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Original Research Article

Effect of diosmetin on young rats with high-fat diet-induced non-alcoholic fatty liver disease

Guoying Zhang*, Yuewu Yan, Xujiao Feng
Department of Infectious Diseases, Heping Hospital Affiliated to Changzhi Medical College, Changzhi 046000, China

For correspondence:* ***Email:* *ytwus9@163.com*

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Abstract

Purpose: *To determine the effect of diosmetin on young, non-alcoholic fatty liver disease (NAFLD) rats.*
Methods: *Five groups of SD rats were used: control group, high-fat diet group, low-dose diosmetin group, medium-dose diosmetin group, and high-dose diosmetin group, each with 10 rats. After 3 months, interleukin 6 (IL-6), IL-1β) and TNF-α) were assayed. Protein expressions of p-AMPKα, CPT-1 and PPAR-α, AMPKα, SREBP-1c and FAS were assayed.*
Results: *In the high-fat diet group, the levels of p-AMPKα, CPT-1 and PPAR-α were lower than the corresponding control values, while p-AMPKα, CPT-1 and PPAR-α levels were dose-dependently higher in all diosmetin groups than in NAFLD group ($p < 0.05$). There were higher levels of SREBP-1c and FAS in the high-fat diet group than in control group, while SREBP-1c and FAS levels in all diosmetin groups were dose-dependently lower than the corresponding levels in NAFLD group. Serum IL-6, IL-1β and TNF-α levels in NAFLD group were raised, relative to control values ($p < 0.05$).*
Conclusion: *Diosmetin alleviates NAFLD lesions induced by high-fat diet, slows down liver cell apoptosis, and inhibits inflammation via activation of AMPK pathway. Thus, diosmetin has potentials for use in the repair of hepatic damage induced by high-fat diet.*

Keywords: *Diosmetin, High-fat diet, Nonalcoholic fatty liver disease, NAFLD*

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INTRODUCTION

It is known that NAFLD is a clinicopathological syndrome characterized by excessive fat deposition in liver cells due to alcohol and other liver-damaging factors, and it is an acquired acute liver injury [1]. With improvements in living standards and changes in diet and lifestyle in recent years, obesity and its related metabolic syndrome have become a global epidemic. It has been reported that NAFLD is a predisposing factor for chronic liver disease worldwide, with serious and adverse impact on human life and health [2]. Diosmetin is an aglycone of the flavonoid glycoside geraniol. Studies have revealed that diosmetin reduces levels of total cholesterol (TC), low-density lipoprotein (LDL-C) and triglycerides (TGs) [3]. In recent years, it has been found that diosmetin also exerts a variety of pharmacological effects such as anti-tumor, anti-oxidant, anti-inflammatory and antibacterial properties. However, not much is known about the anti-NALFD effect of diosmetin [4]. The aim of this research was to study the anti-NAFLD

effect of diosmetin in young rats with high-fat diet-induced NAFLD.

EXPERIMENTAL

Animals

Sprague-Dawley (SD) rats aged 3.15 ± 0.25 days (mean body weight = 12.26 ± 2.12 g) were obtained from Hebei Experimental Animal Center (certificate number: 1306107). The rats were kept in a room with average temperature of 22 – 24 ºC, relative humidity of 40 - 60 % and noise level ≤ 45 dB. The rats were given normal drinking water, and were fasted for 12 h before the experiment. This animal research was approved by the Animal Ethical Committee of Heping Hospital Affiliated to Changzhi Medical College (approval no. 201937452) and was conducted according to the guidelines of Principles of Laboratory Animal Care [5].

Reagents

Diosmetin was provided by Shanghai Yuanye Bio-Technology Co. Ltd. Enzyme-linked immunosorbent assay kits were produced by Wuhan Proteinab Biotech Co. Ltd. Hematoxylin and eosin were obtained from Shanghai Zhenyu Biological Technology Co. Ltd. Terminal deoxynucleotidyl transferase-mediated dUTP nick labeling (TUNEL) apoptosis detection kit was obtained from Wuhan Yipu Biotechnology Co. Ltd. The antibodies (anti-AMPKα, anti-p-AMPKα, anti-CPT-1, anti-PPAR-α, anti-SREBP-1c and anti-FAS) were products of Wuhan Yipu Biotechnology Co. Ltd, China.

Animal groups and model establishment

Five (5) groups of 10 young rats were used: control group, high-fat diet group, low-dose diosmetin group, middle-dose diosmetin group, and high-dose diosmetin group. Rats in control group received standard feed. Rats in high-fat diet group were fed a diet composed of standard feed (73%), lard (20%), sucrose (4%), milk (2%) and cholesterol (1%). Animals in low-, medium-, and high-dose diosmetin groups were raised on a high-fat diet containing diosmetin at doses of 10, 20, and 40 mg/kg, respectively. After 3 months of treatments, follow-up experiments were conducted.

Biochemical assays and histology

Blood sample (1 mL) was taken from the tail vein of each young rat after 3 months. After standing for 20 min at laboratory temperature, the blood was subjected to centrifugation to obtain serum which was kept refrigerated at -70ºC prior to analysis. Freezing and thawing were avoided. Serum concentrations of TC, TGs, LDL-C, HDL-C, IL-6, IL-1 β and TNF-α in each group of young rats were measured with ELISA.

Histology

Rat liver tissue was fixed in 4 % paraformaldehyde for 24 h at 4ºC, washed thrice with PBS, and dehydrated in alcohol gradient (30, 50 and 70 % alcohol) for 10 min. Tissue blocks were prepared and dehydrated in a dehydrator, and then embedded in paraffin and sectioned using a microtome. Then, H & E staining was carried out, and the liver tissue lesions were examined under a light microscope.

TUNEL staining

The paraffin sections of rats in each group were sequentially deparaffinized with xylene and hydrated with gradient ethanol, and then reacted with proteinase K at 20 - 37°C for 15 - 30 min, followed by rinsing thrice with PBS and incubation with 3 % H_2O_2 for 20 min at room temperature. After washing thrice with PBS, the tissue sections were subjected to biotin labeling and color development. Re-staining was done with hematoxylin, followed by dehydration in gradient ethanol, and clearing in xylene. The slices were sealed with neutral gum, examined under a microscope, and photographed. Cells with brown granules in the nucleus were apoptotic cells.

Western blotting

The tissue cells to be tested were washed 3 times with PBS. Total protein was extracted from the cells using TRIzol reagent containing protease inhibitor. Protein denaturation was done at 100°C for 5 min. The proteins were resolved using SDS-PAGE, followed by transfer to PVDF membranes which were then blocked by incubation with 5% BSA for 1 h. Thereafter, incubation of the membranes with appropriate primary antibodies was done overnight at 4 ºC, followed by washing and incubation with horseradish peroxidase-labeled secondary antibody at room temperature for 1.5 h. Grayscale values were calculated to determine the relative expression levels of the various genes.

Statistics

Measurement data are presented as mean ± SD. Two-group comparison was done with *t*-test. Counting data are presented as percentage (%),

and group comparison was done with χ^2 test. Values of $p < 0.05$ were taken as indicative of statistically significant differences.

RESULTS

Liver tissue lesions and apoptosis of young rats

Results of H & E staining revealed that the liver tissue cells in the control group were highly arranged and the cell morphology was relatively regular, with no obvious tissue inflammatory cell infiltration. In contrast, liver tissue cells of rats in the NAFLD group were arranged disorderly, severe necrosis was evident, and steatosis was present. Moreover, there was a large degree of inflammatory cell infiltration. In the low-dose diosmetin group, the liver tissue cells were arranged disorderly, and there were evidence of liver cell necrosis, fatty degeneration and inflammatory cell infiltration. In the middle-dose diosmetin group, the features seen were disorderly arrangement of hepatocytes, necrotic hepatocytes, and vesicular steatosis, while inflammatory cell infiltration was reduced. Liver tissue cells in the high-dose group were tightly arranged and the cell morphology was relatively regular. TUNEL staining showed higher level of hepatocyte apoptosis in the high-fat diet group than in the control group, while the extent of hepatocyte apoptosis in low, medium, and high-dose diosmetin groups were dose-dependently decreased, relative to NAFLD group ($p < 0.05$). These observations are summarized in Figure 1.

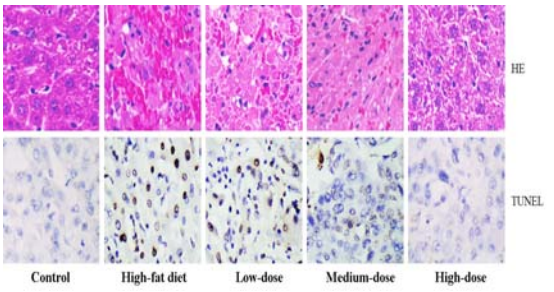

Figure 1: Effect of diosmetin on liver tissue lesions and apoptosis of NAFLD rats

Effect of diosmetin on blood lipid levels of NAFLD rats

Table 1 shows that serum TC, TGs, and LDL-C levels of young rats in NAFLD group were raised, relative to control group, while HDL-C levels were significantly and dose-dependently decreased, relative to control value. However, serum TC, TGs and LDL-C concentrations in low, medium, and high-dose diosmetin-treated rats were lower than the corresponding levels in the high-fat diet group, while HDL-C concentration was significantly and dose-dependently elevated, relative to the NAFLD group ($p < 0.05$).

Table 1: Blood lipid levels of young rats

Group	TC	TG	LDL-C	HDL-C
Control	2.46 ± 0.41	1.21 ± 0.08	2.68 ± 0.28	2.64 ± 0.62
High-fat diet	11.32 ± 1.26[a]	5.41 ± 0.48[a]	16.37 ± 2.41[a]	0.72 ± 0.16[a]
Low-dose	8.72 ± 1.01[ab]	4.65 ± 0.39[ab]	12.38 ± 1.69[ab]	1.21 ± 0.19[ab]
Medium-dose	7.31 ± 0.85[abc]	3.41 ± 0.25[abc]	8.64 ± 1.28[abc]	1.64 ± 0.24[abc]
High-dose	4.51 ± 0.56[abcd]	2.41 ± 0.12[abcd]	4.57 ± 1.05[abcd]	2.41 ± 0.28[abcd]

[a]$P < 0.05$, vs control; [b]$p < 0.05$, vs NAFLD group; [c]$p < 0.05$, vs low-dose diosmetin group; [d]$p < 0.05$, vs middle-dose diosmetin group

Effect of diosmetin on expressions of proteins related to lipid catabolism

Figure 2 shows that the levels of p-AMPKα, CPT-1 and PPAR-α were lower in NAFLD group than in control rats. However, p-AMPKα, CPT-1 and PPAR-α levels in low, medium and high-dose diosmetin-treated rats were markedly and dose-dependently higher than those in the NAFLD group ($p < 0.05$). No statistically significant differences were seen in AMPKα levels of rats amongst the groups.

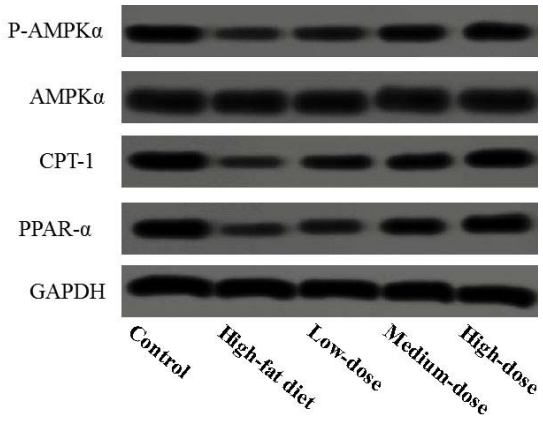

Figure 2: Effect of diosmetin on levels of proteins related to lipid metabolism

Effect of diosmetin on expressions of proteins related to lipid anabolism

Figure 3 shows that SREBP-1c and FAS were upregulated in DAFLD rats, when compared with control rats, while SREBP-1c and FAS levels in diosmetin groups were significantly and dose-

dependently reduced, relative to the NAFLD group.

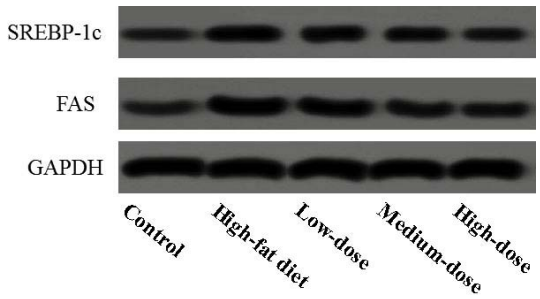

Figure 3: Effect of diosmetin on levels of proteins related to lipid metabolism

Effect of diosmetin on levels of inflammatory factors

Serum levels of proinflammatory factors in the young rats in NAFLD group were higher than those in control group, while their levels in the low, medium, and high-dose diosmetin groups were significantly and dose-dependently lower than those in the high-fat diet group ($p < 0.05$). These results are shown in Table 2.

Table 2: Effect of diosmetin on levels of serum inflammation factors

Control	IL-6	IL-1β	TNF-α
High-fat diet	3.49 ± 0.89	45.38 ± 5.69	24.68 ± 8.31
Low-dose	36.94 ± 5.34[a]	158.64 ± 20.64[a]	86.37 ± 30.49[a]
Medium-dose	26.89 ± 4.31[ab]	116.37 ± 16.34[ab]	72.94 ± 23.68[ab]
High-dose	14.92 ± 2.85[abc]	92.64 ± 13.54[abc]	41.95 ± 20.17[abc]
Control	8.62 ± 1.24[abcd]	60.38 ± 8.64[abcd]	32.95 ± 11.54[abcd]

[a]$P < 0.05$, vs control; [b]$p < 0.05$, vs NAFLD group; [c]$p < 0.05$, vs low-dose diosmetin group; [d]$p < 0.05$, vs middle-dose group

DISCUSSION

The clinical features of NAFLD, a chronic liver ailment, are steatosis, fatty infiltration, steatohepatitis, hepatic fibrosis, cirrhosis and hepatoma, leading to adverse impact on the life and health of patients. The incidence of NAFLD is closely associated with overweight and obesity. Thus, with the prevalence of obesity in China, the incidence of NAFLD is increasing year by year, and it has attracted a lot of attention from medical scholars at home and abroad [6-8]. At present, clinicians use changes in lifestyle, drugs and surgery for reducing blood lipids of NAFLD patients so as to protect their liver cells [9]. The drugs used for treating NAFLD patients are statins, cholanoic acid and pentoxifylline, although these drugs give rise to lots of side effects and cause great pain to patients [10].

In recent years, studies have found that plant extracts play a variety of positive roles in various diseases, the most obvious of which is their lipid-lowering effects [11]. Diosmetin exerts antioxidant, antimicrobial, anti-shock, anti-mutagenic and anti-allergic effects. Recent studies have shown that diosmetin is effective in the treatment of NAFLD, but the related mode of action has not been clearly elucidated [12]. In this study, H & E staining revealed that the liver tissue cells of rats in the control group had no obvious abnormalities. In contrast, liver cells of the rats in NAFLD rats were disorderly arranged, with evidence of severe necrosis, steatosis and large amount of inflammatory cell infiltration. The liver lesions in the diosmetin groups were relieved, relative to those in NAFLD group, and the effect was dose-dependent. Results from TUNEL staining showed that percentage cell apoptosis of rats in NAFLD group was increased, relative to control value, while percentage cell apoptosis values in the diosmetin groups were dose-dependently decreased, relative to the NAFLD group. These results suggest that diosmetin effectively reduces NAFLD lesions induced by high-fat diet and slows down liver cell apoptosis.

A large number of studies have confirmed that diosmetin regulates lipid metabolism [13]. The study by Tang *et al* showed that diosmetin regulated inflammatory response in acute nephrotic damage [14]. In the present study, changes in the levels of TC, TG, LD-C, and HDL-C were used to assess lipid status, while inflammatory factor levels were used to evaluate inflammatory responses in the rats. It was found that the serum TC, TGs, LD-C, IL-6, IL-1β and TNF-α of the young rats in the high-fat diet group were elevated, relative to those of control group, while HDL-C level was markedly low, when compared to control value. Moreover, serum TC, TGs, LD-C, IL-6, IL-1β and TNF-α levels in the diosmetin groups were dose-dependently lower than those in the high-fat diet group, while their HDL-C levels were significantly and dose-dependently higher than that of the high-fat diet group. These results suggest that diosmetin may effectively reduce inflammatory responses in patients with NAFLD and regulate abnormal lipid metabolism.

During tissue ischemia, hypoxia or exercise, the AMPK pathway is activated and it exerts a kinase activity. The proteins related to lipid metabolism

are AMPKα, CPT-1 and PPAR-α, SREBP-1c and FAS. Changes in levels of lipid metabolism-related proteins reflect the body's lipid metabolic state. Phosphorylation of AMPKα converts it to its active form p-AMPKα [15]. Studies have shown that p-AMPKα is related to carbohydrate and lipid metabolism [16]. The levels of p-AMPKα, CPT-1 and PPAR-α in the high-fat diet group were lower than the corresponding control values, while SREBP-1c and FAS levels were raised, relative to control group. The p-AMPKα, CPT-1 and PPAR-α levels of young rats in the diosmetin groups were dose-dependently raised, relative to the NAFLD group. Moreover, SREBP-1c and FAS levels were lower than their corresponding levels in the NAFLD group. These data suggest that the effect of diosmetin on high fat diet-induced NAFLD may be associated to stimulation of AMPK route.

CONCLUSION

Diosmetin slows down liver cell apoptosis, regulates abnormal lipid metabolism, inhibits inflammation and reduces lesions associated with high fat diet-induced NAFLD in rats through a mechanism involving activation of AMPK pathway. Thus, diosgenin has the potential to mitigate liver injury induced by high-fat diet in humans.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

This study was done by the authors named in this article, and the authors accept all liabilities resulting from claims which relate to this article and its contents. The study was conceived and designed by Guoying Zhang; Guoying Zhang, Yuewu Yan, Xujiao Feng collected and analyzed the data, while Guoying Zhang wrote the text. All authors read and approved the manuscript for publication.

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