CTRP9 decreases high glucose-induced trophoblast cell damage by reducing endoplasmic reticulum stress

LIANXIAO ZHANG, HUIQING DING, YUBO SHI, DUOYI ZHANG and XUE YANG

Department of Obstetrics and Gynecology, Ningbo First Hospital, Ningbo, Zhejiang 315000, P.R. China

Abstract. C1q/TNF-α-related protein 9 (CTRP9) is down-regulated in gestational diabetes mellitus (GDM) and may exert a protective effect against GDM, although its mechanism of action is yet to be elucidated. To investigate the specific role of CTRP9 in GDM, the human placental trophoblast cell line HTR8/SVneo was treated with high glucose (HG) to simulate the environment of GDM *in vitro*. The effects of CTRP9 on the HTR8/SVneo cells and endoplasmic reticulum (ER) stress were analyzed before and after CTRP9 overexpression using reverse transcription-quantitative PCR and western blotting. The results obtained demonstrated that CTRP9 alleviated ER stress in the trophoblast cell line. After treating with the ER-stress inducer tunicamycin, cell viability was investigated by performing Cell Counting Kit-8, TUNEL and western blotting assays, which revealed that CTRP9 increased the activity of HTR8/SVneo cells induced by HG through the alleviation of ER stress. Subsequently, ELISA and western blotting assay results demonstrated that CTRP9 inhibited HG-induced inflammation of the HTR8/SVneo cells by the reduction in ER stress. Finally, the detection of reactive oxygen species, nitric oxide (NO) synthase and NO levels confirmed that CTRP9 inhibited the oxidative stress of HTR8/SVneo cells induced by HG through the reduction of ER stress. Collectively, the results of the present study suggested that CTRP9 may decrease trophoblast cell damage caused by HG through the suppression of ER stress, and therefore, CTRP9 may potentially be a therapeutic target in the treatment of GDM.

Introduction

Gestational diabetes mellitus (GDM) is one of the most common complications to occur during pregnancy, posing a serious or even fatal risk to the pregnant woman and their offspring (1). It is estimated that the incidence rate of GDM is ~14% world-wide (2). Maternal diabetes, through transfer of glucose in the placenta, can lead to hyperglycemia in the fetus (1). In addition, since the pancreas of the fetus responds to the increased glucose concentrations by producing and releasing more insulin, as a newborn, they may exhibit hyperinsulinemia and respiratory distress syndrome (1). It has also been shown that these offspring are at greater risk of obesity and diabetes when they grow up (3). Additionally, GDM also has adverse effects on the mother; the condition may lead to perinatal complications, with shoulder dystocia being one of the more prominent perinatal risks. To avoid this risk, the mother is required to have a cesarean section without undergoing labor (4). In addition, both pre-eclampsia (5) and the incidence of cesarean sections (6) are increased in cases of undiagnosed, untreated GDM. At present, the treatment for GDM is usually based on self-monitoring, diet and medication. However, the use of oral antidiabetic drugs in the absence of medical nutrition stimulates the pancreas to produce and release insulin, leading to hypoglycemia in newborns (7). Therefore, there is an urgent need for more scientifically effective diagnostic and therapeutic approaches to meet the requirements for both maternal and neonatal health.

C1q/TNF-associated proteins (CTRPs), especially CTRP9, have been identified as highly conserved homologs of adiponectin (APN), pooling several regulatory functions of APN (8). There has been considerable interest in assessing the metabolic and cardiovascular roles of CTRP9. An increasing number of studies have shown that CTRP9 exerts protective effects on the cardiovascular system by attenuating post-infarction cardiac fibrosis (9), inducing angiogenesis (10) and inhibiting vascular inflammation (11). However, relatively few studies have focused on the role of CTRP9 in GDM. Recently, it has been shown that lipocalin expression is downregulated in GDM (12), and this was suggested to be an early predictor of GDM (13). In addition, it has been reported that CTRP9 is also downregulated in GDM, and that this downregulation of gene expression may have a protective effect (14). Furthermore, CTRP9 is able to interact with the endoplasmic reticulum (ER) molecular chaperone calreticulin in cardiomyocytes to inhibit ER stress (15). Therefore, it is possible to speculate that CTRP9 may be associated with the development of GDM.

ER stress is provoked by a variety of endogenous and exogenous processes that lead to cellular damage, including environmental toxins, viral infections and inflammation (16).

Correspondence to: Dr Xue Yang, Department of Obstetrics and Gynecology, Ningbo First Hospital, 59 Liuting Road, Ningbo, Zhejiang 315000, P.R. China
E-mail: yxuehua121@163.com

Cells experience ER stress when the ability to fold ER proteins is overwhelmed (17). Moreover, ER stress is also a central feature of peripheral insulin resistance, obesity and type 2 diabetes (18). It has also been claimed that ER stress exacerbates the occurrence of GDM (19). Furthermore, the chorionic trophectoderm is an important component of the embryo and, if impaired, this can lead to a variety of complications during pregnancy, including GDM. Therefore, in the present study, expression of CTRP9 was investigated in the HTR8/SVneo human placental trophoblast cell line under conditions of high-glucose (HG) induction, together with any subsequent effects on ER stress, in an attempt to demonstrate whether CTPR9 inhibits HG-induced trophoblast cell injury through the decrease in ER stress.

Materials and methods

Cell culture, treatment and transfection. The human trophoblast cell line HTR8/SVneo was obtained from Procell Life Science & Technology Co., Ltd. HTR8/SVneo cells were grown in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (MilliporeSigma) and 1% penicillin-streptomycin (MilliporeSigma) in a humidified incubator at 37°C in an atmosphere of 5% CO_2. The HTR8/SVneo cells were treated with 25 mM HG for 24 h to mimic the *in vitro* gestational diabetic environment and treated with 78 ng/ml tunicamycin (Abcam) for 16 h. HTR8/SVneo cells maintained in media containing 5 mM glucose were used as the control group.

pcDNA3.1(+) CTRP9 overexpression vector (Oe-CTRP9) and empty vector NC (Oe-NC; Shanghai GenePharma, Co., Ltd.) at a concentration of 20 μM were transfected into HTR-8/SVneo cells using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.), in accordance with the manufacturer's instructions. Cells were transfected at 37°C for 8 h and were used in subsequent experiments 48 h post-transfection.

Reverse transcription-quantitative PCR (RT-qPCR) assay. The extraction of total RNA from HTR8/SVneo cells was performed using TRIzol reagent (Takara Biotechnology Co., Ltd.), following the manufacturer's instructions. The extracted RNA was reverse-transcribed into cDNA using a PrimeScript reverse transcriptase reagent kit (Takara Biotechnology Co., Ltd.) according to the manufacturer's protocol. qPCR was performed using Power SYBR® Green Master Mix (Thermo Fisher Scientific, Inc.), following the manufacturer's instructions. The thermocycling conditions were as follows: 95°C for 5 min, followed by 40 cycles at 95°C for 10 sec, 60°C for 30 sec and 72°C for 30 sec. The samples were analyzed in triplicate. The data were quantified using the $2^{-\Delta\Delta Cq}$ method (20). The following primers were used: CTRP9 forward, 5'-TAGGGT CCAGGTGATGTTTCC-3' and reverse, 5'-CCACCAGAT CCTCATGGTTCAG-3'; and GAPDH forward, 5'-TGGAAG GACTCATGACCACA-3' reverse, 5'-AGGGGTCTACAT GGCAACTG-3'.

Western blot analysis. HTR8/SVneo cells were homogenized and lysed with RIPA buffer (Beyotime Institute of Biotechnology) on ice, followed by centrifugation at 12,000 x g for 10 min

at 4°C. Subsequently, the supernatant was collected, and the protein concentrations were measured using a BCA protein assay kit (MilliporeSigma). The proteins (20 μg/lane) were then separated using SDS-PAGE (10% gels), after which they were transferred onto PVDF membranes (MilliporeSigma). The membranes were blocked with 5% BSA for 1 h at room temperature and incubated overnight at 4°C with primary antibodies against CTRP9 (catalog no. LS-C373857; dilution, 1:400; LifeSpan Biosciences, Inc.), glucose-regulated protein 78 (GRP78; catalog no ab108615; dilution, 1:1,000; Abcam), cyclic AMP-dependent transcription factor ATF-4 (ATF4; catalog no. ab184909; dilution, 1:1,000; Abcam), C/EBP homologous protein (CHOP; catalog no. ab194533; dilution, 1:1,000; Abcam), cleaved caspase-3 (catalog no. ab32042; dilution, 1:500; Abcam), caspase 3 (catalog no. ab32351; dilution, 1:5,000; Abcam), Bcl-2 (catalog no. ab32124; dilution, 1:1,000; Abcam), Bax (catalog no. ab32503; dilution, 1:1,000; Abcam), p65 (catalog no. ab32536; dilution, 1:10,000; Abcam), phosphorylated (p)-p65 (catalog no. ab76302; dilution, 1:1,000; Abcam), cyclo-oxygenase-2 (COX-2) (catalog no. ab179800; dilution, 1:1,000; Abcam) and GAPDH (catalog no. ab9485; dilution, 1:2,500; Abcam). After washing with TBS plus 0.1% Tween-20 (Sigma-Aldrich; Merck KGaA) for 10 min, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG (catalog no. ab6721; dilution, 1:2,000; Abcam) for 1 h at room temperature. The protein blots were visualized using Pierce™ enhanced chemiluminescence western blotting substrate (Thermo Fisher Scientific, Inc.) and images were captured using a GE ImageQuant LAS4000mini biomolecular imager (Cytiva). Protein expression was evaluated by densitometry using ImageJ 1.8.0 software (National Institutes of Health).

Cell viability assay. HTR8/SVneo cells were routinely cultured in 96-well plates ($4x10^4$ cells/well) for 24 h. Subsequently, 10 μl Cell Counting Kit-8 (CCK-8) solution (Dojindo Laboratories, Inc.) was added to the medium, and the cells were incubated for 2 h at 37°C with 5% CO_2. The optical density value was measured at a wavelength of 450 nm with a Spectrafluor™ microreader plate (Tecan Group, Ltd.). These experiments were repeated three times.

TUNEL assay. A total of $1x10^6$ cells were incubated for 24 h in serum-free medium and fixed with 4% paraformaldehyde on slides at 4°C for 10 min. Subsequently, the cells were rinsed briefly with PBS, and then permeabilized with 0.1% Triton X-100 for 2 min on ice. Cells were stained using the TUNEL Assay Kit (Abcam) at 37°C in the dark for 1 h. The cells were subsequently rinsed with PBS and counterstained with 10 μg/ml DAPI at 37°C for 2-3 min, followed by being mounted in an anti-fade reagent (Beijing Solarbio Science & Technology Co., Ltd.). Fluorescence in three random fields of view was measured using a Zeiss LSM 880-Airyscan (University College London) upright confocal multiphoton microscope (Zeiss AG). The relative fluorescence intensity was determined by measuring the ratio of green (TUNEL) to blue (DAPI) using MetaMorph NX 2.5 software (Molecular Devices, LLC).

ELISA. Intracellular levels of TNF-α, interleukin (IL)-1β and IL-6 in HTR8/SVneo cells were respectively detected by
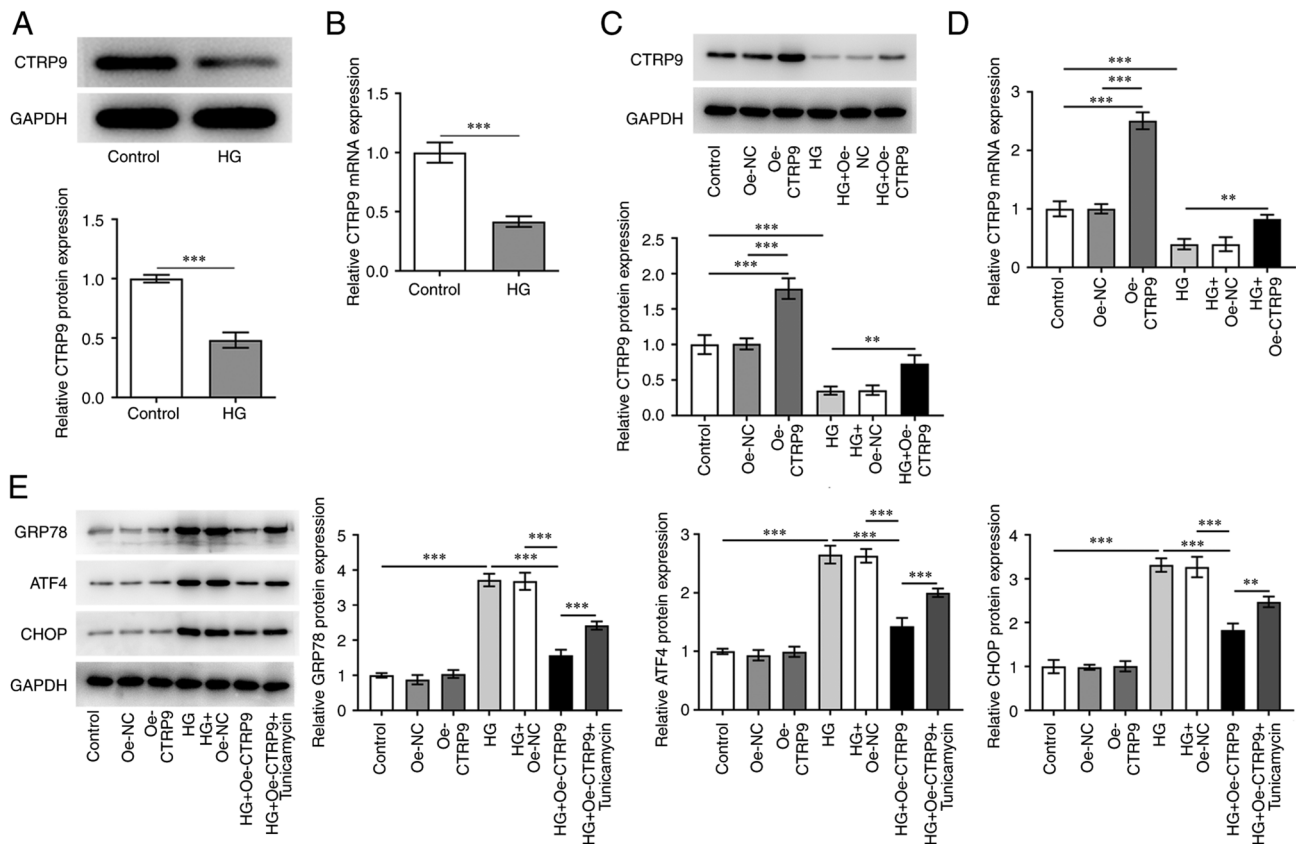
Figure 1. CTRP9 decreases ER stress in a trophoblast cell line. (A) Protein and (B) mRNA expression levels of CTRP9 were detected in HTR8/SVneo human placental trophoblast cells treated with HG using western blotting and RT-qPCR, respectively. The transfection efficiency of Oe-CTRP9 and its effects on HG-treated cells was detected using (C) western blotting and (D) RT-qPCR. (E) Detection of ER stress-related protein expression levels in cells were performed using western blotting. **P<0.01 and ***P<0.001. ATF4, cyclic AMP-dependent transcription factor ATF-4; CTRP9, C1q/TNF-α-related protein 9; CHOP, C/EBP homologous protein; ER, endoplasmic reticulum; GRP78, glucose-regulated protein 78; HG, high glucose; NC, negative control; Oe, overexpression; RT-qPCR, reverse transcription-quantitative PCR.

human TNF-α (catalog no. PT518), IL-1β (catalog no. PI305) and IL-6 (catalog no. PI330) ELISA kits (all Beyotime Institute of Biotechnology). HTR8/SVneo cells (1.0×10^4 cells/well) were digested with trypsin and collected after centrifugation at 300 x g for 5 min at room temperature. After rinsing with washing buffer, the cells were lysed and centrifuged again at 300 x g for 10 min at room temperature. The optical density values were measured at 450 nm using a microplate reader.

Measurement of reaction oxygen species (ROS), nitric oxide synthase (NOS) and NO. The level of ROS generation was assessed using the 2',7'-dichlorofluorescein diacetate probe (MilliporeSigma) according to the manufacturer's instructions. NOS activity was assessed using the NOS Activity Assay kit (AmyJet Scientific, Inc.) in accordance with the manufacturer's instructions. Finally, NO content was measured using a Micro NO Content Assay kit (Beijing Solarbio Science & Technology Co., Ltd.), following the manufacturer's standard protocol.

Statistical analysis. The experimental data were analyzed using SPSS 21.0 software (IBM Corp.) and are expressed as the mean ± standard deviation. An unpaired Student's t-test was used for comparisons between two groups, whereas one-way analysis of variance (ANOVA) followed by Tukey's post hoc

test was applied for comparisons among multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

CTRP9 decreases ER stress in trophoblast cell lines. The effects of CTRP9 on ER stress and the expression levels ER stress-associated proteins were investigated in HTR8/SVneo human trophoblast cells. The cells were treated with HG to simulate the environment of GDM, which resulted in a significant decrease in the protein expression levels of CTRP9 in comparison with the control (Fig. 1A and B). Subsequently, the cells were transfected with Oe-CTRP9, and the transfection efficiency was confirmed using western blotting and RT-qPCR (Fig. 1C and D, respectively). In addition, the HG-induced downregulation of CTRP9 expression was reversed by the transfection of Oe-CTRP9. As shown in Fig. 1E, CTRP9 overexpression had no significant effect on the expression levels of ER stress-associated proteins GRP78, ATF4 and CHOP under normal glucose conditions. However, the expression levels of these proteins were significantly increased under the induction of HG, which were then significantly decreased in cells transfected with Oe-CTRP9. This effect was reversed by the addition of the ER stress
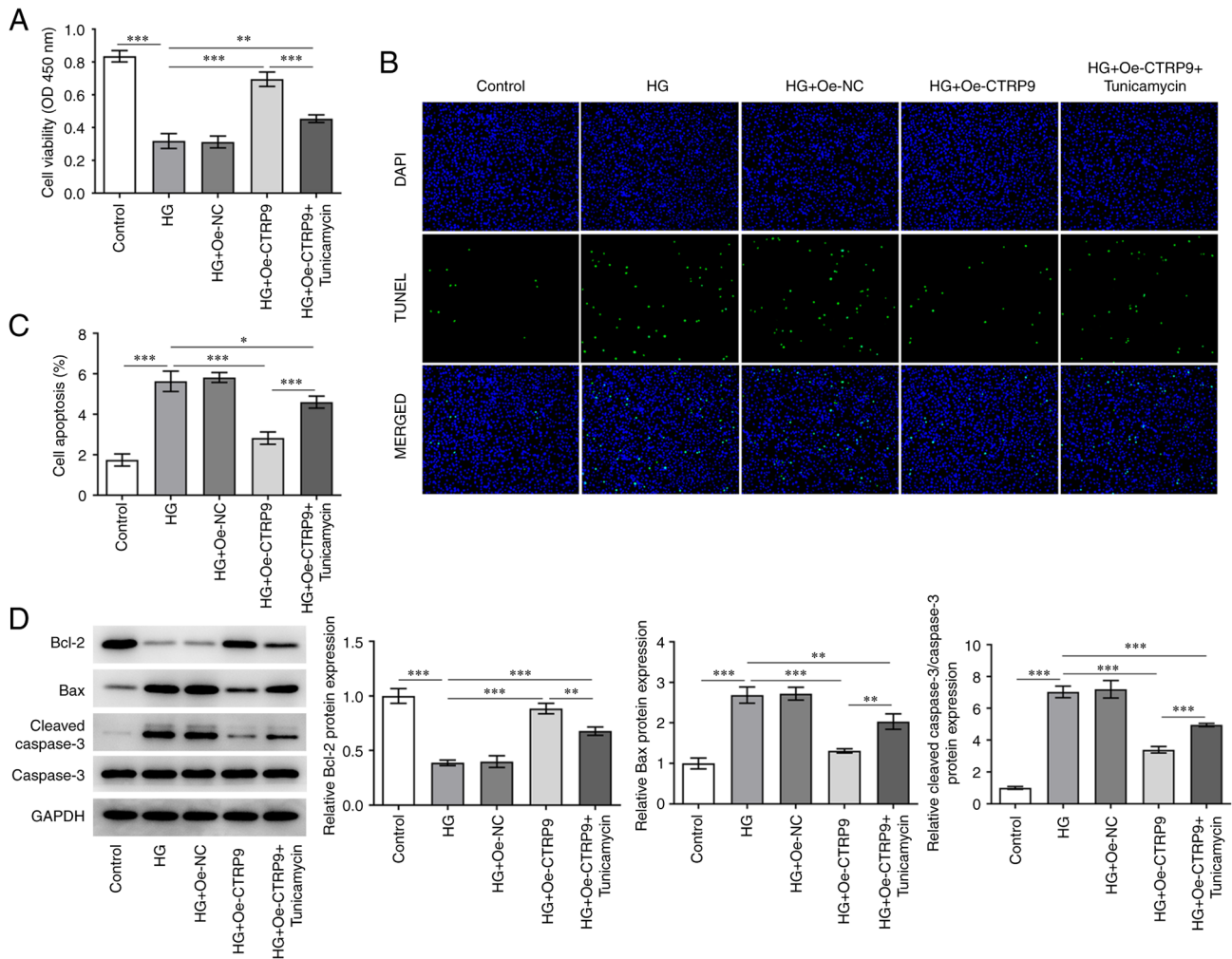
Figure 2. CTRP9 increases the viability of HG-induced HTR8/SVneo cells by decreasing ER stress. (A) Detection of cell viability was conducted by CCK-8 assay after transfection of Oe-CTRP9 and addition of the ER stress inducer tunicamycin. (B and C) Apoptosis levels were detected by TUNEL. Magnification, x200. (D) Expression levels of apoptosis-related proteins were examined by western blotting. $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$. CTRP9, C1q/TNF-α-related protein 9; ER, endoplasmic reticulum; HG, high glucose; NC, negative control; OD, optical density; Oe, overexpression.

inducer tunicamycin. Taken together, these results suggested that CTRP9 had a significant effect on decreasing ER stress in cells induced by HG.

CTRP9 increases the viability of HG-induced HTR8/SVneo cells by decreasing ER stress. To determine the effect of CTRP9 on the viability and apoptosis of HTR8/SVneo cells under HG induction, CCK-8, TUNEL and western blotting assays were performed. As shown in Fig. 2A, cell viability was significantly decreased under HG induction relative to the control group, and was restored to a certain extent following Oe-CTRP9 transfection, but then decreased again after the addition of tunicamycin. Conversely, HG treatment elevated the apoptotic rate of HTR8/SVneo cells in comparison with the control group. In addition, the suppressed apoptosis in HG-induced HTR8/SVneo cells transfected with Oe-CTRP9 was increased after the addition of tunicamycin (Fig. 2B and C). Additionally, Fig. 2D shows that HG treatment downregulated Bcl-2 protein level while up-regulated Bax and Cleaved caspase-3/Caspase-3 protein levels. After CTRP9 was overexpressed, Bcl-2 protein level was elevated while Bax and Cleaved caspase-3/Caspase-3 protein levels were decreased.

There was also a marked decrease in the protein level of Bcl-2 and increase in the protein levels of Bax and Cleaved caspase-3/Caspase-3 in HG + Oe-CTRP9 + Tunicamycin group. Aforementioned results suggested that CTRP9 may increase the viability and hinder the apoptosis of HG-treated HTR8/SVneo cells by decreasing ER stress.

CTRP9 inhibits the inflammatory response in HG-induced HTR8/SVneo cells by decreasing ER stress. To determine whether CTRP9 has a role in the inflammatory response of HTR8/SVneo cells, the intracellular levels of the inflammatory factors TNF-α, IL-1β and IL-6, and the protein expression levels of the inflammation-associated factors, p-p65 and COX-2, were detected by ELISA and western blotting assays, respectively. HG treatment significantly enhanced levels of TNF-α, IL-1β, IL-6 and increased the protein levels of p-p65 and COX-2. After transfection of Oe-CTRP9 into HG-induced HTR8/SVneo cells, a decrease was observed in the concentration levels of TNF-α, IL-1β and IL-6 (Fig. 3A), and a decrease was also observed in the protein expression levels of p-p65 and COX-2 (Fig. 3B) compared with the HG + Oe-NC group. However, the changes caused by Oe-CTRP9 were partially reversed by tunicamycin.
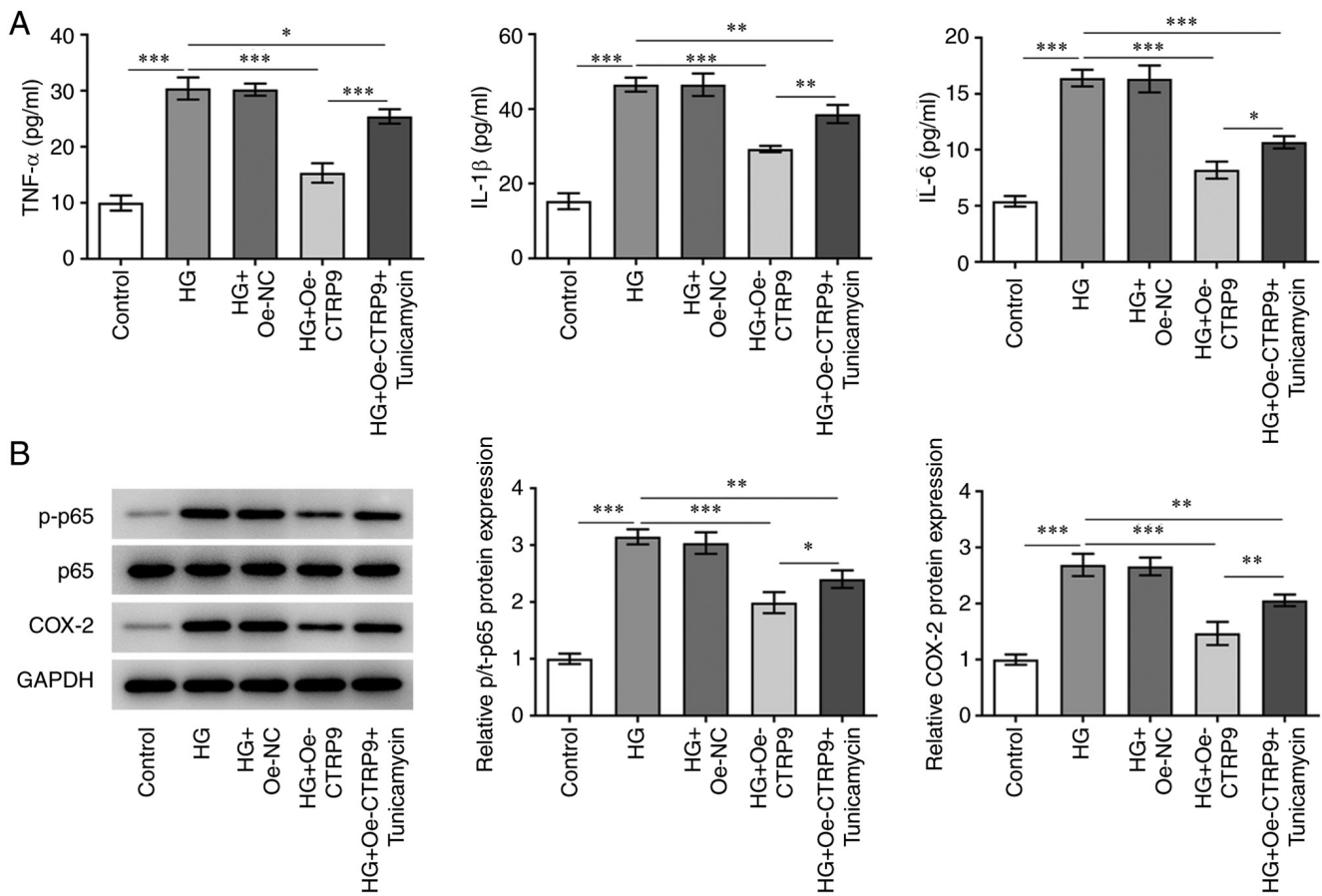
Figure 3. CTRP9 inhibits the inflammatory response in HG-induced HTR8/SVneo cells by decreasing endoplasmic reticulum stress. (A) Concentration levels of inflammatory factors TNF-α, IL-1β and IL-6 were determined using ELISA. (B) Protein expression levels of inflammation-associated factors p-p65 and COX-2 were detected by western blotting. *P<0.05, **P<0.01 and ***P<0.001. COX-2, cyclo-oxygenase-2; CTRP9, C1q/TNF-α-related protein 9; HG, high glucose; IL, interleukin; NC, negative control; Oe, overexpression; p/t-, phosphorylated/total.
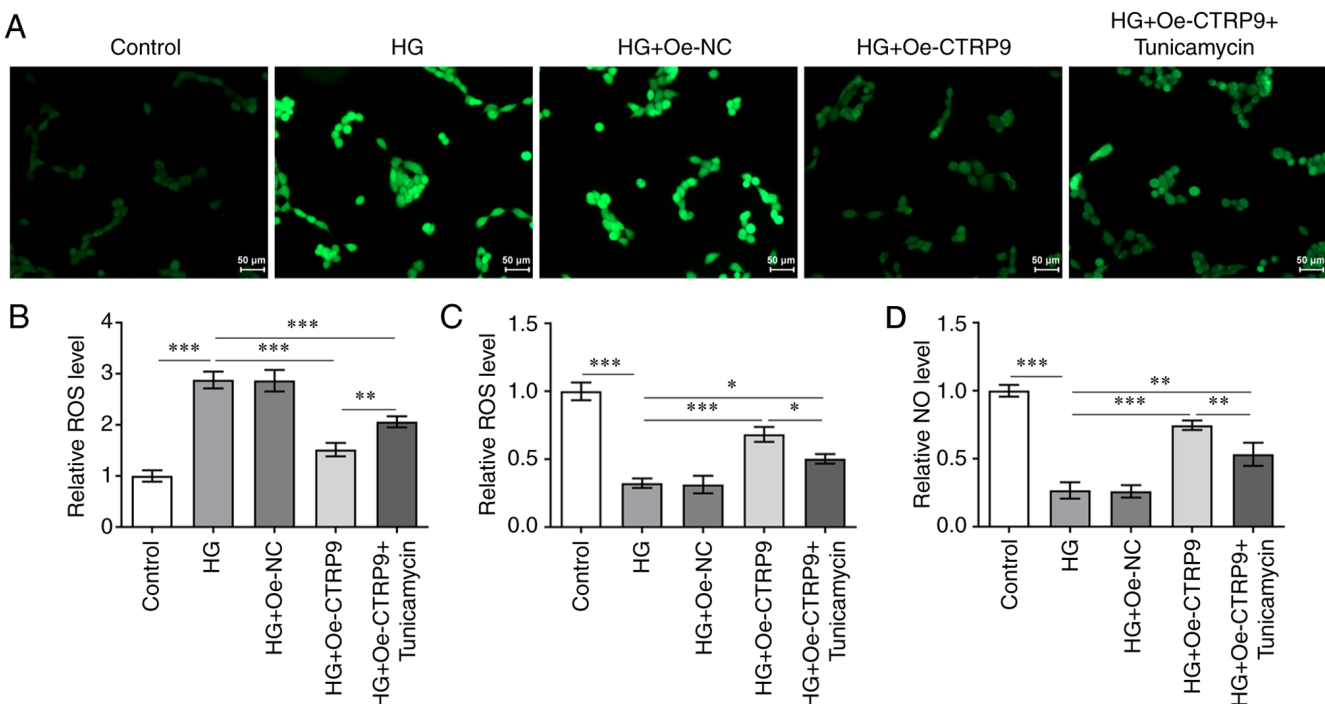

Figure 4. CTRP9 inhibits oxidative stress in HG-induced HTR8/SVneo cells by decreasing endoplasmic reticulum stress. (A) Detection of (B) ROS levels was carried out by the 2',7'-dichlorofluorescein diacetate kit. (C) NOS and (D) NO levels were determined by using the corresponding NOS and NO kits. *P<0.005, **P<0.01 and ***P<0.001. CTRP9, C1q/TNF-α-related protein 9; HG, high glucose; NC, negative control; NOS, nitric oxide synthase; Oe, overexpression; ROS, reactive oxygen species.

Taken together, these results indicated that CTRP9 exerted an inhibitory effect on the inflammatory response in HG-induced HTR8/SVneo cells by decreasing ER stress.

CTRP9 inhibits oxidative stress in HG-induced HTR8/SVneo cells by decreasing ER stress. To study in the effect of CTRP9 on oxidative stress in HG-induced HTR8/SVneo cells, the levels of ROS, NOS and NO associated with oxidative stress in HG-induced HTR8/SVneo cells were examined. Fig. 4A and B show that the HG-induced ROS level was decreased after transfection with Oe-CTRP9 compared with that in HG + Oe-NC group, but were significantly increased after the addition of tunicamycin. Moreover, as shown in Fig. 4C and D, the level of NOS was increased in HG-treated cells transfected with Oe-CTRP9, and this was decreased following the addition of tunicamycin. A similar trend was observed for the NO level. Taken together, these results have provided important insights into the participatory role of CTRP9 in oxidative stress in HG-induced cells.

Collectively, the experiments performed in the present study suggested that CTRP9 inhibited oxidative stress in HG-induced HTR8/SVneo cells through the decrease in ER stress.

Discussion

GDM is a medical complication that may be encountered during pregnancy; it is associated with adverse maternal and neonatal outcomes (1). At present, uniform screening and diagnostic strategies for GDM are lacking globally (21). CTRP9 expression has been reported to be downregulated in GDM, and may have a protective effect against GDM (14). Serum CTRP9 is an independent risk factor for the progression of GDM in pregnant women (22). The present study demonstrated that CTRP9 expression was downregulated in the human placental trophoblast cell line HTR8/SVneo. Furthermore, CTRP9 overexpression was shown to increase cell viability and suppress the inflammatory response as well as oxidative stress in HG-induced HTR8/SVneo cells through the decrease in ER stress.

CTRP9 has been associated with the prevention of atherosclerosis and the development of cardiovascular disease (23). Appari *et al* (24) suggested that upregulation of CTRP9 promotes the poor cardiac remodeling and left ventricular dysfunction associated with hypertrophic heart disease. CTRP3 and CTRP9 were shown to be downregulated in patients with heart failure (25). CTRP9 is also closely associated with the development of ER stress (15). According to Bai *et al* (26), CTRP9 exerts cardioprotective effects by increasing the levels of disulfide-bond A oxidoreductase-like protein to decrease ER stress in the diabetic heart. Cardiac-derived CTRP9 was shown to activate the PKA-CREB signaling pathway and to inhibit ER stress-associated apoptosis signaling during myocardial ischemia-reperfusion injury (15). The present study, to the best of our knowledge, was the first to show that CTRP9 expression was downregulated in HG-induced HTR8/SVneo cells. Moreover, after transfection with Oe-CTRP9, ER stress in HG-induced HTR8/SVneo cells was notably lower.

In addition, CTRP9 is also associated with apoptosis. For example, CTRP9 regulates human keratinocyte growth, differentiation and apoptosis through a TGFβ1-p38-dependent signaling pathway (27). CTRP9 has also been shown to regulate the hypoxia-mediated proliferation, apoptosis and migration of human pulmonary artery smooth muscle cells through the TGF-β1/ERK1/2 signaling pathway (28). In another previous study, CTRP9 also significantly attenuated palmitic acid-induced oxidative stress and ER stress-induced apoptosis in neonatal rat cardiac myocytes (29). Furthermore, CTRP9 was shown to inhibit HG-induced oxidative stress and apoptosis in retinal pigment epithelial cells through the activation of the AMPK/Nrf2 signaling pathway (30). It is well established that the upregulation of Bax, caspase 3 and the downregulation of Bcl-2 are observed in apoptosis (31). In the present study, after CTRP9 had been overexpressed in the HG-treated cells, cell viability was increased and apoptosis was decreased. In addition, the expression levels of cleaved caspase-3 and Bax were decreased, whereas the expression level of Bcl-2 was increased. However, after addition of the ER-stress inducer tunicamycin, the trends observed for the aforementioned genes were reversed. Together, these findings suggested that CTRP9 increases HG-induced HTR8/SVneo cell viability by decreasing ER stress.

Chronic inflammatory processes are associated with an increase in the levels of inflammatory cytokines, including IL-1β, TNF-α and IL-6 (32). The levels of the proinflammatory cytokine IL-1β have been shown to be high in the adipose tissue of women with GDM, which significantly inhibits insulin signaling in the adipose tissue of pregnant women (33). In the present study, it was found that the concentrations of the proinflammatory cytokines TNF-α, IL-1β and IL-6 were elevated in HTR8/SVneo cells upon induction by HG. Additionally, the role of CTRP9 in decreasing ER stress in cells has been demonstrated: A previous study showed that ER stress induced inflammation and insulin resistance associated with obesity and type 2 diabetes (34). In the present study, Oe-CTRP9 was transfected into HG-induced cells, which resulted in decreased expression levels of inflammatory cytokines, indicating that CTRP9 did exert an inhibitory effect on inflammation. Tunicamycin is an ER-stress inducer, which can effectively trigger ER stress by inhibiting protein glycosylation (35). In the present study, elevated expression levels of the proinflammatory cytokines TNF-α, IL-1β and IL-6 were found after adding tunicamycin to the cell culture experiments, as were the protein expression levels of p-p65 and COX-2. Collectively, these experiments confirmed that CTRP9 may inhibit the inflammatory response in HG-induced HTR8/SVneo cells by decreasing ER stress.

ER stress is an important local factor that is not only related to inflammation, but is also closely associated with oxidative stress (36). Oxidative stress is caused by an imbalance between the oxidative and antioxidant systems of cells and tissues as a consequence of the overproduction of oxidative free radicals and associated ROS (37). Oxidative stress is associated with the development of metabolic diseases, including diabetes (38). Additionally, CTRP9 has been shown to inhibit glomerular and tubule glycogen accumulation and fibrosis, and to relieve oxidative stress mediated by hyperglycemia (39). In the present study, it was observed that the levels of ROS were elevated in HTR8/SVneo cells under HG induction, but decreased after overexpression of CTRP9, and the levels then increased again following the addition of tunicamycin, whereas the levels of NOS and NO changed in the opposite direction. These findings

corroborated those of the other experiments, suggesting that CTRP9 inhibited oxidative stress in HG-induced HTR8/SVneo cells by decreasing ER stress.

In conclusion, the present study showed that CTRP9 was expressed at a low level in the HTR8/SVneo trophoblast cell line and may decrease ER stress. In addition, CTRP9 overexpression enhanced cell viability and inhibited the inflammatory response as well as oxidative stress in HG-induced HTR8/SVneo cells through the decrease in ER stress. In summary, CTRP9 decreases trophoblast cell damage induced by HG by decreasing ER stress, and CTRP9 is therefore a promising target for the therapeutic treatment of GDM in the future. However, the present study was limited to a single cell line, and loss of function experiments, as well as *in vivo* experiments, should be undertaken in the future to clarify the precise role played by CTRP9 in GDM.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

LZ and HD designed the study and drafted and revised the manuscript. YS, DZ and XY analyzed the data and searched the literature. LZ, YS and DZ performed the experiments. All authors have read and approved the manuscript. LZ and YS confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Coustan DR: Gestational diabetes mellitus. Clin Chem 59: 1310-1321, 2013.
2. Laredo-Aguilera JA, Gallardo-Bravo M, Rabanales-Sotos JA, Cobo-Cuenca AI and Carmona-Torres JM: Physical activity programs during pregnancy are effective for the control of gestational diabetes mellitus. Int J Environ Res Public Health 17: 6151, 2020.
3. O'Sullivan JB, Gellis SS, Dandrow RV and Tenney BO: The potential diabetic and her treatment in pregnancy. Obstet Gynecol 27: 683-689, 1966.
4. Hill MG and Cohen WR: Shoulder dystocia: Prediction and management. Womens Health (Lond) 12: 251-261, 2016.
5. Crowther CA, Hiller JE, Moss JR, McPhee AJ, Jeffries WS and Robinson JS; Australian Carbohydrate Intolerance Study in Pregnant Women (ACHOIS) Trial Group: Effect of treatment of gestational diabetes mellitus on pregnancy outcomes. N Engl J Med 352: 2477-2486, 2005.
6. Landon MB, Spong CY, Thom E, Carpenter MW, Ramin SM, Casey B, Wapner RJ, Varner MW, Rouse DJ, Thorp JM Jr, et al: A multicenter, randomized trial of treatment for mild gestational diabetes. N Engl J Med 361: 1339-1348, 2009.
7. Lengyel Z, Boér K, Halászlaki C and Németh Z: Diabetes in patients with malignant tumors. Magy Onkol 57: 177-181, 2013 (In Hu).
8. Rezabakhsh A, Sadeghpour Y, Ghaderi S, Rahbarghazi R and Geranmayeh MH: CTRP9: An emerging potential anti-aging molecule in brain. Cell Signal 73: 109694, 2020.
9. Wu D, Lei H, Wang JY, Zhang CL, Feng H, Fu FY, Li L and Wu LL: CTRP3 attenuates post-infarct cardiac fibrosis by targeting Smad3 activation and inhibiting myofibroblast differentiation. J Mol Med (Berl) 93: 1311-1325, 2015.
10. Bossi F, Tripodo C, Rizzi L, Bulla R, Agostinis C, Guarnotta C, Munaut C, Baldassarre G, Papa G, Zorzet S, et al: C1q as a unique player in angiogenesis with therapeutic implication in wound healing. Proc Natl Acad Sci USA 111: 4209-4214, 2014.
11. Yin C, Ackermann S, Ma Z, Mohanta SK, Zhang C, Li Y, Nietzsche S, Westermann M, Peng L, Hu D, et al: ApoE attenuates unresolvable inflammation by complex formation with activated C1q. Nat Med 25: 496-506, 2019.
12. Balachandiran M, Bobby Z, Dorairajan G, Gladwin V, Vinayagam V and Packirisamy RM: Decreased maternal serum adiponectin and increased insulin-like growth factor-1 levels along with increased placental glucose transporter-1 expression in gestational diabetes mellitus: Possible role in fetal overgrowth. Placenta 104: 71-80, 2021.
13. Sweeting AN, Wong J, Appelblom H, Ross GP, Kouru H, Williams PF, Sairanen M and Hyett JA: A Novel early pregnancy risk prediction model for gestational diabetes mellitus. Fetal Diagn Ther 45: 76-84, 2019.
14. Xia L, Zhang H, Shi Q, Zhang X, Wang C and Lin G: Protective role of CTRP3 and CTRP9 in the development of gestational diabetes mellitus. Clin Lab: 66: 200247, 2020.
15. Zhao D, Feng P, Sun Y, Qin Z, Zhang Z, Tan Y, Gao E, Lau WB, Ma X, Yang J, et al: Cardiac-derived CTRP9 protects against myocardial ischemia/reperfusion injury via calreticulin-dependent inhibition of apoptosis. Cell Death Dis 9: 723, 2018.
16. Shen X, Zhang K and Kaufman RJ: The unfolded protein response-a stress signaling pathway of the endoplasmic reticulum. J Chem Neuroanat 28: 79-92, 2004.
17. Tabas I and Ron D: Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. Nat Cell Biol 13: 184-190, 2011.
18. Ozcan U, Cao Q, Yilmaz E, Lee AH, Iwakoshi NN, Ozdelen E, Tuncman G, Görgün C, Glimcher LH and Hotamisligil GS: Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. Science 306: 457-461, 2004.
19. Liong S and Lappas M: Endoplasmic reticulum stress is increased in adipose tissue of women with gestational diabetes. PLoS One 10: e0122633, 2015.
20. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
21. Alfadhli EM: Gestational diabetes mellitus. Saudi Med J 36: 399-406, 2015.
22. Na N and Ji M: Role of first-trimester serum C1q/TNF-related protein 9 in gestational diabetes mellitus. Clin Lab: 66: 200434, 2020.
23. Miyatake N, Adachi H, Nomura-Nakayama K, Okada K, Okino K, Hayashi N, Fujimoto K, Furuichi K and Yokoyama H: Circulating CTRP9 correlates with the prevention of aortic calcification in renal allograft recipients. PLoS One 15: e0226526, 2020.
24. Appari M, Breitbart A, Brandes F, Szaroszyk M, Froese N, Korf-Klingebiel M, Mohammadi MM, Grund A, Scharf GM, Wang H, et al: C1q-TNF-related protein-9 promotes cardiac hypertrophy and failure. Circ Res 120: 66-77, 2017.
25. Gao C, Zhao S, Lian K, Mi B, Si R, Tan Z, Fu F, Wang S, Wang R, Ma X and Tao L: C1q/TNF-related protein 3 (CTRP3) and 9 (CTRP9) concentrations are decreased in patients with heart failure and are associated with increased morbidity and mortality. BMC Cardiovasc Disord 19: 139, 2019.

26. Bai S, Cheng L, Yang Y, Fan C, Zhao D, Qin Z, Feng X, Zhao L, Ma J, Wang X, *et al*: C1q/TNF-related protein 9 protects diabetic rat heart against ischemia reperfusion injury: Role of endoplasmic reticulum stress. Oxid Med Cell Longev 2016: 1902025, 2016.

27. Jung TW, Park HS, Choi GH, Kim D and Lee T: CTRP9 regulates growth, differentiation, and apoptosis in human keratinocytes through TGFβ1-p38-dependent pathway. Mol Cells 40: 906-915, 2017.

28. Li YX, Run L, Shi T and Zhang YJ: CTRP9 regulates hypoxia-mediated human pulmonary artery smooth muscle cell proliferation, apoptosis and migration via TGF-beta1/ERK1/2 signaling pathway. Biochem Biophys Res Commun 490: 1319-1325, 2017.

29. Zuo A, Zhao X, Li T, Li J, Lei S, Chen J, Xu D, Song C, Liu T, Li C and Guo Y: CTRP9 knockout exaggerates lipotoxicity in cardiac myocytes and high-fat diet-induced cardiac hypertrophy through inhibiting the LKB1/AMPK pathway. J Cell Mol Med 24: 2635-2647, 2020.

30. Cheng Y, Qi Y, Liu S, Di R, Shi Q, Li J and Pei C: C1q/TNF-related Protein 9 inhibits high glucose-induced oxidative stress and apoptosis in retinal pigment epithelial cells through the activation of AMPK/Nrf2 signaling pathway. Cell Transplant 29: 963689720962052, 2020.

31. Zhang L and Zhang S: Modulating Bcl-2 family proteins and caspase-3 in induction of apoptosis by paeoniflorin in human cervical cancer cells. Phytother Res 25: 1551-1557, 2011.

32. Duleba AJ and Dokras A: Is PCOS an inflammatory process? Fertil Steril 97: 7-12, 2012.

33. Lappas M: Activation of inflammasomes in adipose tissue of women with gestational diabetes. Mol Cell Endocrinol 382: 74-83, 2014.

34. Liong S and Lappas M: Endoplasmic reticulum stress regulates inflammation and insulin resistance in skeletal muscle from pregnant women. Mol Cell Endocrinol 425: 11-25, 2016.

35. Wu J, Chen S, Liu H, Zhang Z, Ni Z, Chen J, Yang Z, Nie Y and Fan D: Tunicamycin specifically aggravates ER stress and overcomes chemoresistance in multidrug-resistant gastric cancer cells by inhibiting N-glycosylation. J Exp Clin Cancer Res 37: 272, 2018.

36. Brenjian S, Moini A, Yamini N, Kashani L, Faridmojtahedi M, Bahramrezaie M, Khodarahmian M and Amidi F: Resveratrol treatment in patients with polycystic ovary syndrome decreased pro-inflammatory and endoplasmic reticulum stress markers. Am J Reprod Immunol 83: e13186, 2020.

37. Ali SS, Ahsan H, Zia MK, Siddiqui T and Khan FH: Understanding oxidants and antioxidants: Classical team with new players. J Food Biochem 44: e13145, 2020.

38. Zhang P, Li T, Wu X, Nice EC, Huang C and Zhang Y: Oxidative stress and diabetes: Antioxidative strategies. Front Med 14: 583-600, 2020.

39. Hu H, Li W, Liu M, Xiong J, Li Y, Wei Y, Huang C and Tang Y: C1q/Tumor necrosis factor-related protein-9 attenuates diabetic nephropathy and kidney fibrosis in db/db mice. DNA Cell Biol 39: 938-948, 2020.