Title: Nampt/PBEF/visfatin protects PC12 against high glucose-induced neurotoxicity in an in vitro model of diabetic neuropathy via inhibiting oxidative stress, autophagy and apoptosis

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Abstract

Diabetic neuropathy is a well-known complication of diabetes. It has been recently confirmed that hyperglycemia-induced toxicity participates in multiple cellular pathways that are typical for neural deterioration. Nampt/PBEF/visfatin is a novel endogenous ligand, which some studies have shown its neuroprotective effects on neurodegenerative disease. Therefore, we hypothesized that visfatin might prevent high glucose (HG)-induced neurotoxicity via the inhibition of apoptosis, autophagy, and reactive oxygen species (ROS) responses properly. In this study, Pheochromocytoma Cell Line 12 (PC12) cells were exposed to both HG concentrations (50, 75, 100, 125, 150 mM) and visfatin (50, 100, 150 ng/ml) in different time-points to determine the optimum time and dose of glucose and visfatin. To investigate the effects of visfatin on HG-induced damage in PC12 diabetic neuropathy model, we examined ROS response, apoptosis, and autophagy by using ROS detection kit, flow cytometry, and Real-time PCR/western blot, respectively. We determined that HG concentration significantly increased ROS level and apoptosis of diabetic PC12 cells. However, visfatin treatment significantly decreased ROS production ($P < 0.05$) and apoptosis of diabetic PC12 cells ($P < 0.0001$). *Beclin-1* mRNA level ($P < 0.05$) and *Lc3-II* protein level ($P < 0.05$) showed that autophagy pathway is impaired by HG concentrations. We concluded that visfatin could sufficiently decrease neural damage caused by ROS production and apoptosis under HG-induced toxicity.

Keywords: Visfatin, Diabetic Neuropathy, Antioxidant, Apoptosis, Autophagy

Introduction

Diabetes mellitus (DM) is a metabolic disorder that is featured by hyperglycemia. The hyperglycemia results from a failure in insulin secretion, insulin action, or both and is accompanied by carbohydrates, lipids, and proteins metabolism impairment (American Diabetes, 2010). It is increasingly recognized as a serious worldwide public health concern. Various studies have investigated several factors that contributed to diabetes, including population growth, aging, and lifestyle (Li et al., 2015). One of the most significant discussions of diabetes is how it causes failure in the vital organs of human body. Peripheral neuropathy is the most common and life-threatening complication that has recently received considerable attention. Recent examinations have shown that there are some dominant mechanisms at molecular levels that explain how hyperglycemia causes disruptions in pathways and leads to neuropathy. These pathways include activation of polyol signaling, reactive oxygen species (ROS) formation, apoptosis, advanced glycation end product (AGE), and aggregation (Zeng et al., 2017). Whereas each of these pathways can be detrimental to nerves, they can change mitochondrial redox state and cause the excess production of ROS (Feldman et al., 2017).

Recently, the Polyol pathway has been thorough investigation. Studies have shown that when intracellular glucose concentration rises, the polyol pathway of glucose metabolism is activated. Moreover, aldose reductase converts excess glucose into sorbitol and then destroys normal neuron physiology by osmotic shock (Lorenzi and Oates, 2008). In addition, excess glucose causes an elevation in mitochondrial electron transport chain and leads to the production of harmful ROS (Callaghan et al., 2012). Once the ROS level rises, it induces various cytokines production, inflammatory signs, and apoptotic pathways (Kumar, 2017). Besides, incremented glycolysis dysregulates many enzymatic cascades and promotes neural damages. These demolished pathways lead to a series of events such as inflammation, insulin resistance, and VEGF expression, all of which can cause neural damage by inducing hypoxia (Feldman et al., 2017). another side effect of

hyperglycemia is the formation of AGE. This product is produced by the addition of reactive carbohydrates groups to substrates. AGEs attach to their receptors for advanced glycation endproducts (RAGE), so they can trigger inflammatory cascades and activate NADPH oxidases that produce oxidative stress (Duran-Jimenez et al., 2009, Vincent et al., 2007). Besides all of these mechanisms, research shows that autophagy is a major event that plays an essential role in diabetic neuropathy. Autophagy has a critical role in eliminating components of the cells that have become destroyed or inefficient via facing cellular stress. Since different kinds of stress will happen under the diabetic condition, autophagy is considered as one of the primary mechanisms of diabetic neuropathy (Gonzalez et al., 2011, Kume et al., 2012).

So far, several efforts have been conducted to determine a proper treatment for diabetic neuropathy, and researchers indicated that natural adipokines might have positive effects on decreasing neuropathic symptoms. Adipokines are secreted from adipose tissue and have an enzymatic role in metabolism pathways. One of these adipokines is visfatin, which is a highly evolutionary protected 52-kD protein. Fukuhara et al. (2005) introduced visfatin as a new adipokine. At first, it was recognized as nicotinamide phosphoribosyltransferase (Nampt) synthetase and then rediscovered as a pre-B cell colony-enhancing factor (PBEF) that induce the formation of pre-B cell colony (Skop et al., 2009).

Further investigations revealed that visfatin (Nampt/PBEF) has insulin-mimetic effects. It is predominantly produced by visceral adipose tissue and has been linked to various cellular processes such as biosynthesis of nicotinamide dinucleotide, inhibition of neutrophil apoptosis, lowering blood glucose, and improvement of insulin sensitivity by its insulin-mimetic effects. Besides, researchers have shown that it has an impact on the immune and vascular system (Erfani et al., 2015, Skop et al., 2009). The neuroprotective effect of visfatin was reported by some researchers. For example, Yan Zhao et al. demonstrated that visfatin has neuroprotective effects against cerebral ischemia (Zhao et al., 2014). Furthermore, it has been reported that visfatin can

utilize its antiapoptotic effects (Jia et al., 2004) via activating intracellular kinases such as PI3K-Act and MAPK (Fukuhara et al., 2005).

In addition, it has been discovered that visfatin can upregulate antioxidant enzymes and decrease the higher amount of the ROS and lipid peroxidation process (Bułdak et al., 2012b). However, none of these studies has elucidated visfatin effects on diabetic neuropathy. Therefore, we examined the significance of the neuroprotective effects of visfatin on glucose-induced Pheochromocytoma Cell Line 12 (PC12) cells as a diabetic neuropathy model.

Materials and Methods

Materials and Reagents

PC12 cells were purchased from Buali (Avicenna) Research Institute. Dulbecco's Modified Eagle's Medium (DMEM) and Penicillin-Streptomycin were obtained from PAN-Biotech (GmbH, Aidenbach, Germany). D-(+) glucose and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT), and fluorometric intracellular ROS kit were obtained from Sigma Chemical Crop (St Louis, MO, USA). Fetal Bovine Serum (FBS) and 0.25% Trypsin-EDTA (1x) were purchased from Gibco (Carlsbad, CA, USA). Visfatin was purchased from Biolegend (San Diego, California, United States). DMSO and ECL (western blotting luminol reagent), MAP LC3 β antibody (G-9), m-IgG κ BP-HRP, and β -actin antibody were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Annexin V-FITC Apoptosis Detection kit was obtained from Invitrogen (Carlsbad, CA, USA). The PrimeScript RT-PCR Kit and BCA assay kit were purchased from Takara Biotechnology (Shiga, Japan). RealQ plus 2x Master Mix Green was obtained from Ampliqon (5230 Odense M, Denmark). PARIS™ Kit was purchased from Life Technologies, Thermo Fisher Scientific Inc. (Waltham, MA, USA).

Cell culture

PC12 cells were cultured at high glucose (HG) Dulbecco's Modified Eagle's Medium (DMEM) (25mM glucose/ml) with 10% (v/v) heat-inactivated Fetal Bovine Serum (FBS), and 1%(v/v)

penicillin-streptomycin (100 U/ml penicillin and 100 mg/ml streptomycin) at 37°C (BINDER, Tuttlingen, Germany) and 90% humidified atmosphere containing 5% CO₂. In addition, the cells were sub-cultured every 48-72 hours.

Cell viability assay

Cell viability was assessed using MTT assay. In brief, PC12 cells were plated at a density of 5000 cells/well in a 96-well flat-bottom microplate. After 24 hours, to determine HG concentration and time needed to induce diabetic neuropathy, cells were exposed to 4.5 mg/ml HG media (25mM) as the control group and 50, 75, 100, 125, 150mM as HG concentration in presence and absence of visfatin (50 ng/ml, 100 ng/ml, 150 ng/ml), for 24, 48, 72 hours. Afterward, 10 µl MTT solution (5mg MTT powder which liquefied in 1ml PBS) was added to each well, and then the cells were incubated at 37°C for 3 hours after that medium was removed. Formazan crystals were dissolved by adding 100 µl DMSO to each well. After 20 minutes incubation at 37°C, optical density was read at 570 nm and 690 nm (in order to background subtraction) using Biotek Cytation 5 (Biotek Instruments, Winooski, VT, USA). The experiment was repeated for three independent times.

Evaluation of ROS production

In order to evaluate ROS formation under HG condition using Fluorometric Intracellular ROS kit (Sigma-Aldrich; #MAK143-KT), PC12 cells were seeded in the wells of a black microplate (clear bottom) at an initial density of 10⁴ cells and incubated for 24h. The culture media was then changed with the proper dosage of visfatin and master reaction mix. Afterwards, the fluorescence intensity was evaluated at $\lambda_{ex} = 490/\lambda_{em} = 525$ nm using Biotek Cytation 5.

Apoptosis assay

The apoptosis level was measured by Flow Cytometry using annexin V-FITC assay kit. Briefly, 5×10⁵ cells/well were seeded at a 6-well culture plate and incubated at 37°C for 24h. The cells were treated with glucose in the presence and absence of visfatin (100 ng/ml). The cells were then incubated for 48h. Next, they were trypsinized, collected, and washed in cold PBS twice. The

process continued with adding Annexin V-FITC and propidium iodide (PI) according to the Annexin V apoptosis detection kit instructions. The samples were evaluated by FACS analysis with Attune NxT acoustic focusing cytometer (life technologies).

RNA extraction and cDNA synthesis

Total RNA was extracted from cells by RNA-Protein extraction PARIS kit according to the manufacturer's protocol (Life Technologies, Thermo Fisher Scientific Inc., Waltham, MA, USA). In order to evaluate the quantity and quality of the mRNA samples, their optical density was determined using the Picodrop microliter spectrophotometer (OEM, UK). Then, the samples were loaded on 0.8% agarose gel, and electrophoresis was performed. Subsequently, cDNA was synthesized from 1 µg of the total RNA using PrimeScript™ RT reagent kit (Takara Bio, Shiga, Japan). Briefly, cDNA was synthesized by incubating 5 µl of RNA samples of each group with 4 µl standard synthesis buffer, 1µl reverse transcriptase, 1µl oligo (dT) primers, and 1µl random primers in a total volume 25 µl for 15 minutes at 37°C, 5 seconds at 85°C, and 2 minutes at 4°C.

Real-time PCR

Quantitative real-time PCR was carried out using the Bio-Rad CFX96™ Real-Time System (Bio-Rad, Foster City, CA). PCR reaction was performed using RealQ Plus 2x Master Mix Green Ampliqon. Briefly, 12.5 µl SYBER Master Mix without ROX, 10.5 µl ddH₂O, 0.5µl of each primer, and 1 µl template (in total volume 25 µl) were mixed. The amplification profile for *Beclin-1* and *GAPDH* was as follows: hot start initiation at 95°C for 15 minutes and 40 cycles of 5 seconds at 95°C and 30 seconds at 60°C. A melting curve was performed at the end from 65°C to 95°C (0.5°C/second). All tests were performed in duplicates. *GAPDH* was used as a housekeeping control gene. The method for calculating the relative gene expression and the fold changes were $2^{-\Delta\Delta C_t}$. The primer sequences were as follows: *Beclin-1* forward: 5'-ATC CTG GAC CGA GTG ACC ATTC-3'. *Beclin-1* reverse: 5'-GGA GGA AGA GGC TAA CTC AGG AGA-3'. *GAPDH*

forward: 5'-GGC TGC CTT CTC TTG TGA CAA-3', *GAPDH* reverse: 5'-TGC CGT GGG TAG AGT CAT ACT G-3'.

Western blot

Western blotting was performed using a standard protocol. Briefly, after treating the cells, PC12 cells were collected, and total protein was extracted using RNA-Protein extraction PARIS kit. Protein quantity of the cell lysate was evaluated by the Bicinchoninic Acid test (BCA, Takara Bio, Inc., Tokyo, Japan). Then, the samples were mixed with loading buffer at 3:1 ratio and boiled for 10 minutes. Twenty µg/ml of each sample was loaded on 15% SDS-polyacrylamide gel. Afterwards, the proteins were transferred onto the PVDF membrane via wet transfer. In the blocking step, the membrane was placed at 3% BSA (in TBST) for 1 hour at room temperature. Then, the membrane was placed in anti-LC3B and anti-beta-actin antibody solutions (1: 1000 dilution, Santa Cruz, Dallas, TX, USA) and incubated overnight at 4°C on a shaker. After washing the membrane with TBST, it was incubated with HRP-conjugated secondary antibody (1: 1000 dilution, peroxidase-labeled Mouse IgGk) at room temperature for 2 hours, and washed in TBST. Finally, *LC3I/II* and β-Actin bands were visualized using ECL Western blot kit via chemiluminescence reaction. β-Actin was used as a loading control.

Statistical Analysis

Graph Pad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA) was used for statistical analyses. Data were demonstrated as means ± standard error of the mean (mean ± SEM). One-way ANOVA and Tukey's post hoc test were used to analyze the results. A $p < 0.05$ was considered as statistically significant. All of the experiments were performed three times.

Results

The effects of different concentrations of glucose on PC12 cells viability

In this study, to induce hyperglycemic condition, the glucose level of the medium increased gradually to six times of the standard (4.5 mg/ml or 25mM) concentration (50, 75, 100, 125, 150

mM). PC12 cells were exposed to increased levels of glucose for 24, 48, 72 hours, and cell viability was assessed by MTT assay. The analysis determined that cell viability decreased in a dose-dependent manner. Previous studies have shown that 150mM glucose concentration could cause osmotic shock and decrease cell viability (Wang et al., 2017). In order to avoid such effects, 150mM was not chosen. Based on the results shown in figure 1.A, treatment of the cells with 125mM glucose for 48 hours significantly decreased cell viability to 70% (with $P < 0.001$). Therefore, it was selected for further analysis.

The effects of visfatin on hyperglycemia-induced cell toxicity

Different concentrations of visfatin (50, 100, 150 ng/ml) were applied on cultured PC12 cells under the co-treatment with HG to evaluate the neuroprotective effects of visfatin using MTT assay. After 48 hours, cell viability of 125mM-glucose group was significantly decreased compared with the control group, while all doses of visfatin significantly increased cell viability with ($p < 0.0001$) (Fig. 1B). The maximum dose of visfatin (150 ng/ml) did not have any toxic effect on cell viability (Fig. 1B). Since the effects of 100 and 150 ng/ml visfatin on cell viability of HG-induced PC12 neurotoxicity cells were approximately equal, 100 ng/ml was chosen for further studies.

The effect of visfatin on intracellular ROS level in HG-induced PC12 cells

Given that oxidative stress has the primary role in neural injury caused by hyperglycemia, we analyzed the effect of visfatin on ROS formation under high-glucose condition using Fluorometric Intracellular ROS Kit. The data showed that (Fig. 2A) exposure to 125mM glucose dramatically increased the level of intracellular ROS ($p < 0.001$) while co-treatment with 100 ng/ml visfatin in comparison to HG-induced PC12 reduced intracellular ROS production ($p < 0.05$).

Visfatin showed protective effects against HG-induced apoptosis of PC12 cells

In order to find out whether visfatin has inhibition effects on HG-induced apoptosis on PC12 cells, cell death was evaluated by flow cytometry using double staining with annexin-V-FITC and PI. As figure 2B shows, the cells that were exposed to 125mM glucose concentration showed a

significant increase of apoptosis rate compared with the untreated group. Moreover, the cells that were treated with visfatin (100 ng/ml) alone did not show significant apoptosis in comparison to other groups. However, when the cells were co-treated with visfatin and HG medium, visfatin suppressed the toxicity of the hyperglycemic condition (figure 2B).

Hyperglycemia decreased autophagy via reducing Beclin-1 gene expression and Lc3-II protein levels

In order to evaluate the autophagy level in the hyperglycemic PC12 cells, the gene expression level of *Beclin-1* was measured using Real-Time PCR. The results showed that *Beclin-1* mRNA expression in glucose, visfatin, and the visfatin-glucose co-treated groups was lower than that of the control group. In addition, the results showed that the mRNA expression level of *Beclin-1* significantly decreased in the HG group in comparison to the control group ($p < 0.05$) (Fig. 3A). For further investigation, we carried out western blot analysis and evaluated the Lc3-II protein level. In the same way, western blot results showed that glucose treatment significantly decreased Lc3-II protein levels ($p < 0.05$) in HG-induced PC12 cells compared with the control group (Fig. 3B). However, Lc3-II protein expression of the control group and the visfatin group was not significantly different. Besides, the data showed that *Lc3-II* levels under HG-visfatin condition were not significantly different compared with the HG group.

Discussion

The results of this study showed that the HG condition induces a significant elevation of ROS and apoptosis in PC12 cells, while the autophagy process does not initiate under this condition. Based on current results, applying 100 ng/ml of visfatin on HG-induced PC12 cells dramatically decreases ROS and apoptosis.

Hyperglycemia is considered as a significant pathogenic factor in diabetic neuropathy development. The increasingly large number of research have shown that the activation of oxidative stress is essential to the development of metabolic syndrome, obesity, diabetic

neuropathy, and several neurodegenerative disorders such as Parkinson's and Alzheimer's diseases (Feldman et al., 2019, Aljanabi et al., 2020).

Hyperglycemia induces neurotoxicity via some well-known molecular pathways, and the most critical one is ROS generation and oxidative stress (Tomlinson and Gardiner, 2008, Wang et al., 2021). Excessive amounts of glucose in neurons lead to more glycolysis, which is followed by excessive mitochondrial electron transportation. Increased production of ROS and protons in mitochondrial electron chain cause uncoupling mitochondrial proton gradient. Generally, all of these events are followed by mitochondrial complex dysfunction, apoptosis, and disruption in neural function (Callaghan et al., 2012, Feldman et al., 2017). Cell death via oxidative stress is the most critical mechanism in diabetic neuropathy (Kaeidi et al., 2013, Volpe et al., 2018a). In addition, elevated ROS formation and reactive nitrogen elements are reported to cause neural elimination by oxidizing proteins, disruption of DNA integrity, and increasing the amount of lipid peroxidation products in cell membranes (Edwards et al., 2008, Rolo and Palmeira, 2006). Numerous studies have suggested that the release of ROS under acute hyperglycemia condition both *in vivo* and *in vitro* elevates the level of TNF α , IL-6, and other interleukins, which can trigger apoptosis (Esposito et al., 2002, Volpe et al., 2018b). Moreover, it has been shown that ROS and cytokine elevation cause caspase activation and lead to apoptosis (Taburee et al., 2011). Previous studies have cleared that visfatin can increase the activity of antioxidant enzymes in Me45 melanoma cells and suppress ROS activity (Buldak et al., 2012a).

Buldak et al. reported that Nampt/PBEF/visfatin increased the activity of identified antioxidant enzymes (e.g. CuZnSOD, catalase, superoxide dismutase isoenzymes, and glutathione peroxidase) in Me45 human malignant melanoma cells at a concentration of 10–100 ng/ml. Furthermore, they proposed that Nampt/PBEF/visfatin caused a redox adaptation response, resulting in the upregulation of antioxidant potential and reduced the levels of lipid peroxidation in Me45 melanoma cells (Buldak et al., 2012b).

Phosphoinositide 3-kinases (PI3Ks) and their subsequent target serine/threonine kinase Akt play a significant role in the pathway of cell death/survival. Several downstream targets of the survival and apoptotic pathways are regulated by activated AKT to impede apoptosis, including glycogen synthase kinase-3 (GSK-3 β). The dysregulation of the AKT signaling pathway has been shown to be implicated in hyperglycemic pathology (Kolluru et al., 2012, Liu et al., 2020). AKT activation tends to inhibit GSK-3 β by increasing GSK-3 β phosphorylation, which has been demonstrated to boost cell viability and metabolism (Brunet et al., 1999, Yamaguchi et al., 2001). Numerous studies have found that Nampt/PBEF/visfatin prevents apoptosis in many types of cells, including cardiomyocytes (Lim et al., 2008), lymphocytes (Revollo et al., 2007), endothelial cells (Borradaile and Pickering, 2009), and hepatocytes (Dahl et al., 2010). In a study conducted by Cheng et al., visfatin was revealed to inhibit cytochrome c and caspase-3 by ERK1/2 and phosphatidylinositol-3-OH kinase (PI3K), cellular Akt/protein kinase-B (Akt), increasing the Bcl-2/Bax. Visfatin also prevents islet cells from apoptosis caused by palmitate (Qun et al., 2011). Moreover, Wu et al. have reported that visfatin can block apoptosis by activating PI3K/Akt signaling pathway in acute lung injury (Wu et al., 2019).

The results of the current study confirm the previous studies' results (Wang et al., 2012, Russell et al., 2002). On the one hand our results proposed that HG conditions increase ROS and apoptosis. On the other hand, visfatin significantly decreased HG-induced apoptotic cells.

In the next step of the experiment, we investigated autophagy as a "self-eating" process that usually occurs to reduce damaged organelles as well as to maintain cellular homeostasis under starvation and other kinds of cellular stress (Yerra et al., 2016). Previous studies were controversial. Several studies have explored the levels of autophagy in diabetic neuropathy. They were not able to achieve reasonable conclusions about autophagy under diabetic neuropathy conditions (Yerra et al., 2016). For instance, R Town et al. found that when neuroblastoma cells were exposed to sera from type 2 diabetic neuropathic patients, an increased level of *Lc3-II* expression was observed. Therefore,

they have reported that autophagy was elevated under hyperglycemic conditions (Towns et al., 2005).

On the contrary, Ling Qu et al. have shown that *Beclin-1* and Lc3 proteins were downregulated under HG conditions in Schwann cells (Qu et al., 2014). In addition, various studies have reported that autophagy is activated under starvation and cellular stress (Scherz-Shouval et al., 2007, Ding, 2015). Regulation of autophagy under starvation is well-investigated, and mTOR, ULK1, AMPK proteins are the key regulators in this pathway. AMPK is activated when cellular energy is low and initiates autophagy via phosphorylation, the mTOR-ULK1 protein complex. On the contrary, under diabetic conditions, cells face lots of nutrients. Previous researches have clarified that this condition is the main reason that autophagy is impaired in diabetic nerves. Karen A. Weikel et al. have shown that facing HG condition diminishes the AMPK activity and harnesses the autophagy and switches the cellular signaling to apoptosis pathway (Ding, 2015).

In this study, to clarify whether HG condition and visfatin can exert changes in the autophagy status of diabetic PC12 cells, we evaluated *Beclin-1* RNA level and *LC3-I/II* protein level as valuable markers of autophagy. Real-time PCR results of *Beclin-1* showed that when cells were treated in HG media, *Beclin-1* RNA level significantly decreased in comparison with the control group. Consistent with the *Beclin-1* expression results, the examination of Lc3-II protein level by western blot showed that after treating the cells with HG condition, the expression of this protein dramatically decreased. Therefore, In accordance with the previous studies (Satoru Kobayashi 2012, Ling Qu 2014), our results revealed that hyperglycemia not only does not induce autophagy but also inhibits it.

Conclusion

To the best of our knowledge, our research investigated visfatin effects on the PC12 diabetic neuropathic model for the first time. We showed that hyperglycemia could dramatically affect

neural cell viability by influencing cellular pathways such as apoptosis. In addition, we demonstrated that the treatment of cells with visfatin (100 ng/ml) could dramatically reduce the toxic effects of the hyperglycemic condition. Furthermore, our results indicate that visfatin can be a new promising treatment for diabetic neuropathy. However, more investigations are needed on animal models to make these possibilities clear. Also, investigating multiple signaling pathways can be subjected to further researches to elucidate the mechanisms of function of visfatin.

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Author contributions

Asghar Shayannia, Mehdi Khaksari, and Sarvin Jahanbani designed the study. Sarvin Jahanbani and Fatemeh Sadat Bitaraf conducted the experiment. Majid Rahmati and Asghar Shayannia analyzed the data. Sarvin Jahanbani, Kobra Foroughi, Mehdi Khaksari, and Asghar Shayannia wrote the paper.

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

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Figures

Fig. 1 (A) The effects of different concentrations of glucose on PC12 cell viability. The cell viability of each group was normalized to the control group. The analysis determined that cell viability decreased in a dose-dependent manner. **(B)** The effects of visfatin on high-glucose damaged PC12 cells. The viability of damaged PC12 cells was significantly increased in a dose-dependent manner. Data are shown as mean $\pm$ SEM; * $P < 0.05$, ** $P < 0.01$ vs control, # $P < 0.001$, ## $P < 0.0001$ vs HG treatment.

Fig. 2 (A) The effect of visfatin on HG-induced intracellular ROS level in PC12 cells. The data showed that exposure to 125mM glucose increased the level of intracellular ROS while co-treatment with 100 ng/ml visfatin in comparison to HG-induced PC12 reduced intracellular ROS production. (B) The effects of visfatin on HG-induced apoptosis of PC12 cells. Cells that were exposed to 125mM glucose concentration showed a significant increase in apoptosis rate (10.9%) compared with the untreated group (1.59%). Moreover, the cells treated with visfatin (100 ng/ml) alone did not show significant apoptosis (1.43%) compared to other groups. However, when the cells were co-treated with visfatin and HG medium, visfatin suppressed the toxicity of the hyperglycemic condition (5.92%). (C) The apoptosis evaluation of PC12 cells under different treatments. Data are shown as mean $\pm$ SEM. $^* < 0.05$ vs. control, $^{\#} < 0.01$, $^{##} < 0.0001$ vs HG treatment.

Fig. 3 (A) The role of visfatin on Beclin-1 expression level. The results showed that Beclin-1 mRNA expression in glucose, visfatin, and the visfatin-glucose co-treated groups was lower than that of the control group. In addition, the results showed that the mRNA expression level of Beclin-1 significantly decreased in the HG group in comparison to the control group. The results are shown as mean $\pm$ SEM. * $P < 0.05$ vs control. **(B)** Investigating autophagy level under the hyperglycemic condition and visfatin treatment through Lc3-II protein levels. Results showed that high-glucose condition caused a significant reduction in autophagy level by decreasing Lc3-II protein expression. Protein bands were analyzed by Image J software. * $P < 0.05$ compared with control group.