

Plumbagin attenuates high glucose-induced trophoblast cell apoptosis and insulin resistance via activating AKT/mTOR pathway

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Abstract

Plumbagin, a bioactive phytoconstituent, is isolated from the root of *Plumbago zeylanica* L. Plumbagin possesses antidiabetic effect to mediate glucose homeostasis, wound healing and diabetic nephropathy. However, the involvement of plumbagin in gestational diabetes mellitus (GDM) has not been reported yet. Trophoblast cell line (HTR8/SVneo) was incubated with high glucose to establish cell model of GDM. Cell viability and proliferation were detected by MTT and EdU staining. Flow cytometry was used to investigate cell apoptosis. Cell viability of HTR8/SVneo was reduced by high glucose or incubation of plumbagin. Plumbagin restored reduced cell viability and proliferation of HTR8/SVneo induced by high glucose. Plumbagin attenuated high glucose-induced cell apoptosis in HTR8/SVneo cells through upregulation of Bcl-2 and down-regulation of Bax, cleaved caspase-3 and cleaved caspase-9. Protein expression of glucose transporter type 4 (GLUT-4), insulin receptor (INSR)-B and INSR substrate (IRS1) was decreased in high glucose-induced HTR8/SVneo but increased by plumbagin. The suppressive effects of high glucose on phosphorylation of AKT and mTOR in HTR8/SVneo were reversed by plumbagin. Plumbagin improved high glucose-induced cell apoptosis and insulin resistance of HTR8/SVneo through activation of AKT/mTOR pathway, suggesting that plumbagin might be used as a potential strategy for the prevention of GDM.

Keywords: plumbagin; high glucose; trophoblast cell; insulin resistance; AKT/mTOR

Introduction

Gestational diabetes mellitus (GDM) is a disease that occurs during pregnancy, usually accompanied by hyperglycemia and insulin resistance, which seriously affects the health of mother and baby (Agarwal, 2016). The incidence of GDM in China increased rapidly and reached about 15% (Yan *et al.*, 2019). GDM usually causes a series of serious metabolic diseases, such as type 2 diabetes mellitus, abnormal fetal development, abortion and fetal asphyxia (Malaeb *et al.*, 2020; Vince *et al.*, 2020). Dietary or pharmacological interventions have been used for the prevention of GDM (Tanase-Nakao *et al.*, 2017). Early

diagnosis and effective prevention are still urgent for patients with GDM.

The placenta functions as an organ for blood circulation, nutrition and exchange of gases between mother and fetus during pregnancy, and ensures a healthy pregnancy and embryonic development (Kaiser, 2014). Abnormal functioning of the placenta is associated with adverse pregnancy outcomes caused by GDM (Ilekis *et al.*, 2016). During development of GDM, the placenta secretes hormones to enhance insulin resistance and antagonize insulin, thus promoting GDM (Tang *et al.*, 2020). In addition, GDM also leads to placenta dysplasia

and vascular abnormalities to aggravate progression of GDM (Madazli *et al.*, 2008). Therefore, regulation of placental function is important for amelioration of GDM. Trophoblastic cells are vital for the development of the placenta through differentiation, invasion and migration to vascular tissue (Chang and Vivian Yang, 2013). A study has demonstrated that high glucose induces impaired the proliferation, invasion and migration activities of trophoblast cells, leading to dysplasia of the placental tissue, and resulting in abortion, spontaneous abortion and premature birth (Chang and Vivian Yang, 2013). Strategy to mediate high glucose-induced trophoblast cell dysfunction was established as an effective treatment for GDM.

Plumbagin, isolated from the root of *Plumbago zeylanica* L., is a naphthoquinone derivative, and exerts antioxidant, anti-inflammatory, antiviral, anti-tumor and neuroprotective effects. For example, plumbagin promotes reactive oxygen species, apoptosis and autophagy of cancer cells while suppressing cell proliferation and survival (Yin *et al.*, 2020). Plumbagin modulates neuro-inflammatory and apoptotic pathways to attenuate cerebral ischemia (Chen *et al.*, 2018). Plumbagin also regulates oxidative stress, lipid inflammation and metabolism to reduce nonalcoholic fatty liver disease and obesity (Pai *et al.*, 2019). In addition, plumbagin promoted expression of glucose transporter-4 to contribute to glucose homeostasis of streptozotocin-induced diabetic rats (Sunil *et al.*, 2012), and ameliorated diabetic nephropathy (Yong *et al.*, 2013) and wound healing (Shao *et al.*, 2019) in diabetic rats. However, the role and mechanism of plumbagin in GDM are rarely reported.

In this study, high glucose-induced trophoblast cell line (HTR8/SVneo) was established, and the effects of plumbagin on cell proliferation, apoptosis and insulin resistance of HTR8/SVneo were investigated. Therefore, meaningful results could provide potential strategy for the prevention of GDM.

Materials and methods

Cell culture and treatment

HTR8/SVneo cells were purchased from Jining Cell Culture Center (Shanghai, China) and cultured in RPMI 1640 medium containing 1% streptomycin–penicillin and 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, CA, USA) in a humidified incubator at 37°C. Cells were treated with 5-, 10-, 20-, 40- or 80-μM plumbagin (Sigma-Aldrich, St Louis, LA, USA) for 24 h before MTT colorimetric assay. Cells were also treated with 5.5-, 12.5-, 25- or 50-mM glucose for 24 h before MTT assay. In line with previous study that *in vitro* cell model of GDM was established through incubation with 25-mM

glucose (Zhou, Xiang, & Zheng, 2019), HTR8/SVneo cells were treated with 25-mM glucose in the presence of 5-, 10- and 20-μM plumbagin for 24 h.

Cell viability and proliferation assays

HTR8/SVneo cells with indicated treatment were plated in a 96-well plate for 48 h. The MTT solution (Sigma-Aldrich) was added to the well. Cells were cultured for 4 h, and incubated with 100-μL dimethyl sulfoxide (DMSO) for 10 min. Optical density at 490 nm was measured on microplate reader (Biotek, Winooski, VT, USA). For incorporation of 5-ethynyl-2'-deoxyuridine (EdU), HTR8/SVneo cells with indicated treatment were plated in a 96-well plate and incubated with 50-μM EdU (RiboBio, Guangzhou, China) for 12 h. Cells were fixed with 4% paraformaldehyde, incubated with 2-mg/mL glycine followed by 0.5% Triton X-100. EdU antibody from EdU Cell Proliferation Detection (RiboBio) was added and immunostaining was performed with Apollo staining reaction buffer. Cells were measured under fluorescence microscopy (Nikon, Tokyo, Japan) followed by staining with DAPI (4',6-diamidino-2-phenylindole fluorescent stain).

Cell apoptosis assay

HTR8/SVneo cells with indicated treatment were harvested and resuspended with 1× buffer from FITC Annexin V Apoptosis Detection Kit (BD Biosciences, Franklin Lakes, NJ, USA). The cell suspension was then incubated with Annexin V-fluorescein isothiocyanate and propidium iodide before analyzed by FACSCanto II™ flow cytometry system (BD Biosciences, Franklin Lakes, NJ, USA).

Western blot

Proteins were isolated from cultured HTR8/SVneo cells through RIPA lysis buffer (Beyotime, Beijing, China), and electrophoresed on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The membranes were blocked with 5% skim milk after transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were incubated with the following specific primary antibodies: anti-cleaved caspase-3 and anti-cleaved caspase-9 (1:2,000; Cell Signaling, Boston, MA, USA), anti-Bax and anti-Bcl2 (1:2,500; Abcam, Shanghai, China), anti-GLUT4 and anti-INSR-B (1:3,000; Cell Signaling), anti-IRS1 and anti-β-actin (1:3,500; Abcam), anti-AKT and anti-p-AKT (1:4,000; Cell Signaling), anti-mTOR and anti-p-mTOR (1:4,500; Abcam). The immunoreactive signals were measured by enhanced

chemiluminescence reagent kit (Millipore) followed by incubation with the corresponding secondary antibodies.

Statistical analysis

All experimental data at least in triplicate were manifested as mean \pm standard deviation. Differences between groups were analyzed by Student's *t*-test or one-way analysis of variance under the Graphpad Prism 7 software (GraphPad Inc., La Jolla, CA, USA). $P < 0.05$ was established as statistically significant.

Results

Plumbagin promoted cell proliferation of high glucose induced HTR8/SVneo

In vitro cell model of GDM was established through incubation of HTR8/SVneo cells with high glucose. High glucose reduced cell viability of HTR8/SVneo in a dose-dependent manner (Figure 1A). Plumbagin also reduced the cell viability of HTR8/SVneo in a dosage-dependent manner (Figure 1B). Less than 20- μ M plumbagin had no significant effect on the cell viability of HTR8/SVneo (Figure 1B). Therefore, high glucose-induced HTR8/SVneo cells were incubated with 5-, 10- or 20- μ M plumbagin to investigate the effects of plumbagin on GDM. Plumbagin enhanced the cell viability of high glucose-induced HTR8/SVneo (Figure 1C), and promoted cell proliferation (Figure 1D) with increased EdU-positive cells (Figure 1E). These results demonstrated the proliferative effect of plumbagin on GDM.

Plumbagin repressed cell apoptosis of high glucose-induced HTR8/SVneo

High glucose-induced cell apoptosis of HTR8/SVneo cells (Figure 2A), and plumbagin incubation repressed the apoptosis of high glucose-induced HTR8/SVneo (Figure 2A). Moreover, reduced Bcl-2 and enhanced Bax, and cleaved caspase-3 and cleaved caspase-9, in high glucose-induced HTR8/SVneo cells were restored by plumbagin incubation (Figure 2B), suggesting the anti-apoptotic role of plumbagin in GDM.

Plumbagin improved insulin resistance of high glucose-induced HTR8/SVneo

High glucose decreased protein expression of GLUT-4, INSR-B and IRS1 in HTR8/SVneo cells (Figure 3). However, the levels of insulin-signaling and glucose utilization-associated factors, GLUT-4, INSR-B and IRS1,

in high glucose-induced HTR8/SVneo were restored by plumbagin (Figure 3), indicating that plumbagin improved the insulin response of trophoblast cells during the development of GDM.

Plumbagin promoted activation of AKT/mTOR in high glucose-induced HTR8/SVneo

High glucose or plumbagin incubation had no significant effects on the protein expression of AKT and mTOR in HTR8/SVneo (Figure 4). High glucose reduced protein expression of AKT and mTOR phosphorylation in HTR8/SVneo (Figure 4). Plumbagin attenuated high glucose-induced decrease of AKT and mTOR phosphorylation in HTR8/SVneo (Figure 4), revealing that plumbagin promoted activation of AKT/mTOR to ameliorate GDM.

Discussion

Naphthoquinones are phytochemicals isolated from medicinal plants that possess pharmacological potential of antidiabetic activity through increasing glucose uptake and inhibition of protein tyrosine phosphatase 1B and α -glucosidase (Shah *et al.*, 2018). Naphthoquinone-based hybrids exert antagonistic activity against type-2 diabetes (Paul *et al.*, 2019). Plumbagin has been depicted as a naphthoquinone derivative that can reduce blood glucose to attenuate streptozotocin-induced diabetes (Sunil *et al.*, 2012). The antidiabetic effect of plumbagin on GDM has been investigated in this study.

The placenta is considered a target organ of GDM with ability to maintain immunologic, endocrine and nutritional functions between mother and fetus (Carrasco-Wong *et al.*, 2020). Hyperglycemia in mother alters structure of the placenta and facilitates fetus malformation and abortion during the development of GDM (Carrasco-Wong *et al.*, 2020). Functions of trophoblast cells are key regulator of placenta development, and the abnormal growth, migration and invasion are implicated in placenta maldevelopment (Zhang *et al.*, 2021). High glucose, with ability to induce impairment in the proliferation, invasion and migration activities of trophoblast cells, has been widely used for the establishment of *in vitro* cell model of GDM (Zhang *et al.*, 2021). Results in this study demonstrated that high glucose reduced cell viability of HTR8/SVneo, suppressed cell proliferation and promoted cell apoptosis. Moreover, suppression of high glucose-induced biological dysfunction of trophoblast cells facilitated for the amelioration of GDM (Zhang *et al.*, 2021). Plumbagin incubation restored the cell viability of high glucose-induced HTR8/SVneo, promoted cell proliferation and suppressed cell apoptosis, suggesting anti-apoptotic and proliferative effects against GDM.

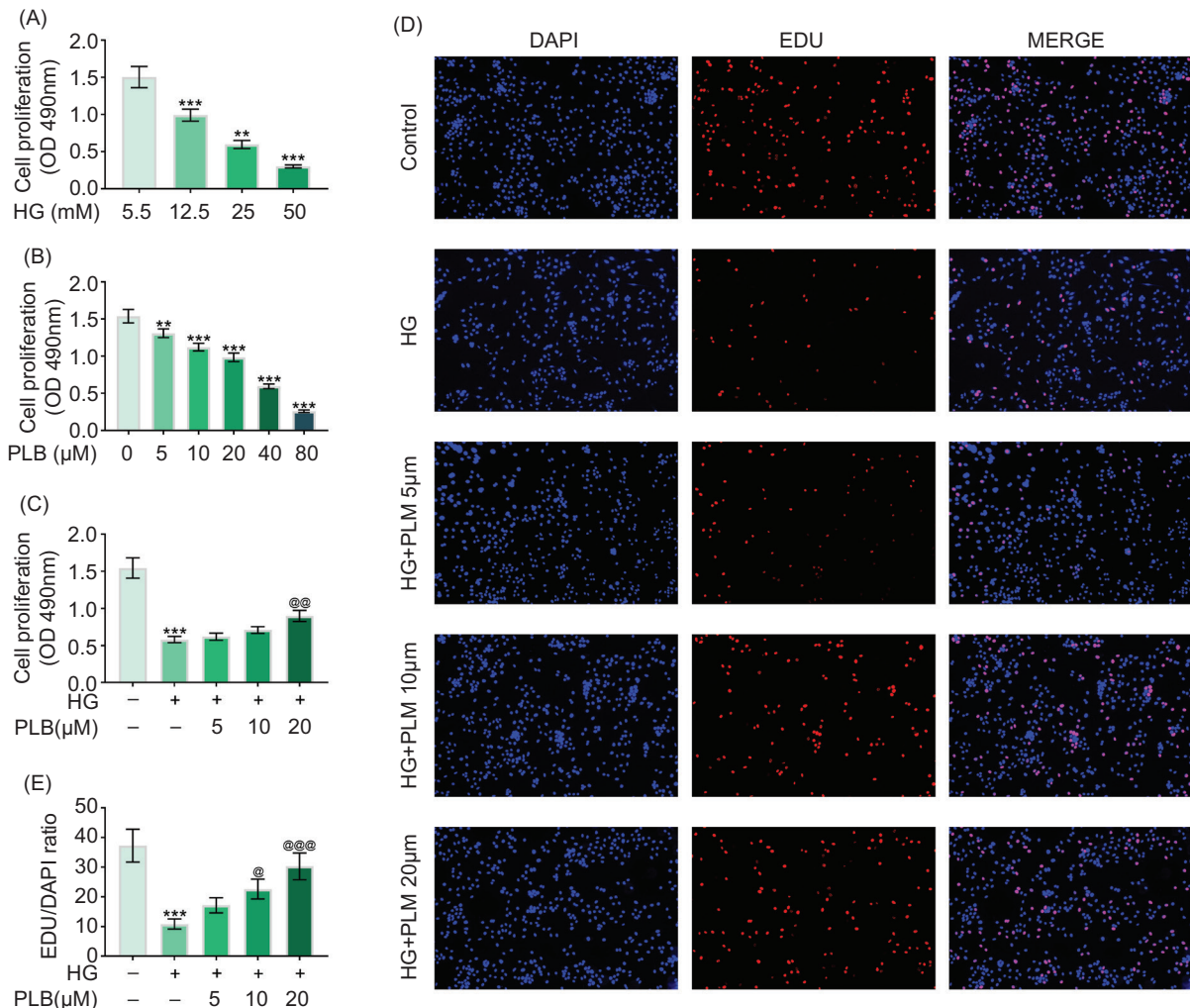


Figure 1. Plumbagin promoted cell proliferation of high glucose-induced HTR8/SVneo. (A) High glucose reduced cell viability of HTR8/SVneo in a dose-dependent manner. (B) Plumbagin reduced HTR8/SVneo cell viability in a dose-dependent manner. (C) Plumbagin enhanced cell viability of high glucose-induced HTR8/SVneo. (D) Plumbagin promoted the cell proliferation of high glucose-induced HTR8/SVneo. (E) Plumbagin promoted EdU-positive cells of high glucose-induced HTR8/SVneo. @ $P < 0.05$; ** $P < 0.01$; * $P < 0.001$.**

In addition, migration and invasion of trophoblasts were also implicated in the pathogenesis of GDM (Sun *et al.*, 2020), and plumbagin exerted anti-invasive effect against breast cancer cells (Lee *et al.*, 2012). The effects of plumbagin on cell migration and invasion of high glucose-induced HTR8/SVneo cells must be investigated in the future research.

During the development of GDM, insulin signal transduction and glucose metabolism in the placenta were disturbed, leading to pathological changes in the placenta tissue (Peng *et al.*, 2020). Increase in insulin sensitivity is established as strategy to improve GDM (Peng *et al.*, 2020). Plumbagin has been reported to regulate GLUT4 translocation to reduce blood glucose, and attenuate streptozotocin-induced diabetes (Sunil *et al.*, 2012). Results of this study demonstrated that plumbagin

increased protein expression of GLUT4 and insulin signaling molecules, including IRS1 and INSR-B, in high glucose-induced HTR8/SVneo, thus promoting insulin sensitivity and suppressing insulin resistance to protect against GDM. Imbalance of inflammation in the placenta with increase in pro-inflammatory cytokines and decrease in anti-inflammatory cytokines mediated proliferation and invasion of trophoblasts and activities of insulin and glucose receptors, thus contributing to the pathogenesis of GDM (Li *et al.*, 2021). Therefore, the anti-inflammatory effect of plumbagin on high glucose-induced HTR8/SVneo must also be investigated in the future studies.

A previous study has demonstrated that AKT/mTOR pathway was involved in the development of GDM through regulation of insulin resistance and trophoblastic

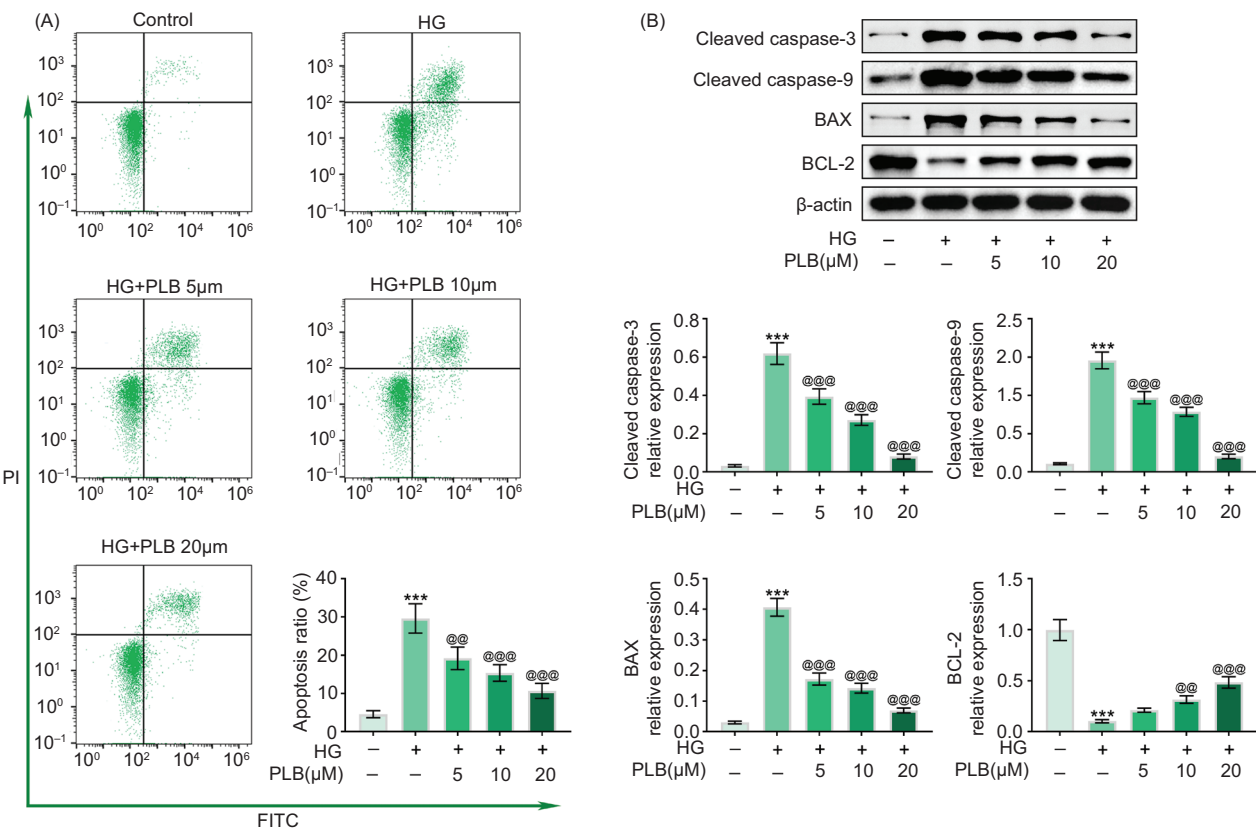


Figure 2. Plumbagin repressed cell apoptosis of high glucose-induced HTR8/SVneo. (A) Plumbagin suppressed the cell apoptosis of high glucose-induced HTR8/SVneo. **(B)** Plumbagin attenuated high glucose-induced decrease of Bcl-2, increase of Bax, cleaved caspase-3, and cleaved caspase-9 in HTR8/SVneo. @ $P < 0.01$; *** $P < 0.001$.

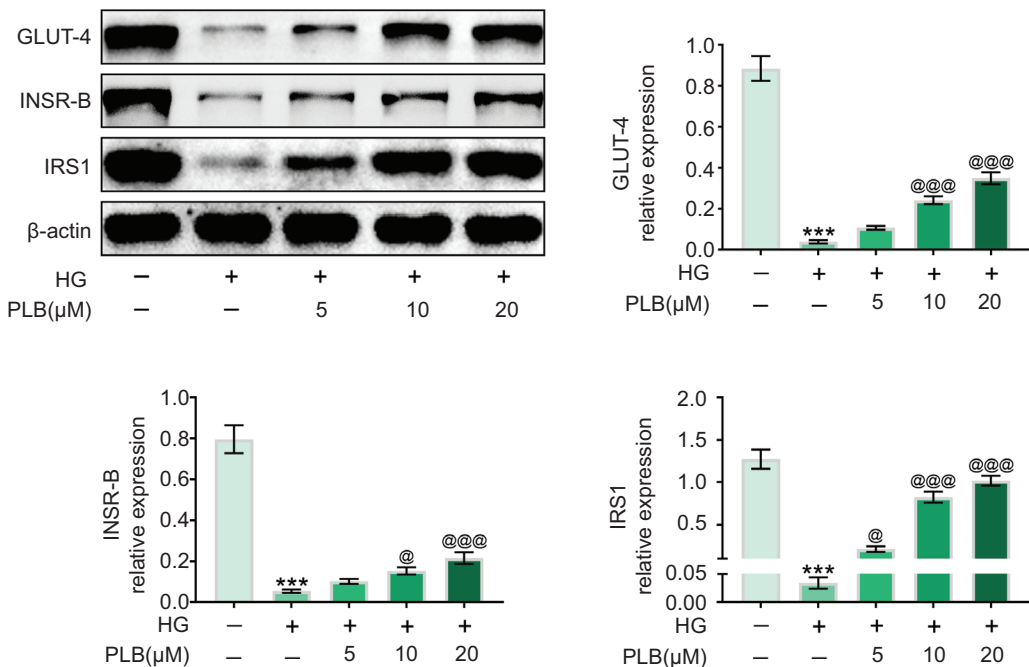


Figure 3. Plumbagin improved insulin resistance of high glucose-induced HTR8/SVneo. Plumbagin attenuated high glucose-induced decrease of GLUT-4, INSR-B and IRS1 in HTR8/SVneo cells. @ $P < 0.05$; * $P < 0.001$.**

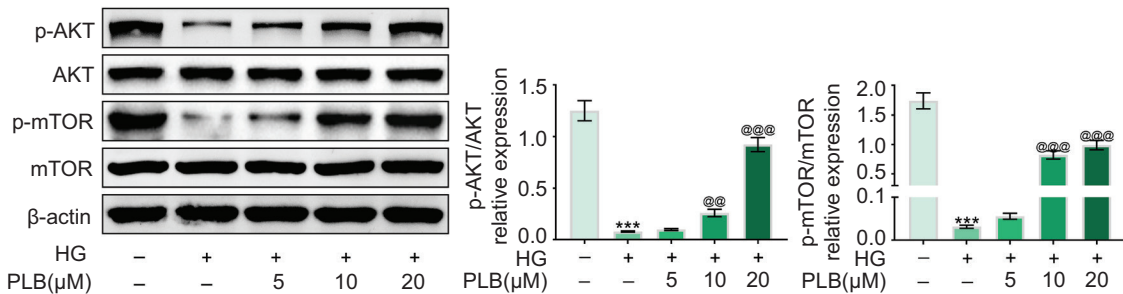


Figure 4. Plumbagin promoted activation of AKT/mTOR in high glucose-induced HTR8/SVneo. Plumbagin attenuated high glucose-induced decrease of AKT and mTOR phosphorylation in HTR8/SVneo. @@ $P < 0.01$; *** $P < 0.001$.

function (Tsai *et al.*, 2021). High glucose induced down-regulation of mTOR phosphorylation in trophoblasts (Tsai *et al.*, 2021), and activation of AKT/mTOR impeded the progression of GDM (Han *et al.*, 2020). Therefore, regulation of AKT/mTOR pathway provided avenues to alleviate GDM. PI3K/Akt/mTOR was implicated in the plumbagin-mediated cancer cell proliferation and survival (Cai *et al.*, 2020) and neuroprotection (Yuan *et al.*, 2017). Here, the protein expression of AKT and mTOR phosphorylation was reduced in high glucose-induced HTR8/SVneo but enhanced by plumbagin incubation, indicating that plumbagin promoted activation of AKT/mTOR pathway to attenuate GDM.

Conclusion

Plumbagin promoted cell proliferation of high glucose-induced HTR8/SVneo cells, suppressed cell apoptosis and promoted insulin resistance through activation of AKT/mTOR pathway. These results could provide a novel therapeutic strategy for treating GDM.

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