Therapeutic Effect of Puerarin on Non-Alcoholic Rat Fatty Liver by Improving Leptin Signal Transduction through JAK2/STAT3 Pathways

Peiyong Zheng, Guang Ji, Zansong Ma, Tao Liu and Lianjun Xin
Institute of Digestive Diseases, Longhua Hospital
Shanghai University of Traditional Chinese Medicine
Shanghai 200032, China

Hongzhong Wu, Xin Liang and Jianwen Liu
Shanghai Key Laboratory of Chemical Biology
School of Pharmacy, East China University of Science and Technology
Shanghai 200237, China

Abstract: In order to investigate the mechanism of the therapeutic effect of puerarin on non-alcoholic fatty liver disease, a non-alcoholic fatty disease male rat model was induced by a high fat diet, all rats were randomly divided into a blank group, model group, simavastatin group and puerarin group. After 4 weeks of drug treatment, the liver was sliced to investigate pathological morphology. Elisa was used to measure the total cholesterol (TC), triglyceride (TG) in liver, and leptin content in serum. RT-PCR and Western blotting were employed to detect liver leptin mRNA receptor expression and P-JAK2, P-STAT3 expression levels in the liver respectively. The results showed that puerarin significantly decreased the TG, TC content in liver of the non-alcoholic fatty disease rats, ameliorated steatosis in liver, lowered liver inflammatory reaction, decreased leptin level in serum, and enhanced the expression of leptin receptor mRNA and P-JAK2/P-STAT3 level. All the results demonstrated that puerarin can exhibit therapeutic effect on non-alcoholic fatty liver disease by improving leptin signal transduction through JAK2/STAT3 pathways.

Keywords: Puerarin; Non alcoholic Fatty Disease; Leptin; Leptin Resistance; P-JAK2/P-STAT3.
Introduction

In recent years, there has been an increasing appreciation for the significance of non-alcoholic fatty liver disease (NAFLD). Although the true prevalence is unknown, estimates of the prevalence of NAFLD in the general population range from 5% to 20% and up to 75% of patients with obesity and diabetes (Varman et al., 2004). NAFLD is a pathologic state of triglyceride over-accumulation caused by dysfunction of fat metabolism in the liver (Benlhabib et al., 2004). This disease is closely correlated with centricity obesity, type II diabetes, metabolic syndrome (obesity, dyslipidemia, insulin resistance, hypertension, etc), polycystic ovary syndrome and hypertriglyceridemia (Benlhabib et al., 2004). The pathogen of non-alcoholic fatty liver disease are presently considered to be the dual hit theory: the first hit is the fat deposit in liver resulted from insulin resistance; the second hit is the fatty hepatitis caused by lipid peroxidation, oxidative stress, and abnormal cytokines participation after the fat deposition (Marchesini et al., 2001; Guerra et al., 2000; Yan et al., 2006; Zhang et al., 2006). Presently, the precise mechanisms for this disease are still unknown and there is no specific medicine for this disease. Therefore, it is urgent to develop an effective drug for the treatment of this disease. Pueraria lobata, a commonly used Chinese herb, exerts sedative and antipyretic actions and is often used to treat influenza, wrist stiffness and headache, expand coronary arteries, depressurization, heart muscle ischemia, ameliorate microcirculation, adjust lipid metabolism and scavenge free radicals. Puerarin (4’,7-dihydroxy-8-ß-D-glucosylisoflavone), a C-glycoside compound, is an abundant active component of Pueraria lobata. Recent research have demonstrated that puerarin could protect B cells from insulin resistance and increase the sensitivity to insulin by correcting the fat metabolism dysfunction through decreasing the blood pressure and TG, TC content in the serum of the insulin-resistant-hypertension rats (Xu et al., 2005) and enhancing the expression of protein kinase B (Han et al., 2007; Xiong et al., 2006). Another report showed that puerarin could increase the SOD content in the serum and liver homogenate and lower the MDA content in acute hepatic injury rats induced by carbon tetrachloride, which implied that puerarin exerted protection on liver injury induced by lipid peroxidation, and the resultant mechanism was possibly related to the radical scavenging ability and anti-lipid peroxidation (Han et al., 2007).

However, there still have been no reports whether puerarin exhibits favorable therapeutic effects on non-alcoholic fatty liver, therefore, in this experiment, a non-alcoholic fatty liver model was induced by high fat diet to evaluate the therapeutic effect of puerarin on non-alcoholic fatty liver and the effect on the leptin receptor mRNA and signal transduction molecules P-JAK2/P-STAT3 in the liver to explore the possible mechanism of the therapeutic effect of puerarin on NAFLD.

Materials and Methods

Materials

Puerarin was provided by Shanghai University of Traditional Chinese Medicine (Shanghai, China). Simvastatin was purchased from Hangzhou MoSaDong Pharmaceutical Company (Hangzhou, China). Cholesterol was obtained from Shanghai Chemical Company.
Animals and Treatment

Male Wistar rats (150–170 g, SPF, Shanghai Center of Experimental Animals, Chinese Academy of Sciences) were housed in conventional cages with free access to water and rodent chow at 20–22°C with 12-hour light/dark cycles. All procedures involving the use of laboratory animals were in accordance with National Institutes of Health guidelines. After 1 week of acclimatization, rats were randomly divided into 5 groups: puerarin groups (0.8 g/kg and 0.4 g/kg respectively), simvastatin group, model group and normal group. All groups, except the normal group which was fed with fundamental diet, were fed with high fat diet (88% fundamental diet + 10% lard + 2% cholesterol) (Cervellati et al., 2002). All rats were given free access to food and water. One rat from the model group was sacrificed in the 4th, 6th and 8th week and the liver was sliced and pathologic HE staining was conducted to inspect the effect of high fat diet on the liver. On the 4th week, the rats in the puerarin groups were administrated with 0.8 g/kg or 0.4 g/kg puerarin by lavage every day, the rats in the simvastatin group were administrated with 4 mg/kg simvastatin by lavage every day, the normal and the model groups were administrated with saline. Four weeks after the therapy, rats in every group were sacrificed and their livers were dislocated, weighed and stored for further experiments.

General Observation of the State of Health

In order to evaluate the high fat diet and therapeutic effect of different treatments on rats, the behavior, morphous, fatality, pelage luster and weight were recorded from the onset to the end.

TC and TG Determination in the Liver

After sacrifice of rats, the liver homogenate were prepared, and the TC and TG content were measured by chromatometry.

Serum Leptin Level Evaluation

After sacrifice of rats, the serum was collected and leptin was evaluated by using a commercial clinical test kit (Shanghai Rongsheng Biotech Co., Ltd.).

Histopathology Observation

The slices of liver from each mouse were prepared with the HE staining and were observed under microscope for the evaluation of steatosis and inflammation. The criterion for hepatocyte steatosis was based on the method proposed by Diehl (Friedman, 2000). Criterion for inflammation in the liver was accorded to HAI (histology activity index) for chronic hepatitis produced by Hnodell (Diehl et al., 1988).

RT-PCR for Leptin Receptor Gene Transcription in Liver

Total RNA was isolated by using TRIZol™ reagent (Promega Corporation, USA) and reverse-transcribed by using Reverse Transcription System (Promega Corporation). An
aliquot (4 µl) of RT product was used for PCR amplification in a total volume of 20 µl. Leptin receptor cDNA (440 bp) was amplified by using the sense primer 5'-CAG GCA ACA CTG AAG GGA AGA CG-3' and the antisense primer 5'-CAT CAT CTG TGA CTT CTA GC-3' and β-actin was used as the control. The thermal cycle profile used in this study was (1) denaturing for 30 sec at 94°C (2) annealing for 90 sec at 55°C and (3) extending for 30 sec at 72°C. PCR amplification was performed for 30 cycles and a portion (10 µl) of the PCR mixture was visualized by electrophoresis in 1.2% agarose gel containing 0.2% ethidium bromide. The gel was photographed and the products were quantified by scanning densitometry.

Western Blotting

Liver fractions were washed with PBS twice and extracted in a lysis buffer [10 mM Tris-HCl (pH 7.5) containing 50 mM NaCl, 50 mM NaF, 10 mM EDTA, 1 mM DTT, 1% (v/v) Triton X-100, 0.1% (m/v) SDS, 1% (m/v) sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 5 mM leupeptin, and 10 mg/ml aprotinin] for 30 min on ice. The lysates were centrifuged to remove insoluble materials, normalized according to their protein content. Then equal amounts of proteins (30 µg) were separated by SDS-polyacrylamide gel electrophoresis and blotted onto a PVDF membrane (Amresco). After blocking for 1 hour at room temperature by using PBST buffer (PBS buffer plus 0.1% (v/v) Tween-20) containing 3% (m/v) BSA, the filter was then applied sequentially with mouse monoclonal anti-STAT3, anti-JAK2 antibodies (diluted 1:500; Santa Cruz Biotechnology), and secondary antibody conjugated horseradish peroxidase (diluted 1:500; Santa Cruz Biotechnology). The blots were visualized with enhanced chemiluminescence detection and imagined on KodakTM X-OMAT film.

Statistics

Experimental values are expressed as the mean ± SE. By using scientific statistic software GraphPad Instat version 2.04, one-way ANOVA assay was used to evaluate the significance of differences between groups with statistical significance considered as *p < 0.05, **p < 0.01.

Results

Health Observation

During the experiments, the health state was observed, which showed that the rats in the normal group were invigorative, nimble, active, with normal diet and luster fur. However, the rats in the model group were depressed, dull, with docile disposition and messy fur without luster, after the treatment with puerarin and simvastatin, the condition was ameliorated compared to the rats in the model group.

Effect of Puerarin on the Liver Index of the Non-Alcoholic Fatty Liver Rats

The high fatty diet caused the liver weight and liver index to increase significantly compared to the normal rats; The treatment with puerarin and simvastatin both decreased the liver
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Figure 1. The effect of puerarin on liver index of non-alcoholic fatty liver SD rats. *p < 0.05, **p < 0.01, compared to the control group, $p < 0.05, S^p < 0.01$, compared to simvastatin group.

Index of the fatty liver rats, and the effect of puerarin was better than simvastatin (p < 0.05, Fig. 1).

Morphology Observation of the Liver

The livers from the normal group showed bright red, sharp edge, and smooth surface. After the rats took a high fat diet, the livers differed from those in normal group; the liver volume was increased greatly; the edge got blunt; the liver felt soft and with earth like flavedo, there was conglutination between the liver and the periphery tissues and the cross section showed grease. After the administration of puerarin and simvastatin for 4 weeks, the liver appearance was improved remarkably, when pressed with fingers, it felt springy, showed shallow yellow. Furthermore, greasy outlook was ameliorated compared to that in mode rats (Figs. 8 and 9).

Light Microscope Observation of the Liver

When observed under a light microscope, the liver slides with HE staining contained no obvious abnormality. However, there existed hepatocyte diffuse steatosis in all the liver slides from the model group. Meanwhile, there was considerable mononuclear cell inflammatory cell infiltrate in the lobules and vessel convergence region of the liver. Partial necrosis region in lobules of the liver was coalesced together. Treatment with puerarin and simvastatin improved the symptoms to some different extent; the adipose degeneration was ameliorated, the liver hepatocytes were almost normal and the nucleus was located in the cell center,
the fat drops were reduced and diffused evenly in the liver. Taken together, there was a significant difference between puerarin group and simvastatin group as anti-inflammatory was concerned (Figs. 2 and 3).

Figure 2. The effect of puerarin on inflammation index of non alcoholic fatty liver SD rats. *p < 0.05, **p < 0.01, compared to the control group, $p < 0.05, $$$p < 0.01, compared to simvastatin group.

Figure 3. The effect of puerarin on fat pathological index of non alcoholic fatty liver SD rats. *p < 0.05, **p < 0.01, compared to the control group, $p < 0.05, $$$p < 0.01, compared to simvastatin group.
Effect of Puerarin on TC, TG in the Liver

High fat diet obviously increased the TG and TC contents in rat liver in model group. The administration of puerarin and simvastatin decreased the TG and TC levels in the liver tissues. Moreover, there was significant differences between the effects of puerarin and simvastatin on TC and TG ($p < 0.05$) as indicated in Figs. 4A and 4B.

![Bar chart showing the effect of puerarin and simvastatin on TG and TC levels in the liver.](image)

**Figure 4.** The effect of puerarin on TC and TG in the serum of non-alcoholic fatty liver SD rats. (A) Effect on TG content in liver; (B) Effect on TC content in liver. *$p < 0.05$, **$p < 0.01$, compared to the control group, $^5p < 0.05$, $^$$p < 0.01$, compared to simvastatin group.**
Effect of Puerarin on Serum Leptin and Liver Leptin Receptor mRNA Expression

High fat diet significantly increased the leptin content in the serum and decreased the leptin receptor mRNA expression in the liver, after administration of puerarin and simvastatin for 4 weeks, the leptin content was decreased in the serum and the leptin receptor mRNA expression was increased in the liver as compared to the model group (Figs. 5, 6A, 6B). Furthermore, the effect of puerarin on leptin and its receptor was better than that of simvastatin ($p < 0.05$).

Evaluation of Puerarin Influence on P-JAK2 and P-STAT3 in the Liver

After administration of the high fat diet to the rats, the P-JAK2 and P-STAT3 content in the liver was significantly lowered in the model group than that in the normal group ($p < 0.01$). Puerarin and simvastatin could significantly increase the P-JAK2 and P-STAT3 protein expressions in the liver when compared to the control group ($p < 0.01$). Meanwhile, the effect of puerarin on leptin in the serum and leptin receptor expression in the liver were improved more than that of simvastatin ($p < 0.05$, Figs. 7A, 7B, 7C).

Discussion

The mechanism for non-alcoholic fatty liver is unclear up through now. Many researchers thought it was associated with insulin resistant metabolism syndromes such as obesity, diabetes, hypertension, and hyperlipemia (Knodell et al., 1981). Recent research showed that non-alcoholic fat liver disease often accompanied with insulin resistance and
Figure 6. The effect of puerarin on liver leptin mRNA expression of non alcoholic fatty liver SD rats. (A) Electrophoresis image of leptin receptor mRNA; (B) Density scanning quantification of leptin receptor mRNA. *p < 0.05, **p < 0.01, compared to the control group, $p < 0.05$, $$p < 0.01$, compared to simvastatin group. B: normal group; M: model group; P (H): high dose puerarin group; P (L): low dose puerarin group; S: simvastatin group.

hyperinsulinemia, and was correlated to the decrease of leptin sensitivity (Lieber, 1999). Therefore, leptin resistance regulation was chosen to unveil the therapeutic effect of puerarin on NAFLD in this experiment.

In this study, after administration of high fat diet for 4 weeks, the rats’ health condition was damaged as judged from the appearances. The liver index, inflammation index, TG and TC levels in serum were increased remarkably compared to that in the normal rats (Figs. 1–4).
Figure 7. The effect of puerarin on P-JAK2/P-STAT3 of non-alcoholic fatty liver SD rats. (A) Western blotting scanning image; (B) Density scanning quantification of western blotting. $^* p < 0.05$, $^{**} p < 0.01$, compared to the control group, $^* p < 0.05$, $^{**} p < 0.01$, compared to simvastatin group.
The pathological slides of the liver further demonstrated the causative of high fat diet on formation of NAFLD (Figs. 8 and 9). After the successive treatment of puerarin, all these symptoms were ameliorated compared to the model rats, which signified that puerarin can exhibit therapeutic effect on NAFLD.

Leptin is the main regulator of fat in the organism. It is released from the fat tissue into blood, then dispensed into the tissues (musculi skeleti, adipose tissue, peripheral lymphoid tissue, central nervous system, gastrointestinal tract, and liver) over the body by circulation and combines with its receptors, which then interact with Janus family protein tyrosine kinase/signal transduction and activates transcription factors (JAK/STAT), mainly JAK2/STAT3, to cause the related biological effects (Chitturi et al., 2002; Iredale et al., 1998; Saxena, 2002) and to exhibit the function of diet control and energy metabolism regulation (Rizk et al., 2001; Larsson, 1998). In this study, it was found that leptin level in serum was significantly increased while the leptin receptor expression was decreased in rats administrated with high fat diet for 4 weeks, when compared to the normal rats. In the following 4 weeks of puerarin administration, the leptin level in serum was moderately decreased, while the leptin receptor expression was increased significantly, which signified that puerarin antagonized the leptin resistance caused by high fat diet.

It is presently thought that leptin participating in glycometabolism and fat metabolism in liver mainly depends on the regulation of gene expression of phosphoenolpyruvic acid
Figure 9. Pathological slides of liver with HE staining after treatment at 8th week. (HE × 400). BL: normal rats without any treatment; M: model group; P (H): high dose puerarin group; P (L): low dose puerarin group; S: simvastatin group.

and efficiency of glyconeogenesis, then stimulates liver intake of lactic acid and genesis of hepatic glycogen; simultaneously, activates the carnitine acyl transferase through protein kinase A, then regulates fat metabolism; leptin can also decrease triglyceride by reducing diet intake (about 50%) and enhance energy consumption (namely increase the fat metabolism in liver). Furthermore, leptin also interferes with the role of insulin in the liver, on one hand, it counteracts the downregulation of phosphoenolpyruvate carboxykinase (PEPCK) induced by insulin, restrains the phosphorylation of insulin receptor substrate-1 to be involved in the signal transduction; on the other hand, leptin restricts triglyceride synthesis and elevates the insulin sensitivity of liver and peripheral tissues (Larsson, 1998). The subsequent multiple regression analysis implied that leptin was the independent risk factor for the genesis of fat liver, which exhibited close correlation to BMI, serum steroid, LDL, and triglyceride. Besides, when obesity occurred, the biological function of leptin would be decreased significantly, whereas leptin level would be increased significantly, and insulin resistance could occur, which showed that insulin resistance may be involved in the liver adipose degeneration (Tobe et al., 1999; Chitturi et al., 2002a).

Leptin regulates energy metabolism signals by combining to its receptor. When the receptor expression is in dysfunction, leptin resistance occurs. It was found that the serum leptin level and nomadic leptin receptors were increased dramatically; it implied there were
obstacles for leptin to combine to its receptors. The receptor down-regulates for the abnormality of mRNA of leptin receptor in the liver (Campillo et al., 2001), the leptin in the serum will not possess physiological functions, namely leptin resistance (Bartek et al., 2001), the existence of postreceptor defects, such as the defect of leptin JAK2/STAT3 signal transduction, would cause the leptin resistance as well (Koteish and Diem, 2001; Carpenter et al., 1998). After administration of puerarin for 4 weeks, leptin receptor expression was elevated. Meanwhile, JAK2/STAT3 protein was higher than that from the model group, which strongly implied that JAK2/STAT3 was involved in the leptin resistance regulation.

Taken together, puerarin presented favorable therapy for NAFLD by ameliorating leptin resistance. Puerarin is a major isoflavonoid compound isolated from Pueraria lobata, an edible vine used widely for various medicinal purposes. It has been used for centuries in China to counteract alcohol intoxication. However, the effects of puerarin on chemical-induced liver fibrosis have not been reported. In the present study, puerarin could effectively reverse chemical-induced liver fibrosis in experimental rats, via the recovery of hepatic injury as well as the induction of apoptosis in activated HSC (Zhang et al., 2006).

After NAFLD was caused by a high fat diet, there was a higher leptin level in rats of the model group, while the lipid metabolism disorder and NAFLD were still present, which indicating that the sensitivity of organism to leptin dwindled, existing leptin resistance. After treatment with puerarin or simvastatin, leptin level in the serum was significantly decreased when compared to the model group. Besides that, the slides of liver showed some pathological relieves, in all, these results demonstrated that puerarin can rectify the leptin resistance and enhance the sensitivity. Since leptin biological effect depends on its combination to its receptor and phosphorylation of JAK2 and STAT3, as from the obtained results, puerarin could exert influence on leptin receptor expression and phosphorylation of JAK2 and STAT3.

The effect of puerarin on leptin receptor mRNA of the NAFLD rats showed that the liver leptin receptor mRNA expression in the model group was weaker compared to the groups treated with puerarin and simvastatin. Although there was no direct evidence, it was reported that free fatty acids could suppress the activity of protein tyrosine kinase, partially restrict the signal transduction of leptin, down-regulate the expression of leptin receptor (Carpenter et al., 1998). The reduction of leptin receptor mRNA possibly was mediated by fatty acids. Considering the increase of leptin in serum was paralleled with the decrease of leptin receptor, it was inferred that high level leptin-emia has a degenerative feedback effect on leptin receptor (Crouse, 1998). Nevertheless, whether the high level of leptin-emia leads to the decrease of its receptor or the decrease of leptin receptor provokes the high leptin-emia still needs further confirmation. In our experiment, after treatment with puerarin, the serum leptin level was decreased while the liver leptin receptor mRNA was increased significantly in NAFLD rats, which indicated that puerarin could regulate leptin resistance by interfering with leptin receptor mRNA expression.

Since the combination of leptin to its receptor and subsequent phosphorylation of JAK2 and STAT3 play a pivotal role in the implementation of leptin biological function (Guo, 2004), Western blotting method were employed to investigate the effect of puerarin on expression of JAK2/STAT3, our results showed that puerarin enhanced P-JAK2/STAT3 expression.
significantly when compared to the model group, which implied puerarin could regulate the signals transduction of JAK2/STAT3. Until now, there was no report that puerarin was involved in the signal transduction of JAK2/STAT3 in the non-alcoholic fatty liver. In order to further understand the therapeutic effect and mechanism of puerarin on non-alcoholic fatty liver, more research need to be done.

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References


Guerra, M.C., E. Speroni, M. Broccoli, M. Cannini, P. Pasini, A. Minghetti, N. Crespi-Peregino, M. Mirasoli, G. Cantelli-Forti and M. Paolini. Comparison between chinese medical herb


