EXERCISE PROMOTES THE BENEFICIAL EFFECTS OF POMEGRANATE PEEL EXTRACT ON LIVER DYSFUNCTION IN RATS FED A HIGH-FAT DIET

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ABSTRACT: Obesity is one of the most serious public health problems worldwide. Physical activities and life style intervention are considered the best prevention strategies. Recently, we reported that pomegranate extract containing 40% punicalagin could significantly prevent high-fat diet induced obesity. In the present study, we reported that the combination of pomegranate and exercise showed additive benefits on liver function. After 8 weeks of feeding and treatment, pomegranate or exercise only showed trends for decreasing liver weight and liver function markers aspartate aminotransferase and alanine transaminase, whereas the combination group showed more significant reductions compared with either pomegranate or exercise group. Liver tissue analyses revealed that the combination group showed more significant effects on decreasing the liver triglyceride contents, increasing the protein expression of mitochondrial complex I, II, and III, cellular ATP and reactive oxygen species levels and reducing the liver malondialdehyde levels. Additionally, the combination was more effective on decreasing serum insulin and increasing HDL-cholesterol. Taken together, our study indicates that exercise promotes the beneficial effects of pomegranate on high-fat diet induced liver dysfunction and that the combination of both could be an effective strategy for improving metabolic health.

KEY WORDS: Exercise, Fatty liver, Mitochondrial function, Obesity, Punicalagin,
which is also an indicator of disrupted lipid homeostasis that is normally controlled by sterol regulatory element binding proteins (SREBPs). The SREBPs transcriptionally activate a cascade of enzymes required for endogenous cholesterol, fatty acid, triglyceride (TG) and phospholipid synthesis (Eberle et al., 2004). Additionally, mitochondria, which are known as the power factory of living cells, regulate lipid degradation through beta-oxidation, and mitochondrial dysfunction and associated oxidative stress was highly suggested to be among the main contributors of NAFLD (Paradies et al., 2014). Through exercise, mitochondrial biogenesis can be induced in adipose tissue (Trevellin et al., 2014) and skeletal muscle (Rockl et al., 2007), which was shown to contribute to improved glucose uptake and weight loss in experimental mice. Additionally, a study in obese women indicated that exercise could effectively restore mitochondrial physiology toward that of lean, insulin-sensitive individuals (Konopka et al., 2015). Although extensive studies have been performed, the exact mechanisms are still limited, and the effects of exercise vary due to the different exercise types and intensities. 

*Punica granatum*, which is commonly known as pomegranate, has been used as a folk medicine for the treatment of various diseases, such as ulcers, fever, diarrhea and microbial infections (Endo et al., 2010). Recently, pomegranate juice has received increasing attention because of its important biological actions, such as antioxidative activity (Gil et al., 2000), cardiovascular protection (Aviram et al., 2002), and hyperlipidemia amelioration (Esmailzadeh et al., 2004). Pomegranate flower extract was also reported to improve cardiac lipid metabolism and diminish cardiac fibrosis in diabetic rats (Huang et al., 2005a; Huang et al., 2005b). In a previous study, we found that pomegranate peel extract (PE) could effectively prevent high fat-diet (HFD)-induced obesity in rats (Zou et al., 2014) and that the combination of exercise and PE had additive benefits, such as the inhibition of HFD-induced body weight increase and improvement of HFD-induced immune dysfunction in rats (Zhao et al., 2016). Punicalagin (PU), the most abundant and highest molecular weight ellagitannin, was suggested to be the major active compound in pomegranate (Zou et al., 2014). Because both exercise and pomegranate have shown benefits in obesity, we investigated whether exercise could promote pomegranate’s effects on HFD-induced NAFLD in the current study.

**MATERIALS AND METHODS**

**Chemicals**

Tissue triglyceride and cholesterol assay kits were purchased from Beyotime (Nanjing, China); PCR primers were synthesized by Baiaoke Biotech (Beijing, China); (PE containing 40% PU was purchased from Tianjin JF-Natural (Tianjin, China)); and antibodies against the complex I, II, III, IV, and V subunits as well as other reagents were purchased from Invitrogen (Carlsbad, CA, USA).

**Animals and experimental design**

Specific pathogen free Sprague-Dawley (SD) male rats were purchased from a commercial breeder (SLAC, Shanghai, China). The rats were housed in a temperature (24-27°C)- and humidity (60%)-controlled animal room and maintained on a 12-h light/12-h dark cycle (light from 08:00 a.m. to 08:00 p.m.) with food and water provided during the experiments. Male rats weighing from 180–200 g were used. After 1 week of acclimatization, the rats were randomly distributed into the following five groups: (1) control rats fed a standard chow (Control, 12% kcal fat content); (2) rats fed a HFD (45% kcal fat content); (3) rats fed a HFD and administered a daily oral gavage of 150 mg/kg/day PE; (4) rats fed a HFD and performed daily exercise (Exe); and (5) rats fed a HFD, administered a daily oral gavage of 150 mg/kg/day PE, and performed daily exercise (PE + Exe). Prior to beginning the exercise, male rats were selected by their ability to perform one week of running exercise at low speed (10 m/min, 20 min/day), and those exhibiting high exercise activity were chosen for the experiments. The exercise speed was started at 10 m/min and gradually reached 20 m/min; the exercise lasted for 30 min on treadmill (Jide, Shanghai, China). For the fifth group, exercise was performed 45 min later after the PE gavage. In total, 60 rats were used for the experiments. After 8 weeks of feeding and exercise training, the rats were fasted overnight and sacrificed. All procedures were performed in accordance with the United States Public Health Services Guide for the Care and Use of Laboratory Animals, and all efforts were made to minimize the suffering and the number of animals used in this study. The formula of control and high fat diets were presented in Table 1.

**TABLE 1. Composition of the control and high-fat diets.**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Control, g/100g diet</th>
<th>High-fat diet, g/100g diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cornstarch</td>
<td>58</td>
<td>37</td>
</tr>
<tr>
<td>Sucrose</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Casein</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Cellulose</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Lard</td>
<td>-</td>
<td>21</td>
</tr>
</tbody>
</table>

**Blood sample preparation**

After the rats were sacrificed, blood samples were obtained by cardiac puncture, and the serum was separated by centrifugation (1500 g, 10 min). The levels of LDL-cholesterol, HDL-cholesterol, aspartate aminotransferase (AST) and alanine transaminase (ALT) were analyzed using an automated biochemistry analyzer (Hitachi Ltd., Tokyo, Japan). The serum...
insulin levels were measured using a commercial ELISA kits according to the manufacturer’s standards and protocols (RD systems, Shanghai, China).

**Western blot analyses**

Samples were lysed with western and IP lysis buffer (Beyotime, Jiangsu, China). The lysates were homogenized, and the homogenates were centrifuged at 13,000 g for 15 min at 4 °C. The supernatants were collected, and the protein concentrations were determined with a BCA protein assay kit. Equal aliquots (20 μg) of the protein samples were separated with 10% SDS-PAGE gels, transferred to pure nitrocellulose membranes (PerkinElmer Life Sciences, Boston, MA, USA), and blocked with 5% non-fat milk in TBST buffer. The membranes were incubated with anti-Complex I (NDUFA9), II (30IP), III (Core II), IV (Subunit IV) or V (Subunit α); or anti-β-actin (1:10,000) at 4°C overnight. Then, the membranes were incubated with anti-rabbit or anti-mouse antibodies at room temperature for 1 hour. Chemiluminescent detection was performed using an ECL western blotting detection kit.

**Liver tissue ATP measurement**

ATP was measured in samples from fresh liver tissue with an ATP bioluminescent assay kit (Sigma), as previously described (Zou et al., 2014). Briefly, tissues were lysed with 0.5% Triton X-100 in 100 mM glycine buffer, pH 7.4. Supernatants were collected after centrifugation at 14,000 g for 10 min at 4°C.

Forty microliter samples were then transferred to an appropriate bioluminescence plate. Luciferase activity was measured after the addition of 160 μL reaction solution. When ATP was consumed, light was emitted due to firefly luciferase catalyzing the oxidation of D-luciferin.

**Evaluation of oxidative stress**

Malondialdehyde (MDA), total antioxidant capacity (T-AOC), reactive oxygen species (ROS), superoxide dismutase (SOD) activity and glutathione peroxidase (GPx) levels were measured using commercial assay kits (Beyotime, Jiangsu, China) according to the manufacturer’s instructions.

**Statistical analysis**

Normal distribution was assessed using the Shapiro-Wilk test (SPSS inc, Chicago, IL, USA). All data are reported as the means ± S.E.M. Statistical analysis was performed using one-way ANOVA followed by a LSD post hoc analysis. In all of the comparisons, the significance level was defined as p < 0.05.

**RESULTS**

**The effects of PE and exercise on liver weight and function**

The HFD-induced obese model was used to investigate the potential additive effects of PE supplement and exercise training on obesity and its associated liver disorders. The HFD feeding as well as PE administration and exercise training lasted for 8

**FIGURE 1. The effects of PE and exercise on serum insulin, liver weight and liver function.** After 8 weeks of feeding and exercise training, the rats were sacrificed and analyzed. (A) The serum insulin level, (B) liver weight, (C) serum ALT activity, (D) and serum AST activity are shown. Values are mean values ± S.E.M. n ≥ 9, *p< 0.05, **p< 0.01.
Exercise on pomegranate benefits

weeks, the changes of body weight gain was shown in Table 2, which indicates significant body weight gain induced by HFD and synergetic inhibition effect by PE and Exe

**TABLE 2. Body weight gain (g).** To elucidate the diet induced obesity and effects of PE and exercise, body weight gain of five groups rats were calculated. All Values are mean values ± S.E.M. n ≥ 9, ’p< 0.05 vs. Control group, **p< 0.01 vs. HFD group.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HFD</th>
<th>HFD + PE</th>
<th>HFD + Exe</th>
<th>HFD + PE + Exe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight</td>
<td>229.7 ± 9.2</td>
<td>262.7 ± 10.8’</td>
<td>201.3 ± 16”</td>
<td>7.3”</td>
<td>146.2 ± 5.1”</td>
</tr>
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</table>

As shown in Fig 1A, the HFD induced a significant increase in blood insulin levels, which indicated possible insulin insensitivity in the obese rats. Both PE and exercise significantly inhibited the blood insulin increase and the combination showed an even more significant inhibition than PE alone. The PE and Exe groups showed a trend for reduced liver weight increase; however, only the combination of PE and exercise treatment caused a significant reduction in liver weight increase compared with the other groups (Fig. 1B). As a major liver damage marker (Hoekstra et al., 2013), ALT increased after HFD feeding and was significantly decreased in the PE and PE + Exe groups (Fig. 1C). The AST activity showed a similar pattern as ALT, except the combination of PE and exercise produced an additive effect on reduced ALT activity compared to PE or exercise alone (Fig. 1D).

**The effects of PE and exercise on liver TG and cholesterol content**

Consistent with a previous study (Zou et al., 2014), PE supplementation efficiently reduced the HFD-induced increase in both the liver TG and total cholesterol content, which was also observed in the exercise group (Fig. 2A, 2B). The combination of PE and exercise produced an additive effect on the liver TG contents (Fig. 2A). As supporting evidence, the HFD induced a significant increase in the serum LDL-cholesterol, and it decreased the HDL-cholesterol, which were all efficiently restored to the normal levels in the PE and Exe groups (Fig 2C, 2D). Additionally, the combination of PE and exercise treatment only produced an additive effect for the increased serum HDL-cholesterol level (Fig. 2D), and it did not affect the serum LDL-cholesterol levels.

**The effects of PE and exercise on liver mitochondrial function**

To investigate the involvement of mitochondria in HFD-induced obesity and its associated liver dysfunction, we measured the protein expression levels of the mitochondrial electron transport chain complexes in liver tissue. HFD feeding induced a significant reduction in the Complex I expression, but however, only the combination of PE and exercise treatment caused a significant reduction in liver weight increase compared with the other groups (Fig. 1B).

**FIGURE 2. The effects of PE and exercise on liver TG and cholesterol.** After 8 weeks of feeding and exercise training, the rats were sacrificed and analyzed. (A) The liver TG content, (B) liver cholesterol content, (C) serum LDL-cholesterol content, and (D) serum HDL-cholesterol content levels are shown. Values are mean values ± S.E.M. n ≥ 9, *p< 0.05, **p< 0.01.
expression levels (Fig. 3A, B, C, D). The combination of PE and exercise effectively increased the Complex I, II, and III expression levels compared with the HFD group (Fig. 3A, B, C, D), whereas the PE supplement or exercise showed no

**FIGURE 3. The effects of PE and exercise on liver mitochondrial protein expressions and ATP levels.** Liver mitochondrial complex subunit expression levels were assessed by western blot, and ATP levels were assayed with an ATP bioluminescent assay kit (Sigma). (A) A western blot image, (B) statistical analysis of complex I, (C) statistical analysis of complex II, (D) statistical analysis of complex III, and (E) liver ATP levels are shown. Values are mean values ± S.E.M. n ≥ 8, *p< 0.05, **p< 0.01.

**FIGURE 4. The effects of PE and exercise on liver oxidative stress.** After 8 weeks of feeding and exercise training, the rats were sacrificed and analyzed. (A) The liver MDA content, (B) liver T-AOC activity, (C) liver ROS level, (D) liver SOD activity, and (E) liver GPx activity are shown. Values are mean values ± S.E.M. n ≥ 9, *p< 0.05, **p< 0.01.
significant effects. Furthermore, HFD feeding reduced the liver ATP content (Fig. 3E), and PE supplement, exercise or the combination of PE and exercise all efficiently restored the liver ATP content (Fig. 3E).

The effects of PE and exercise on liver oxidative stress

It has been suggested that oxidative stress is involved in the progression of obesity and associated liver fat deposits (Sutti et al., 2013). Therefore, we looked into the redox status in the liver tissue. MDA, which is a product of lipid peroxidation, was not significantly increased by HFD feeding; however, the combination of PE + Exe significantly decreased the MDA level in the liver (Fig. 4A). Total antioxidant capacity (T-AOC) was increased after HFD feeding, which was considered an oxidative stress response. The PE and PE + Exe treatments showed a trend for inhibition, whereas exercise had no effect on the increased TAOC (Fig. 4B). Interestingly, the ROS level was decreased by the HFD feeding (Fig 4C). Both PE and exercise alone had no effect, but the combination of PE and exercise showed a significant effect on the increased ROS level (Fig 4C).

For the antioxidant enzymes, we measured the activities of SOD and glutathione peroxidase (GPx) in the liver. HFD feeding had no effect on SOD activity (Fig. 4D) but it did increase the liver GPx activity (Fig 4E). Although exercise had an effect on the increased SOD activity, PE, exercise, or PE+Exe did not produce significant effects on either the SOD or GPx activities (Fig. 4D, E).

DISCUSSION

The incidence of NAFLD, which is the hepatic manifestation of metabolic syndrome, is rising rapidly due to the increasing epidemic of obesity worldwide (Than and Newsome, 2015). Therefore, there is a great need to find effective prevention and treatment strategies. Aerobic exercise is usually considered the most beneficial strategy, but it depends primarily on the aerobic energy generating process. As noted by many studies, exercise has shown benefits in reducing the risk of obesity (Poirier and Despres, 2001), type 2 diabetes mellitus (Barengo and Tuomilehto, 2012), breast and colon cancer (Newton and Galvaõ, 2008), and aging-associated cognitive impairment (Berchtold et al., 2010). In animal studies, various exercise models have been developed, among which treadmill running is still the most popular. In the current study, the treadmill exercise was set at 10-20 m/min for 30 min/day, which is moderate and did not cause any physical damage.

We previously reported that PU from pomegranate peel extract exerted beneficial effects on HFD-induced metabolic disorders, including obesity, hyperlipidemia and fatty liver (Zou et al., 2014). To prove our assumption that moderate exercise could promote the benefits of pomegranate peel extract on fatty liver, rats were gavaged with the same dose of pomegranate extract 30 min before exercise. Consistent with a previous study showing that 37 days of feeding of diet containing 6% PU (4.8 g/kg/day) to rats did not cause any tissue alterations (Cerda et al., 2003a), 150 mg/kg PE containing 60 mg/kg PU in the current study did not provoke any toxicity in the rats based on blood and tissue analysis (data not shown). Obesity was induced following 8 weeks of HFD feeding, which increased the body weight gain by nearly 30% compared with a normal diet. It has been well established that adipose tissue is a major endocrine organ that can produce hormones such as leptin and adiponectin (Tschatzis et al., 2006), which play critical roles in regulating body weight (Oswal and Yeo, 2010) and glucose and lipid metabolism (Tschirrter et al., 2003). As shown in a previous study, PE alone showed no significant effects on adipose tissue mass, whereas the leptin and adiponectin levels were sufficiently restored to the normal levels by exercise or PE, which suggests that PE could modulate adipokine secretion, independent of adipose tissue mass (Zhao et al., 2016). Consistent with this study, the serum lipid levels, including LDL- and HDL-cholesterol as well as the fasting insulin level, were also restored to normal levels by the PE treatment or exercise. Additionally, the additive effects of PE and exercise were observed in the serum HDL-cholesterol and insulin levels, which might attribute to the body weight reduction and fatty liver amelioration.

In the presence of metabolic syndrome and exclusion of alternative chronic liver disease, such as alcoholic liver disease, hepatitis B or C infection, elevated AST or ALT levels were proposed to be predictive of the presence of NAFLD (Clark et al., 2003; Yu and Keeffe, 2003), which was observed in the current HFD model. Together with increased liver TG and CHO content, we proposed that HFD induced significant NAFLD in the current study. The combination of PE and exercise showed an additive reduction on the ALT and AST levels, but not the liver TG and CHO content, because PE or exercise alone already sufficiently reduced them to the normal levels. Although the detailed mechanisms underlying NAFLD development remain obscure, extensive studies have suggested that mitochondrial dysfunction that is associated with excessive oxidative stress plays an important role (Mantena et al., 2008; Paradies et al., 2014; Wei et al., 2009). Interestingly, we observed low levels of ROS in the liver tissue of the HFD group, which could be the result of the increased T-AOC observed in the current study. Alternatively, it may have been the result of UCP2 expression, as we reported previously (Zou et al., 2014). The increased antioxidant capacity after HFD feeding was also considered an early response to HFD stress. Nevertheless, the combination of PE and exercise sufficiently promoted the production of both ATP and ROS to normal levels. More importantly, the combination treatment only significantly inhibited mitochondrial complex I loss, and it increased the expression of complex II and III, suggesting that the exercise promoted the beneficial effects of PE by regulating mitochondrial function.

Pomegranate is becoming a well-known fruit, with several health benefits having been discovered in recent years. Although both ellagic acid and punicalagin are enriched in pomegranate...
fruit, ellagic acid was considered the major active component, as a previous report indicated that intact punicalagin might not be absorbed into the blood stream and is instead hydrolyzed to ellagic acid (EA) in the intestine over several hours (Heber, 2008). However, a previous report has confirmed the direct absorption of punicalagin and its presence in the plasma (Cerda et al., 2003b). We also found that after PE gavage (150 mg/kg), the serum punicalagin concentration increased time-dependently and reached 17.5 μg/mL at 2 h (Zou et al., 2014), which suggests that punicalagin could indeed circulate in the body and exert potential beneficial effects. In a previous study, we reported that punicalagin, not ellagic acid, was the major active component in mitochondria protection and fatty liver progression prevention. Here, we found that exercise could significantly enhance the beneficial effects in HFD-induced liver dysfunction. Together with the fact that exercise could efficiently accelerate nutrient absorption and metabolism, we thereby proposed that exercise could promote punicalagin absorption for increased benefits. Our results suggest that the combination of exercise and pomegranate supplement could be an efficient strategy for treating obesity-associated NAFLD.

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**REFERENCES**


Kmietowicz, Z. (2014). Overweight and obesity are linked to 10 common cancers and more than 12,000 UK cases. BMJ 349, g5183.


Steinberger, J., Daniels, S.R., American Heart Association Atherosclerosis, H., Obesity in the Young, C., and American Heart Association Diabetes, C. (2003). Obesity, insulin resistance, diabetes, and cardiovascular risk in children: an American Heart Association scientific statement from the Atherosclerosis, Hypertension, and Obesity in the Young Committee (Council on Cardiovascular Disease in the Young) and the Diabetes Committee (Council on Nutrition, Physical Activity, and Metabolism). Circulation 107, 1448-1453.


