Chemical Inhibition of Mitochondrial Fission Improves Mitochondrial Function and Subdues Hyperglycemia Induced Stress in Placental Trophoblast Cells

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Abstract

Gestational diabetes mellitus (GDM) is a metabolic complication that affects millions of pregnant women in the world. Placental tissue function is endangered by hyperglycemia during GDM, which is correlated to increased incidences of pregnancy complications. Recently we showed that due to a significant decrease in mitochondrial fusion, mitochondrial dynamics equilibrium is altered in placental tissues from GDM patients. It is not known whether the decrease in mitochondrial fusion causes a decrease in mitochondrial function in placental cells. Here we show that chemical inhibition of mitochondrial fission in cultured placental trophoblast cells leads to an increase in mitochondrial fusion and improves the physiological state of these cells and hence, their capacity to cope in a hyperglycemic environment. Specifically, mitochondrial fission inhibition led to a reduction in reactive oxygen species (ROS) generation, mitochondrial unfolded protein marker expressions, and mitochondrial depolarization. It supported the increase in mitochondrial antioxidant enzyme expressions as well. Mitochondrial fission inhibition also increases the placental cell insulin sensitivity during hyperglycemia. Our results suggest that mitochondrial fusion/fission equilibrium is critical for placental cell function and signify the therapeutic potential of small molecule inhibitors of fission during GDM.

Introduction

Gestational diabetes mellitus (GDM) is an important metabolic complication affecting nearly 10% of pregnant women [1]. It is also suggested that GDM is linked with some long-term metabolic effects on women and fetuses in a significant number of cases [2]. Although a majority of the GDM cases resolve postpartum, there is a strong correlation between impaired glucose tolerance during pregnancy and various birth complications. As a functional interface between the mother and the fetus, the placenta plays a central role in mediating maternal-fetal communication and largely determines the successful outcome of the pregnancy. The placenta supports fetal growth by providing nutrients and oxygen from the maternal circulation, removing fetal waste products, and protecting the fetus from maternal inflammatory responses [3]. Additionally, the placenta senses and responds to changes in the metabolic environment and secretes various hormones that alter maternal physiology to optimize fetal growth [4]. Therefore, it is not surprising that placental dysfunction triggered by GDM is associated with pregnancy complications such as preeclampsia, fetal growth restriction, and fetal macrosomia [5].

The trophoblasts of the human placenta are highly dynamic cells, which perform many functions required to ensure a healthy pregnancy [6]. These functions include active transport of amino acids, ion pumping, synthesis of steroid and peptide hormones, and secretion of growth factors and cytokines. Trophoblasts are equipped with a high number of mitochondria, which are responsible for adaptive processes that maintain cellular homeostasis [7]. Mitochondria, a dynamic organelle, undergo morphological changes to adapt to cellular energy demands. These changes occur continuously through mitochondrial fusion and fission that allow adequate distribution of mitochondria within cells. Mitochondrial fission increases the number of smaller organelles, while fusion produces large interconnected networks of mitochondria [8, 9]. Several dynamin-related GTPases constitute the main

factors of mitochondrial fusion/fission processes. Mitofusin (MFN) 1 and 2 are responsible for outer mitochondrial membrane fusion [10]. Whereas optic atrophy protein 1 (OPA1) carries out the fusion of the inner mitochondrial membrane, which is also involved with the preservation of the mitochondrial cristae structure [11]. Proteins responsible for fission include dynamin-related protein 1 (DRP1) and fission protein 1 (FIS1) [12]. Fusion and fission processes are required for the maintenance of important cellular functions such as the mitochondrial respiration activity, the change in mitochondrial antioxidant enzyme levels responsible for scavenging reactive oxygen derivatives (ROS) [13], and the control of mitochondrial protein folding [14]. Many studies have emphasized the critical role of mitochondrial dynamics in glucose metabolism, regulation of insulin signaling (insulin / AKT pathway), and thus in the development of obesity and type 2 diabetes mellitus (T2DM) [15].

Recently we showed that due to a significant decrease in mitochondrial fusion, mitochondrial dynamics equilibrium is altered in placental tissues from GDM patients. It is not known whether the decrease in mitochondrial fusion causes a decrease in mitochondrial function in placental cells [16]. In this study, we examined the effect of mitochondrial fission chemical inhibition on the cellular homeostasis of human trophoblasts (BeWo) that are subjected to a hyperglycemic environment similar to the metabolic condition of the placental cells face during GDM. We applied three different small molecule inhibitors of mitochondrial fission (mdivi-1, dynasore and p-110) to placental trophoblast cells in a high glucose medium. The biological effect of fission inhibition on mitochondrial antioxidant response, mitochondrial protein folding mechanism, mitochondrial membrane potential, apoptosis, and insulin signaling were determined.

Materials And Methods

Cell Culture and Inhibitor Applications

Human placental trophoblast cells were used in the study (BeWo, ECACC, Cat. No: 86082803). Cells were seeded in six-well cell plates in DMEM (10% FBS, 1% penicillin/streptomycin, 1% sodium pyruvate, and 1% glutamine) medium containing 5.5 mM glucose (normal glucose) for optimization of glucose administration, and were incubated at 37 °C and 5% CO_2. As in our previous study, cells were incubated with glucose-free medium (DMEM) for 2 hours [17]. Then, one group of cells was incubated with DMEM containing 25 mM high glucose for 24 and 48 hours, and the other group of cells was incubated in normal glucose medium (5.5 mM) for 24 and 48 hours. After the administration of glucose onto the cells in normal (5.5 mM) and hyperglycemic (25 mM) media, two different doses of 50 µM and 100 µM mdivi-1 (Cat. no: A14316, Adooq, USA) dissolved in DMSO were applied for 3 hours [18,19]. Likewise, two different doses (50 µM and 100 µM) of dynasore (Cat no: A12726, Adooq, USA) dissolved in DMSO were applied to the cells incubated in normal and hyperglycemic media for 3 hours [20,21]. P-110 (Cat no: 6897, Tocris, UK) at doses of 0.5 µM and 1 µM was dissolved in DMSO and applied for 24 hours [22]. Each inhibitor was administered in two doses in order to determine the dose-dependent changes of the inhibitors and to optimize the inhibition of mitochondria fission, which was shown for the first time in

trophoblast cells. DMSO levels were kept below 1% to prevent cytotoxic effects and applied to the control group.

Protein Isolation and Western Blot

RIPA buffer (1 ml per 5 x 10^6 cells) was added to six-well plates from the stock containing 1% phosphatase and 1% protease inhibitor cocktail. The plates were shaken gently until the bottoms were completely covered with buffer and incubated on ice for 15 min. Lysates were removed from the plates with the help of a cell scraper and homogenized by pipetting. The cell lysates were then freeze-thawed 3 times and were centrifuged at 4 °C, 14000 g for 15 min and the supernatants were transferred to clean tubes. The samples were divided into tubes at equal volumes and kept at –80 °C until the experiment was conducted. Western Blotting was performed as previously described [16]. The antibodies used in the study were: MFN1 (1:1000, FineTest, China, Cat. No: FNab05150), (MFN2 1:1000, BTLAB, China, Cat. No: BT-AP05382; FIS1 1:1000, FineTest, China, Cat.No: P7047), p-DRP1 (ser616, 1:1000, ThermoFisher, Cat. No: PA5-64821), AKT (1:1000, FineTest, China, Cat. No: FNab00271; IRS1, 1:1000, FineTest, China, Cat. No: FNab10261), p-IRS1 (ser323, BT Lab., China, Cat. No: BT-PHS00881), p-AKT (ser473, BT Lab., China, Cat. No: BT-PHS00864), β-Tubulin (Elabscience Cat. No: ENT5051). Membrane images were recorded with the Syngene G: BOX Chemi XX6 instrument and analyzed with the ImageJ program.

RNA Isolation and qPCR

Isolation of RNA from cells in group was performed with “RiboEx RNA isolation solution (GeneAll; catalog no: 301–001; Republic of Korea)”. RNA concentrations were determined by Nanodrop (Micro Digital, Nabi). The WizScript kit (WizScript; catalog no: W2211, Republic of Korea) was used for cDNA synthesis. The RT-PCR reaction was performed with the WizPure qPCR kit [WizPure™ qPCR Master, SYBR, catalog. no: W1711R-5 with ROX; with a program of a 5 min. pre-denaturation step at 95 °C, 30 s. denaturation at 95 °C, 60 s. annealing and extension (40 cycles) at 58 °C]. The sequences of the primers used in the reaction are provided in Table 1.

Methylthiazole Diphenyl Tetrazolium (MTT) Test

In 96-well plates (5000 cells per well), all groups were treated with the inhibitors and the glucose at the relevant dose. Then, 5 µl of MTT solution was transferred to each well and incubated for 1.5 hours. The media at the plates were removed and 200 µl of DMSO was added to each well. Absorbances were measured at 570 nm via a spectrophotometer.

Determination of Mitochondrial Potential

Mitochondrial potential in cells was determined with Muse MitoPotential Kit (Part Number: MCH100110, Luminex, USA) in MUSE Cell Analyzer in accordance with the manufacturer's protocol. Cells were determined as live, dead, depolarized live and depolarized dead, and the cell ratios with depolarized mitochondrial membrane were detected.

Determination of Oxidative Stress

The determination of oxidative stress in the groups was evaluated with the Muse Oxidative Stress Kit (Part number: MCH100111, Luminex, USA) using the MUSE Cell Analyzer in accordance with the manufacturer's protocol. The ratios of ROS (+) and ROS (-) profiles in all cells were determined.

Statistical Analysis

Continuous variables are given as mean ± standard error. The control of the distributions of the variables was determined by the Shapiro Wilk test. In the comparison of normally distributed groups, one-way analysis of variance (One-Way ANOVA) was used for cases with three or more groups. The Kruskal-Wallis H test was used for the cases with three or more groups in the comparison of the groups that did not conform to the normal distribution. IBM SPSS Statistics 21.0 (SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.) program was used for the analysis. The p value of ≤ 0.05 was accepted as a criterion for statistical significance.

Results

Effect of High Glucose Administration on Mitochondrial Fusion and Fission

In order to establish an *in vitro* system that would mimic the physiological effects of gestational diabetes on placental tissue cells, we treated human trophoblast BeWo cells to high glucose in the growth medium. The effect of high glucose treatment on the expressions of fusion and fission proteins were determined in trophoblast cells after the 24 and 48 hours of administration of 5.5 mM / 25 mM glucose were normalized to β-Tubulin control (Fig. 1A). High glucose administered for both 24 and 48 hours led to a significant reduction in the expressions of fusion proteins MFN1 (Fig. 1B) and MFN2 (Fig. 1C). In contrast, high glucose increased the expression of the fission protein FIS1 (Fig. 1E) and also increased DRP1 phosphorylation (Fig. 1D). Overall, the high glucose treatment for 48 hours had the strongest effect on mitochondrial dynamics markers and this treatment regime was used for the rest of the experiments.

Effect of Fission Inhibitors on Mitochondrial Fusion and Fission

Next, we determined the effect of three different specific inhibitors of mitochondrial fission (mdivi-1, dynasore and p-110) on BeWo cells under normal glucose (5.5 mM) and high glucose (25 mM) conditions on mitochondrial fusion and fission markers (Fig. 2). We used two different concentrations of inhibitors in order to determine the effect of inhibitor dosage on the results. Mdivi-1 treatment significantly reduced DRP1 phosphorylation at both concentrations in normal glucose conditions, compared to the control (C). A similar effect was also detected in high glucose administration. DRP1 phosphorylation, which was increased by the effect of high glucose, was significantly decreased with mdivi-1 treatment (Fig. 2B). Mdivi-1 showed a similar effect on the other fission protein FIS1 in both normal and high glucose environments and significantly reduced the FIS1 expressions (Fig. 2C). Dynasore caused a significant reduction of DRP1 phosphorylation at only 100 μM concentration in

normal glucose medium (Fig. 2B). Likewise, two different concentrations of dynasore and p-110 applied in both normal and high glucose media reduced FIS1 expressions compared to the control (Fig. 2C).

Unlike mitochondrial fission proteins, inhibition of fission increased the mRNA expressions of fusion markers as expected. Mdivi-1 showed a significant elevation in mRNA expression of *Mfn1* (Fig. 2D) and *Mfn2* (Fig. 2E) only at 100 μM concentration in normal and high glucose medium. High concentration of dynasore (100 μM) significantly increased *Mfn1* mRNA expression in both high and normal glucose environment, *Mfn2* expression in high glucose environment, and *Opa1* (Fig. 2F) expression in normal glucose environment. P-110 also showed similar effect. However, significant changes were detected in high concentration of p-110 (1 μM) when cells in high glucose media.

Effect of Fission Inhibitors on Oxidative Stress

In order to investigate the effect of fission inhibition on oxidative stress in trophoblast cells, ROS profiles of cells were detected and mitochondrial antioxidant enzyme mRNA expressions were determined (Fig. 3A). High glucose administration significantly increased the ratio of ROS (+) cells compared to the normal glucose environment. P-110 significantly decreased the ratio of ROS (+) cells in normal glucose media. In high glucose environment, all three inhibitors caused a significant decrease in ROS (+) cell ratios compared to the control (Fig. 3B).

As shown in the Fig. 3C, *MnSod* mRNA expressions of cells in high glucose medium increased significantly with the application of high concentrations of all three inhibitors. High concentration mdivi-1 (100 μM) treatment significantly increased *Gpx1* expression of cells in normal and high glucose medium. While dynasore administration (100 μM) caused a significant increase in *Gpx1* expressions of cells in normal glucose environment, p-110 provided a significant increase in both normal and high glucose media, especially when applied at high concentration (1 μM) (Fig. 3D).

Effect of Fission Inhibitors on mtUPR and Apoptosis

The effect of fission on mtUPR and expressions of apoptosis genes was demonstrated by qPCR. While high glucose increased the expression of mtUPR genes, it caused the apoptotic gene expression of the cells to be stimulated. High concentration application of all three inhibitors to cells in normal glucose medium significantly decreased *Hsp60* mRNA expression compared to control. In the high glucose environment, both concentrations caused a decrease in *Hsp60* expression in all inhibitor applications (Fig. 4A). A similar pattern was also found in *ClpP* expressions. However, low concentrations of dynasore and p-110 also significantly decreased the *ClpP* expression of cells in normal glucose medium compared to control (Fig. 4B).

Antiapoptotic *Bcl2* expression, which was decreased due to high glucose, elevated significantly in the high concentration treatment of all three inhibitors compared to control (Fig. 4C). A decreased proapoptotic *Bax* expression was determined when inhibitors were applied at both high and low concentrations in high glucose environment (Fig. 4D).

The effect of mitochondrial fission inhibition on cell viability was determined by MTT assay. According to MTT assay analysis, high glucose administration significantly decreased cell viability compared to normal glucose (T-test, p=0.024). However, inhibitor treatments did not show a significant difference on the cell viability (Fig. 4E).

Effect of Fission Inhibitors on Mitochondrial Potential

Next, mitochondrial membrane polarization of inhibitor-treated cells was determined by flow cytometry in order to determine the effect of mitochondrial fission on mitochondrial membrane potential. High glucose treatment led to a significant increase in the ratio of depolarized mitochondria in the living cells (Fig. 5A). Inhibition of the mitochondrial fission decreased the ratio of depolarized mitochondria in both normal glucose and high glucose medium. Likewise, total depolarized cell density was significantly lower in the inhibitor-administered high glucose groups compared to the control (Fig. 5B) suggesting that mitochondrial fission inhibition suppresses the depolarization effect of the high glucose treatment of the cultured placental cells.

Effect of Fission Inhibitors on Insulin Signaling

Finally, we determined the effect of chemical inhibition of mitochondrial fission on insulin sensitivity in BeWo cells. We analyzed IRS1 and AKT phosphorylation as markers for insulin action (Fig. 6A). The change in IRS1 and AKT phosphorylation by inhibition of fission was determined by western blot separately in insulin-administered and non-insulin-administered groups. Mdivi-1 and dynasore reduced inhibitory IRS1 phosphorylation of cells in normal glucose medium when administered at high concentration (100 μM). On the other hand, p-110 provided a significant decrease in both concentrations compared to the control. Inhibitors reduced IRS1 phosphorylation of cells in high glucose medium compared to control. Mdivi-1 and p-110 were found to be significant at both concentrations (insulin-administered groups, Fig. 6B). Similarly, AKT phosphorylation of cells in normal glucose medium increased significantly with the application of high concentrations of dynasore (100 μM) and p-110 (1 μM) compared to the control. AKT phosphorylation of cells in high glucose environment increased significantly in the groups where all three inhibitors were applied at high concentrations compared to the control. In addition, m-divi1 appeared to be effective at both concentrations (insulin-administered groups, Fig. 6C).

Interestingly, fission inhibitors also improved insulin signaling in cells that were not stimulated by insulin. IRS1 phosphorylation of cells in normal glucose medium was significantly reduced when inhibitors were administered at high concentration compared to control. On the other hand, increased IRS phosphorylation of cells in high glucose environment compared to normal glucose environment decreased significantly with both concentrations of three inhibitors. (non-insulin-administered groups, Supplement Fig. S1B). Application of 100 μM mdivi-1 and dynasore to cells in high glucose environment significantly increased AKT phosphorylation compared to control (non-insulin-administered groups, Supplement Fig. S1C).

Discussion

We investigated the effect of mitochondrial fission inhibition in placental trophoblast cells in order to determine the effect of fission inhibition during high glucose treatment and the therapeutic potential of chemical inhibition of fission during gestational diabetes. Placental trophoblast cells were cultured in high glucose (25 mM) medium and fission inhibitors were applied to the cells. Next, the status of insulin signaling molecules, apoptosis markers, mitochondrial antioxidant enzyme expressions and mtUPR markers in the cells were detected. In addition, cell viability test was performed and the mitochondrial potential and oxidative stress in the cells were determined.

Many studies have shown that high glucose exposure in tissues and high glucose administrations in cells affect mitochondrial dynamics. It has been found that high levels of glucose and palmitate in the cultured pancreatic β cell line stop mitochondrial fusion and decrease respiratory function in cells [23]. In another study, it was determined that high glucose administration during reperfusion caused a decrease in fusion proteins OPA1 and MFN2 and an increase in fission proteins DPR1 and FIS1 in mice [24]. Consistent with the literature, we also demonstrated that high glucose administration caused a decrease in MFN1 and MFN2 protein expressions at both 24 and 48 hours, while an increase in FIS1 expression with DRP1 phosphorylation.

Our results regarding the ROS generation in the cultured cells showed that the fission inhibition improved the ROS profile of cultured placental trophoblasts in high glucose treatment, suggesting that mitochondrial fission may be linked to mitochondrial ROS synthesis. In addition to their role as the "powerhouse" of the cell, mitochondria also play a role in the regulation of energy balance through the induction of mitochondrial biogenesis. Pyruvate is oxidized by the tricarboxylic acid cycle to produce the electron donors NADH and FADH2, which flow through the mitochondrial electron transport system formed by the inner membrane-associated enzyme complexes. It has been shown that if the electrochemical potential difference is too high under high glucose conditions, high amounts of ROS are produced at complex I and at the interface between ubiquinone and complex III [25, 26]. Hyperglycemia is a potent inducer of oxidative stress through increased ROS production at mitochondrial and non-mitochondrial levels, and pathways activated by oxidative stress have an important role in the pathogenesis of diabetes complications. In addition, excessive mitochondrial ROS production has been shown to cause acute and chronic muscle insulin resistance [27, 28]. Similarly, it has been reported that increased mitochondrial fission is causally involved in hyperglycemia-induced excessive ROS production in liver and muscle cells [29]. Our data suggested that increased glucose exposure promotes suppressible ROS increase by fission inhibition. It also showed that inhibition of fission produced an increase in mitochondrial antioxidant enzyme mRNA expressions. The increase in these enzyme levels may have contributed to the decrease in the amount of ROS.

Activation of mtUPR is induced by intense environmental stress. High glucose and high fat exposure can cause an increase and accumulation of unfolded and misfolded proteins in mitochondria, resulting in mito-nuclear protein imbalance that activates mtUPR. Caseinolytic mitochondrial matrix peptidase

proteolytic subunit (CLPP) is also involved in the process of glucose metabolism. Knockdown of CLPP leads to recovery of impaired glucose metabolism in different tissues of CLPP-deficient mice [14]. Another study documented evidence of a significant increase in protein level of HSP60 when human HeLa cells were grown in the presence of mitochondrial inhibitors (such as hydrogen peroxide and high glucose) [30]. Although further detailed studies are needed to support our findings, it has shown that mtUPR is associated with mitochondrial dynamics in placental cells. In our study, we showed that high glucose increases *ClpP* and *Hsp60* mRNA levels in trofoblast cells. We also found that inhibition of fission resulted in a significant decrease in these elevated expressions of mtUPR markers.

The tight link between apoptosis and mitochondrial dynamics has been investigated in many studies and has been clarified by many experimental models. Upon induction of apoptosis, the outer mitochondrial membrane is cleaved by translocation of DRP1 from the cytosol to the mitochondria, where it preferentially localizes to the site of organelle cleavage [31]. DRP1 further promotes BAX/BAK-induced cytochrome c release during apoptosis [32]. MFN1 and MFN2 inhibit cytochrome c release, BAX translocation, and oligomerization caused by apoptotic stimuli. Accordingly, BAX and BAK are also required for the fusion of long mitochondria through the functional activity of other outer membrane proteins, including MFN1 and MFN2 [33]. In this study, the apoptosis-stimulating effect of the high glucose administration in trophoblast cells was demonstrated. In line with the literature, it was determined that suppression of fission caused a significant increase in anti-apoptotic *Bcl-2* mRNA expressions, especially in groups with high dose inhibitors, and a significant decrease in pro-apoptotic *Bax* expressions. The link between apoptosis and mitochondrial dynamics demonstrated in previous studies has also been confirmed in trophoblast cells.

Finally, we determined that high glucose-induced insulin resistance in trophoblast cells was alleviated by fission inhibitors. The delicate balance between mitochondrial fusion and fission can be disrupted by a variety of stress responses, including nutrient stress and simulated ischemia. Moreover, improving unbalanced mitochondrial dynamics reduces cellular damage and disease severity, emphasizing the importance of mitochondrial dynamics in the pathogenesis of neuron and β-cell-mediated diseases [34]. Quality control may not be the only task driven by mitochondrial dynamics [35]. Recent studies show that mitochondrial dynamics play a crucial role in maintaining the balance between energy demand and nutrient supply in the cell [36]. Regulation of mitochondrial dynamics has been proposed as a bridge between mitochondrial dysfunction and insulin resistance during the pathogenesis of T2DM [37]. One of the studies on this subject is especially important in revealing this connection. In the study, it was reported that overexpression of MFN1 and MFN2 resulted in decreased mtROS production and increased IRS1 and AKT activator phosphorylation. At the same time, it was determined that the fission inhibitor mdivi-1 showed the same effects [37]. In another study, mdivi-1 administration in obese mice was shown to improve impaired insulin signaling [34]. Similarly, we determined that high glucose-induced insulin resistance in trophoblast cells was alleviated by fission inhibitors. Consistent with the literature, shifting the balance of mitochondrial dynamics to the fission side due to high glucose increased the inhibitory IRS1 phosphorylation and decreased the activating AKT phosphorylation with the effect of increasing ROS. This change was reduced to normal levels in both insulin-administered and non-insulin-

administered groups by inhibiting fission. As expected, AKT phosphorylation did not occur with normal glucose administration in the non-insulin-administered groups. There was no difference between the control group and the inhibitor applied groups. Surprisingly, high-dose inhibitor administration was found to significantly increase AKT phosphorylation in high glucose groups (in high-dose mdivi-1 and dynasore groups for pAKT/AKT) compared to the control group. This situation brings to mind the view that fission dynamics and AKT signal are mechanistically related. The same situation was observed more clearly in IRS1 expressions. It was determined that inhibitor applications in both normal glucose and high glucose environments caused a decrease in inhibitory IRS1 phosphorylation, although the cells were not stimulated with insulin. This indicates that IRS1/AKT signaling pathway is controlled by the fission of mitochondria in trophoblast cells. It is obvious that further mechanistic studies are needed to support this view. On the other hand, the situation was as expected in insulin-administered cells, and the findings were quite parallel in p-IRS1/IRS1 expressions and p-AKT/AKT expressions. Increasing the inhibitor dose resulted in a stronger improvement in insulin signaling. It supported our hypothesis that there is a connection between mitochondrial dynamics and insulin signaling.

Conclusion

Our study showed that fission inhibitors regulate mitochondrial biology and improve insulin responsiveness in cultured placental cells possibly through the suppression of oxidative stress and ROS synthesis. This result supported our view that inhibition of mitochondrial fission might improve impaired insulin signaling in trophoblast cells. In addition, it caused a greater increase in the mRNA expressions of mitochondrial antioxidant enzymes. In our study, inhibition of fission was also effective on mRNA levels of apoptosis markers in trophoblast cells. The chemicals used to inhibit mitochondrial fission are important for elucidating the effects of new potential therapeutics against metabolic diseases during pregnancy.

Declarations

Author contributions

U.K.K. design and conduction of the research, analysis and interpretation of the data and writing of the initial draft of the manuscript. A.Y. and G.D.Y. analysis and interpretation of the data and writing of the initial draft of the manuscript. All the authors have read and agreed to the final version of the manuscript.

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Conflict of interest

The authors have no conflicts of interest to declare.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Table

Table 1. Primer base sequences used in qPCR

Gene	Forward (5'-3')	Reverse (5'-3')
Mfn1	CCTGTTTCTCCACTGAAGCAC	CCTCACCAATGATGGAAAGC
Mfn2	ACACATGGCTGAGGTGAATG	CGTCCAGAACCTGTTCTTCTG
Opa1	GGATTGTGCCTGACATTGTG	AAGGCTTTCAACAATCTTGTCA
Drp1	CAGTGTGCCAAAGGCAGTAA	GATGAGTCTCCCGGATTTCA
Fis1	CTTGCTGTGTCCAAGTCCAA	GCTGAAGGACGAATCTCAGG
Sod2	AAGGGAGATGTTACAGCCCAGATA	TCCAGAAAATGCTATGATT
Gpx1	GGGACTACACCCAGATGAA	TCTCTTCGTTCTTGGCGTTC
ClpP	GCCAAGCACACCAAACAGA	GGACCAGAACCTTGTCTAAG
Hsp60	CACCGTAAGCCTTTGGTCAT	CTTGACTGCCACAACCTGAA
Bcl-2	CATGTGTGTGGAGAGCGTCAA	GCCGGTTCAGG TACTCAGTCA
Bax	TCGCCCTTTTCTACTTTGCC	AGTCTCACCCAACCACCCT
β-actin	AACTGGGACGACATGGAGAA	GAAGGTCTCAAACATGATCTGG

Figures

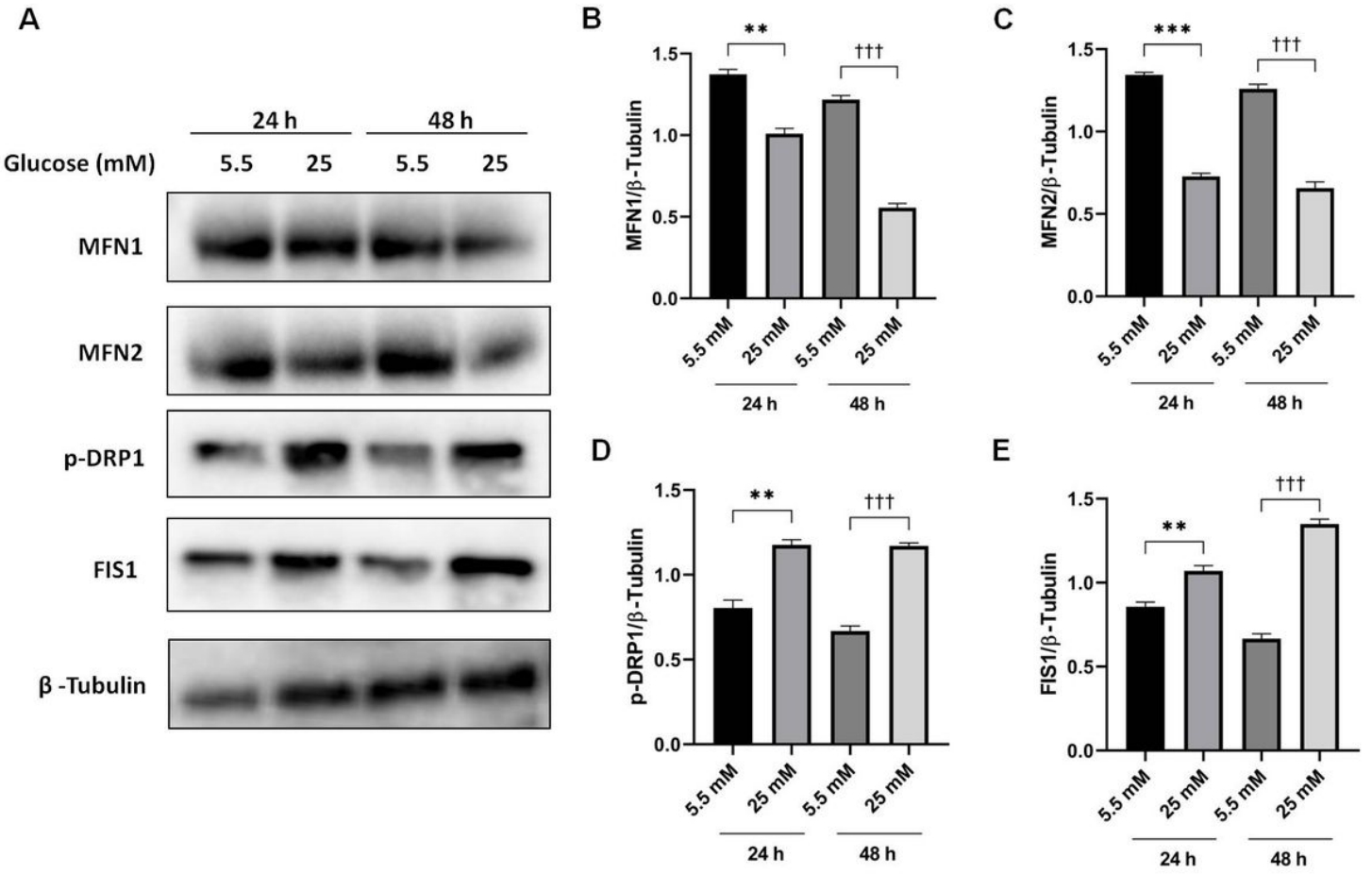

Figure 1

Mitochondrial fission and fusion protein expressions of cells in high glucose environment. A) Representative immunoblot expressions of MFN1, MFN2, FIS1 and p-DRP1 of trophoblast cells in normal (5.5 mM) and high (25 mM) glucose medium at 24 h and 48 h. Quantification of MFN1 (B), MFN2 (C), p-DRP1 (D) and FIS1 (E) expressions demonstrated by bar graph. Expressions were normalized to β-tubulin. Data are presented as mean ± SEM. ***$P < 0.001$, **$P < 0.01$ vs. 5.5 mM glucose (24 h), $^{†††}p < 0.001$ vs. 5.5 mM glucose (48 h). MFN1, mitofusin1; MFN2, mitofusin2; FIS1, mitochondrial fission 1; DRP1, dynamin-related protein 1.

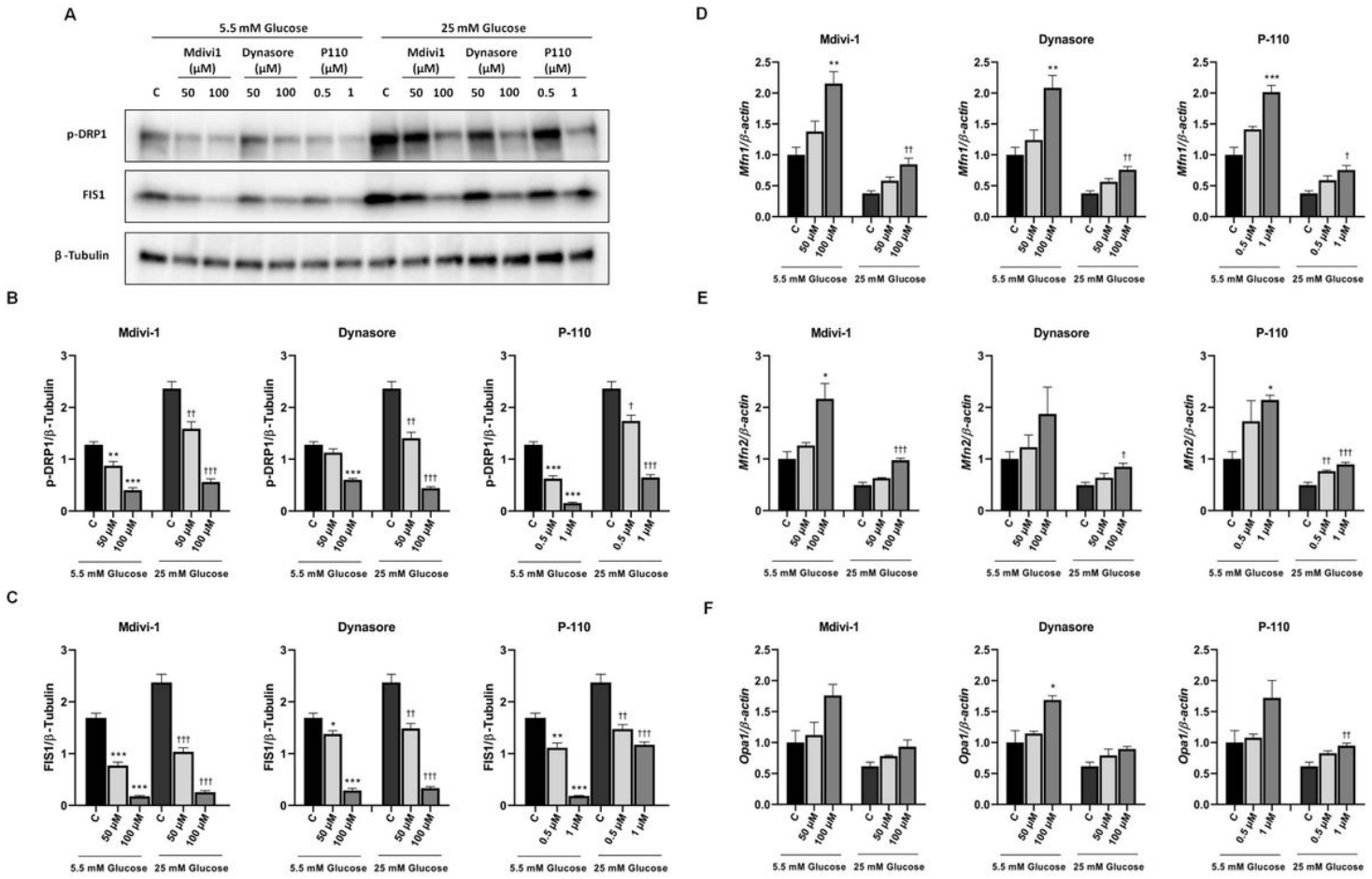

Figure 2

Fission protein expressions and fusion mRNA expressions of trophoblast cells treated with fission inhibitors. A) Representative western blot image of two different concentrations of three inhibitors (mdivi-1, dynasore and p-110) applied to trophoblast cells in normal (5.5 mM) and high glucose (25 mM) medium. Control cells were treated with DMSO. Quantification of p-DRP1 (B) and FIS1 (C) expressions demonstrated by bar graph. Expressions were normalized to β-tubulin. mRNA expressions of mitochondrial fusion markers *Mfn1* (D), *Mfn1* (E), *Opa1* (F) after inhibitor administration. Fold changes were normalized to *β-actin* and calculated relative to 5.5 mM glucose control. Data are presented as mean ± SEM. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ vs. 5.5 mM glucose control, $^{\dagger\dagger\dagger}P < 0.001$, $^{\dagger\dagger}P < 0.01$, $^{\dagger}P < 0.05$ vs. 25 mM glucose control. FIS1, mitochondrial fission 1; DRP1, dynamin-related protein 1, *Mfn1,* mitofusin1; *Mfn1,* mitofusin2; *Opa1,* optic atrophy 1.

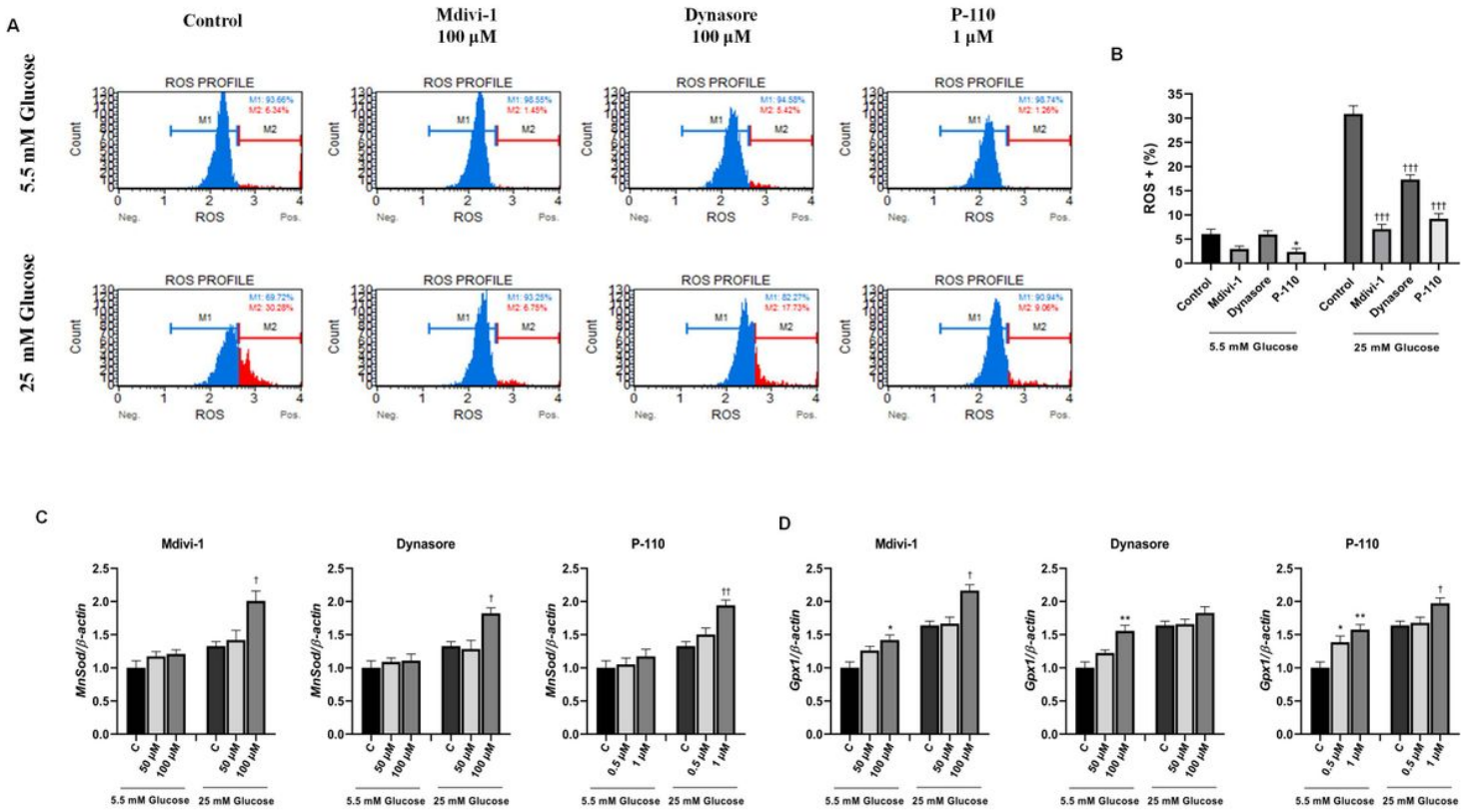

Figure 3

Reactive oxygen species (ROS) profiles and mitochondrial antioxidant enzyme mRNA expressions in trophoblast cells. A) Quantitative measurement of oxidative stress profiles of cells treated with fission inhibitors (mdivi-1, dynasore and p-110) in normal (5.5 mM) and high glucose media (25 mM) was evaluated by cytometry using Muse Oxidative Stress Kit. Control cells were treated with DMSO. B) The bar graph represents the ratio of ROS positive cells. mRNA expressions of *MnSod* (C) and *Gpx1* (D) after inhibitor administration. Data are presented as mean ± SEM of three independent experiments. Fold changes were normalized to *β-actin* and calculated relative to 5.5 mM glucose control. $^{**}P < 0.01$, $^{*}P < 0.05$ vs. 5.5 mM glucose control, $^{\dagger\dagger\dagger}P < 0.001$, $^{\dagger\dagger}P < 0.01$, $^{\dagger}P < 0.05$ vs. 25 mM glucose control. *MnSod,* manganese superoxide dismutase; *Gpx1,* glutathione peroxidase 1.

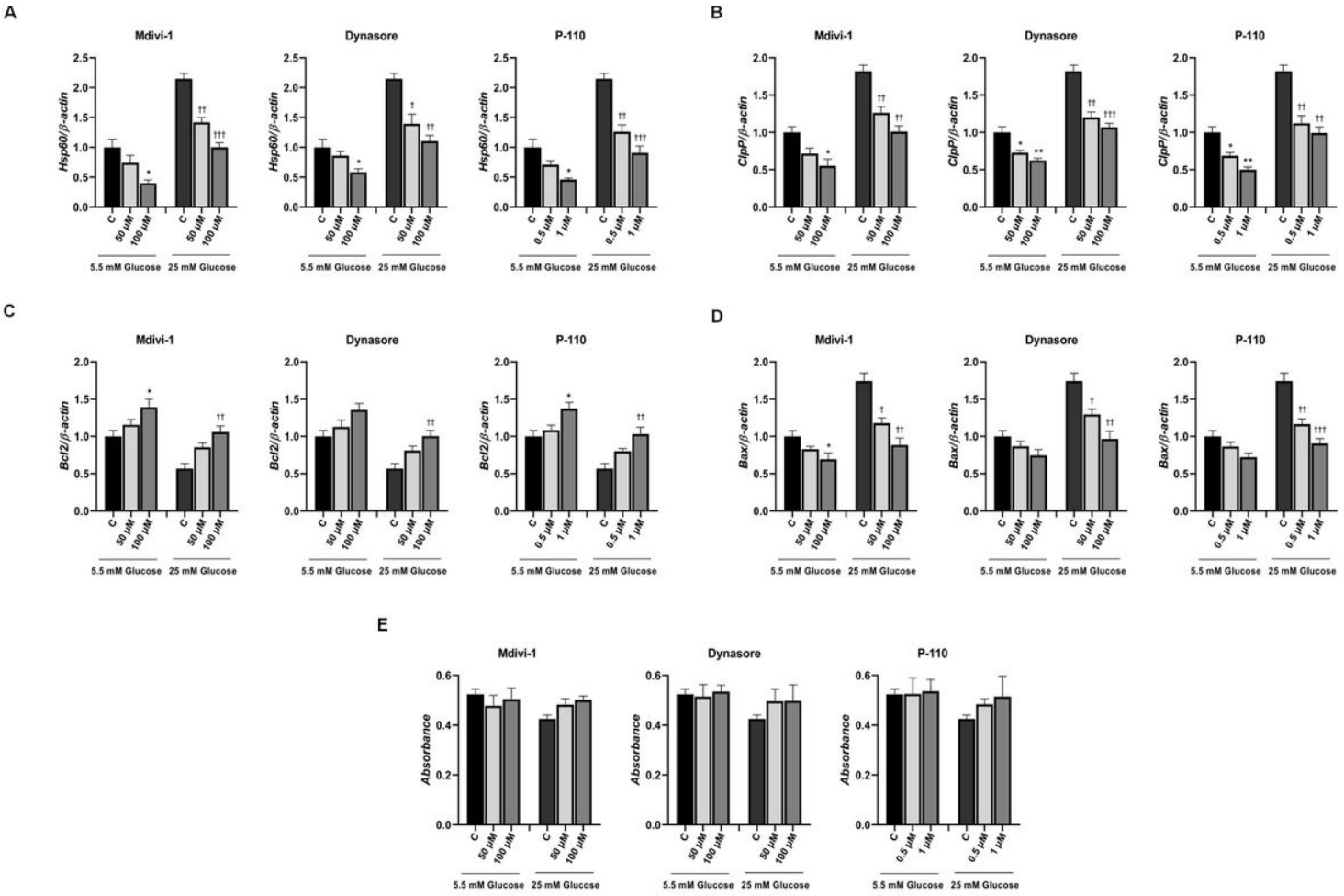

Figure 4

mRNA expressions of mtUPR and apoptotic markers and cell viability analyses in trophoblast cells. mRNA expressions of *Hsp60* (A), *ClpP* (B), *Bcl2* (C) and *Bax* (D) after inhibitor (mdivi-1, dynasore and p-110) administration to cells in normal (5.5 mM) and high glucose media (25 mM). Control cells were treated with DMSO. Fold changes were normalized to *β-actin* and calculated relative to 5.5 mM glucose control. E) Analysis of MTT cell viability after inhibitor application to cells in normal and high glucose medium. Data are presented as mean ± SEM of three independent experiments. $^{**}P < 0.01$, $^{*}P < 0.05$ vs. 5.5 mM glucose control, $^{\dagger\dagger\dagger}P < 0.001$, $^{\dagger\dagger}P < 0.01$, $^{\dagger}P < 0.05$ vs. 25 mM glucose control. *Hsp60,* heat shock protein 60; *ClpP,* caseinolytic mitochondrial matrix peptidase proteolytic subunit; *Bcl2,* B-cell lymphoma-2; *Bax*, Bcl-2-associated X protein.

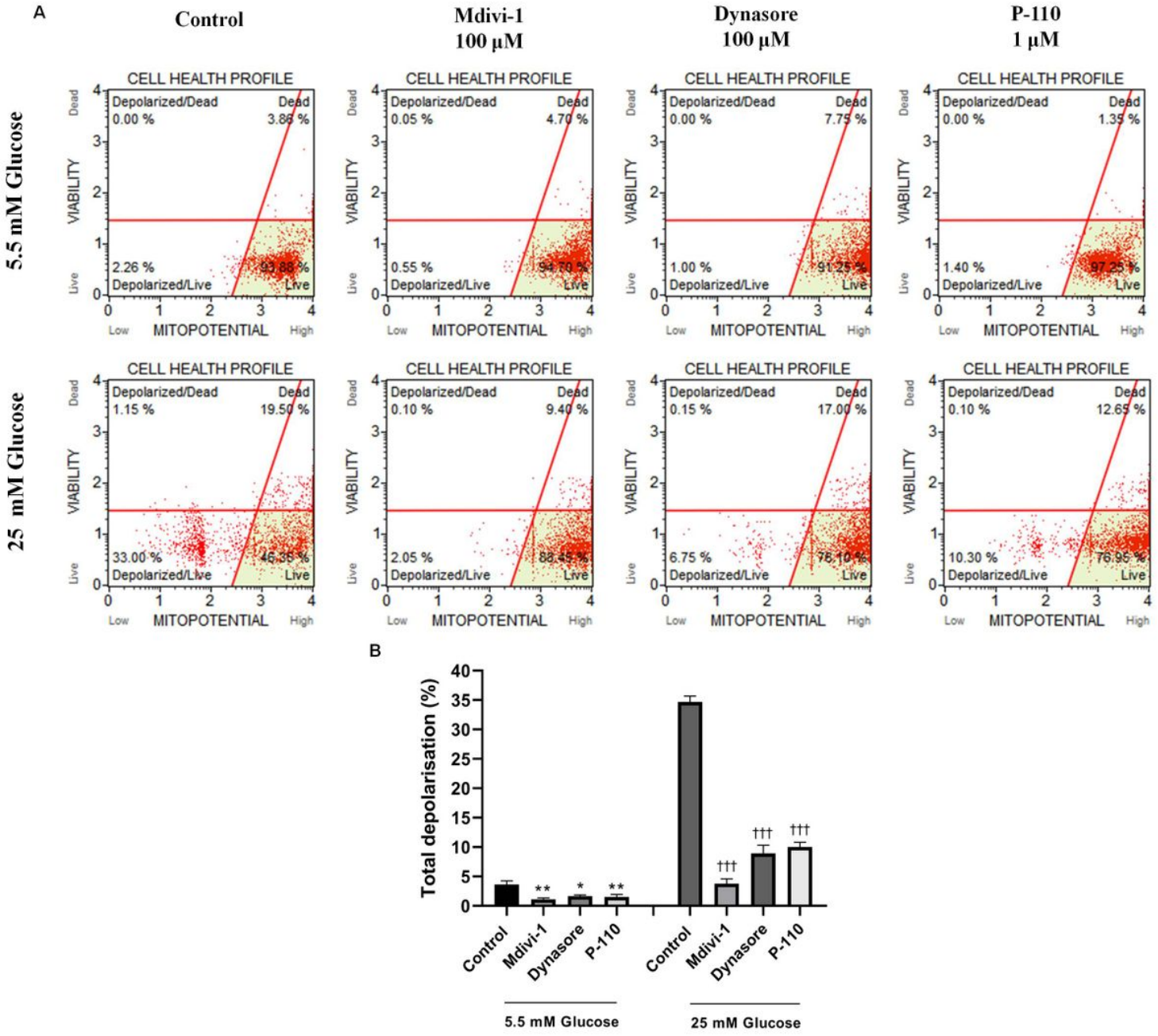

Figure 5

Mitochondrial potential analysis in trophoblast cells treated with fission inhibitors. A) Quantitative measurement of polarized/depolarized cells after inhibitor administration (mdivi-1, dynasore and p-110) in (5.5 mM) and high glucose media (25 mM). Control cells were treated with DMSO. B) The bar graph shows the ratio of total depolarized cells. Data are presented as mean ± SEM of three independent experiments. $^{**}P < 0.01$, $^{*}P < 0.05$ vs. 5.5 mM glucose control, $^{\dagger\dagger\dagger}P < 0.001$ vs. 25 mM glucose control.

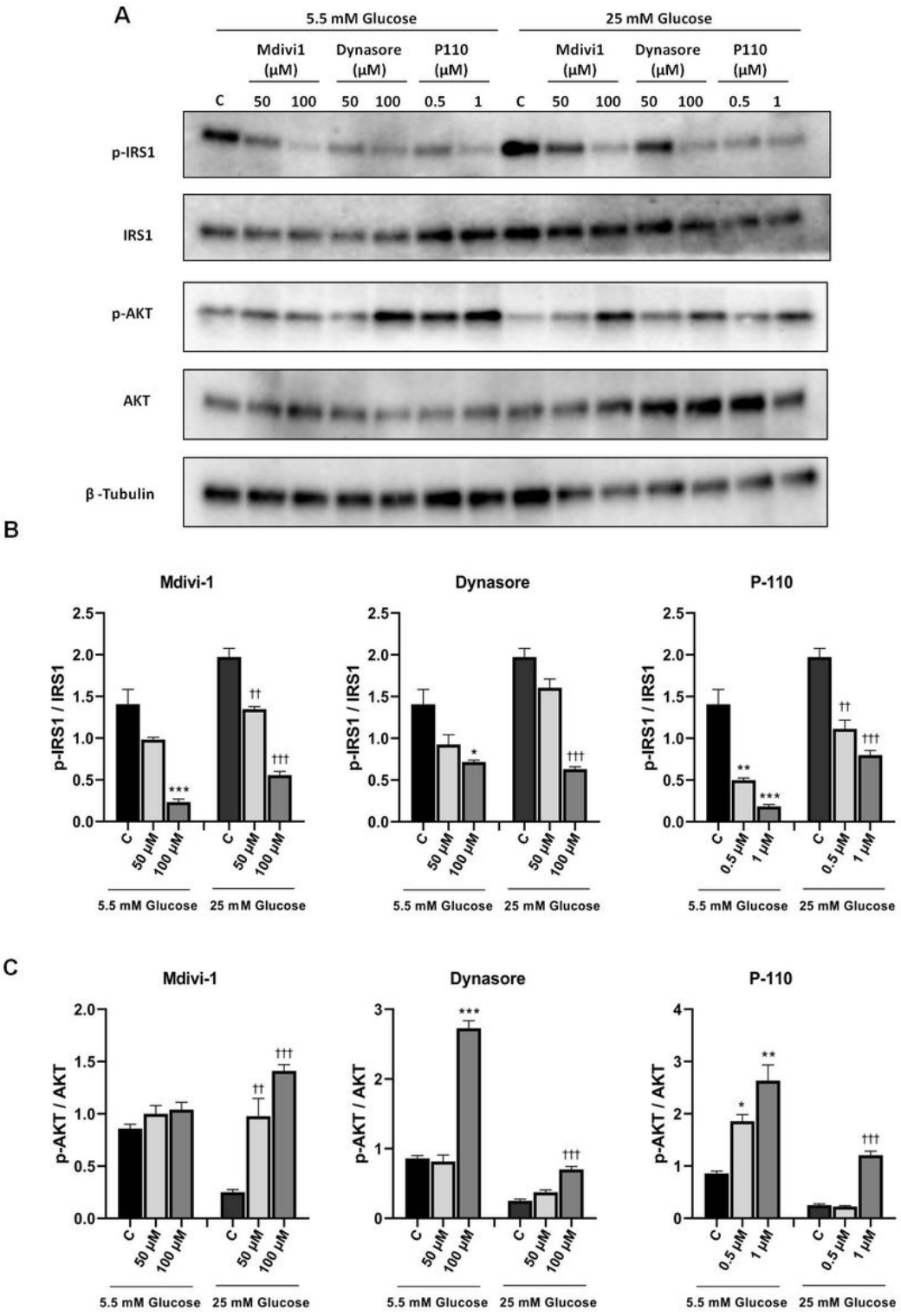

Figure 6

Insulin signaling in fission inhibitor-treated trophoblast cells. A) Representative western blot image of two different concentrations of three inhibitors (mdivi-1, dynasore and p-110) applied to trophoblast cells in normal (5.5 mM) and high glucose (25 mM) medium. Control cells were treated with DMSO. After inhibitor applications, cells were stimulated with 100 nM insulin for 20 minutes. Quantification of p-IRS1/IRS1 (B) and p-AKT/AKT (C) expressions demonstrated by bar graph. Expressions were normalized

to β-tubulin. Data are presented as mean ± SEM. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ vs. 5.5 mM glucose control and $^{\dagger\dagger\dagger}P < 0.001$, $^{\dagger\dagger}P < 0.01$ vs. 25 mM glucose control. AKT, protein kinase B; p-AKT, phosphorylated protein kinase B (ser473); IRS1, insulin receptor substrate 1 (IRS1); p-IRS1 phosphorylated insulin receptor substrate 1 (ser323).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementFigure1.jpg