

The effects of *Oldenlandia diffusa* water extract on glucose metabolism and inflammation level in rats with streptozotocin-induced gestational diabetes mellitus

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Received: 18 September 2021; Accepted: 21 October 2021; Published: 20 January 2022

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RESEARCH ARTICLE

Abstract

To reveal the effects of *Oldenlandia diffusa* (OD) on relieving the progression and development of gestational diabetes mellitus (GDM), and explore the underlying mechanism. A rat model of GDM was established by streptozotocin injection. The effects of OD on GDM rats were evaluated by measuring the levels of fasting blood glucose (FBG), insulin, and hemoglobin A1c (HbA1c), and exposing to oral glucose tolerance test (OGTT) and histological evaluation of the pancreas. The levels of insulin and inflammation response-related factors (tumor necrosis factor [TNF]- α , Interleukin [IL]-6 and IL-1 β) were evaluated by enzyme-linked immunosorbent assay (ELISA). Additionally, immunoblot assay was performed to investigate the effects of OD on the nuclear factor- κ B (NF- κ B) pathway and 5' adenosine monophosphate-activated protein kinase (AMPK) pathway. OD decreased blood glucose level, pancreatic tissue damage, and insulin secretion in GDM rats. OD also reduced serum inflammatory levels (TNF- α , IL-6, and IL-1 β) in GDM rats. Mechanically, OD could inhibit NF- κ B pathway and activate AMPK pathway in the pancreatic tissue of GDM rats. OD affected glucose metabolism and inflammation level in rats with streptozotocin-induced GDM, and the underlying mechanism was through AMPK pathway. OD might serve as a promising and potential drug for the treatment of GDM.

Keywords: gestational diabetes mellitus (GDM); blood glucose; inflammation; insulin secretion; AMPK pathway

Introduction

As one of the most common metabolic disorders during pregnancy, gestational diabetes mellitus (GDM) has become a serious risk to the health of pregnant women and their fetuses (Mierzynski *et al.*, 2021). GDM is characterized by glucose intolerance during the second trimester (Eckstein *et al.*, 2021). In pregnant women, adverse outcomes because of the pathogenesis of GDM include insulin resistance (IR), hyperinsulinemia, hyperglycemia,

and abnormal embryonic development (Zhang *et al.*, 2021). Diseases associated with GDM during embryonic development include stillbirth, metabolic disorders, and fetal macrosomia. In patients with GDM, the increased insulin resistance could reduce insulin secretion (Ye *et al.*, 2021). Pregnancy-related hyperinsulinemia and insulin resistance promote diabetes in some pregnant women (Davis *et al.*, 2021). The pathogenesis of GDM is triggered by both environmental and genetic factors; however, its precise mechanism is still unclear.

At present, medicinal plants are widely used to treat diabetes (Zhu *et al.*, 2018). Oxidative stress and inflammation are related to the occurrence of diabetes and its complications; hence, the prevention of diabetes complications by medicinal plants with strong antioxidant and anti-inflammatory properties has attracted great attention. *Oldenlandia diffusa* (OD), also known as snake-needle grass, belongs to Rubiaceae (madder/bedstraw) family, and is a famous medicinal plant of ancient China (Kim *et al.*, 2011a). Existing studies have demonstrated a variety of biological functions of OD, including antiangiogenic, anti-inflammatory, antioxidant, and pro-apoptotic activity (Kim, *et al.*, 2011b; Sunwoo *et al.*, 2015; Zhu *et al.*, 2014). It has been reported that OD improved sodium sulfate-induced colitis by inhibiting nuclear factor- κ B (NF- κ B) activation. However, there are few studies establishing the effects of OD on GDM and its related regulatory mechanisms.

The activation of impaired 5' adenosine monophosphate-activated in GDM mice, which may contribute to insulin resistance (Lu *et al.*, 2016). Naringin therapy increases AMPK activation, which may lead to increased glucose uptake and reduced insulin resistance (Gravandi *et al.*, 2021). Research has established that olinquin can reduce oxidative stress and inflammation by activating AMPK signaling pathway and effectively reduce GDM symptoms and improve pregnancy status in mouse models (Zhang *et al.*, 2017). Studies have also proved that AMPK is a key metabolic enzyme that regulates glucose metabolism (Wang *et al.*, 2015).

In this study, it is hypothesized that OD treatment after the establishment of GDM rat model reduces the levels of blood glucose and inflammation, and alleviates insulin secretion and pancreatic tissue damage through AMPK pathway in GDM rats. The *in vivo* experiments have confirmed that OD could serve as a promising drug for treating GDM.

Materials and methods

Establishment of GDM model

Experimental Wistar rats (females = 40, males = 20) weighing 150–200 g were obtained from Shanghai Slac Laboratory Animal Technology Company Limited (Shanghai, China). The mice were housed in the temperature-controlled condition under a 12-h light–dark cycle. Ethical approval for the animal procedures of this study was obtained from the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University (Approval No. wyd2016-0083).

After adaptation for 1 week, mice were fed with consecutive high-fat diet for 8 weeks. Vaginal smears were performed every day to determine rats' oestrous cycle, and mice in oestrous stage were paired with healthy male rats in 2:1 ratio. Next day, the rats were examined for pregnancy by the appearance of sperm, or by a mucus plug that was observed using a microscopy. The pregnant rats were divided into the following five groups: sham, GDM, GDM + 75-mg/kg OD, GDM + 150-mg/kg OD, and GDM + 300-mg/kg OD. Pregnant rats were injected intraperitoneally with 1% streptozotocin (25 mg/kg) in 0.1-mmol/L citrate buffer to induce the GDM model. Blood glucose level of 6.67–16.67 mmol/L was established as GDM rats. Rats in the sham group received the same amount of citrate buffer. OD was orally administrated at the concentrations of 75, 150, and 300 mg/kg body weight twice a day for a week.

Measurement of fasting blood glucose (FBG) level and oral glucose tolerance test (OGTT)

The FBG level was quantified through tail incision method with hemoglucometer (Lifescan, Johnson and Johnson Company, CA, USA). For OGTT measurement, after fasting for 6 h, rats were given glucose orally at 2 g/kg body weight and blood was collected at 0, 10, 20, 30, 60, and 120 min using a hemoglucometer (Lifescan; Johnson and Johnson). Hemoglobin A1c (HbA1c) was determined using reagent kit purchased from BioSystems SA (Foster City, CA, USA). The insulin sensitivity index (ISI) was obtained as follows:

$$\text{ISI} = \ln(\text{FBG} \times \text{FINS})^{-1}, \text{ where FINS is the fasting insulin level.}$$

Haematoxylin & Eosin (H&E) staining

The pancreas tissues were isolated and subjected to H&E staining according to the manufacture's procedures and mounted with Permout mounting medium (Sinopharm Chemistry Reagent Co. Ltd, Beijing, China). The sections were observed under an optical microscope (DP73; Olympus, Tokyo, Japan).

Enzyme-linked-immunosorbent serologic assay (ELISA)

The concentrations of IL-1 β (sensitivity: 6.5 pg/mL), TNF- α (sensitivity: 4.32 pg/ml), and IL-6 (sensitivity: 11.3 pg/mL) in the serum were detected by ELISA according to the protocol of ELISA kits (Abcam, Cambridge, MA, USA). Briefly, serum samples were added into wells. The biotin-conjugated specific antibodies were added before the addition of avidin conjugated horseradish peroxidase (HRP). Subsequently, enzyme substrate was added for

color reaction. Finally, intensity of the color was measured (R&D Systems, Minneapolis, MN, USA).

Insulin level was detected by Mercodia Rat Insulin ELISA kit (Mercodia AB, Uppsala, Sweden) with a detection limit of 0.15 µg/L. The optical density of the samples was read at 450 nm.

Immunoblot assay

Proteins (30 µg/lane) were extracted by RIPA buffer (Cell Signaling Technology, Danvers, MA, USA). The samples were collected and subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto polyvinylidene difluoride (PVDF) membranes, followed by blocking with 5% fat-free milk in a mixture of tris-buffered saline (TBS) and polysorbate 20 (TBST) buffer. Subsequently, membranes were conjugated with primary antibodies targeting phosphorylated p-65 (p-p65, 1:1000; Abcam, Cambridge, UK), p65 (1:1000; Abcam), phosphorylated IκBα (p-IκBα, 1:1000; Abcam), IκBα (1:1000; Abcam), phosphorylated AMPK (p-AMPK, 1:1000; Abcam), AMPK (1:1000; Abcam), and glyceraldehyde 3-phosphate dehydrogenase (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 (San Diego, CA, USA). Significance was assessed by analysis of variance (ANOVA); $P < 0.05$ was considered as significant.

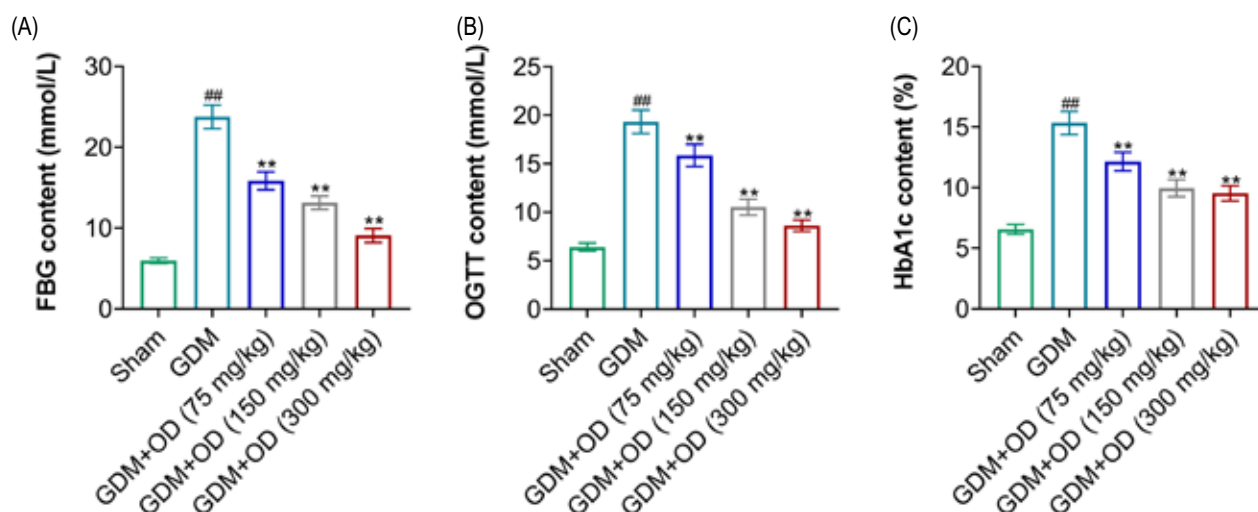


Figure 1. Oldenlandia diffusa decreases blood glucose level in GDM rats. (A–C) FBG, OGTT and HbA1c levels in rats in the sham, GDM, GDM + OD 75, 150, and 300 mg/kg groups. * $P < 0.05$, ** $P < 0.01$ vs. GDM group, # $P < 0.05$, ### $P < 0.01$ vs. sham group.

Results

Oldenlandia diffusa decreases blood glucose level in GDM rats

In order to detect the effect of OD on blood glucose level in GDM rats, the blood samples and pancreas of rats in each group were collected. Compared with that in the sham group, the markedly higher levels of blood glucose, OGTT, and HbA1c were observed in GDM rats ($P < 0.01$). OD treatment effectively reversed the abnormalities of these parameters in GDM rats (Figures 1A–C) ($P < 0.01$). Taken together, the data indicated that OD ameliorates glucose metabolism disorders in GDM rats.

Oldenlandia diffusa reduces pancreas lesion and insulin secretion in GDM rats

H&E staining was used to evaluate tissue lesions in GDM rats. As shown in Figure 2A, GDM rats exhibited mild-to-moderate contraction of the pancreatic islets with inflammatory cell infiltration, disordered pancreas structure, decreased pancreas cells, and pancreatic islet contraction. OD treatment alleviated these phenotypes. Moreover, the reduced insulin secretion and increased ISI in GDM rats were reversed by OD treatment (Figures 2B and C).

Oldenlandia diffusa reduces inflammation in GDM rats

Gestational diabetes mellitus is accompanied with increased inflammation; hence, the effects of OD on proinflammatory cytokines, including IL-6, IL-1β, and TNF-α, were investigated. GDM led to a dramatic increase in the levels of IL-6, IL-1β and TNF-α (Figures 3A–C). OD

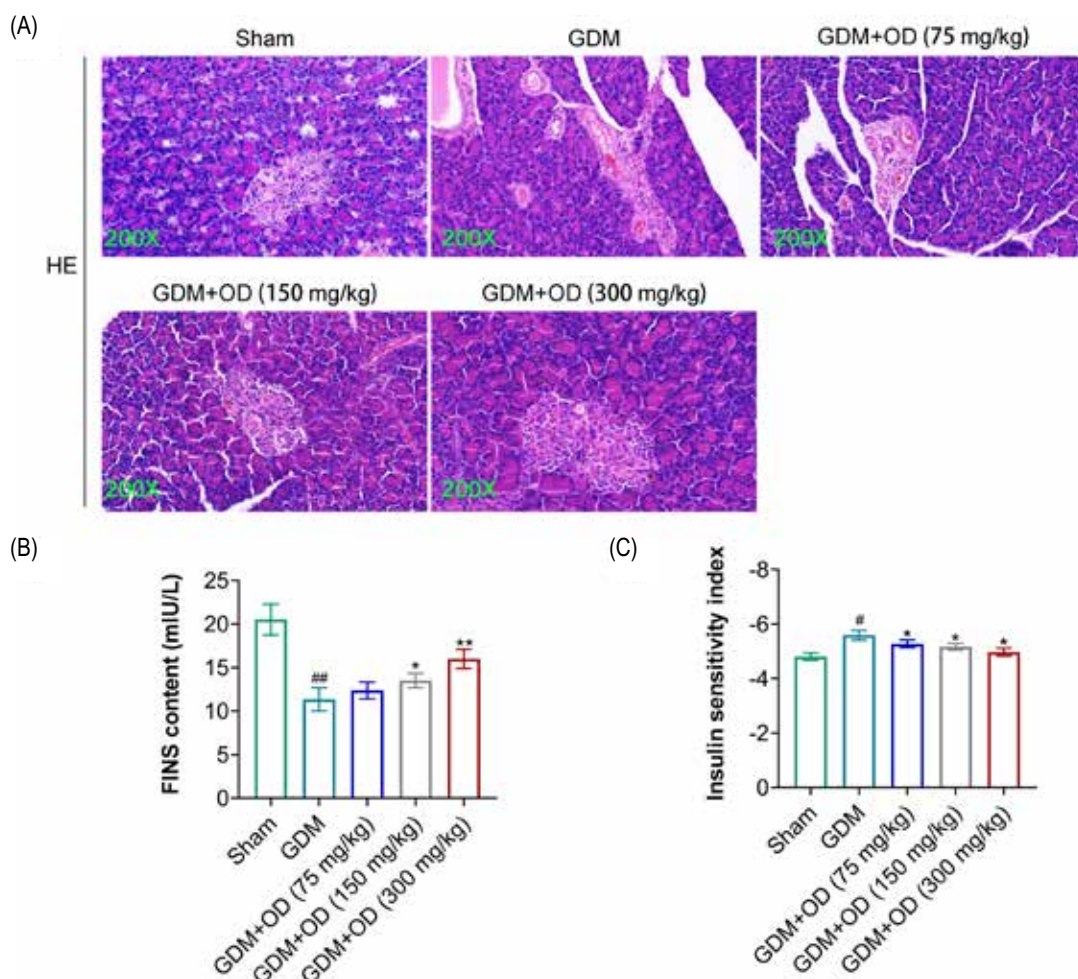


Figure 2. *Oldenlandia diffusa* reduces pancreas lesion and insulin secretion in GDM rats. (A) Histological analysis of rats in the sham, GDM, GDM + OD 75, 150, and 300 mg/kg groups. (B) FINS content and insulin sensitivity index in rats in the sham, GDM, GDM + OD 75, 150, and 300 mg/kg groups. * $P < 0.05$, ** $P < 0.01$ vs. GDM, # $P < 0.05$, ## $P < 0.01$ vs. sham group.

treatment reversed increase in cytokine levels in the blood of GDM rats (Figures 3A–C). Taken together, the data indicated that OD is a promising inhibitor of inflammation in GDM rats.

Oldenlandia diffusa reduces pancreatic tissue damage through AMPK pathway in GDM rats

In order to determine the role of NF- κ B and AMPK pathways in OD-mediated GDM progression, the protein expression levels of p-NF- κ B, p-I κ B α , and p-AMPK in the pancreas tissues of GDM rats were determined by Western blotting testing. The protein expressions of p-NF- κ B and p-I κ B α were significantly increased in GDM rats. OD treatment decreased the protein expressions of p-NF- κ B and p-I κ B α (Figure 4). The protein expression of p-AMPK was also enhanced in the pancreas tissues of GDM rats, and OD treatment suppressed the expression level of p-AMPK (Figure 4).

Discussion

Gestational diabetes mellitus is featured by dysregulated blood glucose and insulin resistance. Multiple factors may contribute to the pathogenesis of GDM, including genetic and environmental factors (Bulut *et al.*, 2021). Notably, therapies targeting inflammation and oxidative stress could alleviate impaired metabolism in diabetes. Previous studies indicated that single nucleotide polymorphism (SNP) in some genes is closely associated with type 2 diabetes and GDM. The occurrence of GDM brings about adverse effects to mothers as well as the offspring (Zhao *et al.*, 2021). GDM during pregnancy might lead to a high tendency of congenital malformations, including the heart, digestive system, and nervous system. Lifestyle and pharmacological interventions have brought about beneficial outcomes in the prognosis in GDM (Wang *et al.*, 2021). In this study, a rat model of GDM was successfully established, and the OD treatment reduced the levels of blood glucose and inflammation, and alleviated

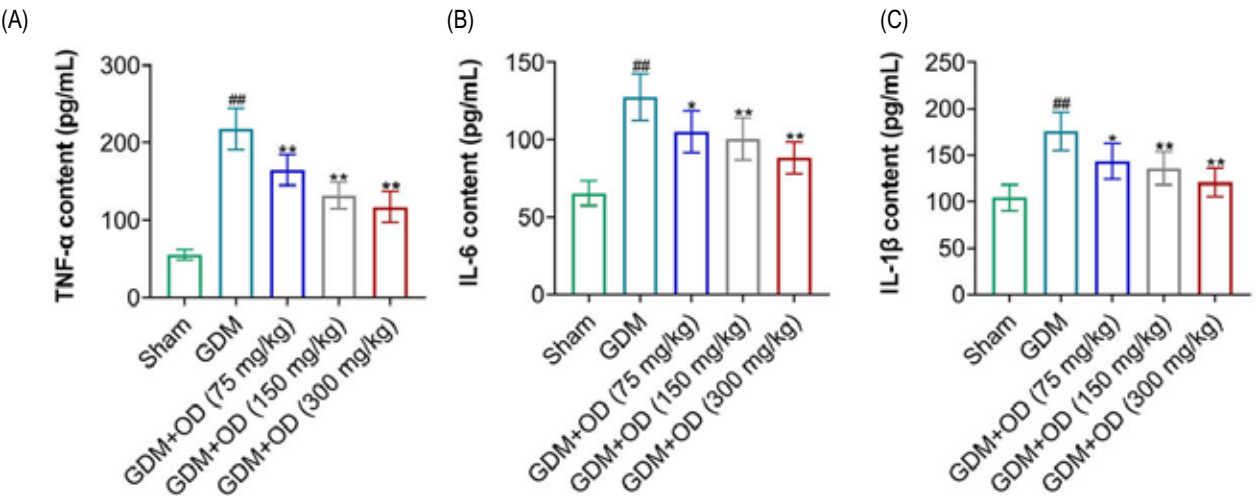


Figure 3. Oldenlandia diffusa improves inflammation in GDM rats. (A–C) TNF- α , IL-6, and IL-1 β levels in the sham, GDM, GDM + OD 75, 150, and 300 mg/kg groups. * $P < 0.05$, ** $P < 0.01$ vs. GDM, # $P < 0.05$, ## $P < 0.01$ vs. sham group.

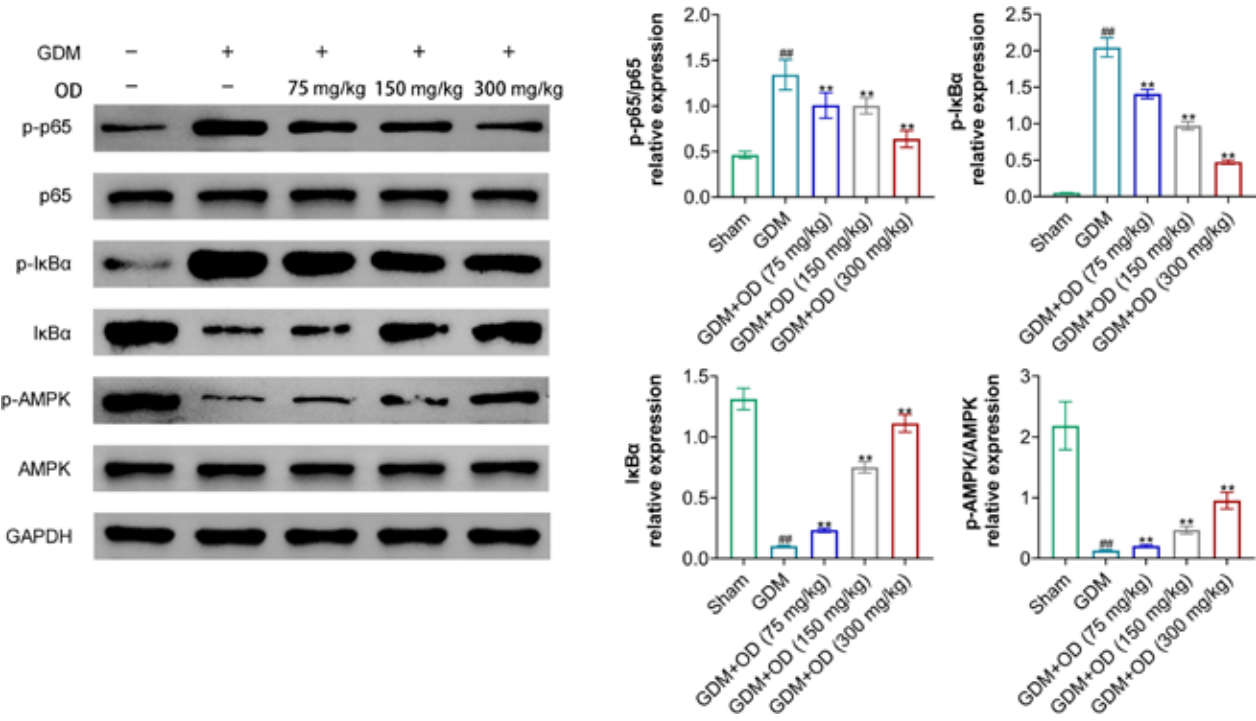


Figure 4. Oldenlandia diffusa improves pancreatic tissue damage through AMPK pathway in GDM rats. The protein expression levels of p-p65, p-IkBa, and p-AMPK in each group. * $P < 0.05$, ** $P < 0.01$ vs. GDM, # $P < 0.05$, ## $P < 0.01$ vs. sham group.

insulin secretion and pancreatic tissue damage through AMPK pathway in GDM rats. Therefore, OD could serve as a promising drug for the treatment of GDM.

As a known medicinal plant in China, OD has exerted various biological activities because of its antiangiogenic, anti-inflammatory, antioxidant, and pro-apoptotic properties according to accumulated studies (Lee *et al.*, 2018; Lin *et al.*, 2018; Zhu *et al.*, 2014), and could serve

as a promising drug for the treatment of several diseases. OD is commonly used in the treatment of cancer, rheumatoid arthritis, and autoimmune diseases (Chung *et al.*, 2017; Gupta *et al.*, 2004; Yadav and Lee, 2006). In addition, OD has proved to be beneficial in improving sodium sulfate-induced colitis by inhibiting NF- κ B activation (Kim, *et al.*, 2011b). In this study, we observed OD treatment alleviated increased blood glucose, insulin level, and impaired pancreas structure. In addition,

the increased OGTT was ameliorated by OD treatment. Whether OD suppresses the progression of other diseases via similar mechanism needs further study.

Many studies have demonstrated a close interaction of inflammatory response and insulin resistance. The insulin signaling transduction was impaired by increasing inflammatory cytokines by inhibiting tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1) (Paneni *et al.*, 2015). As reported, the activation of the NF- κ B pathway contributes to the production of inflammatory mediators. Recent studies have proved that traditional Chinese medicine was efficient in relieving inflammatory response and GDM. For instance, puerarin treatment improved the expression of IRS-1, TLR4, MyD88, and NF- κ B in GDM rats (Ma *et al.*, 2021). Resveratrol treatment brought about improved maternal glucose and lipid homeostasis in both C57BL/KsJ-Lep (db/+) mouse and streptozotocin-induced diabetes animal model. Previous studies have demonstrated that dysregulation in inflammatory response and oxidative stress during pregnancy is induced by altered hormone levels and energy metabolism during pregnancy. In this study, we noticed improved inflammation response in GDM rats after treatment with OD. We also observed the inhibited NF- κ B signaling pathway after OD treatment, indicating the potential mechanism of OD in mediating glucose level and insulin resistance in GDM rats.

According to previous studies, AMPK activation is impaired in GDM animal models, which promotes insulin resistance in GDM animals (Nakamaru *et al.*, 2005). Naringin treatment could induce AMPK activation, subsequently improving glucose uptake and reducing insulin resistance. Other studies have demonstrated that olinquin reduced oxidative stress and inflammation by activating AMPK signaling pathway and effectively alleviated GDM phenotypes and improved pregnancy situations in mouse models (Tajima-Shirasaki *et al.*, 2017). AMPK has been proved as a key metabolic enzyme that regulates glucose metabolism. We observed the impaired AMPK signaling pathway after our construction of GDM rat models. After OD treatment, the AMPK pathway was activated accompanied with enhanced insulin resistance and improved glucose metabolism. In this case, we assume that OD is a promising therapeutic in alleviating GDM symptoms.

Conclusion

In all, we observed that OD treatment alleviated the blood glucose level, insulin resistance, pancreatic tissue damage, and impaired glucose tolerance through AMPK–NF- κ B pathway in GDM rats after the establishment of GDM rat model. Our data, therefore, confirmed

that OD could serve as a promising drug for the treatment of GDM.

Competing interests

The authors stated that there were no conflicts of interest to disclose.

Author contributions

Fengfeng Xie designed the study and supervised data collection. Qianqian Cao analyzed and interpreted the data. Qiuyue Chen, Feng Lin, and Haiou Wang prepared the manuscript for publication and reviewed its draft. All authors have read and approved the final manuscript.

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