Original Article
N,N'-diacetylcystine ameliorates inflammation in experimental non-alcoholic steatohepatitis by regulating nuclear transcription factor kappa B activation

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Abstract: Non-alcoholic fatty liver disease (NAFLD) is one of the most common diseases worldwide that has been continuously increasing recently. NAFLD embraces a spectrum of liver histological alterations, ranging from simple steatosis (NAFL) to severe non-alcoholic steatohepatitis (NASH), that is characterized by fat accumulation, lobular inflammation, and ballooning degeneration in the hepatocytes in the absence of alcohol abuse. The innate immune system has an important role in NASH pathogenesis. Among the components of innate immunity, the nuclear factor kappa B (NF-κB) has been closely associated with NASH. N,N'-diacetylcystine (DiNAC), the disulfide dimer of N-acetylcysteine (NAC), is a potent modulator of the immune system. Previous research has confirmed that DiNAC has beneficial effects in liver injury. In this study, we aimed to investigate the effects of DiNAC on high fat diet (HFD)-induced NASH in rats. Male Sprague-Dawley rats were fed with HFD to produce the NASH model and treated with or without DiNAC for 8 weeks. We assessed serum levels of alanine-aminotransferase (ALT), aspartate aminotransferase (AST), inflammatory cytokines, liver histology, and the expression of NF-κB genes in the liver. The results showed that the levels of ALT and AST were significantly increased in the HFD rat model. DiNAC treatment also resulted in a statistically significant reduction of the levels of ALT and AST. Hematoxylin and eosin (H&E) staining revealed that DiNAC alleviated histological injury. Moreover, DiNAC strongly reduced the generation of inflammatory cytokines, such as interleukin-6 (IL-6), tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β), through NF-κB downregulation. Taken together, these results indicate that DiNAC treatment effectively delayed the progression of NASH by suppressing the expression of NF-κB mRNA in the liver. Our data suggest that DiNAC protects liver injury in HFD-treated NASH rats, which might be a promising drug for the treatment of NASH.

Keywords: N,N'-diacetylcystine, non-alcoholic steatohepatitis, nuclear transcription factor kappa B

Introduction

Nonalcoholic fatty liver disease (NAFLD) is considered a burgeoning health problem and is currently one of the leading causes of chronic liver diseases worldwide, affecting an estimated 25-30% of the general population [1, 2]. NAFLD is strongly associated with obesity and the metabolic syndrome and predisposes patients to hepatic and extrahepatic disorders such as cirrhosis, type 2 diabetes, and cardiovascular diseases [3, 4]. NAFLD comprises a broad spectrum of liver histological alterations, ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), which can cause some serious complications, such as progressive fibrosis, cirrhosis and hepatocellular carcinoma [5, 6]. The pathogenesis of NASH is not yet fully understood, but insulin resistance and inflammatory cytokine production are key mechanisms that lead to steatohepatitis. The pathological states of NASH are characterized by increased inflammation, apoptosis related factors, and increased levels of alanine transaminase (ALT) and aspartate transaminase (AST) concentration in the blood [7].

During NASH progression, the inflammatory reaction is also common and obvious. Inflammatory cytokines cause a recruitment of

macrophages into adipose tissue, which will in turn enhance adipose tissue inflammation. Chronic inflammation has been proposed as an important link between insulin resistance and obesity. Inflammation is also seen as a critical factor in the progression of liver fibrosis and hepatocellular carcinoma (HCC) [8-10], so targeting the reduction of inflammation is a beneficial strategy to combat NASH.

The innate immune system plays an important role in NASH pathogenesis. Among the components of innate immunity, the nuclear factor kappa B (NF-κB) has been closely associated with NASH. NF-κB is a protein complex involved in the up-regulation of many proinflammatory genes and inducible cell adhesion molecules [11, 12]. NF-κB is found in almost all animal cell types and is a pivotal regulator of early response genes, including cytokines, free radicals, inflammatory mediators, and several structural proteins that are involved in infection, inflammation, stress responses, and apoptosis [13]. Inactive NF-κB dimers normally exist in the cytoplasm in an inactive form associated with inhibitor proteins belonging to the inhibitor of NF-κB (IκB) family of related proteins. This interaction blocks the ability of NF-κB to translocate to the nucleus [14]. The activation of NF-κB involves its release from IκB and subsequent nuclear translocation to induce the expression of target genes. NF-κB is a critical player in NASH progression due to its central role in inflammation control by regulating the infiltration of inflammatory cells and the expression of pro-inflammatory cytokines in both Kupffer cells and hepatocytes [15]. NF-κB controls interleukin-1β (IL-1β), and tumor necrosis factor α (TNF-α) productions, which are the most important cytokines involved in NASH progression, and targeting this pathway may have potential benefits for NASH [16, 17].

N-acetylcysteine (NAC) is a thiol containing antioxidant, which has been in clinical use as a mucolytic agent in the treatment of various pulmonary disorders. The well-known role of NAC has acted as the antidote to acetaminophen toxicity for over 30 years [18, 19]. *N,N'*-diacetylcystine (DiNAC), the disulfide dimer of NAC, appears to have beneficial effects in liver injury. It has been shown to be capable of inhibitory activity of the UV-induced NF-κB binding at a much lower concentration [20]. In the present study, we postulated that DiNAC may regulate

NF-κB in experimental NASH induced by a high fat diet (HFD). Our data demonstrated that a high fat diet induced lipid deposition and inflammation in rat hepatocytes and led to hepatic damage as represented by the significantly elevated levels of NF-κB. DiNAC might ameliorate inflammation via downregulating the NF-κB signaling pathway to protect liver injury.

These results identify possible new pharmacological evidence to support the clinical application of DiNAC for NASH.

Materials and methods

Chemical products

DiNAC arginine salt was synthesized at Chia Tai Tianqing Pharmaceutical Group Co., Ltd (Jiangsu, China). All the other chemicals were analytical grade products.

Animals and experimental designs

The animal experiment was conducted following the guidelines for animal experimentation of the Zhejiang Academy of Medical Sciences. Healthy male Sprague-Dawley rats weighing 194-208 g were housed at a temperature of 20-24°C and a relative humidity of 50-60% and were subjected to a light/dark cycle. The animals were allowed free access to standard laboratory chow and tap water. Before the start of the experiment, the animals were acclimatized to laboratory conditions for one week. The rats were randomly divided into five groups (n = 8 per group). The normal group was fed on normal chow for 20 weeks. The HFD group was fed on a HFD with intravenous injections of phosphate-buffered saline (PBS) once a day from 12 weeks. The HFD and DiNAC groups were fed on a HFD with intraperitoneal injections of DiNAC of 12.5 mg/kg, 25 mg/kg, and 50 mg/kg body weight treatment as were the HFD group. The HFD was made up of an ordinary diet (82%) plus 10% lard, 2% cholesterol, 1% bile salts, and 5% sucrose, which was processed by the Zhejiang Academy of Medical Sciences. The DiNAC treatment was started at the twelfth week and continued up to the twentieth week. The animals were weighed once a week. After an eight-week treatment, the rats were put under general anesthesia with sodium pentobarbital and blood was collected from the inferior vena cava. Serum was obtained following blood clotting and centrifugation to analyze,

Table 1. Changes in body weight (g), liver weight, and liver index in different groups of rats

Group	Body weight	Liver weight	Liver index
Control	486.72 ± 26.63	17.57 ± 1.29	3.61 ± 0.32
HFD	638.51 ± 42.36[a]	28.03 ± 2.47[a]	4.39 ± 0.45[a]
HFD+DiNAC (12.5 mg/kg)	634.27 ± 40.73[a]	26.83 ± 2.35[a]	4.23 ± 0.34[a]
HFD+DiNAC (25 mg/kg)	618.95 ± 39.48[a]	24.26 ± 2.16[a,b]	3.92 ± 0.31[b]
HFD+DiNAC (50 mg/kg)	607.14 ± 35.52[a]	22.34 ± 1.92[a,b]	3.68 ± 0.31[b]

Rats were treated with different concentrations of DiNAC (12.5, 25, 50 mg/kg) for 8 weeks, and the body weight, liver weight, and liver index in the different groups were recorded and analyzed. HFD, high-fat diet; DiNAC, N,N'-diacetylcystine. The data were expressed as the means ± standard deviation (n = 8). [a]$P < 0.05$, vs. the control group. [b]$P < 0.05$, vs. the HFD-treated group.

then the remainder was stored at -80°C. At the same time, the livers from the control and DiNAC-treated rats were immediately removed and weighed, and then portions of the livers were snap-frozen in liquid nitrogen and stored at -80°C for further analysis.

Calculation of the liver index

The liver index was expressed using the following formula: liver index = (liver weight/body weight) ×100.

Measurement of ALT and AST production

To assess the biochemical changes in the rats, the blood samples were centrifuged (Beckman GS-6R, Germany) at 4°C for 15 minutes to separate the serum. The levels of ALT and AST were determined using commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocols.

ELISA quantification of inflammatory cytokines

The serum samples from the groups were stored in a refrigeration system at -80°C. Before performing the experiment, the samples were thawed, and 50 μL of serum was transferred from each sample to the test tubes. The reagents were allowed to equilibrate to room temperature at 25°C, and a wash buffer was diluted 10 times with deionized water. The protein levels of IL-1β, IL-6 and TNF-α were analyzed with ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocol. The levels of inflammatory cytokines were determined from standard curves for the combination of these cytokines. Results are expressed as picogram per milliliter (pg/ml).

Histopathological examination

Following standard procedures, pathological analyses were performed on liver tissue sections. The liver sections were stained with hematoxylin and eosin (H&E). The fresh liver tissue samples were fixed in 10% (v/v) buffered formalin and embedded in paraffin. The samples were cross-cut into slices of 5 μm and then stained with H&E for histological assessment by two registered histopathologists unaware of the treatments.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the rat liver tissues using Trizol reagent (Invitrogen, American) to examine the effects of DiNAC on the expression of NF-κB gene. Reverse transcription (RT) of total RNA (2 μg) was performed following the manufacturer's instructions (Promega, Beijing, China) in a final volume of 12 μL. Subsequently, the cDNAs were amplified by using the RT-PCR System (ABI, Stepone plus, American) with SYBR Green kit (Promega, Beijing, China) according to the manufacturer's instructions. The primers used to specifically amplify the genes of interest were: NF-κB forward: 5'-GTGACAAGGTCCAGAAAGATGA-3' and reverse: 5'-TTCGGGGTAGTAGAGAAAGGGT-3', GAPDH forward: 5'-CGGAGTCAACGGATTTGGTC-3' and reverse: 5'-AGCCTTCTCCATGGTCGTGA-3'. The RT-PCR conditions were as follows: initial denaturation at 95°C for 10 min, then 40 cycles consisting of predenaturation at 95°C for 15 s, and extension at 75°C for 30 s. Quantitative values were obtained from the threshold cycle value (Ct). Relative quantification analysis was conducted using the ^ΔΔCt method. An internal control, GAPDH, was used to calculate the relative quantitative values of the target genes of each sample.

Statistical analysis

All results are expressed as the mean ± standard deviation (SD). Between-group differences were evaluated using a one way analysis of variance (ANOVA) followed by Dunnett's test. SPSS
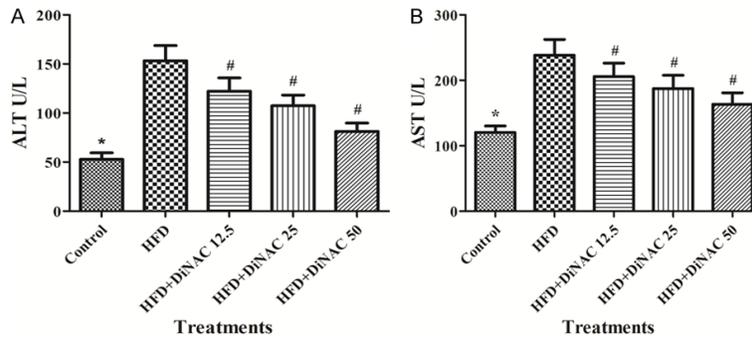
Figure 1. The effects of DiNAC on HFD-induced elevations in serum liver enzyme activities in rats. A. ALT in HFD-treated rats with different doses of DiNAC for 8 weeks; B. AST in HFD-treated rats with different doses of DiNAC for 8 weeks. The data were expressed as the means ± standard deviation of the mean of eight rats in each group. *P < 0.05, vs. the control group, #P < 0.05, vs. the HFD group, as determined by a one-way analysis of variance and Dunnett's test. HFD, high-fat diet; DiNAC, N,N'-diacetylcystine; ALT, alanine aminotransferase; AST, aspartate aminotransferase.
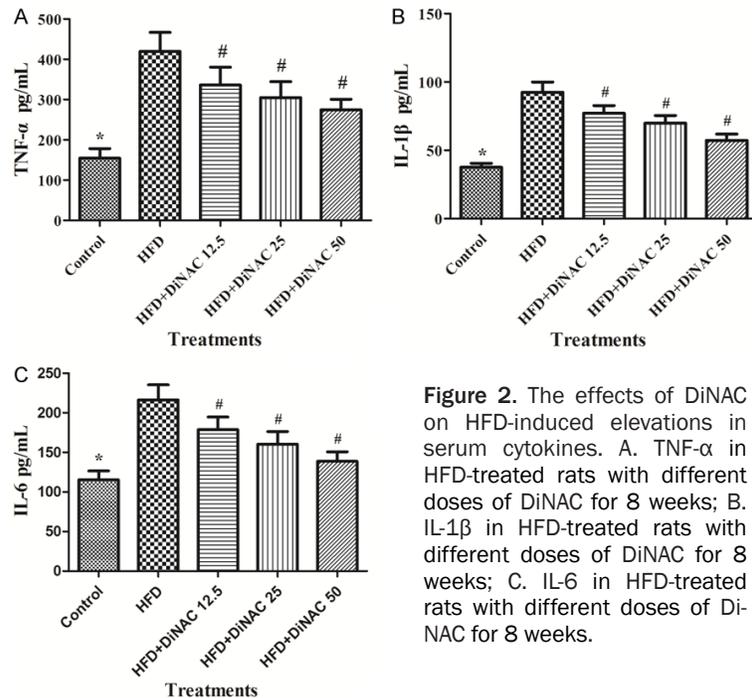
Figure 2. The effects of DiNAC on HFD-induced elevations in serum cytokines. A. TNF-α in HFD-treated rats with different doses of DiNAC for 8 weeks; B. IL-1β in HFD-treated rats with different doses of DiNAC for 8 weeks; C. IL-6 in HFD-treated rats with different doses of DiNAC for 8 weeks.

body weights in the HFD group were increased compared to the weights in the control group (P < 0.05). The treatment with DiNAC produced a slight decrease in body weight, but the decrease was not statistically significant (P > 0.05), which may reflect the effect of DiNAC on the appetite of rats. The liver weight and liver index in the HFD group were higher than those in the control group (P < 0.05). Compared with the HFD group, the liver weight and liver index of the rats that were administered 25 mg/kg and 50 mg/kg DiNAC for eight weeks significantly decreased.

Effects of DiNAC on serum ALT, AST activities in NASH rats

To determine whether DiNAC protected the liver functions induced by HFD in rats, the serum levels of ALT and AST were measured. As shown in **Figure 1**, the levels of ALT and AST were significantly upregulated in the HFD-treated group compared with the control group (P < 0.05). After DiNAC treatment, there were a significant reduction in ALT and AST (P < 0.05) compared with the HFD-induced group. These results suggest that DiNAC could protect the liver from the injury induced by the HFD feeding.

software (version 16 for Windows) was used for the statistical analysis. A value of *P* < 0.05 was considered statistically significant. All the experiments were performed in triplicate.

Results

The effects of DiNAC on body weight, liver weight, and liver index in NASH rats

At the end of the last experimental week, the body weight, liver weight, and liver index of the rats in each group are shown in **Table 1**. The

Effects of DiNAC on serum cytokines in NASH rats

To evaluate the effects of DiNAC on liver injury induced by a HFD in rats, the serum levels of inflammatory cytokines were examined. The results are presented in **Figure 2**.

Compared with the control group, the levels of TNF-α, IL-1β and IL-6 were significantly increased in rats induced by a HFD. After the administration of DiNAC, there was a significant
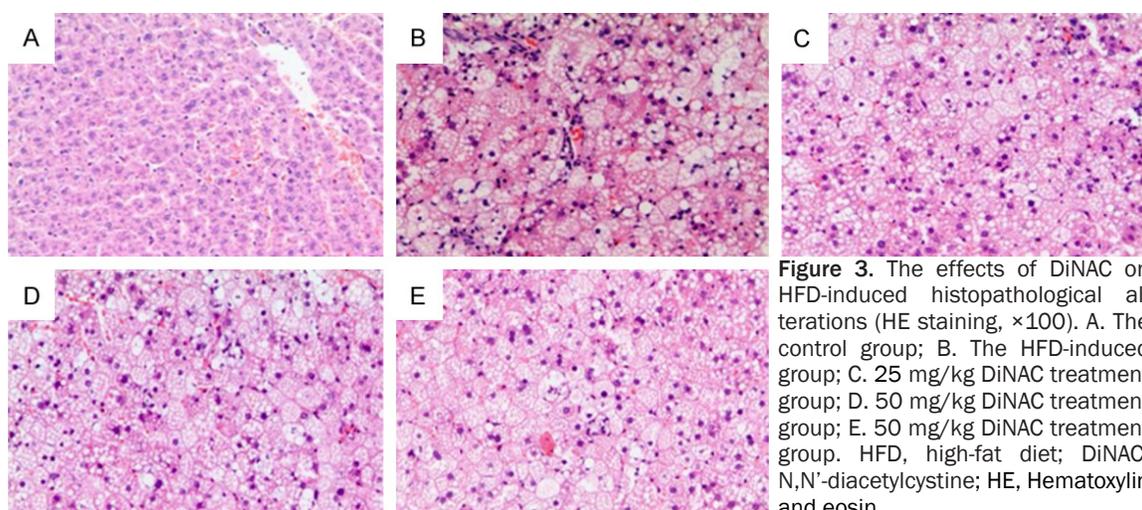
Figure 3. The effects of DiNAC on HFD-induced histopathological alterations (HE staining, ×100). A. The control group; B. The HFD-induced group; C. 25 mg/kg DiNAC treatment group; D. 50 mg/kg DiNAC treatment group; E. 50 mg/kg DiNAC treatment group. HFD, high-fat diet; DiNAC, N,N'-diacetylcystine; HE, Hematoxylin and eosin.
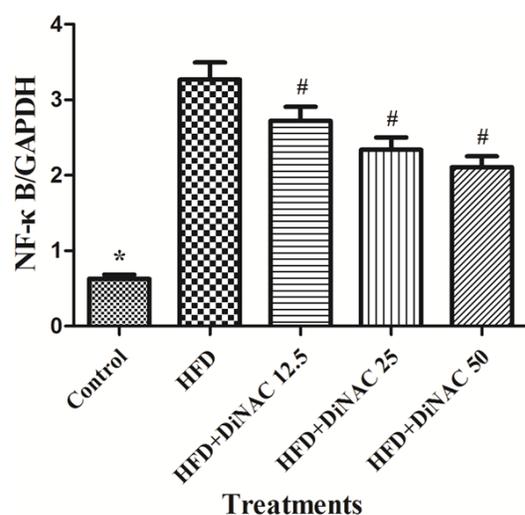
Figure 4. The effects of DiNAC on HFD-induced hepatic NF-κB mRNA expression. NF-κB mRNA expression in HFD-treated rats with different doses of DiNAC for 8 weeks.

reduction in serum cytokine concentrations (P < 0.05) compared with the HFD-induced group. DiNAC suppressed TNF-α, IL-1β and IL-6 production in a dose dependent manner.

The data were expressed as the means ± standard deviation of the mean of eight rats in each group. *P < 0.05, vs. the control group, #P < 0.05, vs. the HFD group, as determined by one-way analysis of variance and Dunnett's test. HFD, high-fat diet; DiNAC, N,N'-diacetylcystine; TNF-α, tumor necrosis factor α; IL-1β, interleukin-1β; IL-6, interleukin-6.

The effects of DiNAC on histopathological alterations in NASH rats

Histopathological examinations of liver tissues were utilized to investigate the protective effect of DiNAC in the present experiment. The results are presented in **Figure 3**. H&E staining of liver tissues showed that the structure was normal in the control group, with normal hepatic lobules, the round shape of the liver cells, and no pathological changes in the area. In the HFD treatment, the structural disorder of hepatic lobules, macrovesicular steatosis, and inflammatory infiltration was noted. Compared with the HFD group, the degree of steatosis and inflammation of the liver samples in the DiNAC treatment groups were significantly decreased. This indicated that DiNAC attenuated the intensity of HFD-induced liver damage.

Effects of DiNAC on NF-κB mRNA in NASH rats

To further study the anti-inflammatory effect of DiNAC, the NF-κB mRNA expression was assessed through qRT-PCR. As shown in **Figure 4**, the expression level of NF-κB mRNA was significantly higher in rats induced by a HFD as compared to the control group rats. Strikingly, with the treatments of DiNAC, the NF-κB mRNA expressions were decreased in the liver. The effects of the DiNAC (12.5 mg/kg, 25 mg/kg and 50 mg/kg) treatments exhibited a dose-effect relationship, indicating that DiNAC might have a strong effect in ameliorating inflammation in HFD rat livers.

The results were analyzed using a one-way analysis of variance and Dunnett's test, and the data were expressed as the means ± standard deviation of the mean of the 3 rats in each group. *P < 0.05, vs. the control group, #P < 0.05, vs. the HFD group, as determined by one-way analysis of variance and Dunnett's test. HFD, high-fat diet; DiNAC, N,N'-diacetylcystine; NF-κB, Nuclear factor kappa B; GAPDH, glyceraldehyde phosphate dehydrogenase.

Discussion

The liver is an important principal organ for the metabolism of carbohydrate, lipids and proteins. The HFD treatment rats demonstrated a pathological condition that mimics the most common features of NASH in humans and provides a useful tool to investigate the role of individual pathogenetic events [21, 22]. Feeding a high fat diet to rats enhances cholesterol and triglyceride (TG) in the liver and can induce steatosis and steatohepatitis, in addition to increasing body weight, adiposity and insulin resistance [23]. In the present study, HFD-fed rats showed significant increases in body weight and liver indexes, which induced obesity. In the HFD group, among the markers of liver damage tested, there was a significant increase in serum biochemical enzyme compared with the control group. In the H&E-stained liver sections, many lipid deposits were observed in the livers from rats induced by HFD, and this pathological finding indicates hepatic steatosis and inflammation. These results were consistent with previous studies showing a chronic intake of HFD induced liver damage and increased serum ALT and AST, which are used as markers of NAFLD [24, 25]. In the DiNAC (25, 50 mg/kg) treatment groups, there were significant decreases in liver weight and in the liver index, and a statistically non-significant decrease in body weight compared with the HFD group. The administration of DiNAC in the HFD-fed group of rats markedly restored levels of the liver enzymes and significantly improved the histopathology of the liver. DiNAC, the disulfide dimer of NAC, attenuates liver injuries in mice induced by D-galactsamine (Gal)/lipopolysaccharide (LPS). Treatment with DiNAC was clearly shown to inhibit Gal/LPS-induced liver failure [26]. DiNAC has also been shown to attenuate acute liver failure induced by Gal in rats. DiNAC can decrease serum ALT and AST, reduce MDA and NO levels in serum, inhibit conjugated diene levels in liver mitochondria, and prevent liver damage and mortality in rats [27].

DiNAC significantly attenuated the elevated serum levels of hepatic enzymes such that the proportion of damaged hepatocytes was reduced as a direct result of DiNAC administration.

NASH is increasingly recognized as a major cause of chronic liver disease, in which fatty infiltration of the liver is accompanied by necro-inflammatory activity. Growing evidence indicates that inflammation is the driving force behind the development of NASH and subsequent fibrosis and cirrhosis [28, 29]. Several studies have confirmed that TNF-α is one of the key factors in the development of NAFLD and NASH in both humans and animals. The use of the anti-TNF-α drug in an experimental model of NASH decreased inflammation, necrosis, and fibrosis in rats [30, 31]. IL-6 is considered a predictor marker of insulin resistance. IL-6 may play a critical role in the systemic effects of insulin resistance and is also increased in NASH patients compared with simple steatosis and control patients [32]. In this study, lipid accumulation and inflammatory foci deposition were observed in the HFD-fed rats for 20 weeks compared to the control rats. The levels of inflammatory cytokines in the serum were significantly higher than those in the normal control group. DiNAC markedly decreased the levels of TNF-α, IL-1β and IL-6, indicating that the inhibition of cytokines was one of the main mechanisms in HFD-induced rats. DiNAC in vivo acts with a potent and effective immuno-modulating property that can either enhance or reduce the CS or DTH response depending on the experimental conditions [33].

DiNAC treatment in corneal transplantation models successfully prolonged graft survival by reducing the mps production of proinflammatory mediators and by inhibiting the ongoing cell immune responses [34]. DiNAC significantly reduced the elevated serum levels of ALT and AST in experimental NASH by alleviating hepatic inflammation. The two main inflammatory pathways JNK/AP-1 and NF-κB/IKK are critically involved in the development of a chronic inflammatory state in NASH. NF-κB is a transcription factor that plays an evolutionarily conserved and critical role in triggering and coordi-

nating both innate and adaptive immune responses. In the liver, NF-κB can induce systemic and profound hepatic insulin resistance associated with an increased expression of proinflammatory cytokines, including TNF-α, IL-1β and IL-6 [35]. Targeting the NF-κB pathway presents an attractive opportunity since this intrinsic pathway can be dynamically modulated. In the present study, the liver NF-κB is activated, and serum TNF-α, IL-1β and IL-6 levels are increased in HFD-fed rats.

Consistent with these findings, the activation of NF-κB has been correlated with HFD-induced hepatic steatosis and inflammation. DiNAC significantly reduced the activation of NF-κB expression and serum cytokine levels in HFD-fed rats. Therefore, we speculate that the regulation of NF-κB may be one potential mechanism for the DiNAC treatment of NASH.

In conclusion, our present study shows that NF-κB participates in the progression of NASH, and the NF-κB inhibitor DiNAC may be effective in the treatment of NASH. But the detailed mechanisms of how NF-κB mediates liver injury and how DiNAC decreases the disease process need further studies.

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Disclosure of conflict of interest

None.

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