Polyphyllin I alleviates inflammatory injury in mice with gestational diabetes through AMPK pathway

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Abstract. Our study aimed to detect the effects of polyphyllin I (PPI) on relieving gestational diabetes mellitus (GDM), and the possible mechanism. A mouse model of GDM was constructed. The effects of PPI on GDM mice were evaluated by detecting blood glucose, insulin level, glucose tolerance test, and insulin tolerance test. The inflammation response in GDM and GDM+PPI group were evaluated by enzyme-linked immunosorbent assay (ELISA). The effect of PPI on the offspring of GDM mice was analyzed. In addition, immunoblot assays were performed to investigate the effects of PPI on the AMPK pathway. We found that PPI improved diabetes-related symptoms and decreased serum inflammatory response in GDM mice. In addition, we also found that PPI reduced the tissue damage of GDM mice. We noticed that PPI alleviated inflammatory injury in GDM mice through targeting AMPK pathway. Our findings showed that PPI has the potential to be explored as the drug for GDM treatment.

Key words: Gestational diabetes mellitus — Polyphyllin I — Diabetes-related symptoms — Inflammatory response — AMPK pathway

Introduction

Gestational diabetes mellitus (GDM) is a common medical condition in pregnant women. According to the population studied, approximately 1.7 to 12% of pregnant women may develop GDM (Pan et al. 2021). During pregnancy, the placenta is the main organ that secretes various hormones, such as estrogen, progesterone and prolactin, which have insulin antagonistic effect (Wang et al. 2021). Blood glucose levels rise when the pancreas does not produce enough insulin to control normal blood glucose levels, and persistent high blood glucose levels contribute to the development of GDM. GDM is closely associated with an increased frequency of large foetus, which may increase the incidence of the development of cardiovascular disease, obesity and diabetes in offspring (Scazzocchio et al. 2021). To better treat the

disease, new and more effective drugs or treatments are still needed to be developed.

Chinese herbal medicine has been used for thousands of years for the treatment of many diseases. Polyphyllin I (PPI), also known as saponin I, a steroid saponin, is a bioactive substance isolated from the roots and stems of Parisian leafy plants (Lou et al. 2017). Previous reports have shown that PPI has anticancer effects in a variety of cancers, and inhibits the growth of non-small cell lung cancer (NSCLC) by activating the adenosine monophosphate-activated protein kinase (AMPK) pathway (Zou et al. 2018). PPI also promoted cell death *via* suppressing unfolded protein response (UPR)-mediated C/EBP homologous protein (CHOP) ubiquitination in NSCLC (Yang et al. 2019). In addition, another study indicated that PPI reduces inflammation and oxidative stress and protects against myocardial ischemia (Huang et al. 2020). Moreover, PPI attenuated cognitive impairments and reduced Alzheimer's disease (AD)-like pathology in 3XTg-AD mice (one of the most commonly used transgenic models of AD) (Zhou et al. 2020). However, its possible effects on the progression of GDM are still unclear.

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It was also revealed that the inhibition of AMPK signal pathway is one of the characteristics of GDM (Zhang et al. 2016). Inhibition of AMPK signaling has been associated with insulin resistance, inflammation, and oxidative stress. For example, activation of AMPK attenuates lipopoly-saccharides (LPS)-induced release of pro-inflammatory cytokines. Furthermore, there is a link between AMPK inhibition and reduced glucose uptake, which is a factor in hyperglycemia (Huo et al. 2013). AMPK has been used as a target for the treatment of GDM in many studies (Zhao et al. 2011).

This study aimed to investigate the possible effects of PPI on the progression of GDM. We found that PPI improved diabetes-related symptoms in GDM mice and we thought it could serve as a promising drug for GDM treatment.

Materials and Methods

GDM mice model establishment

C57BL/6 mice (6–8 weeks, 20–25 g) were obtained from Shanghai SLAC Laboratory Animal Technology Company Limited and were housed in temperature-controlled condition under a 12-h light/dark cycle. In this study, all the animal procedures were approved by The First Affiliated Hospital of Wenzhou Medical University. After adaptation for 1 week, mice were paired with healthy male mice at a ratio of 2:1. The next day, the mice were examined for pregnancy or not. Pregnant mice were randomly divided into 4 groups (5 mice for each group): control, GDM, GDM+PPI (1 mg/kg), GDM+PPI (5 mg/kg). GDM group was *intraperitoneally* (*i.p.*) injected with 0.25% streptozotocin (STZ) solution (Sigma-Aldrich, St. Louis, USA) at 80 mg/kg for 3 consecutive days. No mice were dead upon the experiments. Mice in the control group were given the same volume of saline. Blood glucose higher than 11 mmol/l within 48 h was considered as GDM. PPI was given at the concentration of 1 mg/kg, 5 mg/kg for a week by *i.p.* administration. Body weight, blood glucose and insulin level were monitored at the 0, 10, and 20 days of pregnancy. Meanwhile, the weight and number of fetal mice were recorded for subsequent

analysis. Subsequently, the peripheral blood and pancreas were collected.

Measurement of glucose tolerance test (GTT) and insulin tolerance test (ITT)

Fasting glucose level was quantified from tail incision method with hemoglucometer (Lifescan, Johnson and Johnson, USA). For GTT and ITT measurement, after fasting for 6 h, mice were orally given glucose at 2 g/kg or insulin at 1.0 mU/kg body weight and blood was collected at 0, 10, 20, 30, 60 and 120 min, using hemoglucometer (Lifescan, Johnson and Johnson, USA).

Measurement of insulin level

Insulin level was detected by Mercodia Rat Insulin ELISA kit (Sweden). The optical densities of the samples were read at 450 nm.

ELISA

The concentrations of IL-1β, TNF-α, and IL-6 in the serum were detected by ELISA according to the protocol of the ELISA kits. Briefly, serum samples were added into wells. Biotin-conjugated specific antibody was added before the addition of avidin conjugated horseradish peroxidase (HRP). Subsequently, enzyme substrate was added for color reaction. The optical densities of the samples were read at 450 nm (R&D systems, Minneapolis, MN, USA).

Quantitative PCR

Trizol (Invitrogen, Waltham, MA, USA) reagent were used for total RNA extraction. Then RNA was reverse-transcribed into cDNA using Moloney Murine Leukemia Virus Reverse Transcriptase (Promega, Madison, WI, USA). Fast Start Universal SYBR Green Master kit (Roche, Basel, Switzerland) was used for quantitative mRNA detection on ABI StepOne system (Applied BioSystems, Foster City, CA, USA). The levels of targeted genes were determined by using the $2^{-\Delta\Delta CT}$ method. The primers used were listed in Table 1 (Primer bank).

Table 1. The primers for quantitative PCR

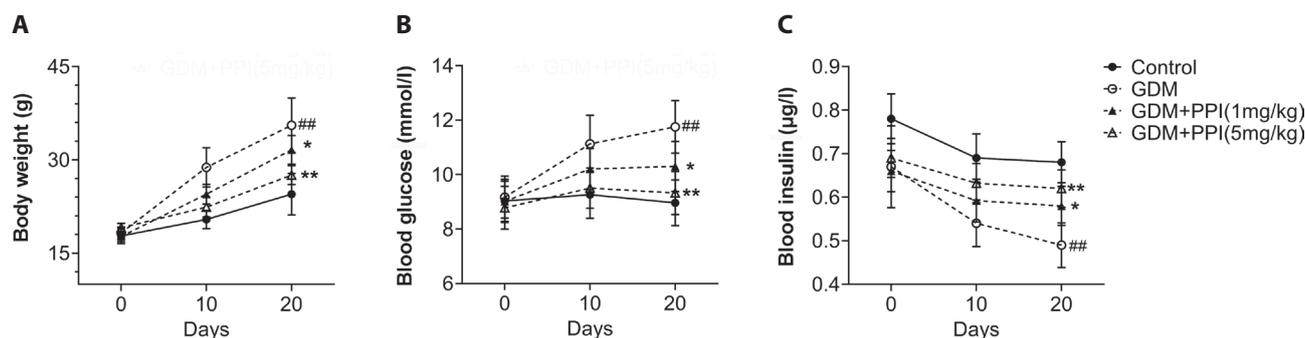| Genes	Forward primers	Reverse primers
TNF-α	GAACTGGCAGAAGAGGCACT	GGTCTGGGCCATAGAACTGA
IL-6	CTGATGCTGGTGACAACCAC	CAGAATTGCCATTGCACAAC
IL-1β	TGGACCTTCCAGGATGAGGAC	GTTCATCTCGGAGCCTGTAGTG
GAPDH	AGTATGACTCCACTCACGGC	CACCAGTAGACTCCACGACA

Figure 1. PPI decreases the body weight (**A**), serum glucose (**B**) and insulin (**C**) level in control, GDM, GDM+PPI (1 mg/kg), and GDM+PPI (5 mg/kg) groups. The experiment was repeated three times. * $p < 0.05$, ** $p < 0.01$ *vs.* GDM group; ## $p < 0.01$ *vs.* control group. GDM group was *i.p.* injected with 0.25% streptozotocin (STZ) solution at 80 mg/kg for 3 consecutive days. PPI, polyphyllin I; GDM, gestational diabetes mellitus.

Immunoblot assay

Proteins were extracted with the use of RIPA buffer (Cell Signaling). Then, the samples were collected and subjected to 10% SDS-PAGE, and transferred onto PVDF membranes, followed by blocking with 5% fat-free milk in TBST buffer. Subsequently, membranes were conjugated with primary antibodies targeting p-HDAC4 (1:1000, Abcam, Cambridge, UK), HDAC (1:1000, Abcam), p-AMPK (1:1000, Abcam), AMPK (1:1000, Abcam), and GAPDH (1:10000, Abcam) for 2 h at room temperature. Subsequently the membranes were incubated with specific secondary antibodies at room temperature for 1 h. The blots were analyzed with ECL kit.

Statistical analysis

Data were displayed as mean ± SD. Statistical analysis was performed using GraphPad. Significance was assessed by analysis of variance (ANOVA). In addition, the results among control, GDM, GDM+PPI (1 mg/kg), GDM+PPI (5 mg/kg) groups are present used two-way Anova statistics. $p < 0.05$ was considered statistically significant.

Results

PPI treatment decreased the body weight, blood glucose and insulin levels in GDM mice

To detect the effect of PPI on the body weight, blood glucose and insulin level in GDM mice, the blood in each group were collected. Compared with the control group, markedly higher body weight and blood glucose levels and lower insulin level were observed in GDM mice. PPI treatment effectively relieved these parameters in GDM mice at 20 days of pregnancy (Fig. 1). Taken together, these data indicated that PPI ameliorated diabetic disorders in GDM mice.

PPI treatment relieves the glucose intolerance and insulin resistance in GDM mice

As shown in Figure 2A, GDM mice exhibited significant glucose intolerance and insulin resistance as detected by much higher blood glucose level after injection of glucose (Fig. 2A) and insulin (Fig. 2B). Therefore, our data confirmed that PPI treatment markedly alleviated glucose intolerance and insulin resistance phenotypes.
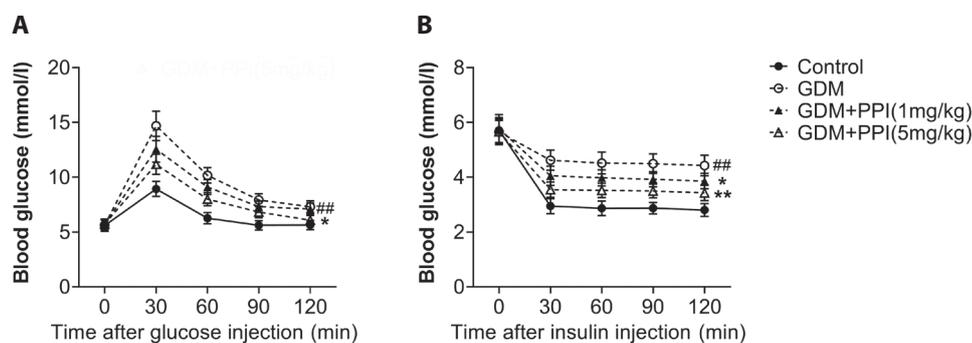

Figure 2. PPI relieves the glucose intolerance (**A**) and insulin resistance (**B**) in control, GDM, GDM+PPI (1 mg/kg), and GDM+PPI (5 mg/kg) groups. The experiment was repeated three times. * $p < 0.05$, ** $p < 0.01$ *vs.* GDM group; ## $p < 0.01$ *vs.* control group. For abbreviations see Figure 1.
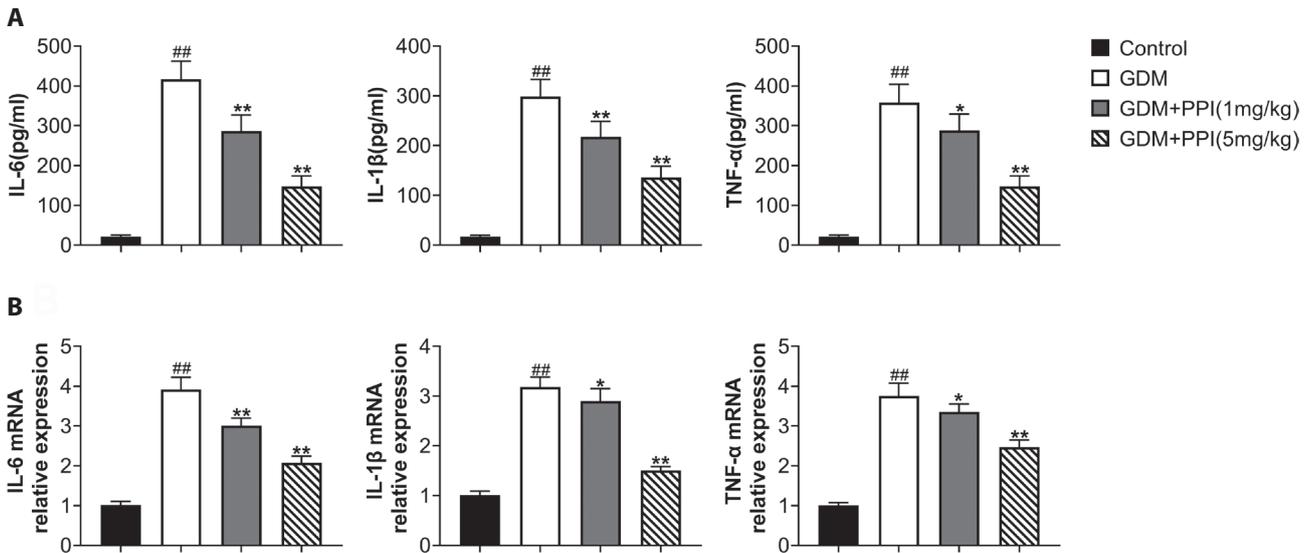
A

B

Figure 3. PPI improves the inflammation in GDM mice. **A.** The IL-6, IL-1β, and TNF-α levels in control, GDM, GDM+PPI (1 mg/kg), and GDM+PPI (5 mg/kg) groups. **B.** The mRNA level of IL-6, IL-1β, and TNF-α in control, GDM, GDM+PPI (1 mg/kg), GDM+PPI (5 mg/kg) groups. The experiment was repeated three times. * $p < 0.05$, ** $p < 0.01$ *vs.* GDM group; ## $p < 0.01$ *vs.* control group. For abbreviations, see Figure 1.

PPI treatment improves the inflammation in GDM mice

Since GDM is accompanied by increased inflammation, we investigated the effect of PPI on proinflammatory cytokines, including IL-6, IL-1β, and TNF-α. Both ELISA and qPCR assays confirmed that GDM led to a dramatic increase in IL-6, IL-1β and TNF-α secretion levels (Fig. 3A) as well as the mRNA levels (Fig. 3B). Importantly, PPI treatment reversed the increase in cytokine levels in GDM animals (Fig. 3A,B). Taken together, these data indicated that PPI is a promising inhibitor of inflammation in GDM.

PPI improves the outcome of pregnant mice

Since GDM has an adverse effect on the quality and quantity of offspring, we investigated the effect of PPI on the body weight and number of fetal mice. Consistent with previous studies, GDM led to reduced number and lower body weight of offspring (Fig. 4A). PPI treatment significantly relieved the adverse effects of GDM (Fig. 4B). Therefore, PPI improved the outcome of pregnant mice.

PPI alleviates inflammatory injury in GDM mice through targeting AMPK pathway in GDM mice

To determine the role of the AMPK pathway in PPI-mediated GDM relieve, we studied the activities of p-HDAC and p-AMPK in pancreas tissues in GDM mice by Western blotting. The protein expression of p-HDAC significantly increased in GDM mice. PPI treatment decreased p-HDAC expression (Fig. 5). Moreover, the expression of phosphorylated AMPK was inhibited in pancreas tissues
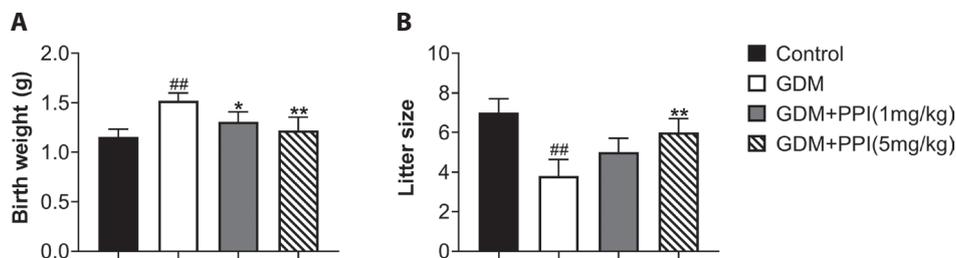

Figure 4. PPI improves the outcome of pregnant mice. The birth weight (**A**) and litter size (**B**) in control, GDM, GDM+PPI (1 mg/kg), and GDM+PPI (5 mg/kg) groups. The experiment was repeated three times. * $p < 0.05$, ** $p < 0.01$ *vs.* GDM group; ## $p < 0.01$ *vs.* control group. For abbreviations, see Figure 1.
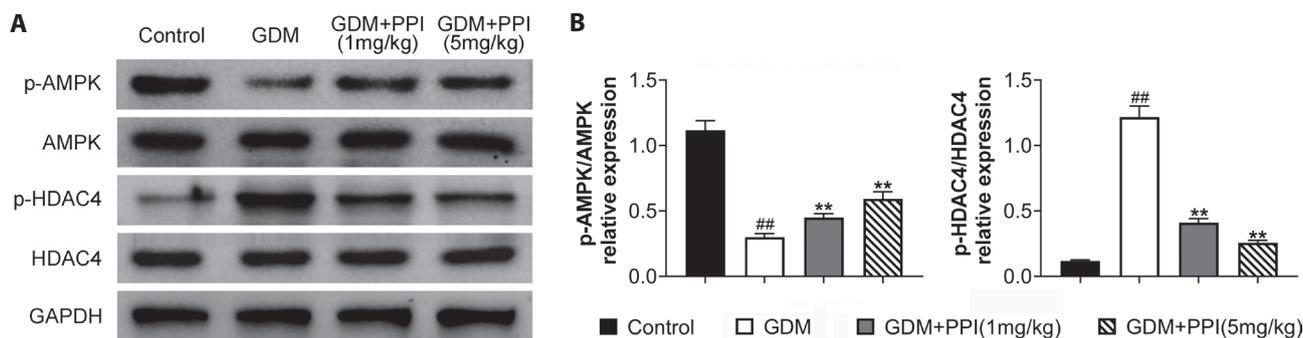
Figure 5. PPI alleviates inflammatory injury in GDM mice through targeting AMPK pathway in GDM mice. The expression level of p-HDAC and p-AMPK in control, GDM, GDM+PPI (1 mg/kg), and GDM+PPI (5 mg/kg) groups. **A.** The results of immunoblot assay. **B.** The quantitative results. The experiment was repeated three times. ** $p < 0.01$ *vs.* GDM group; ## $p < 0.01$ *vs.* control group. For abbreviations, see Figure 1.

of GDM mice, and PPI treatment induced the expression levels of phosphorylated AMPK. In conclusion, we thought PPI alleviates inflammatory injury in GDM mice through targeting AMPK pathway in GDM mice.

Discussion

GDM is a type of diabetes that occurs or is diagnosed during pregnancy in women who have normal glucose metabolism before pregnancy or have underlying glucose tolerance. The reported incidence of the disease in other countries is 1–14%, and the incidence in China is about 1–5%, with an obvious trend of increase in recent years (Najafi et al. 2021). During GDM, blood glucose levels rise when the pancreas does not produce enough insulin to control normal blood glucose levels, and continuous high blood glucose levels contribute to the development of GDM. GDM is closely associated with an increase in the frequency of giant fetuses (Mierzynski et al. 2021). To better improve the outcome of GDM, more effective drugs are needed to be developed. In this study, we noticed that the steroid saponin, PPI, could reduce body weight and blood glucose, increase insulin level, reduce blood lipid and improve pancreatic tissue damage in GDM mice.

The effects of PPI on multiple biological activities, such as anti-tumor and anti-inflammation, have been widely revealed (Liu et al. 2018). For example, PPI could promote autophagy and apoptosis *via* PI3K/Akt/mTOR pathway in melanoma cells (He et al. 2019). Also, the protective effect of PPI on myocardial ischemia/reperfusion injury in mice has been reported. Another study indicated that PPI could attenuate pressure over-load induced cardiac hypertrophy *via* suppressing Wnt/β-catenin pathway (Chai et al. 2018). Notably, a previous study showed that PPI activated AMPK pathway to suppress the progression of NSCLC *via* inducing autophagy. Similarly, we also revealed here that PPI at-

tenuated GDM *via* targeting AMPK pathway (Zhang et al. 2019). Through ELISA, we noticed that PPI improved DM symptoms in GDM mice. Further ELISA and HE assays results indicated that it could reduce serum inflammation and tissue damage in GDM mice. Therefore, our data confirmed that it could serve as a promising drug for GDM treatment.

Previous study indicated that the inhibition of AMPK pathway is one of the features of GDM. The suppression of this pathway has been associated with insulin resistance, inflammation, and oxidative stress. For example, activation of AMPK attenuated LPS-induced release of pro-inflammatory cytokines (Wang et al. 2020). AMPK activation has also been shown to be a cellular reprogramming process that reduces oxidative stress in response to mitochondrial dysfunction. Thus, AMPK is considered a promising target for the regulation of diseases characterized by elevated inflammation and oxidative stress. Furthermore, there is a link between AMPK inhibition and reduced glucose uptake, which is a factor in hyperglycemia (Liu et al. 2019). In several studies, AMPK has been used as a target for the treatment of GDM. We also noticed here that PPI alleviated inflammatory injury in GDM mice *via* AMPK pathway. In a previous study, AMPK activation was found to be attenuated in GDM mice, contributing to higher HDAC4 activation. Reduced HDAC4 expression in the liver strikingly improved glucose tolerance in GDM. Therefore, we detected the activation of HDAC4 in our model (Zou et al. 2018). We thought that this pathway could serve as a promising target for GDM. However, the precise molecular mechanism needs further study.

In conclusion, we investigated the effects of PPI on GDM progression. We found that PPI improved DM symptoms in GDM mice. In addition, PPI could reduce serum inflammation and tissue damage in GDM mice and could alleviate inflammatory injury in GDM mice *via* AMPK pathway. Therefore, we thought it could serve as a drug for GDM treatment.

Availability of data and materials. All data generated or analyzed during this study are included in this published article.

Conflict of interests. The authors state that there are no conflicts of interest to disclose.

Ethics approval. Ethical approval was obtained from the Ethics Committee of The First Affiliated Hospital of Wenzhou Medical University.

Contribution of authors. YC and FX designed the study, supervised the data collection; HW analyzed and interpreted the data; QZ, FL and SP prepare the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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