Tropical Journal of Pharmaceutical Research September 2023; 22 (9): 1863-1869
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v22i9.13

Original Research Article

Procyanidin A1 acts as an antioxidant stressor in gestational diabetes

Mengni Zhu, Liping Liu*
Department of Obstetrics and Gynecology, The Sixth Hospital of Wuhan, Wuhan, Hubei Province 430000, China

For correspondence:* ***Email:* *liuliping3051001@163.com;* ***Tel:*** *+86-18062441008*

Sent for review: 9 June 2023 *Revised accepted: 30 August 2023*

Abstract

Purpose: *To evaluate the mechanism and effect of Procyanidin A1 (PCA1) in gestational diabetes mellitus (GDM).*
Methods: *Human trophoblast cell line HTR-8/Svneo was treated with 25 mM glucose for 24 h, and the effect of PCA1 on HTR-8/SVneo cell viability and proliferation was determined by CCK-8 assay and EdU proliferation kit. The expression of BAX, Bcl-2, cleaved Caspase-3 and cleaved caspase-9 was determined by western blotting. Cell apoptosis was assessed by Annexin V-FITC/ PI staining. Cellular generation of reactive oxygen species (ROS) was determined by ROS assay kit while superoxide dismutase (SOD), malondialdehyde (MDA), and catalase (CAT) were determined by the corresponding assay kits. The effect of PCA1 on Nrf2/HO-1 pathway was evaluated by determining the expression of Keap1, Nfr2, and HO-1.*
Results: *Procyanidin A1 (PCA1) increased the viability of high glucose (HG) -induced HTR-8/SVneo cells and promoted cell proliferation. Furthermore, PCA1 significantly inhibited HG-induced BAX, cleaved caspase-3, and cleaved caspase-9 expression, resulting in further reduction in HG-induced cell apoptosis. High glucose (HG) induced a significant increase in intracellular ROS levels, and this HG-induced oxidative stress was inhibited by PCA1. Furthermore, PCA1 activated Nrf2/HO-1 pathway and this was responsible for its proliferative, anti-apoptosis and anti-oxidative effects.*
Conclusion: *Procyanidin A1 promotes proliferation, and inhibits apoptosis and oxidative stress induced by high glucose in trophoblast cells by activating Nrf2/HO-1 signaling pathway. Therefore, it is a potential drug for the treatment of GDM.*

Keywords: *Procyanidin A1, Antioxidant, Gestational diabetes, Trophoblast damage*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, Web of Science, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Gestational diabetes mellitus (GDM), is a form of glucose intolerance which occurs during pregnancy and disappears spontaneously during late pregnancy period or after delivery [1]. Also, GDM is becoming increasingly common and has become an important issue that affects the health of pregnant women worldwide. If GDM is not treated in time, it leads to serious consequences, such as abnormal fetal development, miscarriage, and fetal asphyxia [2]. As an important exchange organ between the fetus and mother, the placenta plays an important role in transmitting maternal nutrients to the fetus and

excreting fetal metabolic waste [3]. In GDM patients, the placenta and trophoblast cells may be affected by high blood glucose levels. Studies have shown that a high glucose environment inhibits proliferation of trophoblast, thereby restricting the supply of nutrients and oxygen to the placenta [4].

Procyanidin is a class of polyphenolic compounds that is naturally present in plants. It has excellent antioxidant activity and scavenges free radicals, alleviates oxidative stress reactions, and prevents inflammation and oxidative damage [5]. Procyanidin A1 (PCA1) is an A-type proanthocyanidin dimer with strong biological activity. Procyanidin A1 increases activity of antioxidant enzymes [6], glutathione peroxidase (GSH-Px), and reduces the level of oxidative stress markers such as malondialdehyde (MDA) [7]. However, few studies have been done to investigate the effect of PCA1 in GDM, and its mechanism is not clear.

The Nrf2/HO-1 signaling is an important cellular protective mechanism in the oxidative stress and cell apoptosis process [8]. This signaling pathway alleviates oxidative stress and maintains cell survival by inhibiting the apoptotic pathway [8]. In this study, a high glucose-induced (25 mM) HTR-8/Svneo trophoblast cell was used as a cell model. The effect of PCA1 on Nrf2/HO-1 signaling, as well as proliferation, apoptosis, and oxidative stress induced by high glucose in trophoblast cells, were investigated.

EXPERIMENTAL

Cell culture and treatment

The HTR-8/SVneo was grown in RPMI-1640 media (Gibco, Code No. 11875119) and incubated at 37 °C in a 5 % CO_2 incubator. The HTR-8/SVneo cells were treated with 25 mM glucose for 24 h, and cells in control group were cultured in 5.5 mM glucose.

Cell viability assay

The HTR-8/SVneo cell was seeded into 96-well plates at a cell number of 5,000 cells per well. Cell viability was measured by Cell Counting Kit-8 (Beyotime, Code No. C0038).

Cell proliferation assay

The HTR-8/SVneo trophoblast was seeded into 96-well plates. The cell proliferation was determined by an EdU staining proliferation kit (iFluor 647) (Abcam, Code No. ab222421). Deoxyribonucleic acid (DNA) was labeled by DAPI 4’6-diamidino-2-phenylindole) staining solution (Abcam, Code No. ab228549). Observed and captured with fluorescence microscopy, the EdU-positive cells were analyzed.

Cell apoptosis assay

The HTR-8/SVneo trophoblast was digested by trypsin, collected, and resuspended in FACS buffer. Cell apoptosis was determined by Annexin V-FITC/propidium iodide (PI) staining (Invitrogen, Code No. V13242).

Western-blotting assay

Total proteins were extracted from HTR-8/SVneo trophoblast cell pellets with radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Code No. P0013B) containing multiple inhibitors which inhibited protein degradation efficiently during sample lysis. Total protein samples were separated by electrophoresis with 8 % sodium doceyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The primary antibodies and the corresponding dilution ratio used include: β-actin (Abcam, Code No. ab8226, 1:5000), BAX (Abcam, Code No. ab32503, 1:2000), Bcl-2 (Abcam, Code No. ab182858, 1:2000), cleaved Caspase-3 (Abcam, Code No. ab32042, 1:5000), cleaved Caspase-9 (Cell Signaling, Code No. 9505S, 1:1000), Keap1 (Abcam, Code No. ab227828, 1:1000), Nrf2 (Abcam, Code No. ab62352, 1:1000), HO-1 (Abcam, Code No. ab52947, 1:1000). Goat anti-rabbit IgG H&L (HRP) (Abcam, Code No. ab6721, 1:5000) and goat anti-mouse IgG H&L (HRP) (Abcam, Code No. ab205719, 1:5000) were used as secondary antibodies. The semi-quantitative analysis of the western-blotting results was performed by ImageJ.

Cellular generation of reactive oxygen species

The HTR-8/SVneo cell was induced with HG, and a reactive oxygen species assay Kit (Beyotime, Code No. S0033S) was used to assess HG-stimulated generation of oxidant chemical species.

Determination of cellular levels of SOD, MDA, CAT

High glucose-induced HTR-8/SVneo cells were harvested for SOD, MDA, and CAT determination using total superoxide dismutase assay kit (Beyotime, Code no. S0101M), lipid peroxidation MDA assay kit (Beyotime, Code no. S0131M) and catalase assay kit (Beyotime,

Code no. S0051) according to manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using GraphPad. Data was obtained in triplicates and presented as mean ± standard deviation (SD). *P* < 0.05 was considered statistically significant.

RESULTS

Procyanidin A1 enhances proliferation of HG-induced HTR-8/SVneo cell

High glucose (25 mM) significantly inhibited cell viability and proliferation (Figure 1 A and D). The effect of PCA1 on normal HTR-8/SVneo cell viability was also investigated. The results demonstrated that when HTR-8/SVneo cells were cultured in normal medium without high glucose, PCA1 had a negligible effect on cell viability irrespective of treatment groups (Figure 1 B).

However, a significant cell viability reduction was observed in the 160 µM PCA1 treatment group ($p < 0.05$) (Figure 2 B). Therefore, 20, 40, and 80 µM PCA1 were selected to investigate effects of PCA1 on HG-induced HTR-8/SVneo cells in this study.

The effect of PCA1 on HG-induced HTR-8/SVneo cell viability and proliferation was determined by CCK-8 and EdU staining respectively. The results revealed that PCA1 significantly rescued the decrease in cell viability induced by high glucose in a dose-dependent manner. A similar result on cell proliferation was observed in Figure. 1 D. Results of fluorescence microscopy revealed that HGEdU-positive cells accounted for about 40 % of total HG-induced HTR-8/SVneo cells treated with 80 µM PCA1. Furthermore, PCA1 increased the viability of HG-induced HTR-8/SVneo cells and enhanced cell proliferation.

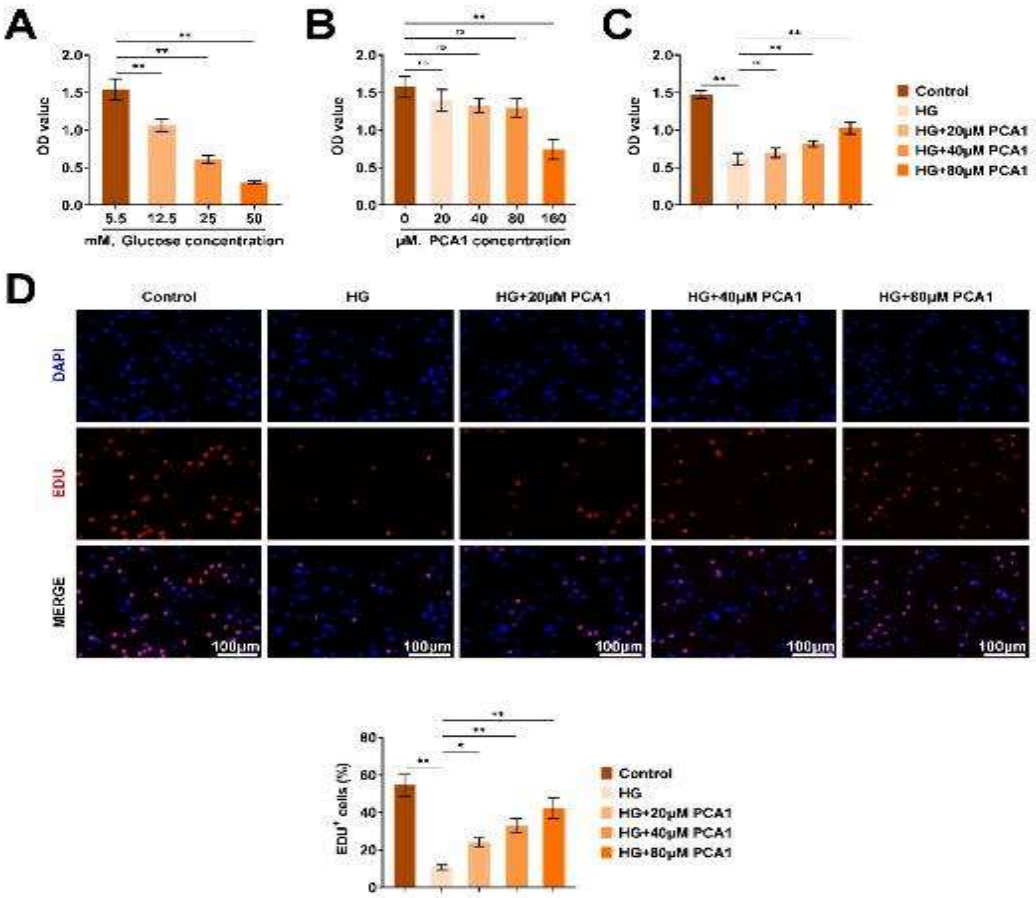

Figure 1: Procyanidin A1 enhanced HG-induced HTR-8/SVneo cell proliferation. (A) Cell viability of HTR-8/SVneo cells after treatment with different glucose concentrations for 24 h (B) Cell viability of HTR-8/SVneo cells after treatment with different PCA1 concentrations for 24 h (C) Cell viability of HG (25 mM glucose) induced HTR-8/SVneo cells after treatment with different PCA1 concentrations for 24 h (D) Cell proliferation of HG (25 mM glucose) induced HTR-8/SVneo cells after treatment with different PCA1 concentrations for 24 h

Procyanidin A1 inhibited HG-induced HTR-8/SVneo cell apoptosis

High glucose (25 mM) induced apoptosis in HTR-8/SVneo cells, while PCA1 significantly inhibited high glucose-induced cell apoptosis. With PCA1 treatment, there was a reduction in both apoptotic and dead cells. Furthermore, high glucose-induced cells plus 80 μM PCA1 treatment reduced apoptosis from 30 to 10 %, compared to the HG group.

The western-blotting results are shown in Figure 2 C. Semi-quantitative analysis of BAX, BCL-2, cleaved Caspase-3, and cleaved Caspase-9 expression are shown in Figure. 2 D. Glucose induction significantly induced BAX expression and reduced BCL-2 expression, while PCA1 inhibited HG-induced BAX expression. Also, PCA1 reduced the BAX/BCL-2 ratio, thereby maintaining mitochondrial membrane potential and reducing the release of apoptotic molecules. Furthermore, PCA1 inhibited expression of cleaved Caspase-3 and cleaved Caspase-9 induced by glucose. As cleaved caspase-3 and cleaved Caspase-9 propagated an apoptotic signal through enzymatic activity on downstream targets, this inhibition expressed by PCA1 further alleviated the degree of cell apoptosis.

Procyanidin A1 inhibited HG-induced oxidative stress in HTR-8/SVneo cells

High glucose (25 mM) induced a significant increase in intracellular ROS levels (Figure 3 A) and reduced SOD and CAT levels (Figure 3 B). Intracellular accumulation of ROS led to membrane lipid peroxidation, resulting in a significant increase in MDA concentration (Figure 3 B), which in turn exacerbated oxidative stress damage. Also, PCA1 inhibited HG-induced oxidative stress in HTR-8/SVneo cells. After PCA treatment, ROS and MDA levels were reduced, and concentration of SOD and CAT raised to a level close to normal (Figure 3 A and B).

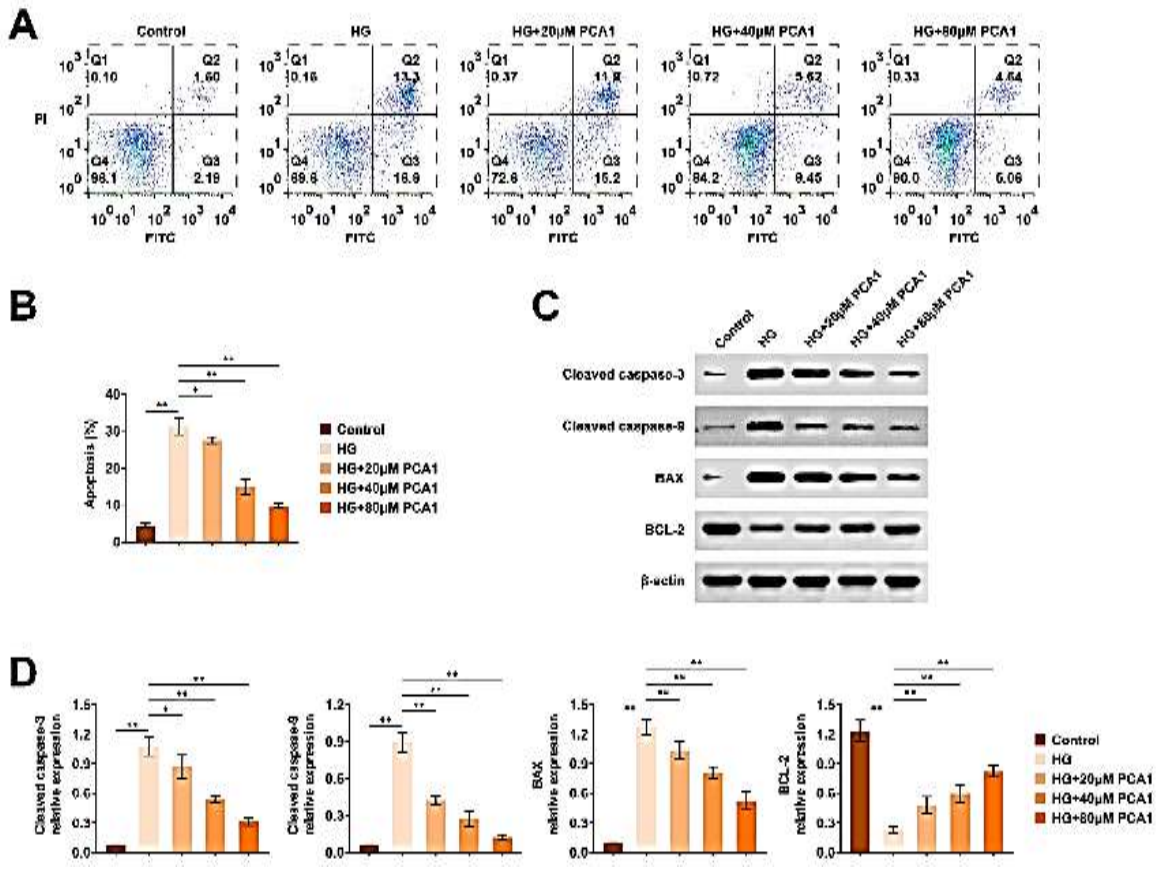

Figure 2: Procyanidin A1 inhibited HG-induced HTR-8/SVneo cell apoptosis. (A) Flow cytometry analysis of HG-induced HTR-8/SVneo cell apoptosis by Annexin V-FITC/ PI staining (B) Statistical analysis of cell apoptosis according to the results of Annexin V-FITC/PI staining (C) The expression of BAX, BCL-2, cleaved Caspase-3 and cleaved Caspase-9 was determined by western blotting. β-actin was selected as reference protein (D) Semi-quantitative analysis of BAX, BCL-2, cleaved Caspase-3 and cleaved Caspase-9 expression through image analysis software

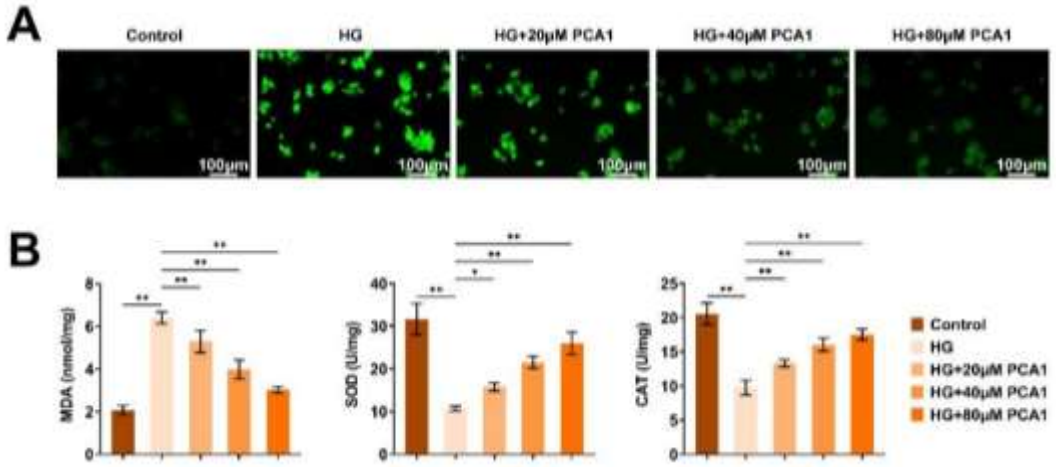

Figure 3: Procyanidin A1 inhibited HG-induced oxidative stress in HTR-8/SVneo cells. (A) Determination of oxidant chemical species in HTR-8/SVneo cells by confocal microscopy (B) SOD, MDA, and CAT level measured by commercially available kit

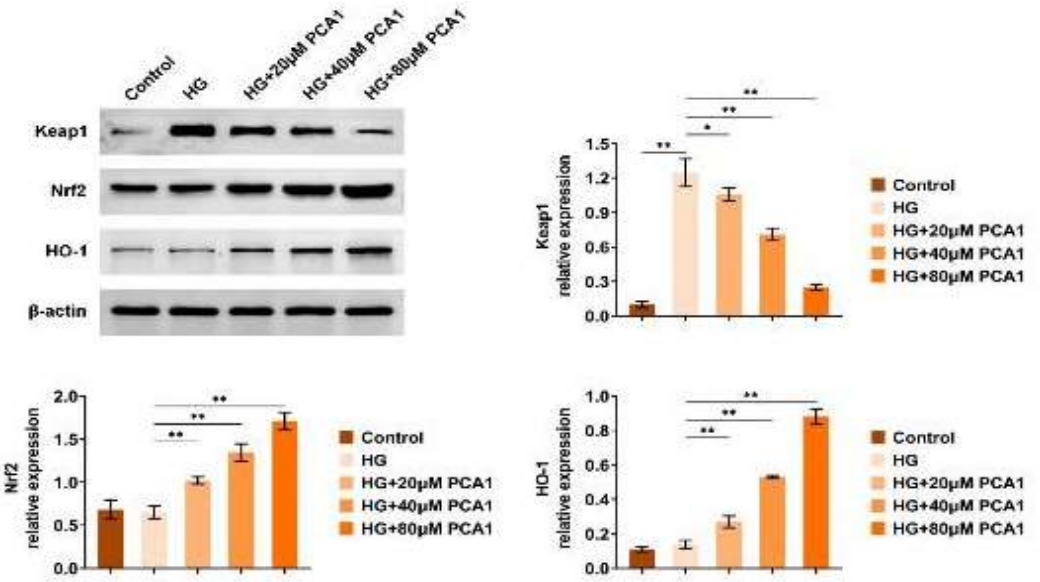

Figure 4: Procyanidin A1 activated Nrf2/HO-1 pathway in HG-induced HTR-8/SVneo cells. Expression of Keap1, Nrf2 and HO-1 were determined by western blotting. β-actin was selected as reference protein. Semi-quantitative analysis was performed through image analysis software to evaluate relative levels of Keap1, Nrf2 and HO-1

Procyanidin A1 activated Nrf2/HO-1 pathway in HG-induced HTR-8/SVneo cells

Western blotting analysis showed that high glucose (25 mM) induced expression of Keap1, resulting in a large amount of Keap1. Keap1 bound to Nrf2, which increased the stability of Nrf2 in the cytoplasm and reduced its degradation, resulting in a decrease in Nrf2 accumulation in the nucleus. Western blotting results also revealed that Nrf2 and HO-1 levels were similar to the control under high glucose induction. Furthermore, PCA1 treatment significantly reduced expression of Keap1, resulting in to increase in Nrf2 and HO-1 levels. Activation of Nrf2/HO-1 pathway by PCA1 may enhance antioxidant stress response function (Figure 4).

DISCUSSION

Recent research has revealed that prevalence of GDM in China increased remarkably from 4 % in 2010 to 21 % in 2020 [9]. Although current treatment options improve the metabolic status of pregnant women with GDM and prognosis of fetuses to some extent, more research is needed to understand its complex pathophysiological mechanisms to find more effective treatment methods. Studies have shown that PCA1 plays a role in pathogenesis of diseases such as diabetes, cardiovascular disease, obesity, and cancer. However, there is currently limited

research on the effect of PCA1 in GDM. Gestational diabetes mellitus (GDM) leads to high blood glucose levels, and a high glucose environment inhibits proliferation of trophoblast cells and promotes cell apoptosis[10]. In this study, HTR-8/SVneo cells were treated with high glucose (25 mM glucose) to mimic damaged trophoblast cells in GDM. This study revealed that PCA1 promoted proliferation and inhibited apoptosis and oxidative stress induced by high glucose in trophoblast cells by activating Nrf2/HO-1 signaling pathway.

Trophoblast cells connect fetus with the placenta and regulate fetal nutritional status and organ development. The normal function of trophoblast cells is crucial for fetal health. However, studies show that apoptosis of trophoblast layer cells in placental tissues of GDM patients is significantly higher than that in non-GDM placental tissues [11]. A shift in BAX/BCL-2 balance and upregulation of cleaved Caspase-3/cleaved Caspase-9 expression are usually regarded as important markers of apoptosis [12]. In this study, mechanism of how PCA1 affected high glucose-induced HTR-8/SVneo cell apoptosis was illustrated by determining expression of key proteins. The results showed that PCA1 inhibited HG-induced BAX expression, and increased BCL-2 expression, therefore reducing the BAX/BCL-2 ratio. The shift in BAX/BCL-2 balance maintained mitochondrial potential and reduced release of apoptotic molecules such as cleaved Caspase-3 and cleaved Caspase-9. In this manner, PCA1 protected trophoblast cells from HG-induced apoptosis and promoted cell survival. In future research, more studies should be done to identify specific signaling pathways through which PCA1 regulates expression of BAX/BCL-2.

During GDM, ROS levels in both maternal and fetal blood are generally increased, while antioxidant capacity is decreased [13]. As ROS are key signaling molecules involved in cell proliferation, death, and antioxidative activity, this high-concentrated ROS environment may be associated with the occurrence, development, and related complications of GDM [14]. Overall, antioxidative treatment is essential in GDM and its development. In this study, PCA1 was an important ROS scavenger with excellent antioxidant activity. The results indicated that PCA1 eliminated ROS through different mechanisms, and activated expression of antioxidant enzymes, such as SOD and CAT, to enhance their efficiency in ROS scavenging. In addition, PCA1 regulated the activity of Nrf2/HO-1 pathway to reduce production and accumulation of ROS. In summary, PCA1 scavenged ROS through multiple pathways and alleviated cell damage caused by oxidative stress. This implies that PCA1 reduced oxidative stress in pregnant women and fetuses, and improved pregnancy outcomes.

Safety of PCA1 is also very important and therefore more preclinical and clinical studies are needed to investigate its safety and effectiveness. More detailed studies on targets and mechanisms of PCA1 are required to guide its application in drug development or clinical trials. Target identification of PCA1 will provide more information on *in vivo* distribution, pharmacokinetics and safety. Furthermore, PCA1 is a procyanidin dimer, and strategies to improve the bioavailability and stability is also a key point in clinical application. This optimization may include nanotechnology and other formulations optimizing drug properties and clinical performance, thus achieving better treatment efficacy and reducing unnecessary risks.

CONCLUSION

PCA1 promotes proliferation, and inhibits apoptosis and oxidative stress induced by high glucose in trophoblast cells by activating Nrf2/HO-1 signaling pathway. Also, PCA1 reduces trophoblast cell damage and maybe a potential lead in the development of a therapeutic strategy for the management of GDM.

DECLARATIONS

Acknowledgements

This work was supported by the Natural Science Foundation of Hubei Province. (Grant no. 2022CFB408).

Funding

None provided.

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Mengni Zhu, and Liping Liu designed the study and carried them out, supervised the data collection, analyzed and interpreted the data, prepared the manuscript for publication, and reviewed the draft of the manuscript. All authors read and approved the manuscript for publication.

Open Access

REFERENCES

1. *Zhang S, Wang Y, Zhou L, Yu Z, Gao A. IRF2BP2 attenuates gestational diabetes mellitus by activating AMPK signaling. Trop J Pharm Res 2022; 21(7): 1459-1465. DOI: PMID:*
2. *Mack LR, Tomich PG. Gestational Diabetes: Diagnosis, Classification, and Clinical Care. Obstet Gynecol Clin North Am 2017; 44(2): 207-217.*
3. *Kaiser J. Reproductive biology. Gearing up for a closer look at the human placenta. Sci 2014; 344(6188): 1073.*
4. *Zhang C, Wang L, Chen J, Song F, Guo Y. Differential expression of miR-136 in gestational Diabetes mellitus mediates the high-glucose-induced trophoblast cell injury through targeting E2F1. Int J Genomics 2020; 2020: 3645371.*
5. *Chacón MR, Ceperuelo-Mallafré V, Maymó-Masip E, Mateo-Sanz JM, Arola L, Guitiérrez C, Fernandez-Real JM, Ardèvol A, Simón I, Vendrell J. Grape-seed procyanidins modulate inflammation on human differentiated adipocytes in vitro. Cytokine 2009; 47(2): 137-142.*
6. *Yan F, Chen L, Chen W, Zhao L, Lu Q, Liu R. Protective effect of procyanidin A-type dimers against H(2)O(2)-induced oxidative stress in prostate DU145 cells through the MAPKs signaling pathway. Life Sci 2021; 266: 118908.*
7. *Yu D, Xu J, Chen Y, Shi B. Procyanidin A1 inhibits insulin resistance and oxidative stress in palmitic acid treated HepG2 cells. Curr Top Nutraceutical Res 2022; 20: 561-566.*
8. *Ma Q. Role of nrf2 in oxidative stress and toxicity. Annu Rev Pharmacol Toxicol 2013; 53: 401-426.*
9. *Zhu H, Zhao Z, Xu J, Chen Y, Zhu Q, Zhou L, Cai J, Ji L. The prevalence of gestational diabetes mellitus before and after the implementation of the universal two-child policy in China. Front Endocrinol (Lausanne) 2022; 13: 960877.*
10. *Wang Z, Wang D, Chen J, Long T, Zhong C, Li Y. Effects of glucose and osmotic pressure on the proliferation and cell cycle of human chorionic trophoblast cells. Open Life Sci 2022; 17(1): 1418-1428.*
11. *Belkacemi L, Kjos S, Nelson DM, Desai M, Ross MG. Reduced apoptosis in term placentas from gestational diabetic pregnancies. J Dev Orig Health Dis 2013; 4(3): 256-265.*
12. *Yang B, Johnson TS, Thomas GL, Watson PF, Wagner B, Furness PN, El Nahas AM. A shift in the Bax/Bcl-2 balance may activate caspase-3 and modulate apoptosis in experimental glomerulonephritis. Kidney Int 2002; 62(4): 1301-1313.*
13. *Shang M, Zhao J, Yang L, Lin L. Oxidative stress and antioxidant status in women with gestational diabetes mellitus diagnosed by IADPSG criteria. Diabetes Res Clin Pract 2015; 109(2): 404-410.*
14. *Schieber M, Chandel NS. ROS function in redox signaling and oxidative stress. Curr Biol 2014; 24(10): R453-462.*