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Research Paper

Effectiveness of Nicotinamide Phosphoribosyltransferase/ Pre-B Cell Colony-enhancing Factor/ Visfatin in preventing High Glucose-induced Neurotoxicity in an In-vitro Model of Diabetic Neuropathy

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ABSTRACT

Introduction: Diabetic neuropathy is a well-known complication of diabetes. Recently, hyperglycemia-induced toxicity has been confirmed to participates in multiple cellular pathways typical for neural deterioration. Nicotinamide phosphoribosyltransferase/pre-b cell colony-enhancing factor (Nampt/PBEF)/visfatin is a novel endogenous ligand that some studies have shown its neuroprotective effects on neurodegenerative disease. Therefore, we hypothesized that visfatin may prevent high glucose (HG)-induced neurotoxicity by inhibiting apoptosis, autophagy, and reactive oxygen species (ROS) responses properly.

Methods: 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) at 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 the PC12 diabetic neuropathy model, we examined ROS response, apoptosis, and autophagy using ROS detection kit, flow cytometry, and real-time PCR/Western blot, respectively.

Results: We determined that HG concentration significantly increased the ROS level and apoptosis of diabetic PC12 cells. However, visfatin treatment significantly decreased the ROS production ($P<0.05$) and apoptosis of diabetic PC12 cells ($P<0.0001$). Beclin-1 messenger ribonucleic acid (mRNA) level ($P<0.05$) and light chain 3 (Lc3)-II protein level ($P<0.05$) showed that the autophagy pathway is impaired by HG concentrations.

Conclusion: We concluded that visfatin can sufficiently decrease neural damage caused by ROS production and apoptosis under HG-induced toxicity.

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Highlights

• High glucose significantly increased the ROS level and apoptosis of diabetic PC12 cells;

• The autophagy pathway is impaired by high glucose;

• Nampt/PBEF/visfatin can significantly reduce neural damage caused by ROS production and apoptosis of diabetic PC12 cells.

Plain Language Summary

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia resulting from a failure in insulin secretion, insulin action, or both. Visfatin (Nampt/PBEF) has insulin-mimetic effects. So far, no study has assessed its effects on diabetic neuropathy. Therefore, we examined the neuroprotective effects of visfatin on cell line 12 (PC12) against glucose-induced neurotoxicity. Based on the results, it was concluded that the Nampt/PBEF/visfatin can significantly reduce neural damage caused by production of reactive oxygen species and apoptosis of diabetic PC12 cell.

1. Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycemia. Hyperglycemia results from a failure in insulin secretion, insulin action, or both and is accompanied by impaired metabolism of carbohydrates, lipids, and proteins (American Diabetes Association, 2010). It is increasingly recognized as a serious public health concern worldwide. Various studies have investigated several factors that contribute 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 the human body. Peripheral neuropathy is the most common and life-threatening complication that has recently received considerable attention. Recent examinations have shown that some dominant mechanisms at molecular levels 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 the mitochondrial redox state and cause the excess production of ROS (Feldman et al., 2017).

Recently, the polyol pathway has been thoroughly investigated. 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 & Oates, 2008). In addition, excess glucose increases the 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 damage. These demolished pathways lead to a series of events such as inflammation, insulin resistance, and vascular endothelial growth factor (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 carbohydrate groups to substrates. AGEs attach to their receptors for advanced glycation endproducts, therefore they can trigger inflammatory cascades and activate nicotinamide adenine dinucleotide phosphate (NADPH) oxidases that produce oxidative stress (Duran-Jimenez et al., 2009). 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 are destroyed or inefficient via facing cellular stress. Since different kinds of stress will happen under the diabetic condition, autophagy is considered one of the primary mechanisms of diabetic neuropathy (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 may 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, 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 induces the colony formation of pre-B cells (Skop et al., 2009).

Further investigations revealed that visfatin (Nampt/PBEF) has insulin-mimetic effects. It is predominantly produced by visceral adipose tissue and is associated with 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 affects the immune and vascular systems (Erfani et al., 2015; Skop et al., 2009). Some researchers reported the neuroprotective effect of visfatin. For example, 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 phosphoinositide 3-kinase (PI3K)-Act and mitogen-activated protein kinases (MAPK) (Fukuhara et al., 2005).

In addition, it has been discovered that visfatin can upregulate antioxidant enzymes and decrease the higher amount of ROS and lipid peroxidation process (Bułdak et al., 2012). 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.

2. Materials and Methods

Materials and reagents

PC12 cells were purchased from the Buali (Avicenna) Research Institute. Dulbecco's modified eagle's medium (DMEM) and penicillin-streptomycin were obtained from PAN-Biotech (GmbH, Aiedenbach, 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). Dimethyl sulfoxide (DMSO) and electrochemiluminescence (ECL) (Western blotting luminol reagent), microtubule-associated protein (MAP) light chain 3 (LC3) β antibody (G-9), mouse IgG kappa binding protein (m-IgGκ BP) conjugated to horseradish peroxidase (HRP), and β-actin antibody were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Annexin V conjugated with fluorescein isothiocyanate (V-FITC) apoptosis detection kit was obtained from Invitrogen (Carlsbad, CA, USA). The PrimeScript reverse transcription polymerase chain reaction (RT-PCR) Kit and bicinchoninic acid (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 in DMEM at high glucose (HG) (25 mM glucose/mL) with 10% (v/v) heat-inactivated 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% carbon dioxide (CO_2). In addition, the cells were sub-cultured every 48-72 hours.

Cell viability assay

Cell viability was assessed using an 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 (25 mM) as the control group and 50, 75, 100, 125, and 150 mM as HG concentration in the presence and absence of visfatin (50 ng/mL, 100 ng/mL, 150 ng/mL), for 24, 48, and 72 hours. Afterward, 10 µL MTT solution (5mg MTT powder which liquefied in 1 mL phosphate-buffered saline [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 of incubation at 37˚C, optical density was read at 570 nm and 690 nm (to background subtraction) using Biotek Cytation 5 (Biotek Instruments, Winooski, VT, USA). The experiment was repeated for three independent times.

Evaluation of ROS production

To evaluate ROS formation under HG conditions using a 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

10^4 cells and incubated for 24 h. The culture media was then changed with the proper dosage of visfatin and master reaction mixture. Afterward, the fluorescence intensity was evaluated at λ_{ex}=490/λ_{em}=525 nm using biotech cytation 5.

Apoptosis assay

The apoptosis level was measured by flow cytometry using an annexin V-FITC assay kit. Briefly, 5×10^5 cells/well were seeded at a 6-well culture plate and incubated at 37˚C for 24 h. The cells were treated with glucose in the presence and absence of visfatin (100 ng/mL). The cells were then incubated for 48 h. 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 fluorescence-activated cell sorting (FACS) analysis with attune NxT acoustic focusing cytometer (life technologies).

Ribonucleic acid (RNA) extraction and complementary DNA (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). To evaluate the quantity and quality of the messenger ribonucleic acid (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 the 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, 1 µL reverse transcriptase, 1 µL oligo (dT) primers, and 1 µL random primers in a total volume of 25 µL for 15 minutes at 37˚C, 5 s at 85˚C, and 2 minutes at 4˚C.

Real-time polymerase chain reaction (PCR)

Quantitative PCR was performed using the Bio-Rad CFX96™ real-time system (Bio-Rad, Foster City, CA). The PCR reaction was performed using RealQ Plus 2x Master Mix Green Ampliqon. Briefly, 12.5 µL SYBER Master Mix without ROX, 10.5 µL ddH_2O, 0.5 µL of each primer, and 1 µL template (volume 25 µL) were mixed. The amplification profile for Beclin-1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was as follows, hot start initiation at 95˚C for 15 minutes and 40 cycles of 5 s at 95˚C and 30 s at 60˚C. A melting curve was performed at the end from 65˚C to 95˚C (0.5˚C/s). 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 was $2^{-\Delta\Delta Ct}$. 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 an RNA-protein extraction PARIS kit. The protein quantity of the cell lysate was evaluated by the BCA test (BCA, Takara Bio, Inc., Tokyo, Japan). Then, the samples were mixed with loading buffer at a 3:1 ratio and boiled for 10 minutes. Twenty µg/mL of each sample was loaded on 15% SDS-polyacrylamide gel. Afterward, the proteins were transferred onto the polyvinylidene difluoride (PVDF) membrane via wet transfer. In the blocking step, the membrane was placed at 3% bovine serum albumin (BSA) (in tris buffered saline buffer with tween 20 [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 horseradish peroxidase (HRP)-conjugated secondary antibody (1: 1000 dilution, peroxidase-labeled mouse IgGκ) at room temperature for 2 hours, and washed in TBST. Finally, LC3I/II and β-Actin bands were visualized using an ECL Western blot kit via chemiluminescence reaction. β-Actin was used as a loading control.

Statistical analysis

GraphPad Prism software, version 6 was used for statistical analyses. Data were demonstrated as Mean±SEM. One-way analysis of variance (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.

3. Results

The effects of different concentrations of glucose on pheochromocytoma PC12 cells viability

In this study, to induce hyperglycemic conditions, the glucose level of the medium was gradually increased to six times the standard (4.5 mg/mL or 25 mM) concentration (50, 75, 100, 125, and 150 mM). PC12 cells were exposed to increased glucose levels for 24, 48, and 72 hours, and the cell viability was assessed by MTT assay. The analysis determined that cell viability decreased in a dose-dependent manner. Previous studies have shown that 150 mM glucose concentration can cause osmotic shock and decrease cell viability (Wang et al., 2017). To avoid such effects, 150 mM was not chosen. Based on the results shown in Figure 1A, treatment of the cells with 125 mM glucose for 48 hours significantly decreased cell viability to 70% ($P<0.001$). Therefore, it was selected for further analysis.

The effects of visfatin on hyperglycemia-induced cell toxicity

Different concentrations of visfatin (50, 100, and 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, the cell viability of the 125 mM-glucose group was significantly decreased compared to the control group, while all doses of visfatin significantly increased cell viability ($P<0.0001$) (Figure 1 B). The maximum dose of visfatin (150 ng/mL) had no toxic effect on cell viability (Figure 1 B). 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 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 conditions using a fluorometric intracellular ROS Kit. The data showed that (Figure 2 A) exposure to 125 mM glucose dramatically increased the level of intracellular ROS ($P<0.001$) while co-treatment with 100 ng/mL visfatin compared to HG-induced PC12 reduced intracellular ROS production ($P<0.05$).

Visfatin showing protective effects against HG-induced apoptosis of pheochromocytoma PC12 cells

To find out whether visfatin has inhibitory 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 2 B shows, the cells exposed to 125 mM glucose concentration showed a significant increase in apoptosis rate compared to the untreated group. Moreover, the cells treated with visfatin (100 ng/mL) alone did not show significant apoptosis compared to other groups. However, when the cells were co-treated with visfatin and HG medium, visfatin suppressed the toxicity of the hyperglycemic conditions (Figure 2 B).

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

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 the control group. In addition, the results showed that the mRNA expression level of *Beclin-1* significantly decreased in the HG group compared to the control group ($P<0.05$) (Figure 3A). For further investigation, we conducted 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 to the control group (Figure 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 the HG-visfatin condition were not significantly different compared to the HG group.

4. 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 a significant pathogenic factor in diabetic neuropathy development. The increasingly large number of research has shown that the activation of oxidative stress is essential to the development of

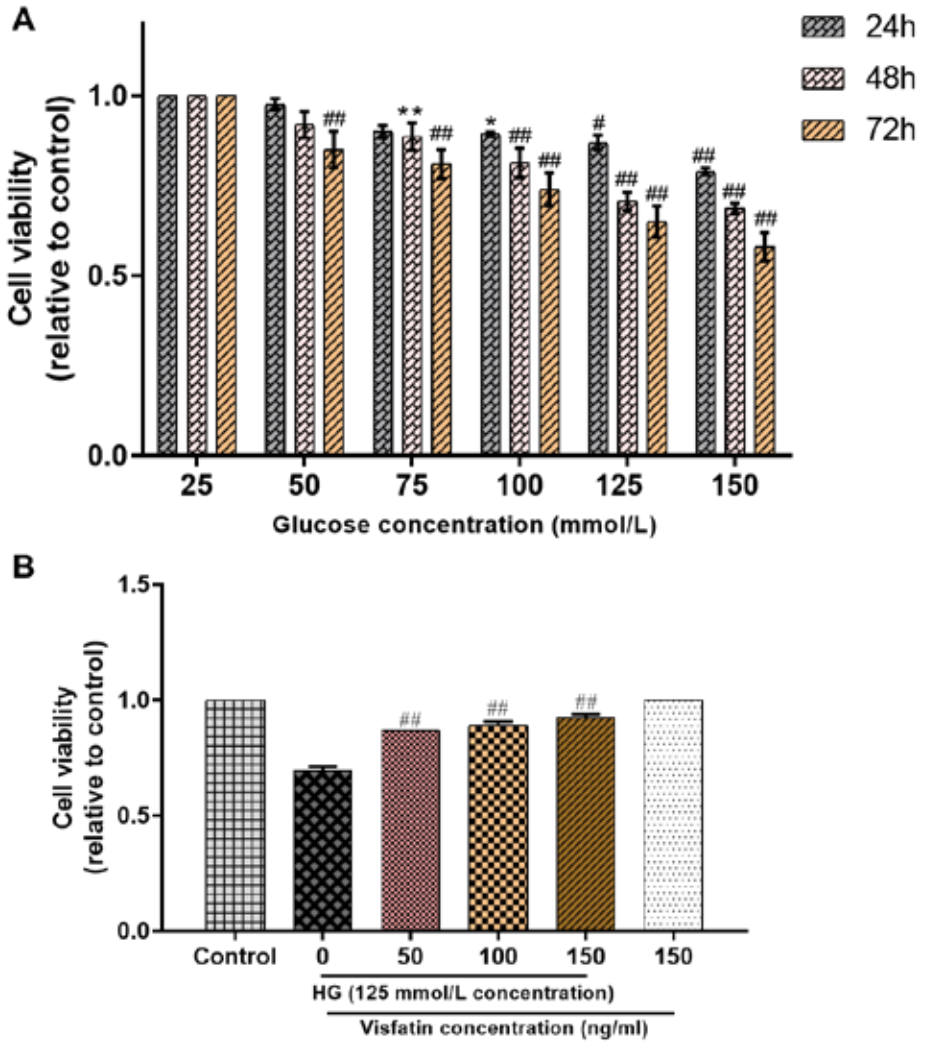

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Figure 1. A) The effects of different concentrations of glucose on pheochromocytoma PC12 cell viability, B) The effects of visfatin on high-glucose damaged PC12 cells

Data are shown as Mean±SEM; *P<0.05, **P<0.01 vs control, #P<0.001, ##P<0.0001 vs HG treatment.

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 & Gardiner, 2008; Wang et al., 2021). Excessive amounts of glucose in neurons lead to more glycolysis, followed by excessive mitochondrial electron transportation. Increased production of ROS and protons in the mitochondrial electron chain causes uncoupling mitochondrial proton gradient. All 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; et al., 2018). In addition, elevated ROS formation and reactive nitrogen elements cause neural elimination by oxidizing proteins, disrupting DNA integrity, and increasing the amount of lipid peroxidation products in cell membranes (Edwards et al.; 2008, Rolo & Palmeira, 2006). Numerous studies have suggested that the release of ROS under acute hyperglycemia conditions both in vivo and in vitro elevates the level of tumor necrosis factor-alpha (TNFα), interleukin-6 (IL-6), and other interleukins, which can trigger apoptosis (Esposito et al., 2002; Volpe et al., 2018). Moreover, increased ROS and cytokine elevation have been shown to activate caspase

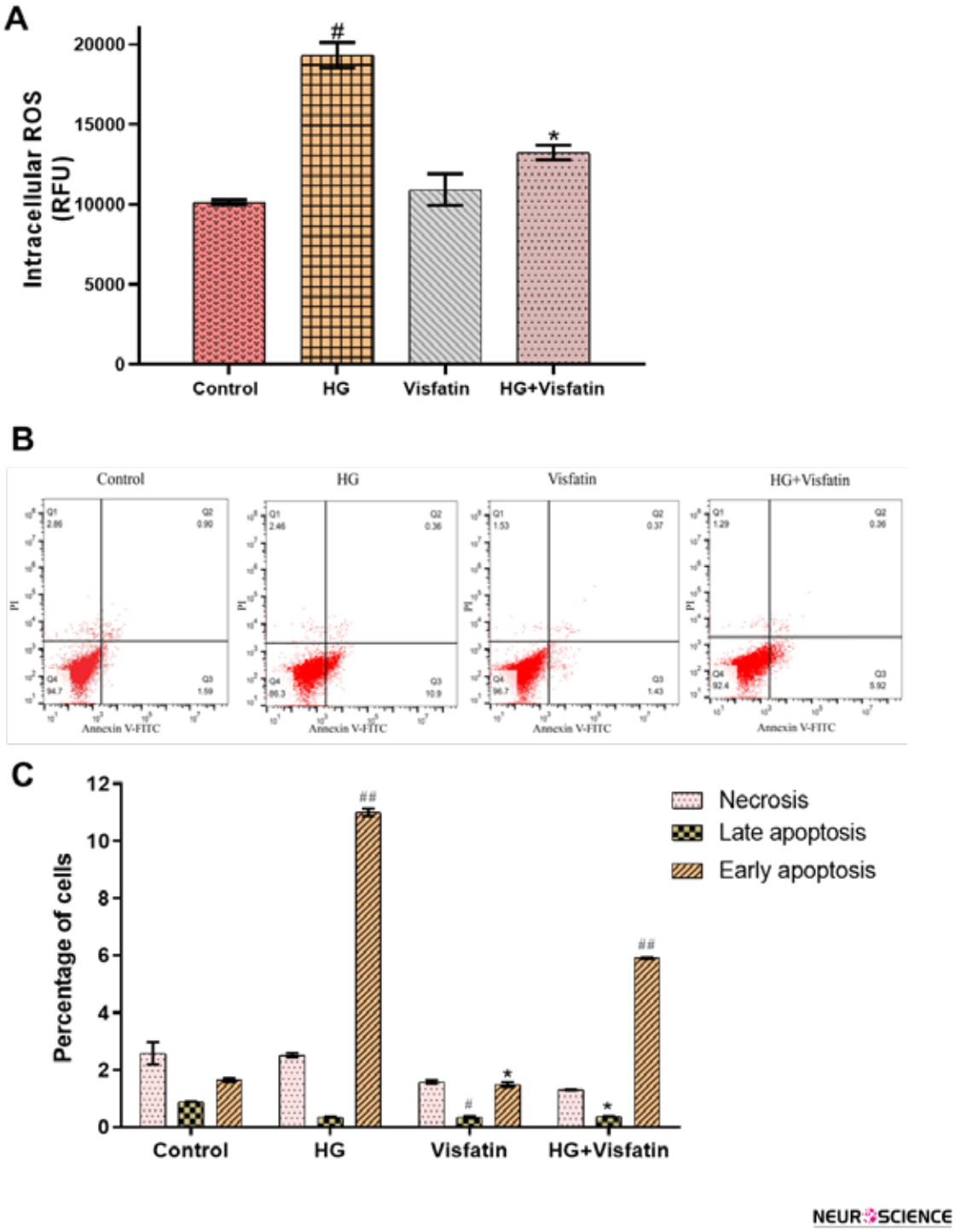

Figure 2. A) The effect of visfatin on HG-induced intracellular ROS level in PC12 cells, B) The effects of visfatin on HG-induced apoptosis of PC12 cell, C) The apoptosis evaluation of PC12 cells under different treatments

Note: Data are shown as Mean±SEM; *P<0.05 vs control, #P<0.01, ##P<0.0001vs HG treatment.

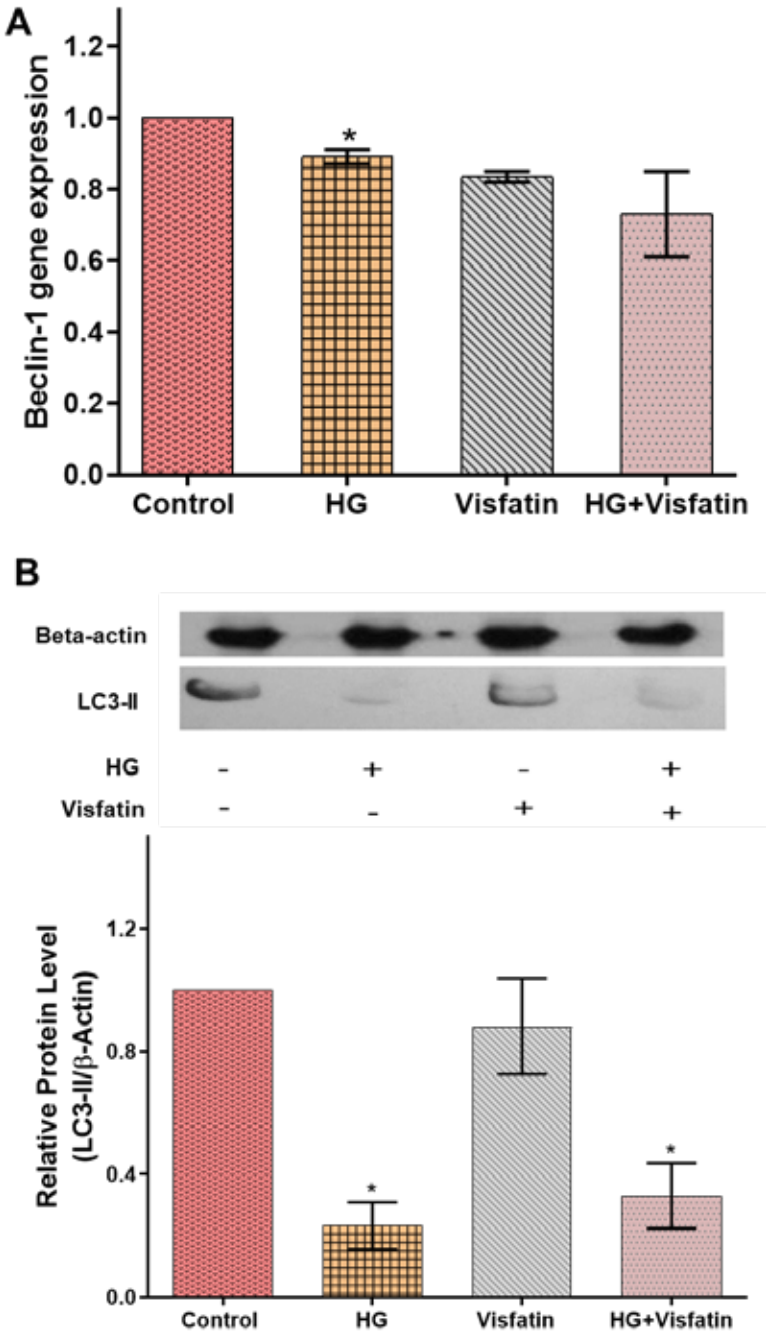

Figure 3. A) The role of visfatin on beclin-1 expression level

The results are shown as Mean±SEM. *P<0.05 vs control.

B) Investigating autophagy level under the hyperglycemic condition and visfatin treatment through Lc3-II protein levels

*P<0.05 compared to control group.

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 (Bułdak et al., 2012).

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 (Bułdak et al., 2012).

Phosphoinositide 3-kinases (PI3Ks) and their subsequent target serine/threonine kinase Akt plays 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-3β). The dysregulation of the AKT signaling pathway is implicated in hyperglycemic pathology (Kolluru et al., 2012; Liu et al., 2020). AKT activation tends to inhibit GSK-3β by increasing GSK-3β 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 & 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 extracellular signal-regulated kinase 1/2 (ERK1/2) and PI3K, cellular Akt/protein kinase-B (Akt), increasing the B-cell (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 the 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 autophagy levels in diabetic neuropathy. They failed to achieve reasonable conclusions about autophagy under diabetic neuropathy conditions (Yerra et al., 2016). For instance, 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, 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 mammalian targets of rapamycin (mTOR), ULK1, and AMP-activated protein kinase (AMPK) proteins are the key regulators in this pathway. AMPK is activated when cellular energy is low and initiates autophagy via phosphorylation, the mammalian target of rapamycin (mTOR)-ULK1 protein complex. On the contrary, under diabetic conditions, cells face lots of nutrients. Previous research has clarified that this condition is the main reason for impaired autophagy in diabetic nerves. Weikel et al. have shown that facing HG condition diminishes the AMPK activity, harnesses the autophagy, and switches the cellular signaling to the 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 the 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 compared to the control group. Consistent with the Beclin-1 expression results, the examination of the Lc3-II protein level by Western blot showed that after treating the cells with HG condition, the expression of this protein dramatically decreased. Therefore, consistent with the previous studies (Kobayashi et al., 2012; Qu et al., 2014), our results revealed that hyperglycemia not only does not induce autophagy but also inhibits it.

5. 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 can 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) can 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 in animal models are needed to clarify these possibili-

ties. Also, investigating multiple signaling pathways can be subjected to further research to elucidate the mechanisms of function of visfatin.

Ethical Considerations

Compliance with ethical guidelines

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

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Authors' contributions

Study design: Asghar Shayannia, Mehdi Khaksari, and Sarvin Jahanbani; Experiment: Sarvin Jahanbani and Fatemeh Sadat Bitaraf; Data analyze: Majid Rahmati and Asghar Shayannia; Writing: Sarvin Jahanbani, Kobra Foroughi, Mehdi Khaksari, and Asghar Shayannia.

Conflict of interest

The authors declared no conflict of interest.

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