group ($P<0.05$). Serum AST level only displayed an increasing trend ($P>0.05$). In addition to the high hepatic triglyceride content, blood lipids including TC and TG levels in model control group remarkably increased compared to those in normal group ($P<0.05$ and $P<0.01$, respectively). In comparison to model control group, serum ALT, AST, TC, TG levels and hepatic TG contents in emodin group were significantly reduced ($P<0.05$ and $P<0.01$, respectively). However, there were no significant difference of parameters between dietary group and model control group (Figure 2).

Changes of FBG, FINS and HOMA-IR

There was no significant difference of FBG, FINS levels and HOMA-IR among four groups (Table 1).

Table 1 Changes of FBG, FINS and HOMA-IR among relevant groups (mean \pm SD)

Group	<i>n</i>	FBG (mmol/L)	FINS(m IU/L)	HOMA-IR
Normal	8	5.67 \pm 0.75	27.26 \pm 3.99	6.86 \pm 2.12
Model	8	6.01 \pm 1.61	29.82 \pm 6.47	8.02 \pm 2.67
Emodin	8	4.84 \pm 0.50	27.56 \pm 8.33	6.26 \pm 2.23
Dietary	8	5.51 \pm 1.07	29.38 \pm 9.47	7.15 \pm 2.38

Expressions of PPAR-gamma mRNA in liver tissue

The expression of PPAR-gamma mRNA in liver tissues of model control group were remarkably reduced compared with those of normal group (0.57 ± 0.05 *vs* 0.80 ± 0.05 , $P<0.01$). In comparison with model control group, the expression of PPAR-gamma mRNA in liver tissues of emodin group were elevated significantly (0.80 ± 0.06 *vs* 0.57 ± 0.05 , $P<0.01$). However, there was no significant difference of the hepatic expression of PPAR-gamma mRNA between dietary group and model control group (0.64 ± 0.03 *vs* 0.57 ± 0.05 , $P>0.05$) (Figure 3).

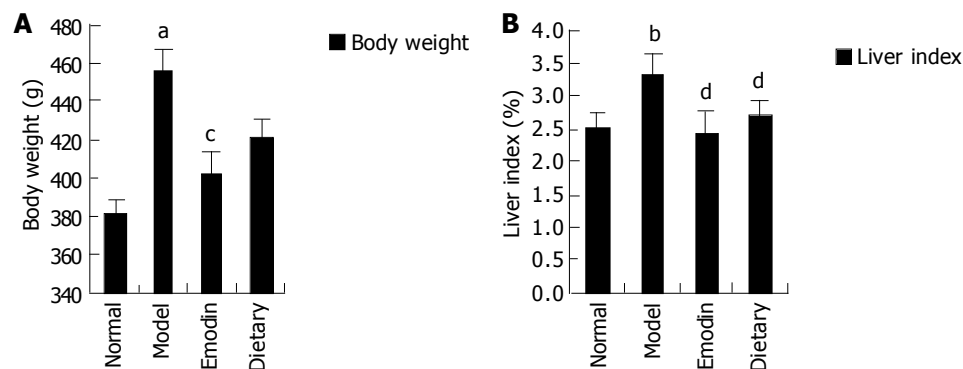


Figure 1 Body weight and liver index changes among four groups. A: Comparison of murine body weight among relevant groups. ^a $P<0.05$ vs normal group, ^c $P<0.05$ vs model control group; B: Comparison of liver index among relevant groups. ^b $P<0.01$ vs normal group, ^d $P<0.01$ vs model control group.

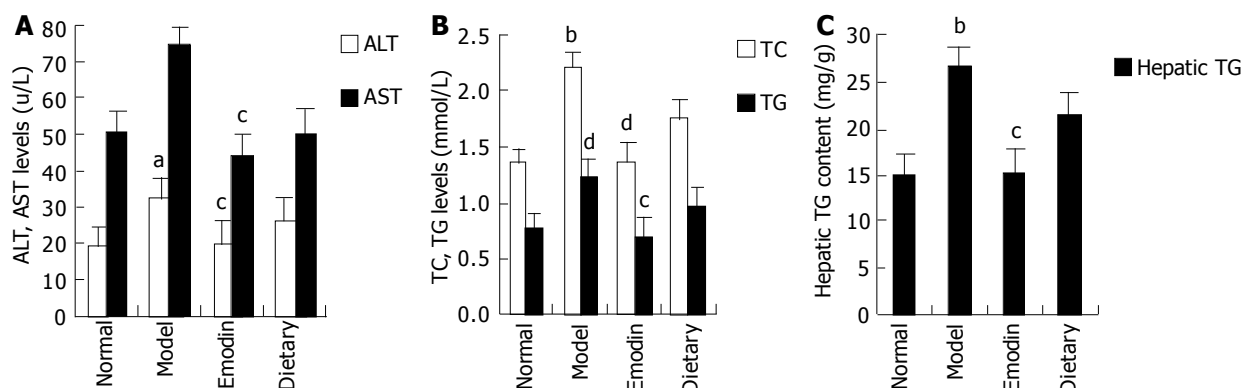


Figure 2 Hepatic enzyme activity, blood lipids levels and hepatic TG contents among four groups. A: Comparison of serum ALT and AST activities among relevant groups. ^a $P<0.05$ vs normal group, ^c $P<0.05$ vs model control group; B: Comparison of serum TC and TG levels among relevant groups. ^a $P<0.05$ vs normal group, ^b $P<0.01$ vs normal group, ^c $P<0.05$ vs model control group, ^d $P<0.01$ vs model control group; C: Comparison of hepatic TG contents among relevant groups. ^b $P<0.01$ vs normal group, ^c $P<0.05$ vs model control group.

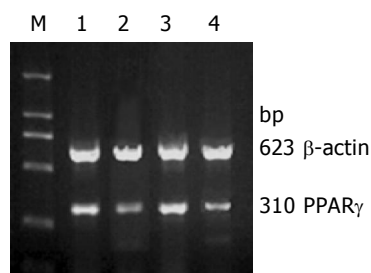


Figure 3 The expression of PPAR-gamma mRNA in murine liver tissues. M: marker. Lane 1: normal group; lane 2: model control group; lane 3: emodin group; lane 4: dietary group.

Hepatopathological manifestations

No specific findings were observed during the hepatohistological examination in the normal rats. Under light microscopy, liver tissue slides stained with HE in model control group showed moderate to severe macro vesicular steatosis, which was diffusely distributed throughout liver lobule, and parenchymal inflammation with both acute and chronic inflammatory cells accompanying focal necrosis. Compared with model control group, the pathological degree of liver steatosis in emodin group was significantly reduced and in dietary group the steatosis was also improved (Figure 4).

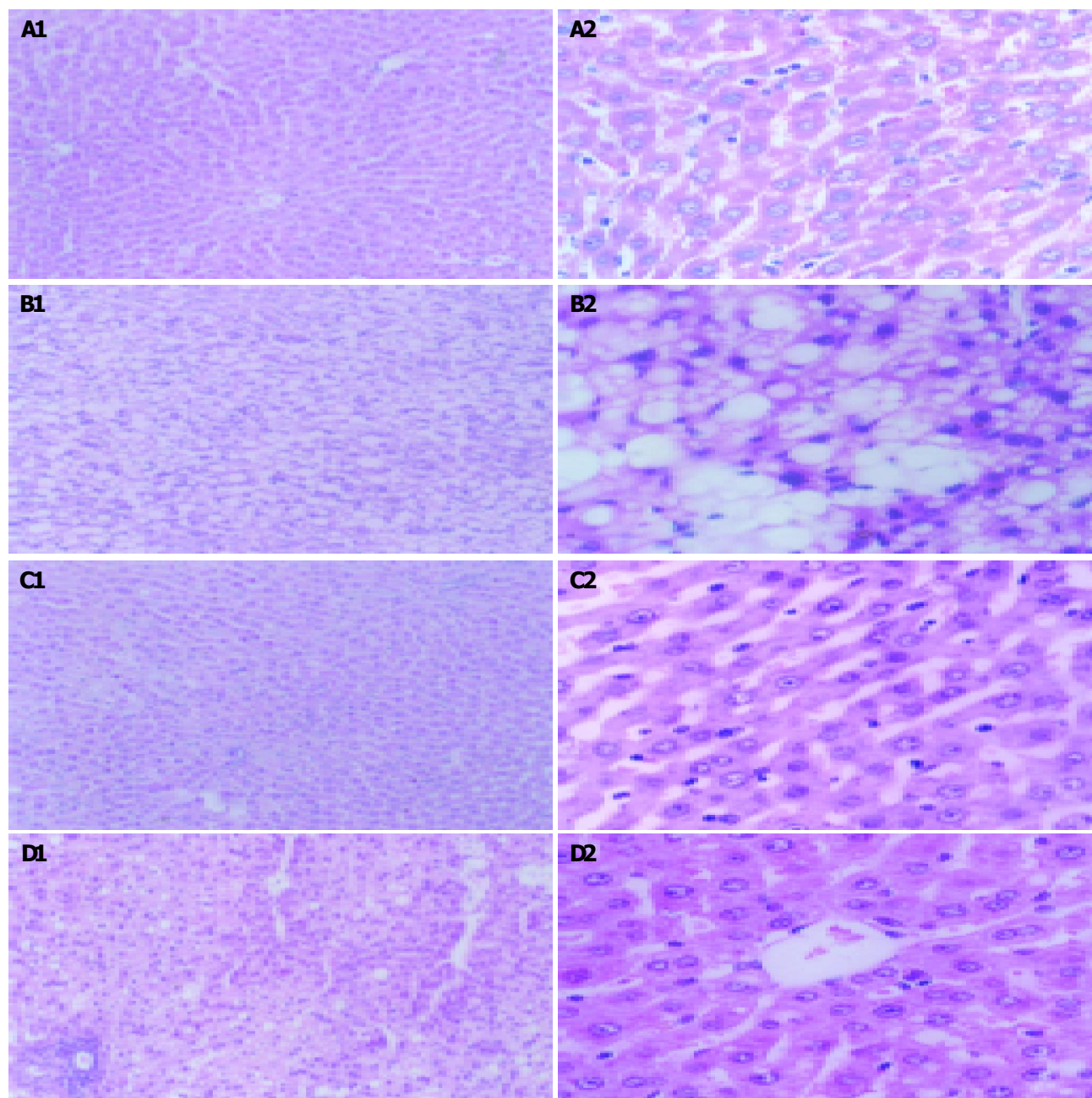


Figure 4 Hepatopathological manifestations among four groups. A: Murine normal liver tissue stained with HE. A1: HE×100, A2: HE×400; B: Demonstration of fatty liver tissues in model control group. Moderate to severe macro vesicular steatosis, diffusely distributed throughout the liver lobule, and parenchymal inflammation with both acute and chronic inflammatory cells accompanying focal necrosis. B1: HE×100, B2: HE×400; C: Significant improvement of liver steatosis in emodin group. There was significant reduction of fatty deposits in liver tissues and the histologic figures restored to nearly normal. C1: HE×100, C2: HE×400; D: Liver steatosis in dietary group. The degree of liver steatosis was improved, but mild steatosis still existed. D1: HE×100, D2: HE×400.

DISCUSSION

Non-alcoholic fatty liver disease is an increasingly recognized condition that may be aggravated to end-stage liver disease. The pathological picture of NAFLD may resemble that of alcohol-induced liver injury, but it occurs in patients who do not abuse alcohol. NAFLD refers to a wide spectrum of liver damage, ranging from simple steatosis to steatohepatitis, advanced fibrosis and cirrhosis^[9]. It is believed that non-alcoholic steatohepatitis represents only a stage within the spectrum of NAFLD. The clinical implications of non-alcoholic fatty liver disease are mostly derived from its high prevalence in the population and its predisposition to aggravate to cirrhosis and liver failure.

Trying to establish an ideal animal model for NASH research is still on the way. There are several animal NASH models, which have provided useful information with respect to the complex behavioral, metabolic and genetic factors that lead to NASH. One of such models is ob/ob mouse, which is deficient in leptin^[10]. Another important animal model of steatohepatitis is the depletion of S-adenosylmethionine with a diet deficient in the methyl donors methionine and choline^[11]. Feeding animals with a fat-rich diet is a simple, reliable and effective method. SD rats fed with a fat-rich diet consisting of 100 g/kg lard oil and 20 g/kg cholesterol for 12 wk showed features of NASH. For 24 wk, all model rats developed perisinusoidal fibrosis^[12]. Therefore we established a model of NASH in rats by continuous feeding on a diet rich in fat and cholesterol for 16 wk. At the end of the experiment, the body weight and liver index of rats in model control group were significantly increased compared with those in normal group. These overweight animals showed remarkably elevated levels of serum TC, TG, ALT and hepatic TG content. Hepatomegaly was obvious and the liver presented yellow color. The moderate to severe steatosis combined with intralobular inflammation and spotty necrosis was characteristically found in their hepatopathological examinations. All the results above demonstrated a successful establishment of murine model for NASH.

Exercise and diet continue to be the cornerstones of therapy against NASH^[13]. In patients with diabetes mellitus or hyperlipidemia, good metabolic control is strongly recommended, but it is not always effective in reversing non-alcoholic fatty liver disease. Improvement in liver-test results is almost universal in obese adults and children after weight reduction. The degree of fatty infiltration usually decreases with weight losses in most fatty patients, although the degree of necroinflammation and fibrosis may worsen^[14-17]. It is reported that the rats fed with a high fat diet for 10 wk followed by 2 wk on low-calorie diet made their overweight and hyperlipidemia alleviated with a trend of increasing serum FFA^[18]. In this study, although the rats with NASH in dietary group shifted to be fed on normal diet, the amount of food intake was not controlled. It was demonstrated that liver index of dietary group decreased significantly as compared with model group. The high serum activities of liver-associated enzymes, hyperlipidemia and high hepatic TG contents still persisted in dietary group, although HE stained liver tissue slides showed only partial improvement of steatosis. Nevertheless, correct components of diet to

get the optimal rate and degree of losing weight still have to be established.

No medications have been proved to directly reduce or reverse liver damage of NASH independent of weight loss. The following NASH targeting medications including gemfibrozil^[19], vitamin E^[20,21], metformin^[22], ursodiol^[23], betaine^[24] and the thiazolidinedione^[25,26] would be effective, but they need to be further evaluated in carefully controlled clinical trials. Emodin (1,3,8-trihydroxy-6-methylantraquinone) is an active herbal component traditionally used in China for treating a variety of diseases. Emodin exerts anti-proliferative, hepatocytoprotective effects in many reports^[27-29]. Our previous research suggested emodin might be effective to prevent and treat murine alcoholic fatty liver. In the present study, it was shown that the weight, liver index, serum activities of liver-associated enzymes, blood lipid and hepatic triglycerides in emodin group decreased significantly and hepatic histology restored to nearly normal. To explain differences between pure fatty liver and NASH, Day and James^[30] proposed a 'two-hit hypothesis' with lipids acting as the first 'hit' and increased lipid peroxidation being the second 'hit'. Then, what is the mechanism of emodin on preventing the development of NASH? It was related to decreasing fat deposits, lipid peroxidation or both?

Peroxisome Proliferator Activated Receptors (PPARs) are initially described as molecular targets for compounds inducing peroxisome proliferation. Among the three PPAR subtypes (alpha, beta, gamma), PPAR-gamma acting as a ligand-activated transcription factor, proved to be an important regulator of adipogenic differentiation and glucose homeostasis^[31]. Recent research indicated that agonists such as rosiglitazone^[26] and pioglitazone^[32] could improve the non-alcoholic steatohepatitis. In our research the expression of PPAR-gamma mRNA in liver tissues of model control group were remarkably reduced compared with those of normal group. In comparison with model group, the expression of PPAR-gamma mRNA in liver tissues of emodin group were elevated significantly. However, the actual molecular mechanisms of emodin in treating murine nonalcoholic fatty liver were not yet illustrated, possibly it might be associated with improving the expression of PPAR-gamma mRNA in liver tissue and increasing the insulin sensitivity similar to Thiazolidinediones.

It is believed that insulin resistance is a common pathophysiological basis in patients with NAFLD and NASH^[33,34]. In view of the hypothesis that insulin resistance and hyperinsulinemia are primary abnormalities in NAFLD, our study was focused on the relation of emodin and insulin resistance in NASH. But in this study, with murine steatohepatitis, there was no significant difference of FBG, FINS and HOMA-IR among four groups. The underlying reason should be investigated in the subsequent study.

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