

## The effect of Nitrochalcone on biochemical indicators and PPAR- $\alpha$ gene expression in nonalcoholic male NMRI mice steatosis model

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### Abstract

**Introduction:** Non-alcoholic fatty liver disease (NAFLD) is associated with oxidative stress, inflammation, and decrease in peroxisome proliferator-activated receptor alpha (PPAR- $\alpha$ ) expression. Nitrochalcone is effective ingredient of chalcones with anti-inflammatory, anti-cancer and anti-hyperglycemic properties. This study examined the effect of intraperitoneal (IP) administration of nitrochalcone in a mouse model with non-alcoholic steatosis.

**Materials and Methods:** In this study, 94 male NMRI mice were assigned to control and experimental groups. The Normal control group (NC) was given normal rodent diet The experimental group was subjected to high fat diet for 4 weeks, which induced NAFLD, then the experimental group was divided in to 5 in vivo subgroups (n=12 in each), High fat (HF) Sham (receiving grapes seed oil), Positive control groups (C<sup>+</sup>: receiving silymarin (80mg/kg) by intra peritoneal injection (IP)) and Experimental Nitrochalcone groups (EN1, EN2, EN3) receiving nitro chalcone (5, 10 and 20 mg/kg) by IP during 4 weeks. Protective groups received high-fat diet and Nitrochalcone 20 mg/kg simultaneously for 4 weeks. At the end of the treatments, biochemical parameters, liver enzymes, antioxidant enzymes and expression of PPAR- $\alpha$  were determined.

**Results:** The serum levels of some biochemical parameters such as cholesterol glucose, liver enzymes, and insulin significantly increased in the HF group in comparison with the control group (P < 0.001). Nitrochalcone (20 mg/ kg) decreased liver enzymes levels as compared with the HF and Sham group (P < 0.001). The highest percentage of increase in PPAR $\alpha$  gene expression was observed in EN3 group, as compared with the controls.

**Conclusion:** HF diet caused steatohepatitis through insulin resistance, impaired lipid profile, increased glucose and liver enzyme levels. Furthermore, the diet decreased antioxidants, adiponectin, leptin and PPAR $\alpha$  levels, and made fibrosis in the liver. Nitrochalcone improved this condition in a dose-dependent manner, and resulted in elevated PPAR $\alpha$  expression.

**Keywords:** NAFLD, NMRI mice, Nitrochalcone, PPAR- $\alpha$

### Introduction

Non-alcoholic fatty liver disease (NAFLD)

is a growing health problem worldwide, especially in advanced countries. NAFLD

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covers a wide range of diseases from steatosis or simple fatty liver to cirrhosis without excessive alcohol consumption. In many patients, NAFLD is associated with metabolic risk factors such as obesity, diabetes mellitus, and dyslipidemia (1). Elevated levels of serum triglyceride (TG) and reduced HDL are common in patients with NAFLD (2). Several studies have also indicated that NAFLD is associated with insulin resistance (IR) (3). In the high-fat diet model (HFD) in rodents, the NF- $\kappa$ B pathway activity increased in order to induce NAFLD, which is associated with an increase in the expression of inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6. It has typically been observed that biochemical parameters change in liver steatosis caused by NAFLD, where the levels alanine aminotransferase (ALT) and aspartate aminotransferase (AST) rise (4). Chalcone compounds are considered as one of the important classes of natural products belonging to the flavonoids family and are reported to have important therapeutic activities including antimicrobial, antioxidant, anti-cancer, and powerful antihyperglycemic activity (5). Chalcone's carbon nitration nitrate leads to the production of nitrochalcone and enhances its anti-inflammatory properties (6). It has been shown that nitrochalcones have mild anti-inflammatory properties and inhibitory effect on free radicals (7). PPAR- $\alpha$  is an intra-nuclear receptor that interferes with the regulation of metabolism and fatty acid storage (FA), as many genes of FA metabolism are regulated by PPAR- $\alpha$  in humans and rats. On the other hand, PPAR- $\alpha$  is a liver beta-oxidation regulator (mitochondrial and peroxisome). High levels of PPAR- $\alpha$  expression have been found in the liver and especially among the parenchymal cell population. Basically, PPAR- $\alpha$  is known for its ability to induce oxidation of fatty acids. A growing body of studies have found PPAR- $\alpha$  to be involved in the regulation of lipogenesis (8). The expression of PPAR- $\alpha$  decreases when a

heavy-fat diet is consumed (9). In this study, we investigated the effect of nitrochalcone on the biochemical and histological characteristics as well as PPAR- $\alpha$  gene expression on non-alcoholic fatty liver disease induced by high-fat diet in NMRI male mice.

## Materials and Methods

### Materials

Nitrochalcone was obtained from Sigma-Aldrich (St. Louis, MO, USA). Commercial kits used for the assessment of high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), total cholesterol, triglycerides (TG), glucose, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline amino transferase (ALP) were purchased from ZistChimi Chemical Company, Tehran, Iran. Serum adiponectin leptin and insulin measurement kits (Yanaihara Institute Inc. Catalog No.: YII-YK052-EX, JAPAN) were used for assaying adiponectin, leptin, and insulin levels. The homeostasis model assessment (HOMA) formula was used as insulin resistance index:  $\text{HOMA} = \text{fasting serum insulin (mU/L)} \times \text{fasting plasma glucose (mM)} / 22.5$  (3).

### High-Fat Emulsion Preparation

High-fat emulsion diet was prepared from Cholesterol (> 95%, SIGMA ALDRICH), Tween 80, sucrose, sodium deoxycholate and propylene glycol (SIGMA), total milk powder (Aptamil), multivitamin (IRAN), and carbohydrates according to a previous study (10).

### Animals and Experimental Protocol

Male NMRI mice (25-30 g) were obtained from Razi Vaccine and Serum Institute, Karaj, Iran, and kept under standard conditions (12-h light-dark cycle with

temperature  $22 \pm 1$  °C). They had ad-libitum access to standard pellet and water. After one week of adaptation in the laboratory, animals were weighed and divided into the following groups (n=12 in each). Sham group which received high-fat emulsion for 4 weeks then was shifted to normal diet with grape seed oil (nitrochalcone solvent) by intraperitoneal injection (IP) for 4 weeks. Experimental Nitrochalcone group 1 (EN1) received high-fat emulsion for 4 weeks and was shifted to normal diet with nitrochalcone (5 mg/kg, solved in grape seed oil) by intraperitoneal injection (IP) for 4 weeks. EN2 group received high-fat emulsion for 4 weeks and shifted to normal diet with nitrochalcone (10 mg/kg, solved in grape seed oil) by intraperitoneal injection (IP) for 4 weeks. EN3 group received high-fat emulsion for 4 weeks and was shifted to normal diet with nitrochalcone (20 mg/kg, solved in grape seed oil) by intraperitoneal injection (IP) for 4 weeks. Protective group (PN) received high-fat emulsion and nitrochalcone 20 mg/kg solved in grape seed oil simultaneously for 4 weeks. In order to reduce the number of animals used in the study, we compared the results with a control group and a high-fat-diet receiving group used in our previous study (10), as well as a positive control (which received 80 mg/kg silymarin instead of nitrochalcone).

During the experimental period, all animals were weighed every week. The experimental protocol was performed in accordance with the guidelines of international organization of the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996). Further, the Animal Ethics Committee of the university approved it.

#### RNA Isolation and cDNA Synthesis

Total RNA was isolated from whole mice liver tissue using a commercial RNA isolation kit (Roche Diagnostics, GmbH Roche Applied Science 68298, Mannheim, Germany). The cells were suspended in

200µl PBS, to which 400µl Lysis/ Binding Buffer was added and mixed for 15 seconds. The purified RNA could be used directly in RT-PCR or stored at  $-80^{\circ}\text{C}$  for later analysis. After transferring it to a high pure filter tube, centrifugation was done for 15 seconds at  $8,000 \times g$ . Flowthrough was discarded and 100µl DNase was added, incubating the mixture for 15 minutes at  $25^{\circ}\text{C}$ . Washing buffer was used twice, followed by centrifugation, and RNA was eluted by 100µl Elution Buffer. The purified RNA can be used directly in RT-PCR or stored at  $-80^{\circ}\text{C}$  for later analysis. The primer sequences for PPAR  $\alpha$  and HPRT (used as housekeeping gene) were obtained from Yekta tajhize Azma website. Specific primers were designed by the primer express program (10).

#### Statistical Analysis

Data were expressed as Mean  $\pm$  standard error of the mean (SEM) and the differences was determined by ANOVA and Tukey's post-test as offered by SPSS statistical software (version 22).  $P < 0.05$  was considered significant.

#### Results

##### Body Weight

After 4 weeks, mice weights of different groups were significantly different compared with the control group (Table 1). Among the experimental groups, the body weight of EN2 (Experimental Nitrochalcone 10 mg/kg), EN3 (Experimental Nitrochalcone 20 mg/kg) and PN (Protective group; Nitro chalcone 20 mg/kg with high fat diet simultaneously) were significantly reduced in comparison to HF ( $P < 0.001$ ). Furthermore, the body weight in EN3 group was significantly lower than that of EN1 and EN2 groups ( $P < 0.001$ ). These results suggested that the highest dose of nitrochalcone used is most efficient in body weight reduction of HF group.

**Table1.** Comparison of the body weight of the mice at the end of the prescribed treatments.

Groups	Normal control	High fat	Positive control	Sham	EN1	EN2	EN3	Protective
Initial weight (g)	27 ± 1	24 ± 0.5	25 ± 0.5	27 ± 0.7	26 ± 0.4	27 ± 0.3	25 ± 0.1	26 ± 0.2
Final weight (g)	40.5 ± 0.4	49.5 ± 0.3 <sup>a</sup>	37 ± 0.6 <sup>b</sup>	40 ± 0.3 <sup>b</sup>	37 ± 0.2 <sup>b</sup>	37 ± 0.2 <sup>b</sup>	35 ± 0.3 <sup>b</sup>	38 ± 0.3 <sup>b</sup>

EN1, EN2 and EN3: Experimental Nitrochalcone groups 1, 2 and 3 which received nitro chalcone 5, 10 and 20 mg/kg, respectively, for 4 weeks.

<sup>a</sup> P < 0.001 compared with normal control group

<sup>b</sup> P < 0.001 compared with high fat group

## Biochemical Parameters

Biochemical parameters measured including cholesterol, glucose, liver enzymes were measured. The serum levels of cholesterol, TG, LDL, very low-density lipoprotein (VLDL), total lipid, phospholipids, and liver enzymes in the HF group increase in comparison with the control group while a significant reduction was observed in cholesterol, TG, and phospholipid levels of experimental groups in comparison to HF and Sham groups (P < 0.001). The HDL-C levels were reduced in the HF group and increased in EN3 group compared with HF and Sham groups (P < 0.001).

Malondialdehyde (MDA) as an oxidative stress biomarker and total protein in the fatty group showed a significant increase compared to the control group (P < 0.001). On the other hand, levels of superoxide dismutase (SOD), ferric reducing antioxidant power (FRAP) and catalase in HF group had a significant reduction compared with the control (P < 0.001).

Treatment with nitrochalcone (dissolved in grape seed oil) for 4 weeks resulted in changes in biochemical parameters compared to HF group. In this regard, cholesterol TG, VLDL, the hepatic enzymes, and MDA levels decreased in the groups EN1, EN2, EN3, PN and sham compared to the HF group (P < 0.001 in experimental groups and P<0.05 in the sham group). There was also a significant decline in these parameters in EN2 and EN3 groups compared to the sham group (P < 0.05) and (P < 0.001) respectively.

The EN3 group indicated a significant decrease compared to the groups EN1 and EN2 (P < 0.001).

Further, HDL levels rose significantly in the sham group (P<0.05), as well as in EN3 and PN groups (P < 0.001).

Levels of LDL, phospholipid, total lipid, Fasting Blood Sugar (Fbs) insulin, homeostatic model assessment (HOMA) in sham group decreased significantly (P < 0.05) compared to HF group, and in EN3 and PN groups compared to HF (P < 0.001). In the EN3 group, there was a significant decrease (P < 0.001) compared to the Sham group.

Tumor necrosis factor alpha (TNF- $\alpha$ ) levels increased in the HF group, as compared with the Control group. However, it significantly decreased in the Nitrochalcone treated group (EN3), as compared with the HF and Sham groups (P < 0.001)

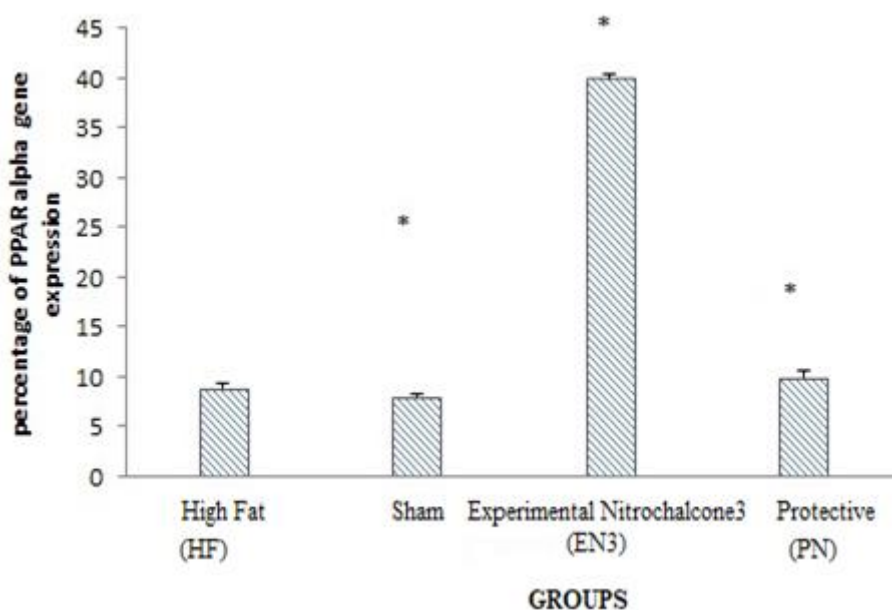
Adiponectin levels increased and leptin levels decreased significantly in the EN3 group as compared with the HF and Sham groups (P < 0.001).

## PPAR- $\alpha$ Expression

PPAR- $\alpha$  gene expression percentages were evaluated in the liver tissue (Figure1). These percentages significantly decreased in HF and sham groups compared with the control (P < 0.001). The changes in the percentage of gene expression in these groups are represented in Figures 1, in comparison to the control and HF groups. The statistical results of the real-time PCR method revealed that treatment of mice with nitrochalcone 20 mg/kg caused

elevated percentage of the PPAR- $\alpha$  gene expression, where in the EN3 and PN groups, the percentage of the expression

was significantly increased compared to the HF group ( $P < 0.001$ ).



**Figure 1.** Comparison of PPAR- $\alpha$  gene expression between HF, Sham and EN3 groups and the PN group. \* $P < 0.001$  compared with the Normal control group.

## Discussion

In this study, using high-fat diet for four weeks, NAFLD was induced in NMRI mice, after what the animals received IP injection of nitrochalcone for four weeks. The effect of this compound on NAFLD disease was determined by several parameters including body weight, lipid profiles insulin, adiponectin, antioxidant enzyme levels, and PPAR- $\alpha$  gene expression.

High-fat diet results in a significant increase in the weight of mice in comparison with the control group with concomitant rises in serum levels of cholesterol, triglyceride, LDL, VLDL, glucose, phospholipid, total lipid, total protein, as well as elevated serum levels of liver enzymes (ALT, AST, ALP), and MDA (10). In general, these changes indicate damage to the liver cells. The increase in the levels of TNF- $\alpha$  is also associated with NAFLD, while TNF- $\alpha$  plays an important role in promoting

hepatic steatosis (11). Decrease in adiponectin levels and increase of leptin levels are also occurring in patients with fatty liver (12).

The excessive supply of lipids to mitochondria promotes beta-oxidation reduction and increases ROS production (2). Excess fatty acids result in high levels of  $\beta$ -oxidation, production of reactive oxygen species (ROS) such as radical superoxide [ $O^{0-}_2$ ] and hydrogen peroxide [ $H_2O_2$ ] in the mitochondrial respiratory tract, along with induction of necrosis. Actually, overeating can lead to the accumulation of excess fatty acids in the liver, inducing high  $\beta$ -oxidation and production of ROS. On the other hand, under NAFLD conditions, levels of antioxidant enzymes such as SOD, FRAP and catalase also diminished (1). According to previous studies, during excessive fat intake, the expression of PPAR- $\alpha$  significantly drops. PPAR- $\alpha$  is a ligand activating variant involved in regulating a spectrum of processes as well

as a range of inflammation and immunity responses to food metabolism and energy homeostasis. In abnormal expression of PPAR- $\alpha$  in the liver, transcription of the target gene is impaired, and excessive fatty acids usually derived from lipolysis are released to the liver of obese individuals, which tend to accumulate more triglyceride-rich proteins (9).

A study observed that PPAR- $\alpha$  moderates the replication of genes involved in inflammatory response pathways. This nuclear receptor can modulate anti-inflammatory activity by regulating the activator-1 signaling pathway and nuclear kB factor via a direct protein-protein reaction. Additionally, it has been shown that PPAR- $\alpha$  activity weakens or suppresses several mediators involved in vascular injury such as lipotoxicity, inflammation, free oxygen species (ROS), angiogenesis, and thrombosis (13).

Studies have demonstrated that silymarin is effective in developing a positive clinical response and treating non-alcoholic steatohepatitis. Silymarin has a significant antioxidant effect, reducing free radicals and liver enzymes (14). In the mice model of NAFLD, the IP injection of silymarin for four weeks attenuates the effects of a high fat diet (10).

In the present study, to enable a better assessment of the compound effect, nitrochalcone solvent effect was also examined on NAFLD disease. Grape seed oil is rich in unsaturated fatty acids and several studies have reported its hepatoprotective activity (15). In the current study, ip administration of grape seed oil for four weeks reduced significantly ( $P < 0.05$ ) parameters such as cholesterol, TG, LDL, VLDL, phospholipid, leptin, insulin FBS, MDA, and total protein. It also showed a significant increase ( $P < 0.05$ ) in HDL, SOD and FRAP levels compared to high fatty groups. Animals receiving high-fat diets revealed the moderating effects of this substance on changes caused by NAFLD. These results are consistent with

previous studies, however, we found that nitrochalcone, especially when used at higher doses has a better overall effect on biochemical parameters an PPAR- $\alpha$  gene expression compared with both silymarin and grape seed oil.

Weight of animals after administration of nitrochalcone for 4 weeks at doses (5, 10 and 20 mg/kg) showed a significant decrease compared to the high fat group. Previous studies reported that the use of chalcones leads to weight loss, and thus chalcone derivatives can be used as a basic structure for the design of drugs to treat obesity (16).

Upon nitrochalcone administration, TG, VLDL, and liver enzymes (ALT, ALP, AST, MDA) showed a significant decrease compared to the high-fat and sham group, while the higher dose of nitrochalcone was more effective. Chalcones can have ALT and AST inhibitory effects, lowering these two enzymes and inhibiting necrosis of liver cells (17). In addition, it has been reported that chalcone derivatives are able to reduce the MDA levels which increases due to high-fat diets (18). Furthermore, the 20 mg/kg dose of nitrochalcone caused a significant decrease in TNF- $\alpha$  levels compared to the high-fat and sham groups (grape seed oil recipient). Previous studies have indicated that nitrochalcone has a strong anti-inflammatory property (7). The anti-inflammatory effects of flavonoids such as chalcone and its derivatives are mainly attributed to NF-kB pathway inhibition. The activation of the NF-kB route has been observed in many animal models affected by NAFLD (19). In addition, chalcones also inhibit regulatory enzymes such as protein kinases that induce inflammatory responses. The findings of this study are consistent with previous studies regarding the anti-inflammatory effects and TNF- $\alpha$  antagonism by Chalcone (20). Flavonoids, to whom chalcones are precursor, have a suppressive activity on free radicals. They can enhance both intracellular antioxidant defense and the expression of SOD and

catalase, which are considered important antioxidant enzymes in mitochondria, finally leading to reduced oxidative stress (21).

Accordingly, there was a significant difference between the group receiving nitrochalcone at 20 mg/kg and high-fat and sham groups in terms of levels of SOD and catalase enzymes.

Serum adiponectin levels had also significantly increased after receiving nitrochalcone, and remarkably so in the 20 mg/kg dose, compared with the high-fat and sham groups. Previous studies have suggested that chalcone and its derivatives can control hyperglycemia by increasing the levels of adiponectin and improving glucose metabolism (22). On the other hand, in this study, nitrochalcone (20 mg / kg) affected adiponectin and leptin levels, where adiponectin levels showed a negative correlation with glucose, leptin and lipid profiles in the high-fat group and nitrochalcone group recipients, which is consistent with previous studies (18). A large number of studies have suggested that chalcones and their derivatives stimulate the expression of PPAR- $\alpha$  gene and enhance the expression of this gene. PPAR- $\alpha$  has the highest expression in the liver and is involved in the oxidation of fatty acids. Chalcones increase

phosphorylation of AMP-activating protein kinase in the liver, which results in an augmented regulation of the PPAR- $\alpha$  gene (23). The results in Figure 1 indicate a significant increase in the percentage of PPAR- $\alpha$  expression in the nitrochalcone receiving group at a dose of 20 mg/kg compared to the high-fat group, which is congruent with previous studies.

## Conclusion

Considering the previous studies and the findings of this study, it can be concluded that nitrochalcone, especially with a dose of 20 mg/kg, with its antihyperlipidemic, anti-inflammatory and anti-oxidant properties, can improve liver function under NAFLD conditions by enhancing the expression of PPAR- $\alpha$  gene, as well as modifying the negative biochemical effects of the NAFLD.

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## Conflict of Interest

The authors declare that they have no conflict of interest.

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