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Protective effects of sulforaphane and aerobic exercise on acute alcoholic hepatic injury in mice

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ABSTRACT

Objective: The paper intends to study the protective effects of sulforaphane (SF) on acute alcoholic hepatic injury in mice by intragastric administration of SF, aerobic exercise and the approach of SF integrated with aerobic exercise.
Methodology: 60 NIH mice were randomly divided into 6 groups of equal number according to their body weight and were intragastrically administrated with 50% ethanol. The serum and liver indexes of each group of mice were detected, and the liver was stained with oil red O for pathological examination.
Results: Compared with the model group, the serum TG and the ratio of liver to body weight of the model mice that suffered from acute alcoholic hepatic injury could be significantly decreased in the group that practiced aerobic exercise, the group administered with SF, and the group treated with the approach of SF integrated with aerobic exercise ($P < 0.05$). The contents of TG and MDA in liver could be significantly decreased ($P < 0.05$) and SOD activity could be significantly increased ($P < 0.05$) both in the group administered with SF and the group treated with the approach of SF integrated with aerobic exercise. Serum VLDL ($P < 0.05$) could also be significantly reduced in the group treated with the approach of SF integrated with aerobic exercise.
Conclusion: Both SF and aerobic exercise could alleviate alcohol-induced acute alcoholic hepatic injury in mice possibly thanks to the working mechanism related to antioxidant stress that reduced the harm posed by alcohol on hepatic cells. In addition, the protective effect of SF on acute alcoholic hepatic injury in mice was stronger than that of aerobic exercise, while the approach of SF integrated with aerobic exercise had the strongest protective effect on acute alcoholic hepatic injury in mice.

1. Introduction

Alcoholic liver disease (ALD) is a disease caused by long-term heavy drinking and leads to hepatic injury, which comprises three most widely recognised forms including alcoholic fatty liver (steatosis), acute alcoholic hepatitis, and alcoholic cirrhosis (Kikuchi et al., 2015). In addition to heavy drinking, obesity or overweight can also increase the incidence of ALD. Therefore, in recent years, the incidence of ALD remains high. Studies have demonstrated that the hepatic parameters of alanine transaminase (ALT), aspartate aminotransferase (AST), total cholesterol (TC), triglyceride (TG), very low-density lipoprotein (VLDL), and malondialdehyde (MDA, a marker of lipid peroxides) are increased, while the activities of antioxidant enzymes such as superoxide dismutase (SOD) are inhibited in ALD patients (Song et al., 2018). Acute alcoholic hepatic injury is an acute form of alcohol-induced hepatic injury that usually occurs if one drinks large amounts of alcohol for a long time. It is a common form of ALD. At present, most of the medications commonly used in the clinical treatment of acute alcoholic hepatic injury still remain controversial in their efficacy, including glucocorticoids, smectate, pentoxifylline, metadoxine and enri (Du, 2006; Gao et al., 2017a). Users of these medications often encounter complications of random adverse reactions and severe side effects (Qu et al., 2014; Gao et al., 2017b; Shipley et al., 2019). Therefore, it is of utmost importance to take proactive steps to research and develop medications for acute alcoholic hepatic injury.

Sulforaphane (SF) [1-Isothiocyanato-4-(methylsulfinyl)butane] is an isothiocyanate, which is generated from the hydrolysis reac-

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tion between glucoraphanin (4-methylsulfinylbutyl glucosinolate) and myrosinase when cruciferous plants such as broccoli, cauliflower, cabbage, kale and mustard are physically damaged by chopping, grinding or chewing. So far, SF has been recognized as one of the natural active components of plants that have the strongest anticancer activity. There have been extensive studies that focus on SF's strong anti-cancer, anti-oxidative, anti-inflammatory and other biological activities, and the protective effects of SF on the liver have also attracted much attention in recent years. In a preliminary study, Chen et al. (2016) suggested that SF could inhibit the activation of hepatic macrophages, reduce hepatic injury and prevent liver tumors. The study of Axelsson et al. (2017) showed that SF could reduce generation of hepatic glucose and improve control of blood glucose in type 2 diabetic patients. Oguz et al. (2015) found that SF could reduce liver oxidative stress caused by ischemia/reperfusion injury through rat experiments. In addition, Kikuchi et al. (2015) suggested that SF-rich broccoli extracts might prove to be quite effective in improving liver function by reducing oxidative stress. However, to the best of our knowledge, there have been no studies or reports on the protective effects of SF on ALD as of now.

In addition, studies have shown that practicing low-to-intermediate intensity aerobic exercise in the long-term can enhance immune function, and can effectively improve recovery from hepatic injury, reserve function and dyslipidemia in patients with non-alcoholic fatty liver disease (Keating et al., 2012; Liang et al., 2018; Wang et al., 2017). However, few studies have focused on the effects of aerobic exercise on ALD.

The method of modeling adopted in mice with one-off alcoholic gavage is easy to replicate, has a short cycle, and is similar to the hepatic injury caused by human alcoholism. Therefore, this model is ideal for studying the working mechanism and preventive treatment of acute alcoholic hepatic injury. In this study, we first established the model of mice suffering from acute alcoholic hepatic injury, and then we treated the mice with intragastric administration of appropriate amount of SF integrated with aerobic exercise. Through examination, we analyzed the biochemical indexes and pathological changes of hepatic injury to explore the protective effect of SF and aerobic exercise on acute alcoholic hepatic injury in mice.

2. Materials and methods

2.1. Main reagents of medications

ALT (alanine transaminase), AST (aspartate aminotransferase), TC (total cholesterol) and TG (triglyceride) kit fabricated by Shanghai Kehua Bioengineering Co., Ltd.; VLDL (very low-density lipoprotein), SOD (superoxide dismutase) and MDA (malondialdehyde) detection kit fabricated by Nanjing Jiancheng Bioengineering Institute; Bifendate pills (BDP; 1.5 mg/pill) fabricated by Guangzhou Baiyunshan Xingqun Pharmaceutical Co., Ltd.; Sulforaphane (SF) standard (purity 95%) fabricated by Sigma company. We prepared 10 mg/mL BDP solution and SF solution before putting them in brown bottles, and we used them right after they were ready.

2.2. Experimental animal

The subjects of the experiment were 60 male healthy NIH (National Institutes of Health) mice of SPF (Specific Pathogen Free) grade, weighing 19.7 ± 1.4 g, provided by Guangdong Medical Laboratory Animal Center. Double identification was performed throught the use of animal hair staining and the numbering of the cage. The purchased NIH mice were quarantined for 3 days. No abnormalities were observed for each of the three days. Subsequently, all mice were included in the experiment. Feeding environment: 5 mice/cage, 12 h of illumination; indoor temperature: 20–26 °C; indoor humidity: 40–70%. During the experiment, mice were given feed and drinking water as required. 10 mice were randomly selected as normal control group A, then 10 mice were randomly selected as model control group B, and the remaining 40 mice were randomly divided into 4 groups, namely, BDP intervention group C, aerobic exercise invervention group D, SF intervention group E, and group F which was treated with SF integrated with aerobic exercise.

2.3. Methodology

2.3.1. Aerobic exercise

Inspired by the methodology adopted by Sha et al. (2018), we placed the mice in medium-intensity treadmill for training. The slope of the running platform was set at 0, and the initial speed was set at 10 m/min, which increased incrementally by 2.5 m/min. Each stage lasted for 5 min, and the speed increased in this manner to 15 m/min when the exercise load peaked. The training time was 60 min a day, 5 days a week, and 8 weeks in total. Every day, the mice were trained on the treadmill according to the set load.

2.3.2. Modeling and administration

We drew insights from and enhanced the methodology adopted by Shen and Li (2014) Mice in model control group and intervention group were intragastrically administrated with 50% ethanol for 8 mL/kg body weight for 12 weeks. Starting from the 5th week, the intervention group C was intragastrically administered with 0.1 mL/g body weight of BDP solution before the ethanol was administered, whereas the intervention groups E and F were intragastrically administered with the same amount of SF solution; and the model control group B was given equal amount of distilled water. In addition, intervention groups D and F were treated with aerobic exercise according to the procedures specified in 1.3.1 after 2 h of ethanol administration. Mice in the normal control group A were given the same amount of distilled water for 12 weeks.

2.3.3. Index detection

(1) Blood collection: After the intervention, the eyeballs of the mice were removed to facilitate collection of blood, and the serum was separated within 2 h after blood collection.
(2) Liver removal: The mice were weighed after anesthesia, and then fixed on their backs. After blood collection, they were dissected along the midline of the abdomen, and their livers were bluntly separated and weighed (Hong et al., 2010)

$$\text{Liver weight ratio}\,(\%) = (\text{Liver weight}\,(\text{g})/\text{mice weight}\,(\text{g})) \times 100$$

The liver was cleansed in cold saline and cut with tissue scissors. A tissue block (about 0.2–1 g) was placed in a 10 mL test tube, and 9 times of the pre-cooled 0.85% normal saline was added to the tube. The tissue homogenate of 10% liver tissue was prepared through its full grinding in ice water bath with high-speed dispersor. Then, it was placed in a low-temperature quick-freeze centrifuge to go through centrifugation at 2000 r/min for 10 min, and the homogenate supernatant was extracted. Part of the supernatant was used for automatic biochemical analyzer to measure TG, and the remaining supernatants were tested to measure their SOD and MDA contents according to the kit instructions. The remaining liver was preserved in a 4% formalin solution, frozen sectioned, oil red O stained, and histopathologically examined.

2.3.4. Pathological examination of the liver

(1) Sectioning and staining: After the liver tissue samples of the mice were drawn, they were sectioned at a thickness of about 6–10 μm. Subsequently, they were directly adhered to the slides, dried at room temperature and cleansed with 60% ethanol. Then the sections were incubated with oil red O staining solution (6 mL of stock solution, 4 mL of DD water) for 8 min before being differentiated with 50% ethanol and cleansed with DD water (with differentiation halted). Subsequently, they were stained with hematoxylin for 1.5 min, then turned blue after being cleansed, and were eventually gelatinized (Hou et al., 2014).
(2) Results of staining: neutral fat showed orange red in tissue block, blue in nucleus and colorless in stroma.
(3) Microscopic examination: the entire pathological section was observed under a 100-fold field of vision, and the pathological changes were recorded. The distribution and area of lipid droplets in the liver were observed and recorded. According to Table 1, each section was scored.

2.3.5. Data processing

The experimental data were shown as "mean + deviation", and the variance test was performed with SPSS 21 software. In case the variance is homogeneous, one-way ANOVA is used; in case the variance is not homogeneous, rank sum test is used.

3. Results and analysis

3.1. Examination results of indexes

3.1.1. Serum indexes

Table 2 shows that:

(1) ALT: Compared with group A, ALT in group B increased significantly ($P < 0.05$). Compared with group B, ALT in group C decreased significantly ($P < 0.05$). There was no significant difference in ALT among groups D, E and F, but a tendency to decrease was observed to some extent.
(2) AST: There was no significant difference in the level of AST between group B and group A. There was no significant difference in AST among groups D, E, and F.
(3) TC: Compared with group A, TC in group B increased significantly ($P < 0.05$). There was no significant difference in TC among among groups D, E, and F.
(4) TG: Compared with group A, TG in group B increased significantly ($P < 0.05$). Compared with group B, the TG of mice in each intervention group decreased significantly ($P < 0.05$), especially in the group treated with the approach of SF administration integrated with aerobic exercise.
(5) VLDL: Compared with group A, VLDL in group B increased significantly ($P < 0.05$). Compared with group B, VLDL in groups C and F decreased significantly ($P < 0.05$). There was no significant difference in VLDL between mice in groups D and E. Therefore, the approach of SF administration integrated with aerobic exercise imposed an effect on VLDL in mice that was closest to BDP intervention.

3.1.2. Liver indexes

Table 3 shows that:

(1) TG: Compared with group A, TG in group B increased significantly ($P < 0.05$). Compared with group B, the levels of TG measured in groups C, E and F decreased by a significant margin ($P < 0.05$). At the same time, it can be seen that the integrated treatment approach of SF administration with aerobic exercise yielded better results than BDP intervention in reducing liver TG in mice.
(2) MDA: Compared with group A, MDA in group B increased significantly ($P < 0.05$). Compared with group B, the levels of MDA measured in groups C, E and F decreased by a significant margin ($P < 0.05$). Moreover, the approach of SF administration integrated with aerobic exercise imposed a better effect on reducing MDA in mice liver than separate intervention of SF administration or aerobic exercise.
(3) SOD: Compared with group A, SOD activity of mice in group B decreased. Compared with group B, SOD activity of mice in groups C, E and F increased, and SOD activity of liver observed in mice treated with SF administration integrated with aerobic exercise was higher than that of BDP.
(4) Ratio of liver to body weight: Compared with group A, the ratio of liver to body weight of mice in group B increased. Compared with group B, the ratio of liver to body weight of mice in each intervention group decreased by a significant margin ($P < 0.05$). In addition, the ratio of liver to body weight of mice treated with SF administration integrated with aerobic exercise decreased to the closest extent to that of BDP intervention.

3.2. Scoring of hepatic pathology

Stained with oil red O, the lipid droplets in hepatocytes were red and other parts were light blue. Histological scores were shown in Table 4, and hepatic pathology sections of each group were

Table 1
Histological scoring criteria for oil red O staining.

Histological changes	Score
Lipid droplets in liver cells are scattered and scarce.	0
Hepatocytes containing lipid droplets do not exceed 1/4	1
Hepatocytes containing lipid droplets do not exceed 1/2	2
Hepatocytes containing lipid droplets do not exceed 3/4	3
Liver tissue is almost replaced by lipid droplets.	4

Table 2
Changes of serum indexes detected in mice.

Groups	ALT (U/L)	AST (U/L)	TC (mmol/L)	TG (mmol/L)	VLDL (μmol/L)
A	51 ± 9	156 ± 26	4.53 ± 0.54	1.13 ± 0.30	0.17 ± 0.10
B	142 ± 104▲	214 ± 104	7.26 ± 1.26▲	2.89 ± 0.90▲	1.76 ± 0.91▲
C	36 ± 16*	152 ± 45	6.86 ± 1.49	1.12 ± 0.34*	0.38 ± 0.22*
D	72 ± 29	178 ± 46	7.31 ± 1.29	1.38 ± 0.13*	0.67 ± 0.42
E	68 ± 25	173 ± 44	6.91 ± 1.24	1.18 ± 0.23*	0.54 ± 0.58
F	64 ± 22	168 ± 35	7.17 ± 1.02	1.09 ± 0.24*	0.42 ± 0.30*

Note: Compared with group A, ▲ means $P < 0.05$; compared with group B, * means $P < 0.05$.
A: normal control group; B: model control group; C: BDP intervention group; D: aerobic exercise invervention group; E: SF intervention group; F: SF & aerobic exercise intervention group.

Table 3
Changes of liver indexes detected in mice.

Groups	TG (mmol/L)	MDA (nmol/mgprot)	SOD (U/mgprot)	Ratio of liver to body weight (%)
A	0.42 ± 0.05	0.73 ± 0.19	270.4 ± 26.5	4.36 ± 0.23
B	0.97 ± 0.27▲	1.54 ± 0.74▲	213.8 ± 26.6	6.61 ± 0.41
C	0.46 ± 0.08*	0.69 ± 0.24*	264.2 ± 28.9	6.16 ± 0.39*
D	0.49 ± 0.08	0.83 ± 0.29	220.1 ± 54.6	6.27 ± 0.49*
E	0.41 ± 0.12*	0.80 ± 0.23*	254.2 ± 48.9	6.22 ± 0.48*
F	0.38 ± 0.04*	0.76 ± 0.25*	283.9 ± 26.3	6.18 ± 0.60*

Note: Compared with group A, ▲ means $P < 0.05$; compared with group B, * means $P < 0.05$.
A: normal control group; B: model control group; C: BDP intervention group; D: aerobic exercise invervention group; E: SF intervention group; F: SF & aerobic exercise intervention group.

Table 4
Histological scores of oil red O staining.

Groups	A	B	C	D	E	F
Scores	1.8 ± 0.63	3.9 ± 0.32*	3.8 ± 0.42	3.9 ± 0.32	3.6 ± 0.52	3.6 ± 0.70

Note: * indicates that compared with group A, $P < 0.05$.
A: normal control group; B: model control group; C: BDP intervention group; D: aerobic exercise invervention group; E: SF intervention group; F: SF & aerobic exercise intervention group.

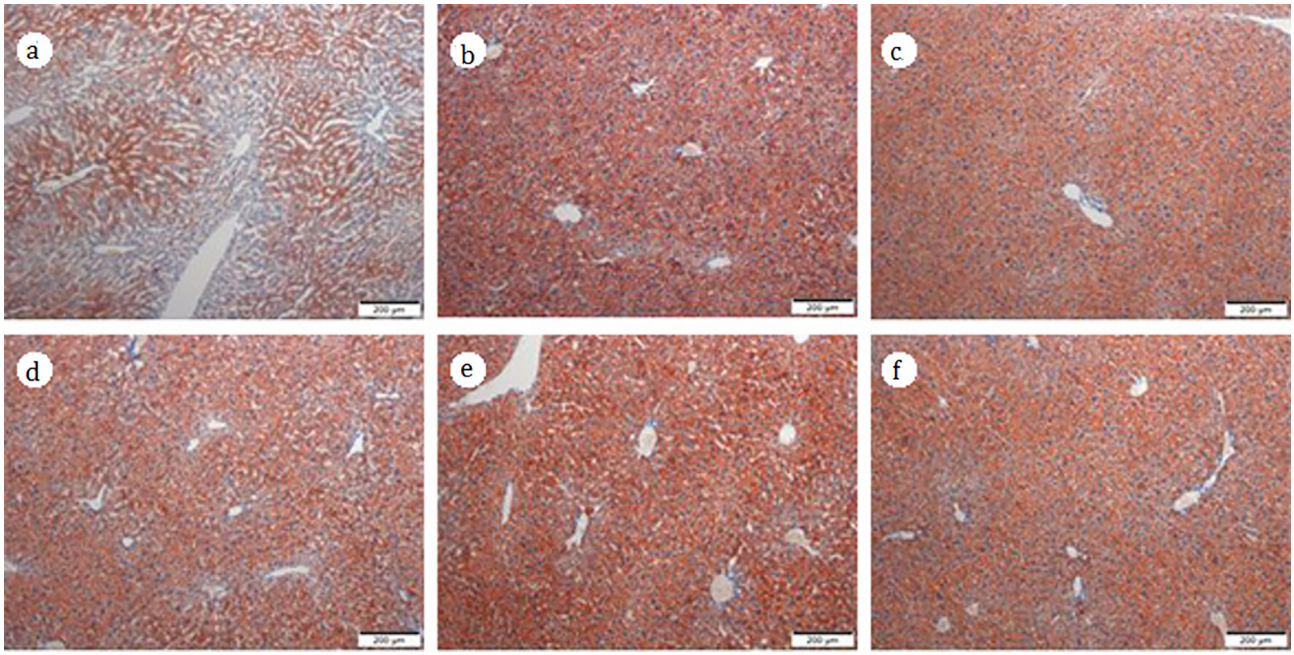

Fig. 1. Pathological sections of liver in mice with alcoholic hepatic injury by oil red O.

shown in Fig. 1. Compared with group A, the pathological score of liver in group B increased by a significant margin ($P < 0.05$). Compared with group B, there was no significant difference in liver pathological scoring between the intervention group and the control group. As shown in Fig. 1a, the lipid droplets in liver of group A did not exceed 1/2 of the total section area. The liver tissues in groups B, C, D, E and F were almost replaced by lipid droplets in Fig. 1b–f.

According to the above-mentioned serum and liver indexes measured in mice and the results of liver tissue pathological scoring, we may conclude that compared with group A, there were increases in the serum ALT, TC, TG, VLDL, liver TG, MDA, SOD, ratio of liver to body weight and pathological tissue scores of mice in group B, and the difference was significant ($P < 0.05$), proving the sucess of the modeling of mice. Compared with group B, there were decreases in the serum ALT, TG, VLDL, liver TG, MDA, ratio of liver to body weight of mice in group C, and there were increases in liver SOD. There were decreases in the serum TG and the ratio of liver to body weight of mice in group D, so were the serum TG, liver TG, MDA, the ratio of liver to body weight of mice in group E, whereas there were increases in the liver SOD. There were decreases in the serum TG, VLDL, liver TG, MDA, liver-to-body weight ratio of mice in group F, whereas the liver SOD increased. The difference was significant ($P < 0.05$), and there was no significant difference in other indexes.

4. Conclusion and discussion

ALT is widely distributed in hepatocyte plasma and hepatocyte mitochondria. When hepatocytes rupture, ALT is released into the blood circulation, and the increase of ALT concentration in the blood is consistent with the severity of hepatocyte injury (Zhao et al., 2005). SOD is an enzyme used in the body to remove oxygen

free radicals. SOD mainly functions to eliminate toxic O2-free radicals existing in the body and to prevent lipid peroxidation and cell membrane damage (Wang et al., 2010) MDA activity usually serves as a critical indicator of membrane lipid peroxidation, which can indirectly show the extent of damage caused by oxygen free radicals attacking body cells. VLDL is mainly synthesized by the liver. In addition, VLDL is easily metabolized into low density lipoprotein (LDL). When there is excessive LDL, it tends to accumulate in the arterial wall and cause atherosclerosis. When alcohol gets access to the blood and goes through systemic circulation with the blood, the ethanol is dehydrogenated in large amounts due to the catalysis of ADH. Such dehydrogenation hinders the tricarboxylic acid cycle and reduces the degree of oxidation of the fatty acid, which affects fat metabolism. Ethanol can promote the synthesis of TG by inhibiting lipoprotein esterase in the body, resulting in a large accumulation of fat in liver cells (Qiu et al., 2014; Zhang et al., 2011). Through the experiment, we found that the content of TG in the liver of group B increased significantly, which was proved by the examination chart of pathological sections stained with oil red O.

Under the conditions of this experiment, interventions of SF administration, aerobic exercise and the approach of SF administration integrated with aerobic exercise could all significantly reduce serum TG and ratio of liver to body weight in the model mice suffering from acute alcoholic hepatic injury ($P < 0.05$). The activity of SOD in liver experienced significant increase ($P < 0.05$) and the contents of MDA and TG experienced significant decreases ($P < 0.05$) in the SF group and the group treated with SF administration integrated with aerobic exercise. The VLDL in serum of mice also experienced significant decreases in the group treated with SF administration integrated with aerobic exercise ($P < 0.05$). It is thus clear that both SF and aerobic exercise can impose a certain protective effect on acute alcoholic hepatic injury in mice, and help reduce the damage caused by alcohol lipid peroxidation in liver cells. Moreover, the SF sdministration has a stronger treatment effect than aerobic exercise alone, and the combination of SF and aerobic exercise has the strongest protective effect on acute alcoholic hepatic injury in mice.

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