Paeony attenuates high fat diet-induced kidney injury via inflammation inhibition

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Abstract: High-fat diet (HFD) feeding is a risk factor for kidney damage with limited treatment options. This study explored the effect of total glucosides of paeony (TGP) for treating obesity-related kidney damage. C57BL/6 mice fed with HFD were used for \textit{in vivo} experiments. Body weight, lipid and renal function indicators were measured. Hematoxylin and eosin, Masson, Sirius Red and F4/80 immunohistochemical staining were performed. Fibrosis levels, inflammatory factors, MyD88, IκB and p-JNK levels were detected. SV40 cells were incubated with palmitic acid, transfected with siRNA MyD88 and/or treated with TGP. The expression of MyD88, IκB, p-JNK, fibrosis and inflammatory factors were detected. TGP considerably decreased HFD-induced body weight gain and serum lipid concentration but improved renal function. In addition, TGP inhibited renal fibrosis and inflammation and reduced MyD88 and p-JNK levels, whereas increased IκB levels. Moreover, silencing MyD88 decreased p-JNK levels while increasing IκB levels which may be the mechanism of TGP treatment. Our findings demonstrated that TGP treatment can ameliorate HFD-induced kidney injury via regulating JNK and IκB signals mediated by MyD88.

Keywords: Total glucosides of paeony, obesity-associated kidney damage, MyD88, inflammation, fibrosis.

INTRODUCTION

The incidence of obesity is increasing annually and at younger and younger ages. Alarming, 493 million people are estimated to be either obese or overweight by 2030 (Hruby and Hu, 2015). After accounting for traditional risk factors like diabetes, hyperlipidemia and hypertension, weight gain also directly increases the risk of obesity-related kidney damage (Lakkis and Weir, 2018; McPherson et al., 2019). High-fat diet (HFD) can cause glomerular structural and functional lesions, such as glomerular inflammation, nodular glomerulosclerosis, mesangial expansion and extracellular matrix deposition leading to kidney fibrosis (Kumar et al., 2021; Hall et al., 2019). Moreover, kidney damage caused by obesity is a substantial cause of end-stage renal disease (ERSD) (Ruiz-Ortega et al., 2020). The prevalence of obesity-induced kidney damage increased from 0.2% in 1986-1990 to 2.0% in 1996-2000 (Kambham et al., 2001). However, treatment options remain very limited due to controlling blood pressure and glycermic index (Wouk, 2021). Developing new therapeutic options is urgently needed to treat HFD-induced kidney injury.

The innate immune system and inflammatory response over activation may contribute to obesity-related kidney disease (Schetz et al., 2019). Since obesity is generally considered to be a systemic, low-grade inflammatory state (Gregor and Hotamisligil, 2011; Saltiel and Olefsky, 2017). Excess nutrition, especially fatty acids, directly damage the renal system. Saturated fatty acids can bind to toll-like receptors (TLRs), myeloid differentiation protein2 (MD2), or fetal bovine serum protein-A (FetA) to initiate inflammatory pathways, promote synthesis and release of chemokine and cause inflammation of the kidney (Jourde-Chiche et al., 2019; Wang et al., 2017). Consequently, HFD can increase the pro-inflammatory cytokines levels, including IL-1β, IL-6 and TNF-α and upregulates adhesion cytokines, leading to immune cells infiltration (Raftar et al., 2022; Duan et al., 2018). However, immuno-suppression attenuated nephropathy by reducing the renal inflammatory response (Zhu et al., 2017). Therefore, reducing inflammation may be a potential therapeutic direction for treating obesity-induced kidney damage.

Paeonia lactiflora Pall is a Chinese traditional herbal medicine. Total glucosides of paeony (TGP), extracted from P. lactiflora Pall roots, have been used for treating gynecological problems, cramps, pain and giddiness for over 1500 years in Chinese medicine (Tan et al., 2020; Wang et al., 2014b). TGP exhibited extensive activities of anti-inflammatory, anti-oxidative, anti-hepatic damage and immune-regulatory activities accompanied by low toxicity and fewer adverse effects (Tang et al., 2018). In 1998, the National Medical Products Administration approved TGP (Pafulin) to treat rheumatoid arthritis. Also, studies have shown that TGP can protect the kidneys against type 1 diabetes-induced damage (Shao et al., 2019). However, whether TGP could mitigate obesity-related kidney injury remains unclear. Therefore, this study aimed to examine the role of TGP on inflammation to cure obesity-induced kidney damage.

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Pak. J. Pharm. Sci., Vol.36, No.4, July 2023, pp.1217-1225
MATERIALS AND METHODS

Materials
Total Glucosides of White Paeony Capsules purchased from Ningbo Lihua Pharmaceutical Co., Ltd. (PaFuLin, 300mg/tablet, H20055058) that was dissolution using 1% sodium carboxyl methyl cellulose (CMC-Na) in the animal experiment or DMSO in the cell experiments. PA was purchased from Sigma (St. Louis, MO). Anti-GAPDH, anti-IκB and anti-MyD88 were obtained from Santa Cruz (Santa Cruz, CA); and anti-p-JNK and anti-JNK were purchased from Cell Signaling (Danvers, MA). The immunohistochemistry kit (PV-9000) was purchased from Beyotime Biotechnology Inc. (China).

Cell culture
SV40 cells were purchased from the Institute of Biochemistry and Cell Biology (Shanghai, China) and grown in RPMI-1640 medium (Gibco, Eggenstein, Germany) containing 10% fetal bovine serum and 1% penicillin and streptomycin solution in an incubator with 95% air and 5% CO2 at 37°C.

Animals
Adult male C57BL/6 mice (18-22g) were purchased from the Animal Center of Academy of Sciences (Shanghai, China). The animals were kept in a stable environment (12:12 h light-dark cycle, 24°C temperature, 40%-60% humidity) and with free access to food and water. The animals were kept adaptively for at least 3 days before use. “The Detailed Rules and Regulations of Medical Animal Experiments Administration and Implementation” were followed (Document NO. 1998-55, Ministry of Public Health, P.R. China.).

Animal experiments
Four groups of twenty-eight male C57BL/6 mice were randomly selected: one group was fed with a regular diet and the remaining three groups were fed with HFD for 8 weeks. In the HFD group mice, vehicle dosing (1% CMC-Na solution) or orally TGP (100mg/kg and 200mg/kg per day, respectively) were administered (Pafulin, 300mg/tablet, H20055058; Ningbo Lihua Pharmaceutical Co., Ltd, Ningbo, China) over 8 weeks. Body weight was recorded every 4 weeks. After 16 weeks, the mice were euthanized under ether anesthesia. Blood samples (300-500μL) were collected via the tail vein. Kidney tissues (50-100mg) was used for RNA extraction previously published methods. Collected cells or homogenized kidney tissue samples were lysed. Lysates were incubated with anti-MyD88, anti-pJNK, anti-IκB and anti-GAPDH antibodies. Overnight at 4°C, the membrane was incubated with secondary antibody against mouse F4/80 (1:500) for 24 hours at 4°C. After washing, secondary antibody was used to incubate kidney tissues at room temperature for 1 h. And then the tissues were stained with 3,3′-diaminobenzidine and the nucleus with hematoxylin. The images were viewed and captured with an Olympus light microscope (400× amplification; Nikon, Tokyo, Japan).

Western blot analysis
Western blotting was carried out according to the previously published methods. Collected cells or homogenized kidney tissue samples were lysed. Lysates were incubated with anti-MyD88, anti-pJNK, anti-IκB and anti-GAPDH antibodies. Overnight at 4°C, the membrane was incubated with secondary antibodies and visualized using enhanced chemiluminescence reagents (Bio-Rad, Hercules, CA) (Kuba et al., 2020).

Serum lipid and renal function index detection
Mice serum was collected and the serum lipid index including the TG, TCH and HDL, was detected. Furthermore, the renal function index including serum Cr,
blood BUN and albumin (ALB), was detected using commercial kits (Nanjing Jiancheng, Jiangsu, China) (Ding et al., 2018).

**Ethical approval**
Ethics approval (include appropriate approvals or waivers) All experiments with C57BL/6 mice were constructed in accordance with the Guidelines for the Care and Use of Laboratory Animals, which was presented by the National Institutes of Health and approved by the Ethics Committee of Zhejiang Cancer Hospital.

**STATISTICAL ANALYSIS**
Statistical differences were analyzed using analysis of variance and independent Students t-test using GraphPad Prism 5 statistic software (La Jolla, CA, USA). Data were expressed as the mean ± standard error of the mean (SEM). A difference of p<0.05 was considered statistically significant.

**RESULTS**

**TGP decrease body weight and TG but increase HDL**
Obese mice fed with HFD were treated with TGP (100 and 200mg/kg) or negative control. Body weight, TCH and TG increased markedly in the HFD-fed group compared to those in the control group (table 2). Furthermore, with TGP treatment, there were considerable decrease in body weight and TG levels. However, HFD-fed and TGP-treated groups did not show significant difference in TCH levels. Moreover, significantly elevated HDL levels were noted. These results suggest that TGP protect mice from obesity-induced hyperlipidemia.

**TGP prevent kidneys damage**
Next, we detected serum Cr, BUN and ALB. HFD-fed mice with kidney disease exhibited elevated levels of Cr, BUN and ALB, which were drastically converted by TGP at a dose of 200mg/kg (figs. 1A-1C). Furthermore, we conducted H&E staining to investigate renal tubules and glomeruli changes. Compared to the control group, the kidney tissue morphology in HFD-fed mice was considerably different, with tubular epithelial cell shedding and vacuolar degeneration, glomerular shrinkage and glomerular basement membrane thickening (fig. 1D). However, when treated with TGP, the lesions improved. These results suggest that TGP protects mice from obesity-induced kidney lesions.

**TGP reduced kidney fibrosis**
Then, we evaluated the fibrosis in the kidneys. Sirius Red and Masson staining for collagen both revealed fibrotic lesions in the kidneys of HFD-fed mice (figs. 2A and 2B). The mRNA levels of collagen I, TGF-β and C-TGF significant increased in HFD-fed mice compared to those in the control group. However, TGP distinctly reversed this increase (figs. 2C-2E). These results suggest that TGP protects mice from obesity-associated kidney fibrosis.

**TGP reduce kidney inflammation**
Inflammation is an essential pathological process leading to kidney damage caused by obesity. Therefore, we examined whether TGP could inhibit HFD-induced kidney inflammation. F4/80 immunohistochemical staining showed that HFD-fed had increased F4/80 levels, indicating high macrophage infiltration, which was significantly decreased with TGP treatment (fig. 3A). Furthermore, we investigated pro-inflammatory cytokines mRNA levels, including TNF-α (fig. 3B), IL-6 (fig. 3C), IL-1β (fig. 3D). TGP treatment reversed the HFD-induced the increase in pro-inflammatory cytokines. These results suggest that TGP protects mice from obesity-induced kidney inflammation.

**TGP inhibited hyperlipidemia-induced activation of NF-κB and p-JNK signals via MyD88**
To explore whether TGP’s anti-inflammatory effect was mediated by MyD88 inhibition mediating NF-κB and JNK, the MyD88, phosphorylated JNK and IκB levels were determined. MyD88 and phosphorylated JNK levels were markedly increased in the HFD-fed group. However, these levels were reduced by TGP treatment. HFD also induced IκB degradation, however, TGP treatment reversed this degradation (fig. 4A). Palmitic acid (PA) is the most abundant and widespread FFA and is commonly used as a representative FFA to stimulate cellular stress response. Therefore, we used PA (200μM) as a stimulant to investigate the effect of TGP. As expected, PA induced an increase in MyD88 and phosphorylated JNK, however, TGP (150μg/ml or 300μg/ml) reversed the decrease in IκB (fig. 4B). We further silenced MyD88 and detected phosphorylated JNK and IκB levels. Silencing MyD88 suppressed PA-induced activation of NF-κB and JNK by decreasing the JNK phosphorylation and degradation IκB (fig. 4C). These results suggest that TGP inhibited hyperlipidemia-induced inflammation via MyD88 mediating NF-κB and JNK.

**TGP reduce the fibrosis and inflammatory factors in PA-stimulated SV40 cells**
SV40 cells were first pre-treated with PA alone (200μM) or combined with TGP (150μg/ml or 300μg/ml). PA induced an increased in fibrosis cytokines TGF-β, collagen I and C-TGF in SV40 cells, while TGP dose-dependently reduced those cytokines (fig. 5A-C). Moreover, TGP also inhibited the mRNA expression of inflammatory markers, such as TNF-α, IL-6 and IL-1β in a dose-dependent manner (figs. 5D-5F). These results suggest that TGP inhibited PA-induced fibrosis and inflammation in SV40 cells.
Table 1: Primer sequences.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
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<tr>
<td>TGF-β</td>
<td>Forward primer: GGACACTGTTACATAGCTCAAGT&lt;br&gt;Reverse primer: TGCATAGCAGATGGATTCCATCC</td>
</tr>
<tr>
<td>Collagen1</td>
<td>Forward primer: AGATGCCTGTAAGGGAATCAGAG&lt;br&gt;Reverse primer: CCAGTGTGCTCTAAAGTCCAG</td>
</tr>
<tr>
<td>Collagen4</td>
<td>Forward primer: GACCCACTATGAGCGAGGCC&lt;br&gt;Reverse primer: CCCCCACAGGTCTTAGAAC</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Forward primer: GTGGAAGCCATTTGTGCTCAAATC&lt;br&gt;Reverse primer: CCCCCTTGAGGTTGATTAAT</td>
</tr>
<tr>
<td>IL-6</td>
<td>Forward primer: GCACGGTACAGTGAGTTGCAT&lt;br&gt;Reverse primer: GAGCCACGATACACAGT</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Forward primer: TGCCACCTTTTGACACGTAGT&lt;br&gt;Reverse primer: AAGTTCACGGGGGAGACAC</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward primer: CGTCATTGCAGGAGACACAA&lt;br&gt;Reverse primer: CCTGGTCCACCATTTAAAGGC</td>
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</table>

Table 2: TGP slowed body weight gain in HFD-mice (n=7).

<table>
<thead>
<tr>
<th>Groups</th>
<th>First month(g)</th>
<th>Second month(g)</th>
<th>Third month(g)</th>
<th>Fourth month(g)</th>
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<tbody>
<tr>
<td>Control</td>
<td>20.1±0.6</td>
<td>23.4±0.2</td>
<td>25.4±0.1</td>
<td>27.9±0.6</td>
</tr>
<tr>
<td>HFD</td>
<td>21.2±0.8</td>
<td>28.1±0.6</td>
<td>37.4±0.8**</td>
<td>50.1±0.5***</td>
</tr>
<tr>
<td>HFD+TGP-100mg/kg</td>
<td>20.3±0.2</td>
<td>25.9±0.2</td>
<td>32.4±0.5</td>
<td>39.8±0.7</td>
</tr>
<tr>
<td>HFD+TGP-200mg/kg</td>
<td>21.8±0.3</td>
<td>25.4±0.3</td>
<td>30.4±0.4*</td>
<td>36.8±0.2*</td>
</tr>
</tbody>
</table>

### p<0.01, ### p<0.001, HFD group vs Control group; *p<0.05, ** p<0.01, TGP group vs HFD group

Table 3: TGP improved hyperlipidemia in HFD-mice (n=7).

<table>
<thead>
<tr>
<th>Groups</th>
<th>TCH (mg/dl)</th>
<th>TG (mg/dl)</th>
<th>HDL (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>278.1±0.6</td>
<td>59.5±0.9</td>
<td>117.8±0.4</td>
</tr>
<tr>
<td>HFD</td>
<td>728.9±0.8***</td>
<td>100.9±0.8**</td>
<td>74.4±0.3***</td>
</tr>
<tr>
<td>HFD+TGP-100mg/kg</td>
<td>708.3±0.2</td>
<td>80.5±0.7</td>
<td>86.1±0.4</td>
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<tr>
<td>HFD+TGP-200mg/kg</td>
<td>664.8±0.3</td>
<td>67.8±0.6</td>
<td>102.3±0.2</td>
</tr>
</tbody>
</table>

### p<0.01, ### p<0.001, HFD group vs Control group; *p<0.05, ** p<0.01, TGP group vs HFD group

Fig. 1: TGP improved the pathology and histology of HFD mice. Mice serum was collected and analyzed. TGP treatment reduced the increased serum Cr (A), BUN (B) and ALB levels (C) in HFD-fed mice. (D) TGP treatment improved kidney histological abnormalities in HFD-fed mice (400× magnification). Histological analysis using H&E staining showed that TGP treatment significantly improved glomerular shrinking and tubular necrosis. Data are presented as the means ± SEM (n=7 in each group; HFD group vs Control group, **p<0.01, ***p<0.001; TGP group vs HFD group, #p<0.05, ##p<0.01) ALB: albumin; BUN: blood urea nitrogen; Cr: creatinine; HFD: high-fat diet; TGP: total glucosides of paeony.
Fig. 2: TGP inhibited kidney fibrosis in HFD mice. (A-B) TGP treatment considerably decreased kidney fibrosis in HFD-fed mice, as indicated by Sirius Red staining (red) and Masson staining (blue) (200×magnification). (C-E) TGP treatment significantly inhibited the expression of fibrosis markers including collagen I (C), TGF-β (D) and C-TGF (E) at the increased level in HFD-fed mice. Data are means ± SEM (n=7 in each group; HFD group vs Control group, ###p<0.001, ##p<0.01; TGP group vs HFD group, *p<0.05, **p<0.01, ***p<0.001). HFD: high-fat diet; TGP: total glucosides of paeony.

Fig. 3: TGP inhibited kidney inflammatory responses in HFD mice. Kidney tissues in each group were collected and analyzed. (A) TGP treatment inhibited kidney macrophage infiltration in HFD-fed mice. F4/80 staining was used to detect macrophage infiltration (400×magnification). (B-D) TGP treatment reduced kidney inflammatory genes expression, including TNF-α (B), IL-6 (C) and IL-1β (D) in HFD-fed mice. Data are presented as the means ± SEM (n=7 in each group; HFD group vs Control group, ###p<0.001, ##p<0.01; TGP group vs HFD group, *p<0.05, **p<0.01, ***p<0.001). HFD: high-fat diet; TGP: total glucosides of paeony.
DISCUSSION

Obesity is a substantial risk factor for chronic kidney disease. Furthermore, obesity can lead to structural and histopathological changes in the kidneys by affecting renal hemodynamics, angiotensin-aldosterone system and insulin resistance thus causing nephropathy (Hall et al., 2021; Hall et al., 2019). However, the treatment options are still limited, resulting in an increased incidence of end-stage renal disease. Therefore, new methods or drugs are urgently needed to treat obesity-induced kidney damage. Traditional Chinese herbal extracts or monomers can effectively delay the progression of obesity-related kidney disease and have a strong potential market in treating chronic diseases. Consequently, we explored TGP to protect the kidneys against obesity-induced renal injury. To the best of our knowledge, this is the first study to investigate the therapeutic effect of TGP on obesity-related kidney disease.
TGP is recognized as a valuable traditional herb to treat rheumatoid arthritis, systemic lupus erythematosus and hepatitis.

Moreover, TGP reportedly cured, Heymann nephritis, IgA nephropathy and diabetic nephropathy. Additionally, TGP extracted from *Radix Paeoniae Alba* can reduce blood lipids and enhance insulin sensitivity (Chang et al., 2021; Chen et al., 2020). In this study, C57BL/6 mice fed with HFD showed obesity, elevated serum Cr, BUN, urea ALB levels and pathological changes, including glomerular enlargement and thickened glomerular basement membranes, renal fibrosis and collagen accumulation. However, TGP administration ameliorated obesity-induced kidney pathology and histological changes, decreasing Cr, BUN and ALB levels. We also observed an inhibiting effect of TGP on inflammation by reducing macrophage infiltration and inflammatory factors release in HFD-fed mice’s kidneys.

Increased cytokine production and inflammatory cell infiltration in tissues promotes the progression of obesity-related kidney damage (Gregor and Hotamisligil, 2011; Manabe, 2011). Inflammation is the early pathology of renal disease, which can promote kidney fibrosis and sclerosis, resulting in renal function disruption, which leads to end-stage renal disease (Arabi et al., 2022; Stone et al., 2017). Macrophage infiltration in kidneys is associated with increased inflammatory level leading to kidney damage. However, reducing the pro-inflammatory macrophages infiltration in kidneys successfully improved renal damage (Kavvadas et al., 2018). Macrophages infiltrating to renal tissue secrete inflammatory factors to stimulate inflammatory kidney lesions and cause apoptosis to induce the kidney necrosis (Liang et al., 2021; Palau et al., 2021). Therefore, anti-inflammatory treatment by inhibiting the inflammatory response in kidneys may be the prevention or treatment of obesity-induced kidney damage.

TGP could inhibit the progression of inflammation-related diseases, such as autoimmune diseases, enteritis and hepatitis, diabetes and had an inhibitory effect on fibrosis and apoptosis caused by inflammation (Jin and Zhang, 2022; Zhang et al., 2022). In addition, TGP was also effective in inducing the production of M2-type anti-inflammatory macrophages production (Jin et al., 2022). In this study, the increase of phosphorylated JNK and degradation of IκB in HFD-fed mice and PA-stimulated SV40 cells could be reversed by TGP. These results support the ability of TGP to protect against hyperlipidemia-induced renal damage.

Transcription factor nuclear activation of factor-kappa B (NF-κB) plays a central role in regulating inflammation by regulating pro-inflammatory cytokines, chemokines and cell adhesion molecules (Yu et al., 2020). Once activated, IκB is phosphorylated and degraded, resulting in its subunit, p65, migrating into the nucleus to activate the expression of its downstream genes expression. Moreover, JNK is another responsive kinase to obesity which represents a possible target for therapeutic intervention in obese patients. Studies demonstrate that JNK is required to establish obesity-induced insulin resistance and inflammation (Solinas and Becattini, 2017). MyD88 is the common upstream gene of NF-κB and JNK signals. MyD88 is a typical adapter of inflammatory signaling pathways for the toll-like receptor (TLR) and interleukin-1 (IL-1) receptors, leading to various functional outputs, including NF-κB, mitogen-activated protein kinases (MAPK) and signal transducer and activators of transcription 3 (STAT3). *In vivo* and *in vitro*, we all found that TGP inhibited MyD88 expression and silencing MyD88 in SV40 cells inhibited the activation of the NF-κB and JNK pathways activation. These results support the hypothesis that MyD88 mediated the anti-inflammatory effect of TGP and maybe a target of obesity-related kidney damage.

**CONCLUSION**

The results of this study indicate that TGP is an effective therapeutic agent for treating obesity-induced kidney damage. TGP treatment inhibited inflammation by blocking inflammatory cytokines release and immune cell infiltration and exhibited substantial improvements in histological abnormalities and fibrosis. The results showed that TGP could reduce the expression of MyD88 and inhibit IκB degradation and JNK phosphorylation and the TGP mechanism may be inhibiting MyD88-mediating JNK and IκB.

**ACKNOWLEDGEMENTS**

We would like to give our thanks to Prof. Luo Fang for valuable discussion. This research was funded by the Medical Health Science and Technology Project of Zhejiang Province (No. 2022KY105 and 2017190933), National Natural Science Foundation of Zhejiang Province (No. LY22H280015, YY19H310003 and LYY18H310006) and National Natural Science Foundation of China (No. 81903898 and 81803585).

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