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D-chiral inositol ameliorated MAFLD by targeting NLRP3-mediated macrophage pyroptosis in db/db mice

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Abstract

Objective: This study aims to explore the molecular mechanism by which DCI improves MAFLD induced by diabetes by pyroptosis, and to bring new ideas for clinical treatment and reversal of MAFLD.

Methods: In this study, we investigated the effect of DCI on macrophage pyroptosis using a diabetic mouse model (db/db mice) and high glucose (HG) and lipopolysaccharide (LPS) -induced mouse macrophage raw264.7 cells. Immunohistochemistry (IHC) and immunofluorescence (IF) analysis were used to evaluate the effect of DCI on NLRP3-mediated pyroptosis of macrophages in vitro and in vivo. The binding ability of DCI to nuclear transcription factor NF-κB and NOD-like receptor pyrin domain-containing protein 3 (NLRP3) was investigated by molecular docking experiments.

Results: DCI can reverse the abnormal liver function and hepatic steatosis in db/db mice, and block the release of inflammatory cytokines. These effects were related to the inhibition of NLRP3/NF-κB/Caspase1/GSDMD signaling pathway by DCI. Molecular docking experiments also suggested that NF-κB and NLRP3 are potential targets of DCI.

Conclusion: Using a combination of animal and in vitro experiments, this study is the first to demonstrate that DCI improves MAFLD by targeting NLRP3-activated macrophage pyroptosis. Therefore, DCI may be a potential therapeutic agent for MAFLD.

Keywords: D-chiral inositol, MAFLD, NLRP3, macrophage pyroptosis, db/db mice

1. Introduction

The International Diabetes Federation Diabetes Atlas, 9th edition, shows that the worldwide prevalence of diabetes is estimated at 9.3% (463 million people) [1]. Data from the 2020 Chinese epidemiological survey showed that the incidence of diabetes among people over 18 years old was about 12.8% (113.9 million people) [2]. About 49.7% of diabetic patients are complicated with Metabolic dysfunction-associated Fatty Liver Disease (MAFLD) [3, 4], and the population affected by MAFLD tends to be younger. MAFLD, formerly known as Nonalcoholic Fatty Liver Disease (NAFLD) [5, 6], is recognized as the most common chronic liver disease. It affects about 1/4 of the population worldwide, and there are about 310 million patients in China. MAFLD causes multiple organ failure, which is life-threatening and seriously affects the quality of life of patients.

A 2019 survey showed that 12%-40% of patients with MAFLD worldwide will progress to non-alcoholic steatohepatitis (NASH), and 15%-33% of NASH patients can progress to cirrhosis [7], which manifests as four

pathological types: There are four types of pathological changes: simple steatosis, lobular inflammation, ballooning, and liver fibrosis [8], and the hepatitis can be reversed before fibrosis, but not [9] after fibrosis. Long-term hyperglycemic conditions lead to the accumulation of bullous lipid droplets and cellular swelling in hepatocytes. Lipid accumulation in hepatocytes leads to hepatic steatosis, lipid peroxidation, and the release of pro-inflammatory factors, which eventually leads to irreversible liver tissue damage. Diabetes mellitus, as an independent risk factor for MAFLD, interacts with MAFLD and synergistically [10, 11]. Currently, there is no drug approved by the Food and Drug Administration for MAFLD [12].

The pathogenesis of MAFLD in diabetes is still unclear. It is mainly related to Aberrant lipid metabolism (ALM), insulin resistance (IR), advanced glycation end product (EDV), and the pathogenesis of diabetes complicated with MAFLD. AGEs) accumulation, oxidative stress, intestinal flora imbalance, genetic factors, etc [13]. Studies have found that pyroptosis-related proteins are highly expressed [14] in the kidney of diabetic model mice, which are closely related to lipid metabolism and inflammatory damage. Pyroptosis is a natural immune defense response, but often due to excessive immunity, the body's damage is greater than its defense, leaving tissues and organs in a pro-inflammatory state. Liver inflammation in MAFLD is mainly due to the influence [13] of innate immunity by adaptive immunity. The accumulation of metabolites such as lipotoxic substances stimulates the activation of immune cells and further activates the immune response [15]. Not all patients progress from the steatosis stage to NASH, and the mechanism of the progression is not clear. In addition, there is currently no treatment for NASH. Therefore, further studies are needed to understand the pathogenesis of MAFLD and design new treatment strategies.

It is now generally accepted that multiple hits from the gut and adipose tissue are critical [16] for disease progression. Liver macrophages (macs) are involved in this process and are activated as proinflammatory cytokines [17] by excessive lipid accumulation in fatty liver, excessive cell damage, and signals from the gut. However, it is not clear which Macs are involved. Kupffer cells (KCS) are the major mac population in the healthy liver. A small proportion of KCS are present in the hepatic sinusoids, where they interact [18-20] with liver sinusoidal endothelial cells (LSECs), hepatic stellate cells (HSCS) and hepatocytes. In recent years. Studies have shown that inositol can regulate the growth, activation and phagocytosis [21-24] of macrophages, effectively reduce triglyceride (TG) content and the expression of fatty acid synthesis genes.

Inositol, also known as vitamin B8, has anti-inflammatory, anti-oxidation and regulating lipid metabolism. D-chiro-inositol (DCI) is one of the major natural isomers, which exists in nature. DCI is a natural compound involved in endocrine signal transduction, which is located on the cell membrane and plays a normal physiological function. Compared with other drugs, DCI is derived from food and has no toxic side effects. Dci is mainly found in [25] crops such as buckwheat, soybean, pumpkin seeds, and has a good hypoglycemic effect. It has been found that DCI can alleviate glucose and lipid metabolism disorders and kidney injury [26, 27] caused by diabetes in db/db mice. DCI diet therapy is expected to become a new treatment for diabetes with MAFLD.

Both hepatocyte and macrophage pyroptosis play a certain role [28, 29] in the pathogenesis of MAFLD complicated by diabetes, but what is the cause of pyroptosis? How pyroptosis interact with each other; Whether more protein factors are involved in the process is rarely reported in the literature. This study aims to explore the molecular mechanism by which DCI improves MAFLD induced by diabetes by pyroptosis, and to bring new ideas for clinical treatment and reversal of MAFLD.

2. Materials and Methods

2.1. Chemicals and Reagents

DCI was purchased from Yuanye Biotechnology Co., LTD. (Shanghai). Anti-NLRP3 and Anti-β-actin antibodies were purchased from (Proteintech, Wuhan), Anti-GSDMD from (CST, USA), Anti-F4/80 from (Abcam, USA), Anti-Caspase1 from (Servicebio, Wuhan), Metformin hydrochloride (MET) was purchased from (Gehuate, Shanghai) and the cck-8 kit was purchased from (LABLEAD, Beijing).

2.2. Animal and experimental design

Male *db*/db mice (6-8 weeks) and *db/m* mice were purchased from GemPharmatech (Nanjing, China). The experimental mice were maintained at a constant temperature of 22°C±2°C and 60% humidity under a 12-h light/dark cycle with AD libitum access to j food and water. All mice were randomly divided into the following four groups: normal control *db/m*, diabetic db/db and *db/db* + MET 300mg/kg. db/*db* + DCI 35mg/kg was administered for 8 weeks. The db/*m* and db/*db* groups received the same amount of normal saline. All animal experiments were approved by the Animal Ethics Committee of North China University of Science and Technology and conformed to the NIH Guidelines for the Care and Use of Laboratory animals.

2.3. Liver tissue staining

The liver was removed and immediately fixed in 4% paraformaldehyde. After dehydration with gradient ethanol, the liver was over-embedded in paraffin over xylene. Then, the liver tissue was cut into 4μm paraffin sections. After deparaffinization, hematoxylin-eosin (HE) staining (Solebo, China) was performed, and the liver structure was observed using a microscope (Olympus, Japan).

2.4. Immunohistochemical analysis

Paraffin sections were heated in an oven at 60°C until wax fusion and then sequentially immersed in xylene and gradient ethanol for antigen repair using citrate buffer in a microwave oven. Subsequently, the tissue sections were incubated with 3% hydrogen peroxide (Biyuntian, China) for 25 min at room temperature, washed three times with phosphate buffered saline (PBS) for 10 min each, and blocked with 3% hydrogen peroxide for 1 h at room temperature. Next, the sections were incubated with Anti-NLRP3 (1:100, Proteintech, Anti-Caspase1 (1:200, Servicebio), Anti-F4/80 (1:100, Abcam) overnight at 4°C. After addition of biotin-labeled goat anti-rabbit IgG antibody (Zhongshan Jinqiao, China), the tissue sections were incubated at 37°C for 30 min, then washed three times with PBS and stained with hematoxylin for 2 min. The sections were finally sealed with neutral resin and recorded under microscopy.

2.5 Cell culture

Raw267.4 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai). The medium was DMEM (gibco, USA) supplemented with 10% fetal bovine serum (gibco, USA) at 37°C and 5%CO_2. The cells were divided into 4 groups: normal control group (NC), high glucose (HG, 30mmol/L), lipopolysaccharide (LPS, 100ng/ml) and lipopolysaccharide +DCI (LPS +DCI).

2.6. cck-8 assay

Raw264.7 cells were seeded uniformly in 96-well plates. After cell attachment, cells were incubated with HG and LPS and different concentrations of DCI (50/100/200mM) for 24 hours. Then, the cells were incubated with 10ml of cck-8 solution for 2 h at 37°. Optical density analysis was performed by adjusting the wavelength of the microplate reader (Thermo Fisher, USA) to 492nm.

2.7. Immunofluorescence assay

Raw264.7 cells were grown on coverslips placed in 24-well plates. The cells were fixed with 4% paraformaldehyde for 15 min and then sealed with 5%BSA (Thermo Fisher, USA) at 37°C for 30 min. They were then incubated with an antibody against TXNIP (1:50, Santa Cruz Biotechnology, CA, USA) at 4°C overnight. Subsequently, they were washed three times with PBS and then incubated with Anti-Rb594 secondary antibody (proteintech, Wuhan) for 30 min at 37°C in the dark. Nuclei were counterstained with DAPI (Solebo, China) for 3 min. Finally, recordings were made using a fluorescence microscope (Zeiss, Canada).

2.8. Molecular docking

Molecular docking was performed using Discovery Studio 2018 (V18.1.0.17334) software. The crystal structures of NF-κB and NLRP3 (PDB ID: 4LL4) were used for docking studies. Prior to docking simulations, ligands (DCI) and proteins (NF-κB and NLRP3) were prepared using standard protocols using Discovery Studio 2018. The drug DCI inserts into the pocket of NF-κB and NLRP3. All docking calculations were performed using default Settings.

2.9. Statistical Analysis

All experiments were performed three times independently, and the results are expressed as mean ± SD. Differences between the two groups were assessed using an independent sample *t-test*. For multiple group comparisons, significant differences were analyzed by ANOVA followed by ANOVA (one-way analysis of variance) using GraphPad Prism 8.3 software. A P value of less than 0.05 was considered statistically significant.

3. Results

3.1. Effects of DCI on the pathological changes of liver tissue in db/db mice

It was observed that the hepatocytes in the hepatic lobules of db/m mice were arranged radially around the central vein, the structure was clear, and the hepatocyte nucleus was round, located in the center of the cell, and there were one or more nucleoli in it. In db/db group, the hepatic lobule hepatocytes were arranged disorderly and the radial hepatic cord structure was lost. There were different degrees of swelling and degeneration and necrosis of hepatocytes. The central veins were absent or multiple, sometimes surrounded by portal areas. The regenerated hepatocytes were large, with large, hyperchromatic nuclei and often binucleated. DCI can improve the pathological changes of liver tissue in db/db mice, and exert similar effects as the positive drug metformin, as shown in Figure 1.

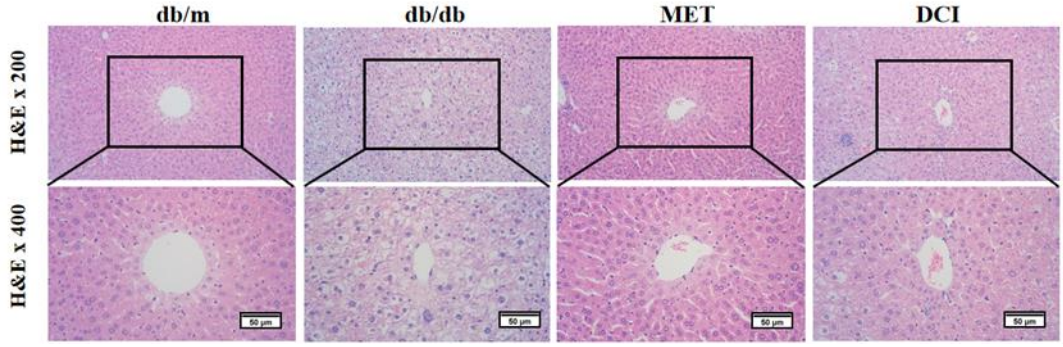

Figure 1: Effect of DCI on the pathological changes of liver tissue in db/db mice

3.2. DCI can improve the infiltration of inflammatory cells in liver tissue of db/db mice

Pyroptosis is a progressive cell death caused by injury stimulation. It is a component of the innate immune system and plays a role in indicating the immune system. However, due to excessive immunity, the damage is greater than the defense, and the inflammatory response of tissues and organs is promoted. Compared with the liver tissue of db/m mice, the number of F4/80 positive cells in db/db mice was significantly increased, which may be due to the increased inflammatory cell infiltration caused by cytokine efflux caused by macrophage pyroptosis. The DCI group had a significant reduction in F4/80 positive cells in liver tissue, and both MET and DCI could improve inflammatory cell infiltration in MAFLD, as shown in Figure 2.

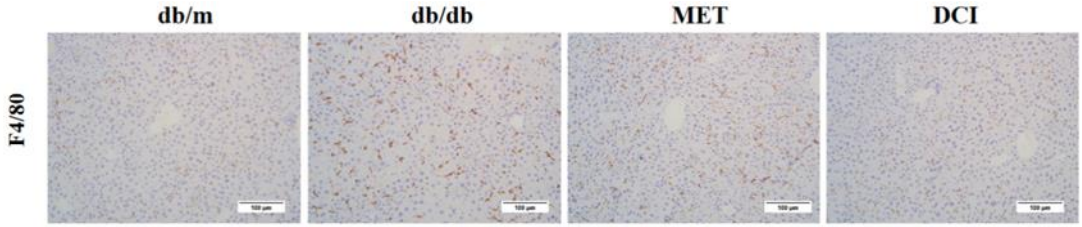

Figure 2: DCI improved macrophage infiltration in liver tissue of db/db mice

3.3. DCI intervention down-regulated CASP1 and NLRP3 protein levels in db/db mice

Cysteinyl aspartate specific proteinase 1 (CASP1, NLRP3) was detected by immunohistochemical staining. Caspase1 and NOD-like receptor thermal protein domain associated protein 3 (NLRP3) protein levels, The results showed that DCI inhibited the expression of these two proteins, and the inhibitory effect was better than that of MET, as shown in Figure 3.

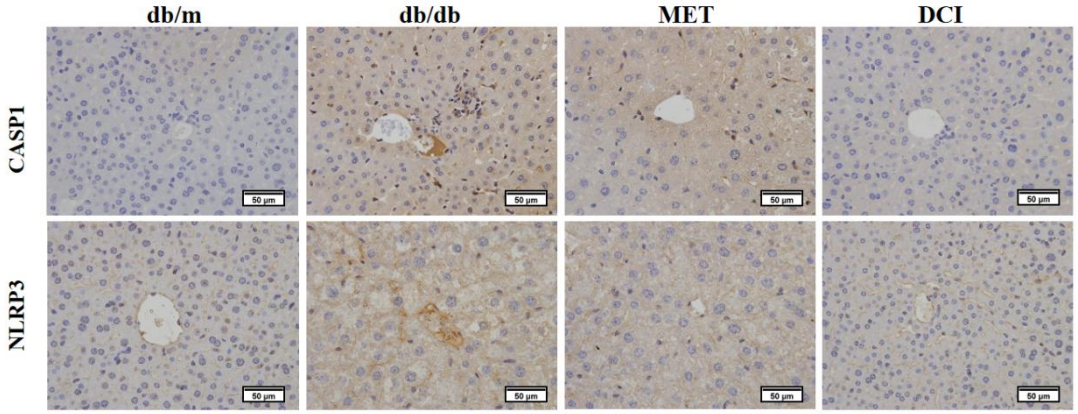

Figure 3: DCI intervention downregulated CASP1 and NLRP3 levels in db/db mice

3.4 DCI and nf-kappa B and NLRP3 molecular docking results

The docking results of DCI and the two protein structures are shown in FIG. 4. DCI can naturally bind to the two target proteins, and there is good docking activity between DCI and each target.

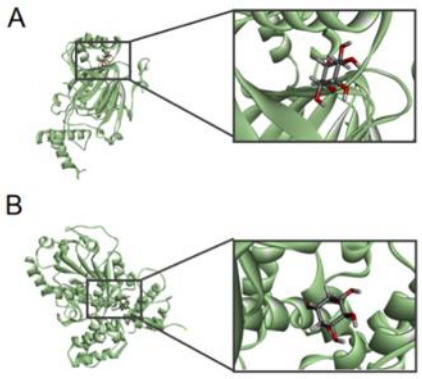

Figure 4: Diagram of docking patterns of DCI with NF-κB and NLRP3
A:DCI and Nf-κb, B: DCI and NLRP3

3.4. CCK-8 method was used to detect the effect of different concentrations of DCI on the survival rate of macrophages induced by HG and LPS

Raw264.7 cells were treated with 30mM high glucose (HG) and 100ng/mL lipopolysaccharide (LPS) for 24h, HG had no effect on the survival rate of mouse macrophages (P=0.5188), LPS had lower cell proliferation rate than the control group (P<0.05). DCI could significantly improve the situation. Dci with a dose of 50mM had the best effect.

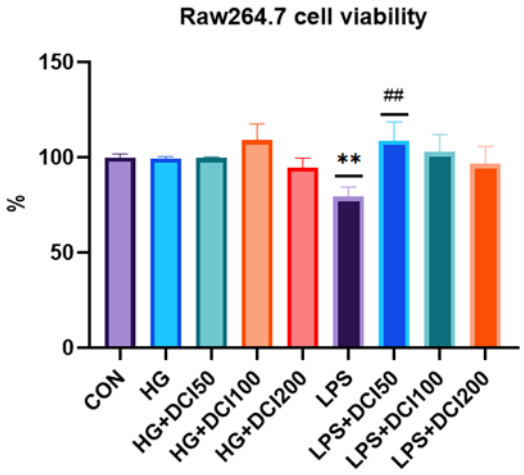

Figure 5: CCK-8 assay was used to detect the effect of different concentrations of DCI on the survival rate of macrophages induced by HG and LPS
***:Compared to CON, ##:Compared with the LPS group*

3.4. Immunofluorescence was used to detect the protein expression of NF-κB/Caspase1/GSDMD in different groups

Compared with the control group, there was no significant difference in the expression of the three proteins in the HG group, but the LPS group had a significant increase in the expression of the three proteins, which was significantly improved by DCI (FIG. 6).

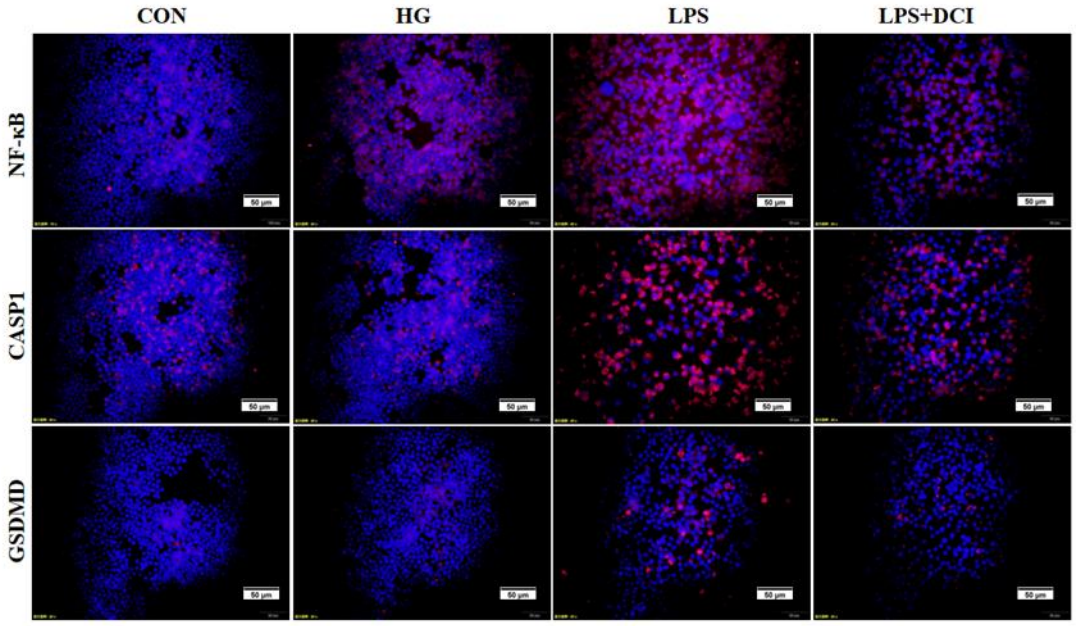

Figure 6: The protein expressions of NF-κB/Caspase1/GSDMD in different groups were detected by immunofluorescence

4. Discussion

The liver is not only a central organ of energy metabolism, but also an immune organ, rich in a variety of immune cells; Therefore, macrophages are abundant in the liver. Innate immune cells and related effectors play an integral

role in the development of fatty liver disease. There are three main different origins of macrophage subsets in the liver: yolk sac-derived tissue-resident macrophage-Kupffer cells, monocyte-derived macrophages (MDM)/myeloid-derived monocyte-derived macrophages, and hepatic cystic macrophages (LCM). Kupffer cells (KCs) are in close contact [17] with hormones, bacterial endotoxins (LPS), metabolites and immune complexes carried by the liver circulation. There are 20 to 40 macrophages in every 100 hepatocytes [30]. Macrophages form a highly active, dynamic and complex immune network system in the liver and play various important roles [31, 32] in the progression of liver fibrosis.

Studies have shown that GSDMD, the key protein of pyroptosis, is highly expressed in db/db mice, which is closely related to lipid metabolism disorders and inflammatory damage, mainly caused [33] by the synergistic activation of GSDMD by NLRP3 and NF-κB. NLRP3 is almost not expressed in hepatocytes in vivo, but its expression is high [34] in macrophages. Macrophage pyroptosis can lead to the release of NLRP3 inflammasomes and promote the progression [35] of MAFLD. In addition, hepatocytes cultured in vitro can also undergo pyroptosis induced by LPS [34].

DCI has therapeutic effects on endocrine and metabolic diseases such as diabetes mellitus, polycystic ovary syndrome and obesity, and its specific mechanism is related [36, 37] to the regulation of insulin signal transduction. A clinical study in 2021 confirmed that DCI can improve insulin resistance, thereby restoring physiological insulin levels[38] in resistant subjects. In addition, in vitro cultured SGBS cells found that DCI could directly regulate human adipocytes to improve glycogen storage and inflammatory factor release.

In summary, in this study, we investigated the role of NLRP3 and NF-κB in the pathogenesis of macrophage pyroptosis MAFLD by using DCI in db/db mice and HG - and LPS-induced macrophages in vivo and in vitro. We found that DCI could significantly inhibit the protein expression of NLRP3/NF-κB/Caspase1/GSDMD in macrophages of db/db mice and LPS-induced macrophages, and reduce the progression of MAFLD caused by macrophage pyroptosis to NASH.

Acknowledgements

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