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RESEARCH ARTICLE

Tetramethylpyrazine attenuates placental oxidative stress, inflammatory responses and endoplasmic reticulum stress in a mouse model of gestational diabetes mellitus

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Abstract Gestational diabetes mellitus (GDM) is a disease characterized by insufficient insulin secretion and glucose metabolic disorder during pregnancy. Tetramethylpyrazine has been reported to inhibit endoplasmic reticulum (ER) stress and high glucose-induced inflammation, which are closely associated with GDM. This study aimed to investigate the effects of tetramethylpyrazine on inflammatory responses, ER stress and oxidative stress of the placenta in a mouse model of GDM. Our results showed that tetramethylpyrazine treatment significantly alleviated the GDM symptoms characterized by low body weight and serum insulin levels, high blood glucose, and decreased β-cell function in pregnant C57BL/KsJdb/+ mice. In addition, tetramethylpyrazine reduced the level of malondialdehyde, and increased the levels of superoxide dismutase, glutathione peroxidase and glutathione. Moreover, tetramethylpyrazine decreased the total serum cholesterol, serum triglyceride, and serum low-density lipoprotein levels and increased the high-density lipoprotein level. Further, tetramethylpyrazine regulated the levels of serum and placental inflammatory factors and the expression of ER stress related proteins. Taken together, the present study demonstrated that tetramethylpyrazine attenuated placental oxidative stress, inflammatory responses and ER stress in GDM mice.

Keywords Gestational diabetes mellitus (GDM) · Tetramethylpyrazine · Oxidative stress · Inflammatory responses · Endoplasmic reticulum stress

Introduction

Gestational diabetes mellitus (GDM) refers to diabetes in pregnant women with abnormal glucose metabolism or potential impaired glucose tolerance during pregnancy (Mishra et al. 2018). In recent years, with the improvement of living standards in China, the prevalence of GDM has also increased and has reached 1% to 3% (Poola-Kella et al. 2018). The quality of blood glucose control during pregnancy is directly related to the health and safety of pregnant women and fetuses (Miettinen et al. 2018). Poorly controlled GDM is likely to cause excessive amniotic fluid, increased incidence of pregnancy-induced hypertension and infection, which is an important cause of increased perinatal mortality (Feghali et al. 2018). Increasing studies have shown that the occurrence of GDM is closely related to the lipid metabolism disorder, genetic factors and placental vascular endothelial injury in pregnant women (Plows et al. 2018). It is not determined by a single factor, but caused by changes in molecular, tissue, organ, and system interactions

Yalou Jiao and Sheng Zhang contributed equally to this work.

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with genetic factors and the environment (Dias et al. 2018). Among them, vascular endothelium lesions caused by oxidative stress, which leads to hypoperfusion of the placenta, blood supply to the placenta and umbilical cord, and insufficient oxygen supply, is the main cause of fetal distress and even fetal death in pregnant women (Lappas et al. 2011; Poulakos et al. 2015). Therefore, in-depth study of the molecular mechanisms and further exploration of better treatments for GDM are of great significance.

Tetramethylpyrazine, also known as ligustrazine, is a nitrogen-containing heterocyclic compound in which a methyl group is attached to each pyrazine ring carbon atom (Wu et al. 2019). Tetramethylpyrazine is the main active alkaloid component of the Chinese medicinal material *Ligusticum wallichii*, which exhibits the functions in dilating blood vessels, improving microcirculation and inhibiting platelet aggregation, and is widely used in clinical practice (Zhao et al. 2016). Tetramethylpyrazine has been reported to inhibit endoplasmic reticulum (ER) stress (Liu et al. 2018; Yang and Wu 2018), which been shown to be caused by GDM in the placenta (Yung et al. 2016). In addition, tetramethylpyrazine is also able to inhibit high glucose-induced inflammation (Yang et al. 2011; Chen et al. 2019). Therefore, tetramethylpyrazine might play an important role in GDM. There is currently no research on tetramethylpyrazine in GDM, and study is also limited with regard to the effect of tetramethylpyrazine on ER stress in the placenta. Therefore, this work focuses on the protective effects of tetramethylpyrazine in a mouse model of GDM from in terms of oxidative stress, inflammation and ER stress in the placenta.

Methods

Animals

All animal studies in this paper were approve by the Ethics Commitment of Xingtai People's Hospital of Hebei Province. For generating GDM mice model, C57BL/KsJ+/+ (wild type) and C57BL/KsJdb/+ (db/+) mice were purchased from iBio Logistics Co. (Changping, Beijing, China). Up to six mice were placed in a ventilated SPF cage. Mice were housed under 22–26 °C and a 12/12-h light/dark cycle with adequate sterile food and water supply. In each group, 18 mice matched for successful pregnancy were selected for further experimental study. Different doses (0, 20, 40, and 60 mg / kg body weight) of tetramethylpyrazine (≥ 98%, W323713, Sigma-Aldrich, St. Louis, MO, USA) were administered once per day by intragastric administration from the time of successful pregnancy to the end of the experiment. The dose of tetramethylpyrazine used in the following experiments was determined according to our preliminary experiments, and no side effects were observed during the entire experimental period. The mice were sacrificed at gestation day (GD) 18 after the last administration.

Blood glucose and serum insulin level measurements

Blood samples were obtained from the tail vein of the mice. A Glucose Detection Kit (GOD-POD Microplate Method) was purchased from Leagene biotech.co. (Haidian, Beijing, China) to measure the blood glucose according to the manufacturers' instructions. An Insulin Mouse Enzyme-Linked ImmunoSorbent Assay (ELISA) Kit (Invitrogen Life Technologies, Carlsbad, CA, USA) was used to determine the serum insulin levels of the mice. The pancreatic islet β-cell function in different groups was examined by homeostasis model assessment (HOMA-β), as follows: β-cell function = (serum insulin, μIU/mL × 20)/(serum glucose, mmol/L) − 3.5.

Serum lipid level measurement

Total serum cholesterol (TCh), serum triglyceride (TG), and serum low-density lipoprotein (LDL) levels, serum high-density lipoprotein (HDL) level in serum were analyzed by ILab Chemistry Analyzer 300 PLUS (Instrumentation Laboratory, Bedford, MA, USA).

Placental oxidative stress measurement

ELISA was used to analyze the activities of malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione (GSH) in placenta on GD 18 among indicated groups according to the previous described methods (Fu et al. 2012a, b).

RNA extraction and qRT-PCR

The total RNA in placenta were extracted by Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). RNA was reverse transcribed into cDNA using a reverse transcription kit (Fermentas, St. Leon-Rot, Germany). SYBR-Green Master mix (Life Technologies, Carlsbad, CA, USA) was used to quantify the expression levels.

Primers used in this paper were listed as follows:

Interleukin (IL)-6 forward 5′-TCCAGTTGCCTTCTTGGGAC-3′; IL-6 Reverse 5′-GTGTAATTAAGCCTCCGACTTG-3′;
Tumor necrosis factor (TNF)-α Forward 5′-CATCTTCTCAAAATTCGAGTGACAA-3′; TNF-α Reverse 5′-TGGGAGTAGACAAGGTACAACCC-3′;
Monocyte chemotactic protein 1 (MCP-1) Forward 5′-TTCACAGTTGCTGCCTGTAG-3′; MCP-1 Reverse 5′-TCTGATCTCACTTGGTTCTGG-3′;

GAPDH Forward 5′-TTCACCACCATGGAGAAGGC-3′;
GAPDH Reverse 5′-GGCATGGACTGTGGTCATGA-3′.

Western blot

Western blot was performed with the standard method. The following antibodies were used: CCAAT/Enhancer-Binding Protein Homologous Protein (CHOP, sc-575, Santa Cruz, Dallas, TX, USA), glucose-regulated protein 78 (GRP78, ab21685, Abcam, Cambridge, UK), phospho-eukaryotic initiation factor 2 (eIF2a, #3398, Cell Signaling Technology, Shanghai, China), eIF2a (#5324, Cell Signaling Technology), and GAPDH (#5174, Cell Signaling Technology).

Statistical analysis

SPSS software version 19.0 was used in the data analysis. All data in the experiment was shown as mean ± standard deviation (SD). One-way ANOVA with a Tukey's post hoc test was used to calculate the differences between each group.

Results

First, we conducted a tetramethylpyrazine safety verification and concentration selection experiment. Different concentrations of tetramethylpyrazine were found to have no significant effect on blood glucose and insulin concentrations in healthy pregnant mice (Fig. S1A and B). In addition, we treated the C57BL/KsJ+/+ (wild type) and C57BL/KsJdb/+ (db/+) mice with different concentrations of tetramethylpyrazine and detected the blood glucose and serum insulin levels. We found that blood glucose, which was increased in GDM mice, was decreased with administration of different doses of tetramethylpyrazine (Fig. S1C). Treatment of tetramethylpyrazine increased the serum insulin levels in GDM mice. Furthermore, 20, 40 and 60 mg/kg of tetramethylpyrazine were selected as the optimum concentrations and were used in the follow-up experiments. In addition, we also studied the toxicity of tetramethylpyrazine on the fetal mice. There was no significant change in fetus survival ratio and fetal weight, which indicated that tetramethylpyrazine had no obvious toxicity to fetal mice (data not shown). Next, maternal body weight, blood glucose and serum insulin were measured on GD 18 following administration of different doses of tetramethylpyrazine. We found that the body weight of in GDM mice was lower than that in wild type mice, and tetramethylpyrazine treatment significantly increased the body weight of GDM mice (Fig. 1a). In addition, our results showed that tetramethylpyrazine treatment rescued the altered blood glucose (Fig. 1b) and serum insulin (Fig. 1c) levels in GDM mice in a dose-dependent manner. Moreover, we examined the pancreatic islet β-cell function in different groups by HOMA-β). We found that tetramethylpyrazine treatment rescued the inhibitory effects on HOMA-β in GDM mice in a dose-dependent manner (Fig. 1d). Taken together, we found that tetramethylpyrazine treatment alleviated the GDM symptoms

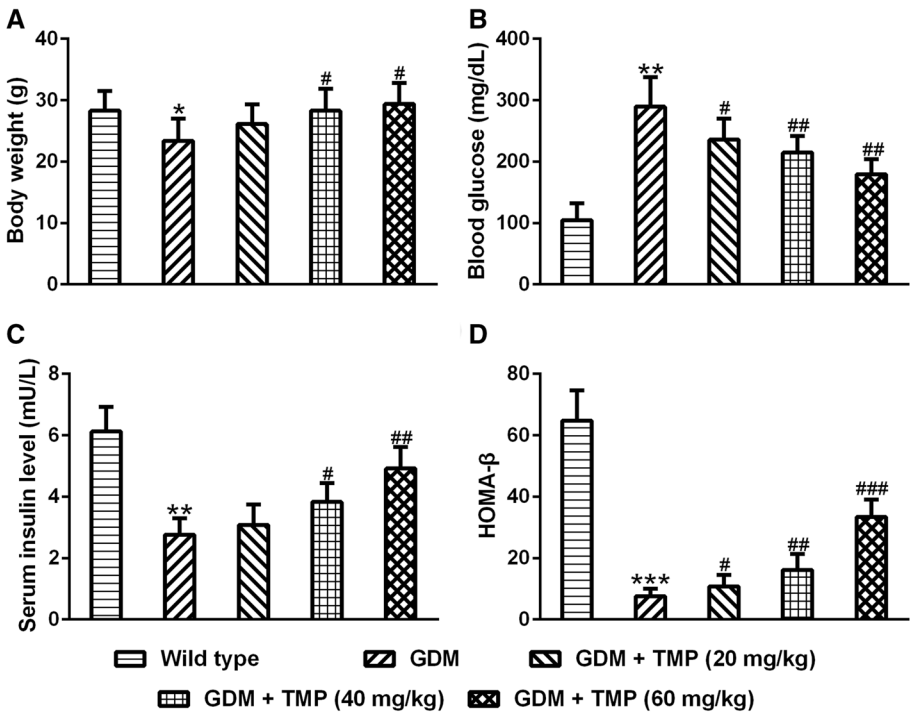

Fig. 1 Tetramethylpyrazine alleviated GDM symptoms in pregnant *db/+* mice. **a**–**c** Maternal body weight (**a**), blood glucose (**b**) and serum insulin (**c**) were measured on gestation day (GD) 18 among different doses of tetramethylpyrazine. **d** Homeostasis model assessment of β-cell function (HOMA-β) were calculated on GD 18. Data are presented as mean ± SD. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to wild type group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared to GDM mice

characterized by low body weight and serum insulin levels, high blood glucose, and decreased β-cell function in pregnant db/+ mice.

To further explore the effects of tetramethylpyrazine in GDM mice, various biochemical parameters were measured among different groups in the late stage of pregnancy. We found that TCh (Fig. 2a), TG (Fig. 2b), and serum LDL (Fig. 2c) levels were significantly upregulated in GDM mice, whereas tetramethylpyrazine treatment decreased this trend in a dose-dependent manner. In addition, tetramethylpyrazine treatment remarkably rescued the down-regulated serum HDL levels in GDM mice (Fig. 2d). Moreover, the atherogenic index, which was significantly elevated in GDM mice, gradually decreased with increasing doses of tetramethylpyrazine (Fig. 2e). Therefore, we confirmed that tetramethylpyrazine treatment ameliorated the biochemical indexes in GDM mice.

It is reported that oxidative stress plays an important role in the pathogenesis of GDM (Liu et al. 2018). Thus, we detected the MDA content and the anti-oxidative enzyme activities in placenta tissues on GD 18 in different groups. We found that tetramethylpyrazine treatment reduced the MDA levels which were increased in GDM mice (Fig. 3a). In addition, we found that the activities of SOD, GPx and GSH were remarkably decreased in GDM mice. Following tetramethylpyrazine treatment, the activities of each anti-oxidative enzyme were enhanced in a dose-dependent manner (Fig. 3b–d). Thus, our data demonstrated that tetramethylpyrazine treatment attenuated the elevated placental oxidative stress in GDM mice.

Previous studies have reported that tetramethylpyrazine possesses anti-inflammatory effects and could inhibit the inflammation responses caused by high glucose (Yang et al. 2011). Thus, we examined the levels of various inflammatory factors in the serum and placenta by ELISA and qRT-PCR, to identify the role of tetramethylpyrazine in inflammatory responses in GDM mice. Our results showed that the levels of IL-6, MCP-1 and TNF-α were significantly increased in GDM mice, whereas tetramethylpyrazine treatment inhibited this trend in a dose-dependent manner (Fig. 4a–i). Therefore, we speculated that tetramethylpyrazine might play a role in GDM by regulating the concentration of serum and placental inflammatory factors.

Previous studies have found that GDM could cause ER stress in the placenta (Yung et al. 2016). Therefore, we evaluated the effects of tetramethylpyrazine on ER stress by detecting the levels of various ER stress related proteins in the placenta. Our results revealed that tetramethylpyrazine treatment decreased the expression of GRP78, CHOP and p-eIF2/eIF2a, which were significantly elevated in GDM

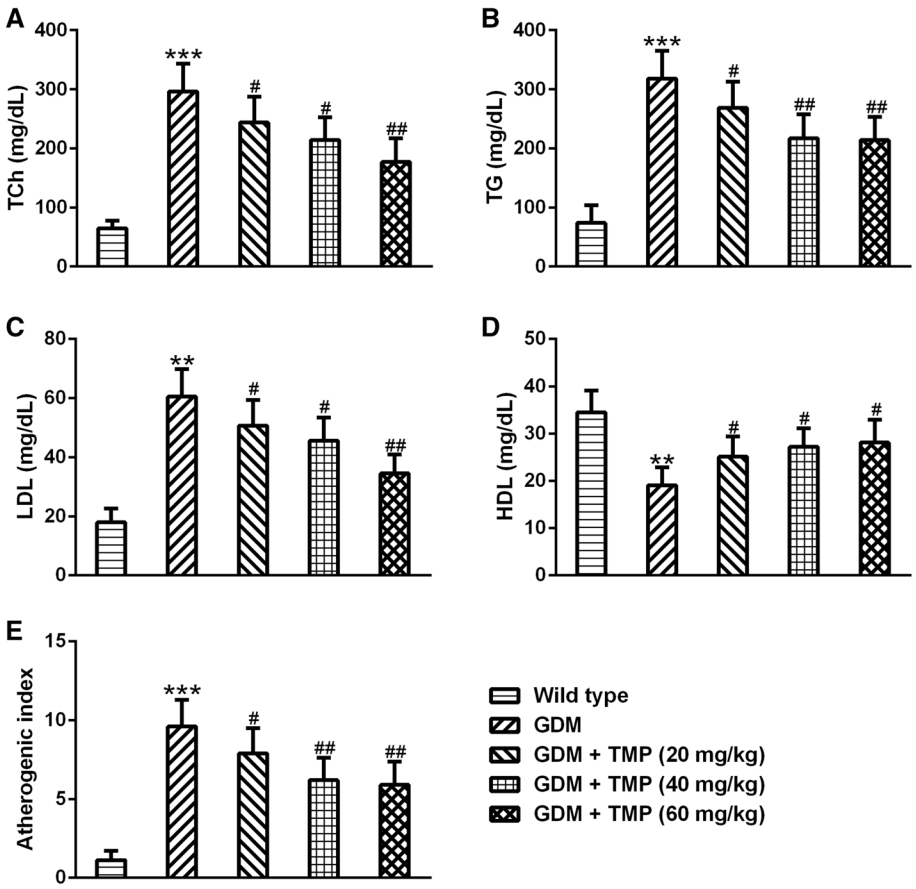

Fig. 2 Tetramethylpyrazine ameliorated biochemical indexes in GDM mice in the late stage of pregnancy. **a**–**e** TCh (**a**), TG (**b**), LDL (**c**), HDL (**d**), and atherogenic index (**e**) were tested on GD 18 among indicated groups. Data are presented as mean ± SD. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to wild type group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared to GDM mice

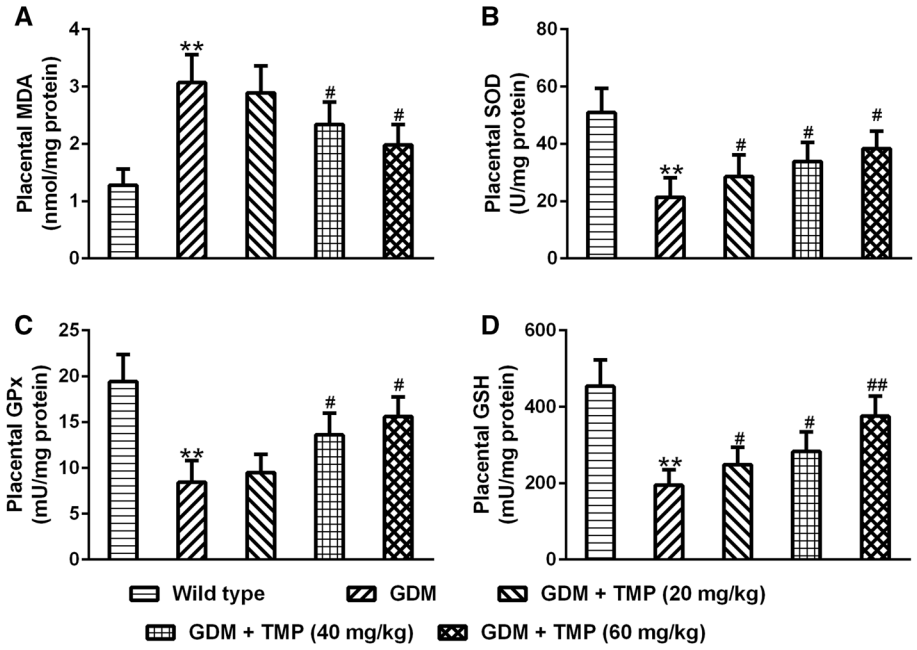

Fig. 3 Tetramethylpyrazine attenuated placental oxidative stress in GDM mice. **a**–**d** ELISA was used to analyze the activities of MDA (**a**), SOD (**b**), GPx (**c**) and GSH (**d**) in placenta on GD 18 among indicated groups. Data are presented as mean ± SD. $^{**}p < 0.01$ compared to wild type group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared to GDM mice

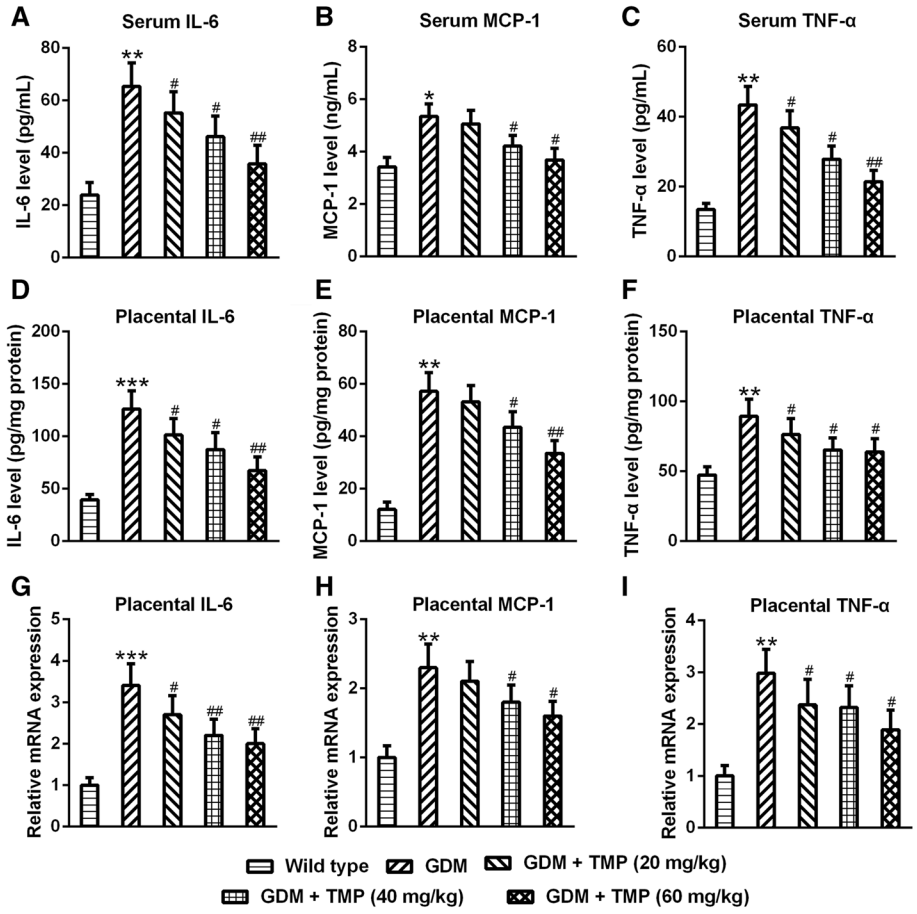

Fig. 4 Tetramethylpyrazine attenuated serum and placental inflammation in GDM mice. **a**–**c** ELISA was used to analyze the levels of IL-6 (**a**), MCP-1 (**b**) and TNF-α (**c**) in serum from indicated mice on GD18. **d**–**f** ELISA was used to analyze the levels of IL-6 (**d**), MCP-1 (**e**) and TNF-α (**f**) in placenta from indicated mice on GD18. **g**–**i** QRT-PCR was used to analyzed the mRNA levels of IL-6 (**g**), MCP-1 (**h**) and TNF-α (**i**) in placenta from indicated mice on GD18. Data are presented as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to wild type group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared to GDM mice

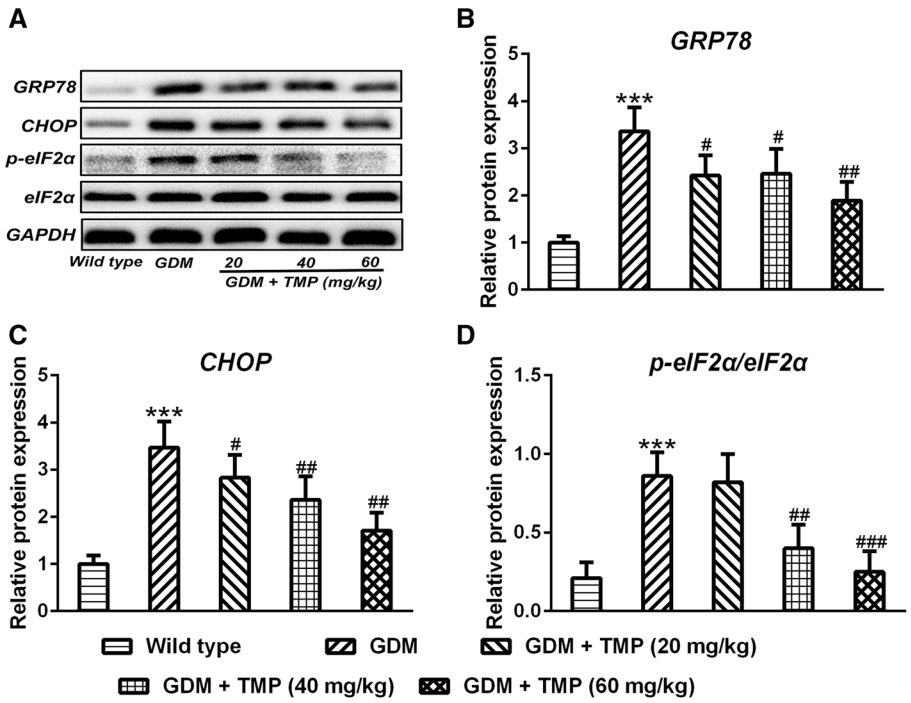

Fig. 5 Tetramethylpyrazine attenuated placental endoplasmic reticulum stress in GDM mice. **a**Western blotting was used to analyze the protein expression of placental GRP78, CHOP, p-eIF2α, eIF2α and relative expression. **b**–**d** Quantification of the band intensity from (**a**). Data are presented as mean ± SD. ***$p < 0.001$ compared to wild type group,$^{\#}p < 0.05$,$^{\#\#}p < 0.01$ compared to GDM mice

mice (Fig. 5a–d). Taken together, our data suggested that tetramethylpyrazine treatment might attenuate the placental ER stress in GDM mice by down-regulating the expression of GRP78, CHOP and p-eIF2a/eIF2a.

Discussion

GDM is one of the common complications during pregnancy. In recent years, with the development of medical diagnostic techniques and living standards, the incidence of GDM has been gradually increasing. GDM has many adverse effects on pregnant women and fetuses (Silva et al. 2017). For instance, GDM women are prone to complications such as pregnancy-induced hypertension syndrome, polyhydramnios, infection, ketoacidosis, and the possibility of diabetes and metabolic syndrome after delivery is higher than health pregnant women (Johns et al. 2018). If GDM patients have poor long-term control of blood glucose, it may cause fetal chronic intrauterine hypoxia, abnormal growth and development, malformation, and neonatal hyperbilirubinemia (Prakash et al. 2017). After leaving the maternal hyperglycemic environment, neonates often have hypoglycemia. Because fetal hyperinsulinemia affects the synthesis of alveolar surfactants, fetal lung development retardation, the prevalence of neonatal respiratory distress syndrome increases (Badon et al. 2017). The neonates of GDM patients are more susceptible to obesity, impaired glucose tolerance, and even diabetes in adolescence (Li et al. 2017). Therefore, it is imperative to further explore the pathogenesis of GDM and find new treatments against GDM.

Tetramethylpyrazine is the main active ingredient of traditional Chinese medicine Chuanxiong. It has the functions in activating blood circulation, improving blood supply to organs, thrombolysis, and scavenging free radicals (Hu et al. 2013). Recent studies have found that tetramethylpyrazine could inhibit ER stress and inflammation caused by high glucose (Yang et al. 2011; Liu et al. 2018; Yang and Wu 2018; Chen et al. 2019). Here, we reported that tetramethylpyrazine alleviated the GDM symptoms in our mouse model. Tetramethylpyrazine attenuated the GDM-induced alterations in body weight, blood glucose, serum insulin and lipid levels. Therefore, tetramethylpyrazine may contribute to alleviating the symptoms of GDM.

The pathogenesis of GDM is still unclear. Researchers believe that its mechanism is similar to that of type II diabetes. Insulin resistance and islet β-cell dysfunction are central to the pathogenesis of GDM (Li et al. 2018). Studies have shown that oxidative stress plays an important role in insulin resistance and islet β-cell dysfunction, leading to impaired glucose regulation and elevated blood glucose (Kaneto et al. 2006). Therefore, oxidative stress may be one of the important pathogenic factors of GDM. Some scholars believe that pregnancy is in a state of persistent low oxidative stress (Coughlan et al. 2004). Compared with non-pregnant women, serum lipid peroxidation levels in normal pregnant women are elevated and peak during mid-pregnancy, and fall to levels comparable to early pregnancy during late pregnancy (Chen and Scholl 2005). During normal pregnancy, as

soon as the maternal fetal blood circulation is established, the concentration of oxygen in the placenta increases rapidly (Zhang et al. 2019). This phenomenon is similar to the ischemia-reperfusion process and produces a large amount of reactive oxygen species (ROS) (Sudharshana Murthy et al. 2018). During normal pregnancy, low levels of ROS in the body play an important role in embryo development and implantation, placental formation and function, and childbirth (Schliefsteiner et al. 2017). As the pregnancy progresses, in order to maintain normal pregnancy, the body's anti-oxidant level is also increased, maintaining a relatively balanced oxidation/anti-oxidation effect (Schliefsteiner et al. 2017). Studies have shown that in GDM placenta, maternal and fetal oxidative stress response is enhanced, suggesting that oxidative stress is not only involved in the occurrence and development of GDM, but may also be involved in the occurrence of GDM complications such as fetal complications. Previous studies have shown that patients with GDM have elevated plasma MDA levels, decreased levels of anti-oxidant markers, and decreased insulin secretion and sensitivity (Rueangdetnarong et al. 2018). MDA is a major factor affecting islet β-cell function, suggesting that islet β-cell dysfunction in patients with GDM may be associated with increased oxidative stress in the body (Kotani et al. 2015). Since the content and activity of anti-oxidative enzymes in islet β-cells are relatively low, it is considered to be one of the main targets of oxidative stress-mediated damage (Kotani et al. 2015). In the present study, we demonstrated that tetramethylpyrazine treatment reduced the GDM-induced MDA levels in mice. In addition, we found that following tetramethylpyrazine treatment, the activities of anti-oxidative enzymes, including SOD, GPx and GSH, were enhanced in a dose-dependent manner. Further, we observed that tetramethylpyrazine could ameliorate islet β-cell dysfunction. Therefore, our research provides a new idea for the treatment of GDM and its complications.

ILs are involved in inflammatory response, and regulating the total amount of fat and muscle tissues by controlling apoptosis (Kuzmicki et al. 2009). Studies have shown that IL-6 is involved in the pathogenesis of GDM (Kuzmicki et al. 2009). In the early stages of diabetes, IL-6 promotes insulin secretion, leading to hyperinsulinemia (Kim et al. 2008). When IL-6 is increased to a certain extent, it inhibits insulin secretion and damages islet β-cells, further aggravating diabetes (Kim et al. 2008). Besides, studies have shown that TNF gene polymorphism is associated with the pathogenesis of GDM (Gao et al. 2008), by increasing plasma TNF levels in GDM pregnant women and causing insulin resistance. The mechanism by which TNF induces insulin resistance may be through its promotion of lipolysis and the resulted increase in free fatty acid levels (Friedman et al. 2008). TNF inhibits the tyrosine kinase activity of the insulin receptor in muscle tissue and suppresses the expression of the serine phosphorylation transporter 4 of the insulin receptor, which can alter the catalytic activity of the insulin receptor (Kuzmicki et al. 2006). In this paper, we found that the levels of IL-6, MCP-1 and TNF-α were significantly increased in GDM mice, whereas tetramethylpyrazine treatment inhibited this trend in a dose-dependent manner. Thus, our results suggested that tetramethylpyrazine might play a role in GDM by regulating the serum and placental levels of inflammatory factors, including IL-6, MCP-1 and TNF-α.

A variety of factors such as hyperglycemia, hyperlipidemia, viral infection, exotoxin, pro-inflammatory cytokines and mutant protein expression may interfere with the homeostasis of the ER, triggering a series of reactions within the cell by activating the corresponding signaling pathway called ER stress (Flamment et al. 2012). Upon ER stress, eIF2α is phosphorylated to inhibit the initiation of protein translations, therefore maintaining the homeostasis of the ER (Kim et al. 2006; Chambers et al. 2008). Previous studies found that the expression of ER chaperones GRP78 and CHOP in islet cells increased significantly during high glucose stress (Yung et al. 2016). It is believed that the ER stress caused by high glucose is due to excessive synthesis of proteins such as insulin in β-cells (Rueangdetnarong et al. 2018). ER stress hinders the downstream signaling of insulin receptor activation, thereby affecting the physiological function of insulin and triggering insulin resistance (Pirot et al. 2007). Therefore, we speculate that tetramethylpyrazine may affect the levels of blood glucose and insulin in GDM by regulating ER stress. Our results showed that tetramethylpyrazine treatment decreased the expression of GRP78, CHOP and the phosphorylation of eIF2a, indicating that tetramethylpyrazine exerted inhibitory effect on ER stress. When ER stress persists or the reaction is too strong, expression of CHOP, an ER stress-specific protein (Oyadomari and Mori 2004), is increased to initiate the relevant apoptosis program, causing apoptosis of islet β-cells and diabetes (Oyadomari and Mori 2004; Pirot et al. 2007). Therefore, tetramethylpyrazine treatment might modulate the blood glucose and insulin levels by affecting ER stress, inhibiting CHOP and apoptosis of islet β-cells.

In conclusion, we reported that tetramethylpyrazine treatment alleviated the GDM symptoms characterized by low body weight and serum insulin levels, high blood glucose, and decreased β-cell function in pregnant db/+ mice. In addition, tetramethylpyrazine reduced the serum lipid levels and increased the activities of anti-oxidative enzymes. Furthermore, tetramethylpyrazine regulated the concentration of serum and placental inflammatory factors and the expression of ER stress related proteins. Our results revealed the protective mechanism of tetramethylpyrazine against GDM, which could be utilized as novel agents for the clinical treatment of GDM.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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