Original Article
A novel herbal treatment reduces liver damage and increases RRAR-γ in the liver of type 2 diabetic rats

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Abstract: We investigated the anti-hyperlipidemia and antioxidant effects of the Radix puerariae and hawthorn fruit in type 2 diabetic rats induced by a high-fat diet followed streptozotocin (STZ) injection. Our results showed that the combination significantly reversed the changes induced by hyperglycemia and hyperlipidemia. The combination decreased sterol regulatory element-binding protein-1 (SREBP-1) and increased insulin receptor substrate-1 (IRS-1). Moreover, the combination markedly upregulated protein levels of nuclear factor-like 2 (NRF-2) and peroxisome proliferator-activated receptor-γ (RRAR-γ) in the liver of diabetic rats. Collectively, our findings suggest that the combination can protect the liver from steatosis in a diabetic rat model by enhancing anti-hyperlipidemia and antioxidant activity. This study indicates a potential therapeutic importance of the combination for reducing liver damage in type 2 diabetes.

Keywords: Radix puerariae, hawthorn fruit, high-fat diet, fatty liver, antioxidation

Introduction

The liver damage consequences of diabetes have been studied by a number of research teams and they have reported bidirectional associations between non-alcoholic fatty liver disease (NAFLD) and type 2 diabetes. NAFLD has become the most common chronic liver disease in developed countries, and its incidence in developing countries such as China has increased rapidly, seriously affecting the quality of life [1-3]. Although the etiology of NAFLD has not been fully elucidated, the ‘two-hit’ theory has been widely accepted [4]. The ‘first hit’ is related to hepatic triglyceride (TG) accumulation and insulin resistance, whereas the ‘second hit’ includes oxidative stress and inflammation [5]. As a result, NAFLD is characterized by steatosis and hepatocellular ballooning, increased levels of low-density lipoprotein cholesterol (LDL-C), total cholesterol (TC), TG, aspartate transaminase (AST), alanine aminotransferase (ALT) and oxidative stress markers [6]. Peroxisome proliferator-activated receptor-γ (PPAR-γ) is a member of the nuclear hormone receptor family [7]. Previous studies have demonstrated that PPAR-γ is associated with insulin resistance, lipid metabolism, and the regulation of inflammation in fatty liver disease [8, 9]. Insulin receptor (IR), a receptor tyrosine kinase, is also related to insulin resistance, and it binds to insulin receptor substrate-1 (IRS-1) to regulate glucose metabolism [10, 11]. Nuclear factor-like 2 (NRF-2) is a transcription factor that is crucial for controlling the cellular defense against oxidative stress [12]. Sterol regulatory element-binding protein-1 (SREBP-1), is an important transcription factor that regulates fatty acid and cholesterol metabolism in liver [13]. In conclusion, IRS-1, NRF2, and SREBP-1 might be associated with the development of NAFLD because they control insulin resistance, inflammation, and lipid metabolism. PPAR-γ agonists, such as thiazolidinediones (TZDs), are able to increase insulin sensitivity and slightly improve cholesterol metabolism in the liver [14]. However, TZDs have side effects such as significant peripheral edema [15, 16]. Therefore, it is essential to develop novel agents that target PPAR-γ but cause few adverse effects.

Previous studies have shown the therapeutic effects of Traditional Chinese Medicine in treating patients with metabolic syndromes [17, 18].
Radix puerariae, the root of kudzu vine, has been reported to increase peroxisome proliferator-activated receptor-γ (PPAR-γ) and reduce liver damage caused by CCl4. However, PPAR-γ agonists has been reported causing significant peripheral edema [19]. On the other hand, Hawthorn fruit, the fruit of crataegus, can relieve peripheral edema and reduce blood lipid effects in type 2 diabetes [20]. Therefore we hypothesize that combination of radix puerariae and hawthorn fruit (CRPHF) may be effective in targeting PPAR-γ without causing severe side effects. In the present study, to elucidate the pathological changes and the molecular mechanism in liver injury in diabetes, and further clarify the potential for natural combination of pharmacological therapies to protect the liver in patients with metabolic disorders, we investigated whether CRPHF could prevent liver injury in a rat model of diabetes induced by a high-fat diet and a low-dose streptozotocin (STZ).

**Material and methods**

**Drugs and reagents**

Radix puerariae and hawthorn fruit were provided by Shanghai Chinese Traditional Medical University. They were extracted separately using the following process: one kilogram of each was reflux extracted twice with 60% alcohol; the amount of solvent used was 5 L, and the extraction time was 90 min for each extraction [21]. The extract was reduced-pressure evaporated until the volume was 590 mL. The radix puerariae and hawthorn fruit solutions were mixed at a volume ratio of 1:1 and used for treating the rats in this study.

**Animals and experimental design**

A total of 24 male Sprague Dawley rats, aged 6 to 8 weeks (200 to 250 g), were obtained from the Experimental Animal Center of Zhejiang Province (Zhejiang, China). Rats were housed at 22 ± 3°C, 55 ± 5% humidity, and a 12 h light/dark cycle (light from 08:00 to 20:00) with free access to water and food throughout the experiment. All the animal handling procedures were in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals. The study was approved by the Ethics Committee of Ningbo University. All the surgical procedures were performed under anesthesia and all efforts were made to minimize suffering.

The entire experimental period was 9 weeks long and rats were fed with a regular or high-fat diet throughout this time according to their group assignment. In the first 4 weeks, we did nothing but to feed the rats with a regular or high-fat diet. At the end of 4th week, rats were given an intraperitoneal injection of 25 mg/kg STZ (Sigma, St. Louis, MO, USA) dissolved in a citrate buffer, pH 4.5 or the same volume of the citrate buffer. In the 5th week, we measured blood glucose levels every day, and extra half-doses of STZ were prepared to the rats whose blood glucose was lower than 11.1 mmol/L to make sure the successes of type 2 diabetic models (RBG>11.1 M). In the last 4 weeks, rats were treated with the CRPHF (2 g/kg/d) or vehicle (equivalent volume) every day via an intragastric feeding tube. The rats were randomly assigned to four groups (n=6 per group) as follows: 1. Normal control group (N group), consisting of rats fed with the regular diet and treated with normal saline; 2. Normal-given-CRPHF group (NC group), consisting of rats fed with the regular diet treated with the CRPHF; 3. Diabetic control group (D group), consisting of rats fed with the high-fat diet followed STZ injection and treated with normal saline; 4. Diabetic-given-CRPHF group (DC group), consisting of rats fed with the high-fat diet followed STZ injection and treated with the CRPHF.

**Measurement of body weight and blood glucose**

Body weights were recorded 7 days after injection of STZ and again at the end of the experiment. Blood was collected from the tail vein and blood glucose was measured with a blood glucose meter once every 4 days in the last four weeks of the experiment (Johnson & Johnson, America). Fasting blood glucose measurement and OGTT were performed on overnight-fasted rats at the end of the study. Rats were fasted for 12 h and then given a glucose solution (2 g/kg body weight), and blood samples were collected before and at 30, 60, and 120 min after the glucose solution was given. AUC for OGTT were calculated to evaluate glucose tolerance.

**Tissue preparation**

After treatment ended, all the rats were euthanized by intraperitoneal injection of a lethal dose of pentobarbital. The abdominal cavity was opened and blood was collected by direct
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The expression of SREBP-1, IRS-1, and GADPH in the liver tissues was measured by quantitative RT-PCR. Total RNA was extracted from the liver with TRIzol reagent (Invitrogen-Life Technologies Co., Ltd.) and cDNA was synthesized using the HiFiScript 1st Standard cDNA Synthesis kit (CWBIO Co., Ltd.) according to the manufacturer’s protocol. Subsequently, SREBP-1, IRS-1, and GADPH were amplified using a Multiplex PCR kit (Roche Biotechnology Co., Ltd.). The PCR (20 μl) conditions were as follows: initial denaturation at 94°C for 2 min; 45 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and elongation at 72°C for 30 sec; followed by an extension at 72°C for 2 min and final cooling to 4°C. The specific primers were designed based on published rat sequences from the GenBank database: SREBP-1 cagtgactctcctggcctat and caggagagcccagagaag, IRS-1 gagaacgagaagttggcg and agtgttcgtctcgggtgtag, GADPH accatacattctacggag and gaggggcggagatgagac.

Protein extraction and Western blot analysis

Proteins were extracted from rat liver tissues that were frozen in liquid nitrogen. 40 mg liver tissues were homogenized in a lysis buffer containing 200 ml RIPA (Solarbio Science & Technology Co., Ltd, Beijing, China) and 1 mM aprotinin and the lysate was centrifuged at 12,000×g for 5 min. The protein concentration of the supernatant was quantified using a Bicinchoninic acid Protein Assay kit (Beyotime Institute of Biotechnology). For Western blot analysis [23], the protein samples were mixed 4:1 with a sample buffer (Beyotime Institute of Biotechnology) and boiled for 5 min. Samples (containing 80 μg total proteins) were resolved by 12% SDS-PAGE. Separated proteins were electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon-P; Millipore Corp., Billerica, MA, USA). Other biochemical indicators were measured with a MODULAR P800 Automation Biochemist Analyzer (Roche, Basel, Switzerland).
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**Statistical analysis**

In each analysis, data are presented as the mean ± standard deviation, and differences among the groups were assessed by one-way analysis of variance (ANOVA), followed by Bonferroni post hoc test multiple comparison test, using the SPSS software package for Windows (Version 18.0; SPSS, Inc., Chicago, IL, USA). P<0.05 was considered statistically significant difference.

**Results**

The CRPHF reduces random blood glucose, restore the body weights reduced and improve glucose tolerance in diabetic rats

A total of 24 rats were used in the in vivo experiment, and no rat died at the end of the experiment. As shown in Figure 1A, between and within group analyses were performed to identify differences in random blood glucose (RBG) across the various groups and time points, respectively. The N group rats exhibited stable blood glucose levels throughout the treatment period, while the RBG of D group rats showed upward trend and the RBG of NC and DC group showed downward trends. The RBG level of DC group rats were significantly lower than that of the D group rats on day 28 (P<0.01). And reduction percent in RBG was 18.7% and 54.9% on day 28 compared with day 1 in NC and DC groups, respectively.

As shown in Figure 1B, at the completion of the study, rats in D group undergo weight loss, however, the mean body weights in the DC group (494±44.4) were significantly higher than in the D group (446±22.1), suggesting that CRPHF could significantly restore the body weights reduced.

Oral glucose tolerance test (OGTT) was conducted to determine whether the CRPHF improved glucose homeostasis in these animals.
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As shown in Figure 1C all groups showed significant increase (P<0.001) in blood glucose 1 hour following oral glucose challenge. We found that blood glucose showed a greater increase in the D group than the DC group from 0.5 h to 1 h, indicating a postponed blood glucose peak. Moreover, DC group rats had a lower blood glucose compared with the D group at 2 hours after the glucose challenge. Interestingly, the glucose levels in the DC group rats remained 52.8% lower than that of the D group rats at 2 hours after the glucose challenge, indicating that rats treated with the CRPHF had improved glucose tolerance. Areas under the

Figure 2. The CRPHF reduced blood lipid in diabetic rats. The CRPHF reduced TC (A) and TG (B), but didn’t significantly change HDL-C (C) and LDL-C (D) in diabetic rats. *, P<0.05, **, P<0.01, ###, P<0.0001, ***, P<0.01; ****P<0.0001.

Figure 3. The CRPHF promote the secretion of insulin in diabetic rats. (A) Fasting serum insulin and (B) homeostasis model assessment of insulin resistance (HOMA-IR) in rats at the end of treatment. Data are presented as mean ± SD (6 rats in each group). *, P<0.05, **, P<0.01, *, P<0.05.
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curves (AUC) of blood glucose response (Figure 1D) were significantly lower in the DC group rats than in the D group rats (P<0.01).

The CRPHF reduced blood lipid in diabetic rats

The CRPHF reduced TG (P<0.01) and TC (P<0.001) in diabetic rats (Figure 2). Similarly, the NC group showed significantly decreased TG (P<0.05) and TC (P<0.001) and significantly increased high-density lipoprotein cholesterol (HDL-C) (P<0.05) compared with the N group. These data indicate that the CRPHF effectively improved liver function and lipid metabolism in type 2 diabetic rats, and lower blood lipid in normal rats to a certain extent.

The CRPHF promote the secretion of insulin in diabetic rats

Figure 3 shows that fasting insulin (FINS) in the DC group was significantly higher than that in the D group (P<0.05), and the HOMA-IR was significantly higher in the D group compared with the N group (P<0.01). These data indicate that the CRPHF stimulated insulin secretion in a certain degree.

The CRPHF reduced serum AST and ALT

As shown in Figure 4, the activities of AST and ALT, markers of abnormal liver function when elevated, were significantly increased in the D group when compared with the N group (P<0.01 and P<0.001, respectively). The CRPHF was effective in protecting the liver function as the enzyme activities were significantly reduced in the DC group compared with the D group (P<0.01 and P<0.05, respectively).

The CRPHF preserved liver morphology in diabetic rats

Histological analyses (Figure 5) were carried out in order to assess whether the CRPHF treatment had any effect on the liver in normal rats,
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and whether it protected the liver from hyperglycemia and hyperlipidemia induced damages in diabetic rats. No steatosis, hepatocyte ballooning, or lobular inflammation was observed in the N and NC groups. The livers from diabetic rats (D and DC groups) presented pronounced steatosis, hepatocyte ballooning, and inflammation, which resemble the liver in diabetic patients. We did not observe fibrosis in any of the groups. The CRPHF did not alter the morphology of the liver in normal control rats, but reduced inflammation, steatosis, and edema in diabetic rats, indicating that the CRPHF preserved liver morphology in diabetic rats.

The CRPHF reduces mRNA expression of SREBP-1 and decreases IRS-1 in liver tissues

The expression of SREBP-1 and IRS-1 in the liver was measured with real-time polymerase chain reaction (RT-PCR). As shown in Figure 6, treatment with the CRPHF significantly reduced the expression of SREBP-1 (P<0.05) and increased the expression of IRS-1 (P<0.01) in DC group as compared with the D group. Moreover, there was a significant difference in SREBP-1 and IRS-1 expression between the D and N groups (P<0.01 and P<0.001, respectively). No significant difference in SREBP-1 or IRS-1 was observed between the NC and N groups.

Figure 6. The CRPHF reduces mRNA expression of SREBP-1 and decreases IRS-1 in liver tissues. A and B represent SREBP-1 and IRS-1 expression, respectively. Data are expressed as the mean ± standard deviation of three independent experiments. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001, ** P<0.05; ** P<0.001.

Figure 7. Effects of CRPHF on PPAR-γ and NRF-2 expression in liver tissues. A and B are representative Western blot images of the PPAR-γ and NRF-2, respectively; C and D represent relative ratio of PPAR-γ and NRF-2, respectively. Data are expressed as the mean ± standard deviation of three independent experiments. ** P<0.01, *** P<0.0001, * P<0.05; ** P<0.001.
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IRS-1 expression was observed between the N and NC groups.

Effects of CRPHF on PPAR-γ and NRF-2 expression in liver tissues

PPAR-γ and NRF-2 protein levels in liver tissues were quantified by Western blot analysis. As shown in Figure 7, the protein levels of PPAR-γ and NRF-2 were significantly higher (P<0.01 for both) in rats of the DC group than in the D group rats. Moreover, the protein levels of PPAR-γ and NRF-2 were lower in rats of the D group than in rats of the N group (P<0.05 and P<0.01, respectively).

Discussion

Experimental studies have reported that radix puerariae and hawthorn fruit lower blood glucose, alleviate inflammation and regulate lipid metabolism among diabetic patients [24, 25]. Here we found that treatment with the combination consisting of radix puerariae and hawthorn fruit markedly increased the body weight, decreased random blood glucose, and improved oral glucose tolerance. These results strongly indicate the antidiabetic effect of the CRPHF. The increased insulin secretion and partially relieved insulin resistance could be the reasons of the antidiabetic effect.

Previous studies shows that AST, ALT, TC, and TG levels are raised in NAFLD patients [26], and their levels are decreased after therapy [27]. In the present study, the serum AST, ALT, TC, and TG were significantly decreased by the CRPHF treatment. Histopathological examination showed reduced swelling and inflammation of the liver following the CRPHF treatment, illustrating its liver protection effect. These results suggest that the CRPHF may become an effective drug for the treatment of NAFLD. Furthermore, we find that the CRPHF treatment increased the expression of PPARγ and NRF2, suggesting its molecular mechanism to prevent NAFLD.

In conclusion, our results suggest that feeding with the combination of radix puerariae and hawthorn fruit may prevent or delay the development of hyperglycemia and protect the liver in type 2 diabetic rats by upregulating PPAR-γ and NRF2 expression.

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Disclosure of conflict of interest

None.

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