Original Article
The effect of polyene phosphatidyl choline intervention on nonalcoholic steatohepatitis and related mechanism

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Abstract: Nonalcoholic steatohepatitis (NASH) has similar clinical pathological changes to alcoholic hepatitis. It shows increased incidence and young trend year by year. Polyene phosphatidyl choline (PPC) is widely used in clinic for liver disease treatment. The effect and mechanism of PPC on NASH have not been fully elucidated. Thirty healthy male Wistar rats were randomly equally divided into control, NASH group, and PPC group. NASH model was established by high fat diet. PPC was intraperitoneal injected to NASH rat from the second week at 80 mg/kg·d for three weeks. Body weight, liver weight index, ALT, AST, TG, and TC were tested. TNF-α and IL-1β levels were detected by ELISA. NF-κB mRNA and protein expression in liver tissue were determined by real time PCR and Western blot. SOD activity and ROS content were measured by colorimetry. NASH rat presented significantly elevated body weight and liver weight index, increased ROS content, declined SOD activity, enhanced liver function and inflammatory factors expression, and upregulated NF-κB mRNA and protein levels compared with control ($P < 0.05$). PPC intervention obviously reduced body weight and liver weight index, declined ROS content, amplified SOD activity, decreased liver function, weakened inflammatory factor TNF-α and IL-1β expression, and downregulated NF-κB mRNA and protein levels compared with NASH group ($P < 0.05$). PPC can play a treatment effect on NASH through regulating oxidative balance, inhibiting inflammatory factors and NF-κB signaling pathway.

Keywords: Polyene phosphatidyl choline, nonalcoholic steatohepatitis, TNF-α, NF-κB, IL-1β

Introduction

Nonalcoholic steatohepatitis (NASH) presents similar clinicopathological changes to alcoholic hepatitis without history of heavy drinking. Its pathological features include fat storage, parenchyma hepatic cells fatty change, liver fatty infiltration, hepatic cells necrosis, ballooning degeneration, and inflammatory cells infiltration [1, 2]. NASH is the most common cause of chronic liver disease. Severe NASH can appear different degrees of liver fibrosis and Mallory corpuscle, even develop to liver cirrhosis and hepatocellular carcinoma (HCC) [3, 4]. Epidemiological investigation demonstrated that the incidence of NASH was more than 20% worldwide. More obesity and metabolic syndrome appeared following life rhythm speeding up, high-fat and high-calorie diet, eating habits changing, and long-term sitting, which caused NASH incidence elevation and young trend [5, 6]. NASH has become a global public health

problem. The pathogenesis of NASH is complex, involving heredity, physics, chemistry, and many other factors. The pathogenesis of NASH is related to a variety of metabolic disorder factors, such as adipocyte factor, oxygen stress, intestinal endotoxin disease, and insulin resistance [7, 8]. Currently, it is considered that NASH is associated with second strike theory. The first strike is insulin resistance, leading to benign lipid deposition in the liver cell. The second strike is oxygen stress and lipid peroxidation, which is the key to disease progression [9]. In spite of multiple treatment choice for hepatitis, there is still lack of drugs with good effect on NASH so far [10].

Polyene phosphatidyl choline (PPC) is extracted from soy by the unique craft, which is rich in polyunsaturated fatty acid, including linoleic acid, linolenic acid, and oleic acid [11]. Polyunsaturated fatty acid, also known as essential fatty acid, cannot be autonomously synthetized

Table 1. Primer sequence

Gene	Forward 5'-3'	Reverse 5'-3'
GADPH	AGTGCCAGCCTCGTCTCATAG	CGTTGAACTTGCCGTGGGTAG
NF-κB	CTCATCTAAGCGGAACAATGG	GCACATTCTCTCCGTAGCG

Table 2. Liver weight and liver weight index comparison

Group	Weight (g)	Liver weight index (%)
Control	401.19±11.53	3.21±0.06
NASH	482.87±14.12*	4.56±0.11*
PPC	418.79±9.54#	3.63±0.09#

*$P < 0.05$, compared with control; #$P < 0.05$, compared with NASH group.

in the human body and must be supplied by food [12]. Therefore, PPC plays a role in numerous aspects, such as anti-inflammation, anti-oxidation, and immune regulation function [13, 14]. In clinic, PPC is widely used in various types of liver disease treatment [15]. However, the treatment effect of PPC and related mechanism on NASH has not been fully elucidated. This article intends to establish rat NASH model to investigate the effect and mechanism of PPC intervention on NASH.

Materials and methods

Experimental animals

Thirty healthy male Wistar rats at 5 weeks old and 120±20 g were purchased from animal center in Zhengzhou University and fed in SPF laboratory. Feeding conditions include constant temperature (21±1°C), constant humidity (50-70%), and 12 h diurnal cycle.

Rats were used for all experiments,and all procedures were approved by the Animal Ethics Committee of Henan Provincial People's Hospital.

Main materials and instruments

Surgical instruments were bought from medical instrument factory in Suzhou. Surgical microscope was from Zhenjiang optical instrument co., LTD. PPC injection was from Chengdu Tiantaishan pharmaceutical co., LTD. Trizol reagent was got from Invitrogen. RNA extraction kit and reverse transcription kit were from Axygen. SOD activity detection kit was purchased from Nanjing Jiancheng bioengineering

institute. PVDF membrane was from Pall Life Sciences. Western blot related chemical reagents were bought from Beyotime. ECL reagent was from Amersham Biosciences. Rabbit anti rat NF-κB monoclonal antibody and HRP-tagged IgG secondary antibody were from Cell Signaling. Microplate reader was from BD Company. DNA amplifier was from PE Gene Amp PCR System 2400. Automatic biochemical analyzer was from Beckman. Other common reagents were purchased from Sangon.

Methods

Experimental animal grouping: Thirty healthy male Wistar rats were randomly equally divided into control, NASH group, and PPC group.

NASH model preparation and PPC treatment: The rats were fed with mixed food containing 84% basal feed, 10% lard, 5% egg yolk powder, and 1% cholesterol disinfected by cobalt 60 irradiation for five weeks after 1 week's adaptive feeding. The rats in control were only gave normal basal feed. PPC was intraperitoneal injected to NASH rat from the second week at 80 mg/kg·d for three weeks.

Sample collecting: After modeling, the blood was extracted from aorta abdominalis and let stand at room temperature for 30 min. The serum was collected and store at -20°C after centrifuged at 4°C and 3600 rpm for 10 min. The rats were euthanatized and the liver tissue was preserved at -80°C. Liver weight index was calculated.

Liver function index detection: Serum ALT, AST, TG, TC, HDL-C, and LDL-C levels were tested by automatic biochemical analyzer.

ELISA: Serum was collected to detect inflammatory factors TNF-α and IL-6 expression according to the manual. 50 μl diluted standard substance was added to 96-well plate to prepare standard curve. 50 μl sample was added to the well with three replicates. After washed for 5 times, 50 μl enzyme-labelled reagent was added and the plate was incubated at 37°C for 30 min. Next, the plate was added with 50 μl color development agent A and B at 37°C for 10 min. At last, the plate was read at 450 nm within 15 min after adding 50 μl stop buffer. The linear regression equation of standard

Table 3. Liver function detection

Group	ALT (U/L)	AST (U/L)	TG (mmol/L)	TC (mmol/L)
Control	61.23±11.06	101.25±12.09	1.08±0.23	1.01±0.17
NASH	148.15±17.33*	214.76±11.41*	2.43±0.31*	2.81±0.48*
PPC	78.22±11.86#	123.63±21.59*	1.17±0.51#	1.21±0.62#

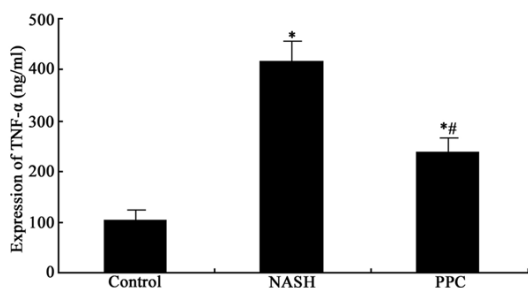*$P < 0.05$, compared with control; #$P < 0.05$, compared with NASH group.

Figure 1. PPC impact on TNF-α secretion in NASH rat. *$P < 0.05$, compared with control; #$P < 0.05$, compared with NASH group.
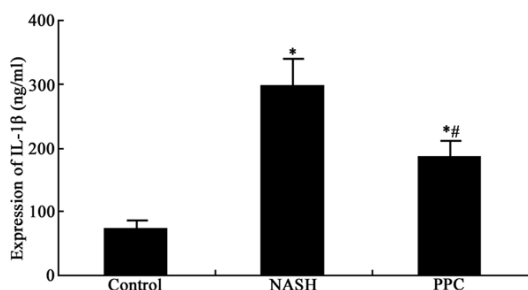

Figure 2. PPC impact on IL-1β secretion in NASH rat. *$P < 0.05$, compared with control; #$P < 0.05$, compared with NASH group.

curve was calculated by OD value and used to test sample OD value.

SOD activity detection: SOD activity in liver tissue was tested according to the kit instruction. Protein was extracted and water bathed at 95°C for 40 min. Next, the protein was cooled and centrifuged at 4000 rpm for 10 min. Ethanol phase in the tissue homogenate was extracted using ethanol-chloroform mixture (v/v 5:3) to detect LDH and total SOD activity.

ROS content detection: Protein was extracted and water bathed at 95°C for 40 min. Next, the protein was cooled and centrifuged at 4000 rpm for 10 min. Then the tissue homogenate was incubated in 2', 7' - dichlorofluorescein diacetate (DCF-DA) for 15 min and centrifuged

at 10,000 rpm for 15 min. The sediment was incubated in PBS at 37°C for 60 min. ROS content was determined by spectrophotometer.

Real time PCR: Total RNA was extracted from liver tissue using Trizol. Primer sequences were synthesized (**Table 1**). Real-time PCR was applied to detect the target genes. The reaction consists one cycle of 52°C for 1 min, followed by 35 cycles of 90°C for 30 s, 58°C for 50 s, and 72°C for 35 s. GAPDH was used as reference, and the results were calculated $2^{-\triangle Ct}$ method.

Western blot

The cells in each group were cracked on ice for 15-30 min and ultrasonicated for 4×5 s to extract protein. After centrifuged at 10,000 g and 4°C for 15 min, the protein was moved to a new Ep tube and store at -20°C. The protein was separated by 10% SDS-PAGE electrophoresis and transferred to PVDF membrane. After blocked by 5% skim milk for 2 h, the membrane was incubated in NF-κB primary antibody at 1:1000 and 4°C overnight. Then the membrane was incubated with secondary antibody at 1:2000 for 30 min and washed by PBST. At last, the membrane was treated with chemiluminescent agent for 1 min and imaged on X-ray. Protein image processing system and Quantity one software were used for data analysis. All experiments were repeated for four times.

Statistical analysis

All the statistical analysis was performed on SPSS16.0 software. The data was presented as $\overline{x}±s$. One-way ANOVA was used for mean comparison. $P < 0.05$ was considered as statistically significance.

Results

Liver weight and liver weight index comparison

After modeling, the rats were euthanized and the liver tissue was collected. Liver wet weight was measured and liver weight index was calculated. The results showed that body weight and liver weight index in NASH group increased significantly compared with control ($P < 0.05$). PPC treatment obviously declined body weight and liver weight index compared with NASH group ($P < 0.05$) (**Table 2**).
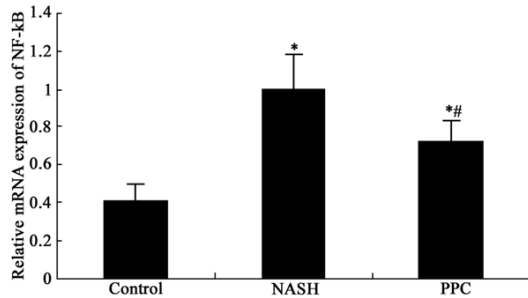
Figure 3. PPC impact on NF-κB mRNA expression in the liver tissue of NASH rat. $^*P < 0.05$, compared with control; $^\#P < 0.05$, compared with NASH group.
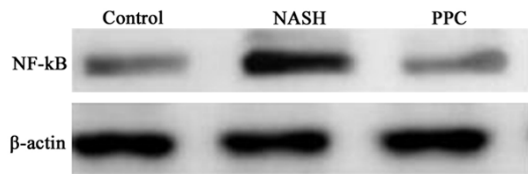

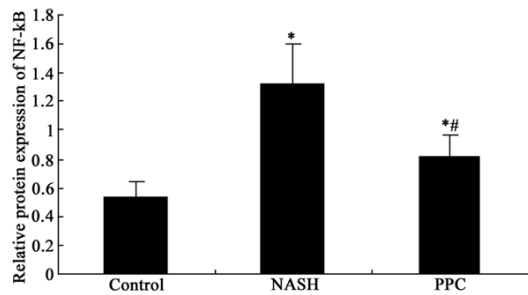**Figure 4.** PPC impact on NF-κB protein level in the liver tissue of NASH rat.

Figure 5. PPC impact on NF-κB protein level in the liver tissue of NASH rat analysis. $^*P < 0.05$, compared with control; $^\#P < 0.05$, compared with NASH group.

Liver function index comparison

Liver function injury was determined by analyzing AST, ALT, TC, and TG levels. It was revealed that ALT, AST, TG, and TC markedly enhanced in NASH group compared with control (P < 0.05). After PPC treatment, ALT, AST, TG, and TC significantly decreased compared with NASH group (P < 0.05) (**Table 3**).

PPC impact on serum inflammatory cytokines expression in NASH rat

ELISA was applied to test PPC impact on serum inflammatory cytokines expression in NASH rat. It was demonstrated that TNF-α and IL-1β secretion obviously elevated in NASH group compared with normal control (P < 0.05). PPC can apparently suppress TNF-α and IL-1β secretion compared with NASH group (P < 0.05) (**Figures 1** and **2**).

PPC impact on NF-κB mRNA expression in the liver tissue of NASH rat

Real time PCR was performed to determine PPC impact on NF-κB mRNA expression in the liver tissue of NASH rat. The results showed that NF-κB mRNA overexpressed markedly in the liver tissue of NASH rat compared with control (P < 0.05). PPC therapy obviously inhibited NF-κB mRNA expression compared with NASH group (P < 0.05) (**Figure 3**).

PPC impact on NF-κB protein level in the liver tissue of NASH rat

Western blot was used to detect PPC impact on NF-κB protein level in the liver tissue of NASH rat. The results showed that NF-κB protein upregulated markedly in the liver tissue of NASH rat compared with control (P < 0.05). PPC therapy significantly restrained NF-κB protein level compared with NASH group (P < 0.05) (**Figures 4** and **5**).

PPC impact on oxidative stress in the liver tissue of NASH rat

ROS production and SOD content in the liver tissue were tested. The results revealed that SOD production obviously increased, while SOD content reduced in NASH group compared with control (P < 0.05). PPC apparently suppress ROS production and enhanced SOD content compared with NASH group (P < 0.05) (**Table 4**).

Discussion

As a common liver inflammatory disease, NASH is featured as high incidence, chronic deferment, and refractory [16]. The pathogenesis of NASH is closely associated with obesity, hyperlipidemia, and insulin resistance [17]. NASH is generally developed from fatty liver, which is mainly caused by fat synthesis increase and fatty degeneration. It leads to insulin resistance and becomes the first strike together with intrahepatic lipid transport decrease. Later, hepatic endotoxin level elevation promotes free fatty acid metabolism in the liver, weakens antioxi-

Table 4. PPC impact on oxidative stress in the liver tissue of NASH rat

Index	Control	NASH	PPC
ROS	51.10±8.21	201.67±31.22[*]	123.18±21.22[*,#]
SOD	132.35±25.18	47.01±15.31[*]	92.21±15.41[*,#]

[*]$P < 0.05$, compared with control; [#]$P < 0.05$, compared with NASH group.

dant activity, and increases mitochondrial decoupling protein activity. Mitochondrial dysfunction accelerates oxidative stress production. Proinflammatory factor can change simple steatosis to steatohepatitis, and even liver cirrhosis, which is the second strike. Thus, oxidative stress and inflammatory factor are the main causing factors of NASH [18, 19].

Our results confirmed that NASH can elevate body weight and liver weight index, injure liver function, and enhance NF-κB expression. NF-κB expression can activate corresponding target genes, including immune receptor, adhesion molecule, inflammatory cytokines, and C-reactive protein, thus regulate immune response and amplify inflammation [20]. NASH can increase ROS content and decline SOD activity. ROS overproduction can lead to oxidative injury, while SOD is an important antioxidase to eliminate free radical. SOD plays a critical role for oxidation balance, and its activity indirectly reflects free radical elimination ability [21]. PPC is reported to play an important role in anti-inflammation and oxidative balance, whereas its effect in eliminating free radical has not been confirmed [13, 14]. This study verified that PPC intervention declined ROS content and amplified SOD activity, thus recovering oxidative balance, downregulating inflammatory factors TNF-α and IL-1β expression, and reducing NF-κB level. It can alleviate liver injury and promote liver function restoration.

In conclusion, PPC plays a therapeutic role on NASH through regulating oxidative balance, inhibiting inflammatory factors, and suppressing NF-κB signaling pathway.

Acknowledgements

Disclosure of conflict of interest

None.

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