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ORIGINAL ARTICLE

Leptin attenuates oxidative stress and neuronal apoptosis in hyperglycemic condition

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ABSTRACT

One of the main pathological mechanisms of neurotoxicity in diabetic situation is oxidative stress promoted by hyperglycemia. It has been shown that leptin has neuroprotective effects and may provide neuronal survival signals. This study was designed to reveal the possible neuroprotective effects of leptin in hyperglycemic conditions. Pheochromocytoma (PC12) cell viability was assessed via the MTT test. Cellular reactive oxygen species (ROS) generation was determined by DCFH-DA analysis. The malondialdehyde (MDA) and glutathione (GSH) levels were measured in high-glucose-treated PC12 cells with and without leptin cotreatment. Western blotting was performed to measure apoptosis markers (Cleaved caspase-3 and Bax/Bcl2 ratio). Elevation of glucose levels (100 mmol/L) consecutively increased intracellular ROS and MDA level, and apoptosis in PC12 cells after 24 h leptin administration (12 and 24 nmol/L) decreased the high-glucose-induced cell toxicity, caspase-3 activation, and Bax/Bcl-2 ratio. Also, cotreatment with leptin (12 and 24 nmol/L) significantly reduced oxidative damage to PC12 cells in high-glucose condition, as reflected by the diminution in MDA and ROS levels and the increase in GSH content. Our finding demonstrates that leptin has protective effects against hyperglycemia-induced neural damage. This could be related to the attenuation of oxidative stress and neural apoptosis.

INTRODUCTION

Diabetes is one of the general metabolic complaints that leads to several central and peripheral nervous system disorders, such as neuropathy [1,2]. Nonetheless, the exact pathogenesis mechanisms of glucose-induced neurotoxicity have not been fully clarified. It has been considered that hyperglycemia is a main key pathogenic factor of diabetes-induced neuropathy and tissue damage [3,4].

One of the main pathological mechanisms of neurotoxicity in hyperglycemic situation is oxidative stress, which promotes the generation of free radicals and diminishes free radical scavenging [2]. ROS production can lead to protein-, lipid membrane- and nucleic acid damage and result in oxidative injury to cells [5]. Oxidative metabolism elevation of glucose constructs hydrogen peroxide in the mitochondria generated by the superoxide dismutase activity on superoxide [6].

Cellular oxidative stress that is generated in hyperglycemic condition stimulates some metabolic pathways of glucose such as sorbitol and fructose elevation, protein kinase C (PKC) activation, NADPH redox imbalances, poly (ADP-ribose) polymerase activation,

promotes hexosamine pathway activity and superoxide generation and diminishes levels of essential anti-oxidative enzymes [2,7].

There are several studies documenting that hyperglycemia condition causes neuronal cell oxidative injury through NADPH oxidase-dependent production of reactive oxygen species (ROS) [8,9]. Furthermore, ROS generation through hyperglycemia promotes apoptosis, a probable mechanism of glucose neurotoxicity [10,11]. High-glucose situation (such as diabetes condition) not only increases ROS production but, through the glycation of antioxidant enzymes, decreases antioxidant protective mechanisms [5,8]. One of the potential mechanisms for hyperglycemia-induced neural cell death is apoptosis that was assessed by both in vitro and in vivo investigations [12,13]. Apoptotic cell death can be stimulated by some chemical and physiological forms of oxidative stress inducers. For instance, in many cell types, hydrogen peroxide can induce apoptosis, and this effect can be prohibited by the enhancement of cellular antioxidant mechanisms [14]. It is now approved that an effective approach to treatment and prevention of hyperglycemia-induced complications such as neuropathy should focus on both glycemic control and decrease oxidative stress-related factors [15].

Leptin is a 16kD protein produced and secreted by adipocytes whose plasma concentration is related to body fat. Leptin has a significant effect on the nervous system and various peripheral tissues. This hormone passes through the blood-brain barrier via its saturation receptors and enters the brain or cerebrospinal fluid [16]. Evidence has shown that defect in the leptin system is closely related to neurodegenerative diseases (Alzheimer's and Parkinson's) that are associated with oxidative stress [17]. Previous investigations have shown that the level of leptin hormone in diabetic patients is lower than in healthy people [18]. In addition, it was found that leptin hormone increased the level of natural antioxidant enzymes and reduced the expression of inflammatory factors in cells, and thereby inhibiting cell damage caused by oxidative stress [19]. Furthermore, leptin can protect cultured dopaminergic cells from 6-hydroxydopamine (6-OHDA) -induced apoptosis in an in vitro model of Parkinson's disease [20].

The PC12 cell line has been widely used as an in vitro model for glucose neurotoxicity investigation [10,11,21–23]. Several studies in literature have indicated the cytotoxicity of hyperglycemia condition in PC12 cells [10,11,21,22,24,25]. We designed this study to examine the possible neuroprotective effect of leptin on PC12 cells cultured under high-glucose conditions.

MATERIAL AND METHODS

Main material and reagents

Cell culture substances, fetal bovine serum (FBS), high-glucose Dulbecco's modified Eagle's medium (DMEM), horse serum (HS), trypsin-EDTA and penicillin-streptomycin solution were bought from Biosera Co. (UK). Culture dishes and flasks were got from SPL Life Sciences (South Korea). Dichlorofluorescein diacetate (DCFH-DA), 3-[4,5-Dimethyl-2-thiazolyl]-2,5-diphenyl-2-tetrazolium bromide (MTT), thiobarbituric acid, Dimethyl sulfoxide (DMSO), leupeptin, aprotinin, phenylmethylsulfonyl fluoride (PMSF) and leptin were obtained from Sigma (USA). D-(+)-glucose, trichloroacetic acid, sodium dodecyl sulfate were obtained from Merck Chemicals (Germany). Anticaspase 3 and anti-β-actin antibodies were acquired from Cell Signaling Technology (USA). Anti-Bax and anti-Bcl-2 antibodies were obtained from Santa Cruz Biotechnology (USA). Polyvinylidene difluoride (PVDF) membrane was obtained from Roche (Germany).

Cell culture

PC12 (Pheochromocytoma) cells were acquired from National Cell Bank of Iran (NCBI) – Pasteur Institute of Iran (Tehran, Iran). PC12 cells were cultured with high-glucose Dulbecco's modified Eagle's medium (DMEM, with 25 mmol/L glucose) combined with penicillin (100 U/mL), streptomycin (100 μg/mL), 5% horse serum and 10% fetal bovine serum that was kept at 37 °C under a humidified atmosphere (90%) including 5% CO2. For MTT assay, PC12 cells were plated at a density of 5 000 per well in a 96 micro-well plate. Control group were grown in normal media suitable for culturing the PC12 cells (with 25 mmol/L glucose), and high-glucose-treated cells were grown in media with 100 mmol/L (20 g/L) glucose. After that, PC12 cells were incubated in media including 100 mmol/L glucose and different concentration of leptin at 3, 6, 12, and 24 nmol/L doses (or 48.6, 97.2, and 194.4 nanogram/liter respectively) for 24 h.

MTT assay

To determine cellular viability, MTT assay was used [22,26,27]. Chemical reaction of this method is based

on reduction of 2- (4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. MTT at 0.5 mg/mL working concentration was added to the 96-well plates and the cells were incubated for 2 h at 37 °C. Then, following media removal, 100 μL dimethyl sulfoxide (DMSO) was added per well. The optical density values were assessed at 570 nm by microplate reader (Eliza MAT 2000, DRG Instruments, GmbH). Results were represented as percentages of control.

Intracellular ROS generation assay

Oxidative-sensitive fluorescent probe DCFH-DA was used for measuring the intracellular ROS generation. PC12 attached cells were treated with high-glucose media and leptin. Then, the cells were loaded with 10 μmol DCFH-DA in culture medium and incubated at 37 °C for 30 min. Additional DCFH-DA was deprived by washing with phosphate buffered saline (PBS) three times, and 100 μL of phosphate buffered saline was added per well. Fluorescence was assessed at 485 nm for excitation and at 530 nm for emission with a microplate reader (Perkin Elmer Victor 2) [22].

MDA and GSH assay

The PC12 cells were washed and homogenized after treatment with different doses of leptin. The homogenate was centrifuged and the supernatant of protein content was evaluated by Bradford method (Bio-Rad Laboratories, Germany). The GSH level was evaluated according to the method of Gulati et al. [28]. In summary, 100 μL of the cellular homogenized supernatant was combinated with 200 μL of trichloroacetic acid (TCA) and centrifuged. The resulting supernatant was blended with phosphate buffer and its absorbance evaluated at 412 nm. For evaluation of malondialdehyde (MDA) content, 100 μL of the supernatant was mixed with 1.5 mL of acetic acid (20%), 200 μL of sodium dodecyl sulfate (SDS) (8%), and 1.5 mL of thiobarbituric acid (0.8%). Each reaction mixture was heated for 60 min at 95 °C and cooled at room temperature. Thence, 5 mL of n-butanol was added. The organic layer was collected after mixing and centrifugation at 3 000 ***g*** for 10 min, and the absorbance was evaluated at 532 nm [23].

Western blot analysis

To detect caspase-3 activation and Bax/Bcl-2 ratio in PC12 cells, western blot analysis was exploited. Briefly, attached cells were collected by trypsin-EDTA (0.5%), following two washes with cold phosphate buffered saline (PBS) and lysed in lysis buffer [10 mM Tris–HCl (pH 7.4), 0.1% Na deoxycholate, 1 mmol EDTA, 0.1% SDS, 1% NP-40; 2 μg each of the protease inhibitors leupeptin, aprotinin and pepstatin A; and 0.5 μmol/L PMSF] and incubated on ice for 30 min. The samples were centrifuged twice at 14 000 rpm at 4 °C for 20 min. Protein concentrations were evaluated with Bradford procedure (Bio-Rad Laboratories, Germany). The protein samples were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS/PAGE) and subsequently transferred onto PVDF membrane which was incubated with antibodies including cleaved caspase-3 (1:1 000 overnight at 4 °C), Bax, Bcl-2 (1:1 000 for 3 h at room temperature) and secondary antibody (1:15 000) at room temperature for 60 min. Antigen-antibody complexes were visualized by ECL system. Beta-actin antibody (1:1 000) was utilized as loading control.

Statistical analyses

The results are presented as the mean ± SEM. The difference in cellular viability (mean MTT assay), ROS production, MDA and GSH levels between different groups was determined via one-way ANOVA followed by Tukey's post hoc test. The values of cleaved caspase-3, Bax, Bcl-2 and Beta-actin band density were expressed as tested protein/Beta-actin ratio for each sample. A probability criterion for significance level was P-value < 0.05.

RESULTS

Analysis of cell viability

At first, the effects of various concentrations of glucose on cellular viability were assessed by MTT assay. Hyperglycemia was induced by increasing the medium glucose level at 50, 75, 100, 125, and 150 mmol/L concentrations for 24 h. As shown in *Figure 1*, glucose could reduce the cellular viability in a concentration-dependent way. The glucose on 100 mmol/L concentration had 50% decreasing effect (IC50) on relative cell viability ($P < 0.001$). The effect of glucose toxicity at concentrations of 125 and 150 mmol/L was similar to 100 mmol/L concentration. So, for inducing cell damage and assessing the protective effects of leptin, 100 mM glucose was added to media culture. It is notable that this concentration for induction of cell viability IC50 is much higher than the value range seen in real diabetic patients. Furthermore, we took into

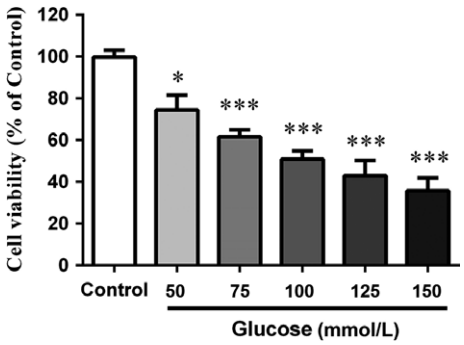

Figure 1 Effects of enhanced glucose levels for 24 h on PC12 cells viability. Data are expressed as mean ± SEM; n = 5–6 wells for each group; * $P < 0.05$ and *** $P < 0.001$ versus control cells.

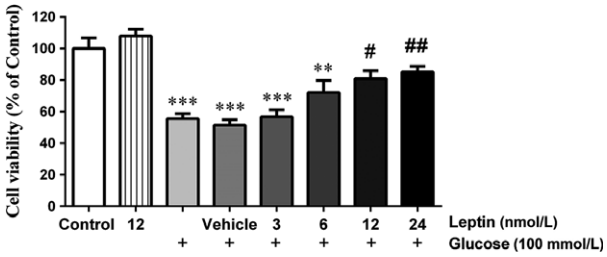

Figure 2 Effects of various doses of leptin on high-glucose-treated PC12 cell viability for 24 h. High-glucose media (100 mmol/L glucose) decreased cell viability and leptin (12 and 24 nmol/L) protected the PC12 cells from high-glucose induced cell injury. Data are expressed as mean ± SEM; n = 5–6 wells for each group; ** $P < 0.01$ and *** $P < 0.001$ versus control cells. # $P < 0.05$ and ## $P < 0.01$ compared to high-glucose-treated cells.

account the same concentration of mannitol for checking the osmotic effect of 100 mmol/L (20 g/L) glucose on the viability of PC12 cells. The mannitol treatment at 100 mmol/L concentration had no significant effects on PC12 cells viability versus control cells. As shown in *Figure 2*, leptin at 12 and 24 nmol/L doses, significantly inhibited high-glucose-induced toxicity after 24 h ($P < 0.05$ and $P < 0.01$, respectively), but could not prevent cell damage in other concentrations (3 and 6 nmol/L). It is notable that we examined the 12 nmol/L leptin effects (as a minimum effective dose in the hyperglycemic situation) on nonglucose-treated (control) PC12 cells (*Figure 2*).

Analysis of ROS generation assay

To examine the intracellular ROS in the hyperglycemic situation, DCFH-DA fluorescent procedure was used. As shown in *Figure 3*, in PC12 cells treated with high-glucose medium for 24 h, DCFH-DA fluorescence levels were elevated compared to control cells. Moreover, treatment of PC12 cells with high glucose and leptin (12 and 24 nmol/L; the effective doses in cell viability test) significantly reduced production of reactive oxygen species (ROS)($P < 0.05$ and $P < 0.01$, respectively for 12 and 24 nmol/L leptin) (*Figure 3*).

Effects of leptin on MDA and GSH

24 h exposure of PC12 cells to high glucose showed a significant increase in MDA versus control cells ($P < 0.001$, *Figure 4*) and reduction in GSH level ($P < 0.01$, *Figure 5*). After leptin treatment (12 and 24 nmol/L), the MDA level significantly decreased ($P < 0.05$ and $P < 0.05$ for 12 and 24 nmol/L, respectively), whereas the GSH level significantly increased (*$P < 0.05$ and **$P < 0.05$ for 12 and 24 nmol/L, respectively) compared with high-glucose-treated PC12 cells.

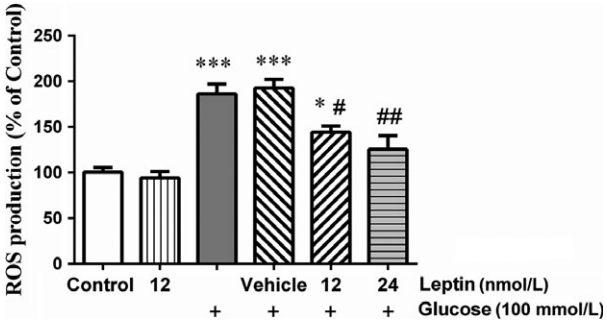

Figure 3 Effects of leptin on glucose-induced ROS formation in high glucose (100 mmol/L) treated PC12 cells for 24 h. ROS formation was assessed by fluorescent probe DCFH-DA assay. Leptin at doses of 12 and 24 noml/L reduced the glucose-induced ROS production. Data are expressed as mean ± SEM; n = 5–6 wells for each group; * $P < 0.05$ and *** $P < 0.001$ compared to control cells. # $P < 0.05$ and ## $P < 0.01$ versus glucose-treated cells.

Western blot analysis of cleaved caspase-3 level, Bax:Bcl-2 ratio in the hyperglycemic situation in PC12 cells

Potential mediators of high-glucose-induced apoptosis were assessed by analysis of the cleaved caspase-3 expression and Bax:Bcl-2 protein ratio. The PC12 cells were divided into control, high-glucose (100 mmol/L) medium, and high-glucose medium plus 12 and

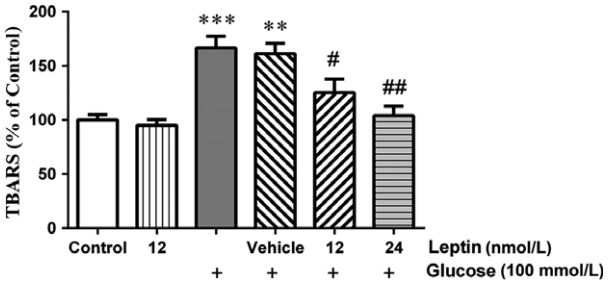

Figure 4 Effects of different doses of leptin on the level of TBARS under hyperglycemic situation (100 mmol/L glucose) in PC12 cells for 24 h. Leptin can reduce TBARS level in glucose-treated cells. Data are expressed as means ± SEM, n = 5–6 wells for each group. ** $P < 0.01$ and *** $P < 0.001$ vs. control. # $P < 0.05$ and ## $P < 0.01$ vs. glucose-treated PC12 cells.

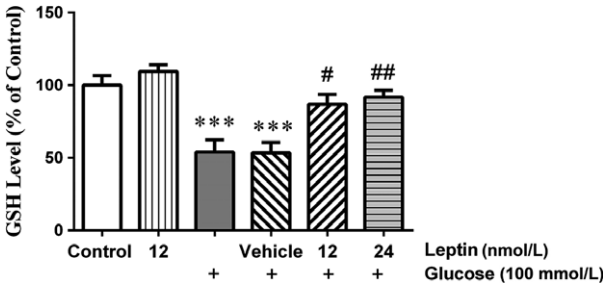

Figure 5 Effects of different doses of leptin on the level of GSH under hyperglycemic situation (100 mmol/L glucose) in PC12 cells for 24 h. Leptin can elevate GSH level in glucose-treated cells. Data are expressed as means ± SEM, n = 5–6 wells for each group. ***$P < 0.001$ vs. control. # $P < 0.05$ and ##$P < 0.01$ vs. glucose-treated PC12 cells.

24 nmol/L concentrations of leptin for 24 h. In high glucose (and vehicle group) exposed PC12 cells, the amount of cleaved caspase-3 expression was enhanced compared to control cells ($P < 0.01$). Leptin-treated cells (12 and 24 nmol/L) significantly ($P < 0.05$ and $P < 0.01$, respectively) opposed high-glucose-induced upregulation of cleaved caspase-3 (*Figure 6*). In the high-glucose situation, a significant enhancement in the Bax:Bcl-2 proteins ratio was seen ($P < 0.01$). Moreover, leptin (12 and 24 nmol/L) had a significant reducing effect on the increased Bax:Bcl-2 ratio ($P < 0.05$) (*Figure 7*).

DISCUSSION

Growing literature reveals several biological effects of leptin hormone. The aim of this study was to provide some cellular evidence about the beneficial effects of leptin on hyperglycemia-induced neuronal damage in an in vitro investigation.

These study findings showed that leptin can exert a protective effect against reactive oxygen species-mediated oxidative stress and apoptosis induced by the hyperglycemic condition in PC12 cells. Our results showed that induction of high-glucose toxicity in PC12 cells is mediated via ROS production and apoptosis. In

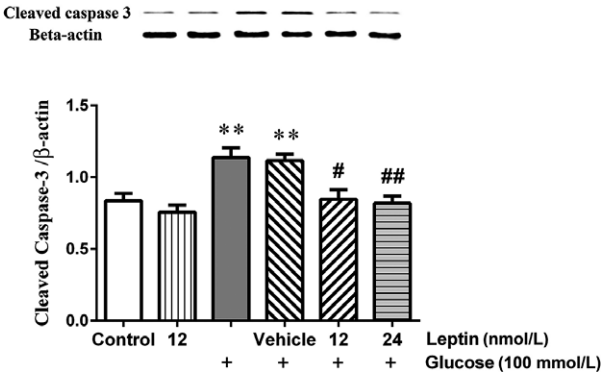

Figure 6 Effects of leptin (12 and 24 nmol/L) on cleaved caspase-3 expression in PC12 cells exposed to high-glucose media (100 mmol/L) for 24 h which was considered by Western blot. Each value in the graph is the mean ± SEM band density ratio for each group. Beta-actin was used as an internal control. ** $P < 0.01$ compared to control cells; # $P < 0.05$ and ## $P < 0.05$ compared to high-glucose-treated cells.

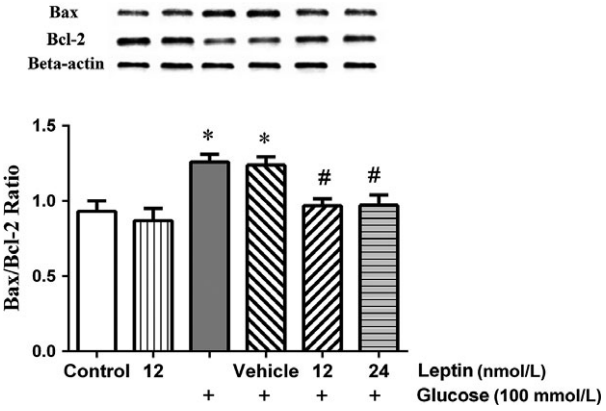

Figure 7 Effects of leptin (12 and 24 nmol/L) on Bax/Bcl-2 protein expression ratio in PC12 cells exposed to high-glucose media for 24 h which was considered by Western blot. Each value in the graph is the mean ± SEM band density ratio for each group. Beta-actin was used as an internal control. * $P < 0.05$ compared to control cells. # $P < 0.05$ vs. high-glucose-treated cells.

this investigation, caspase-3 expression and Bax/Bcl-2 index, hallmarks of apoptosis, were used for evaluation of apoptotic cell death. Leptin (12 and 24 nmol/L) inhibited high-glucose-induced cellular toxicity, elevated cleaved caspase-3 expression, and reduced the ratio of Bax/Bcl-2 in PC12 cells.

To reduce oxidative stress, cells generally scavenge ROS by using antioxidant defense systems [5]. In this regard, our investigation showed that PC12 cells treated with high glucose demonstrated an increase in oxidative stress by excessive ROS and MDA generation and depletion of GSH contents. But, cotreatment with leptin (12 and 24 nmol/L) significantly reduced oxidative damage to PC12 cells in high-glucose situation, as reflected by the reduction of MDA and ROS levels and the increment in GSH content.

It has been shown that, high-glucose-induced oxidative stress and cellular dysfunction in PC12 and other cell types [22,23,26,29]. However, the generation of superoxide in high-glucose condition is a well-defined occurrence that arises through the mitochondrial electron transport chain which can cause additional free-radical production and oxidative stress [30].

In fact, the imbalance between production of radical species and organism's antioxidant potential leads to oxidative stress. The free radical-induced peroxidation of membrane lipids enhances membrane fluidity and permeability with loss of its integrity [31,32]. MDA is a highly reactive compound that is a biochemical marker for cellular lipid peroxidation. To evaluate lipid peroxidation, MDA secondary products, such as thiobarbituric acid reactive substance (TBARS), are commonly used [33,34]. Glutathione (GSH) is the major cellular thiol protein, consisting of three amino acids: glutamine, cysteine, and glycine. It is a nonenzymatic antioxidant cellular factor that plays an important role in the ROS scavenging and reactive species quenching [35,36]. Various studies have shown that hyperglycemia is associated with elevation of oxidative stress and reduction of antioxidant potential [37,38]. A number of antioxidant types have important roles in ROS homeostasis, containing endogenous antioxidant enzymes (e.g., catalase, superoxide dismutase, glutathione reductase, and glutathione peroxidase), dietary natural antioxidants (e.g., vitamins E, C, and A), and antioxidant molecules (e.g., glutathione, bilirubin, and coenzyme Q) [39].

Hyperglycemia produces reactive oxygen species (ROS) that lead to membrane damage and lipid peroxidation through auto-oxidation of glucose [40]. High glucose via glycation of the enzymatic and nonenzymatic antioxidant (such as glutathione) cellular factors reduces antioxidant protective mechanisms [5,8,41].

In addition, ROS can destroy proteins, nucleic acids, lipid membranes and lead to oxidative damage in cells [5]. Also, excessive generation of ROS by high-glucose neurotoxicity enhances apoptotic cell death [10,22,23].

Previous studies have shown that ROS leads to caspase-3 activation and has a specialized role in the promotion of hyperglycemia-induced neuronal damage [7,23]. Furthermore, caspase activation (including caspase-3 and -9) is enhanced in high-glucose situations [10,24,25,42].

Over the past few years, the anti-oxidative and neuroprotective effects of hormones have drawn considerable attention [17,43,44]. It is notable that some hormones can prevent neuronal cell death and apoptosis in various neurodegenerative diseases [44,45].

Leptin is an adipostatic protein hormone that elevates loss of body weight by reduction of food intake and promotes the activation of nervous system [46]. There is evidence demonstrating that leptin has different physiological roles such as immunological function [47], anti-aging [48] and neuroprotective effects [49,50]. There are some in vitro studies that show leptin can reduce cell death [20,46]. Also, some evidence indicated that leptin has a decreasing effect on oxidative stress. For example, in HepG2 ethanol-treated cells, leptin reduced oxidative stress and decreased apoptosis in these cells [51]. It has been shown that leptin changes the activity of various antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) [52].

It has been reported that leptin may have an in vitro neuroprotective effect by stimulation of Mn-SOD (a mitochondrial antioxidant enzyme) upregulation [49]. In addition, leptin prevents apoptosis in some peripheral cell types such as pancreatic beta-cells [53], hepatic stellate cells [54] and lymphocytes [55,56]. Moreover it has been shown that leptin activates some anti-apoptotic pathways in neural cells [17].

It is notable that leptin, via some intracellular signaling pathways, increases cell proliferation and decreases apoptosis in SK-N-SH-SY5Y cell line. This anti-apoptotic reaction involves downregulation of two key genes of the death cascade such as caspase-10 and caspase-3 profusion [46]. Furthermore, in murine dopaminergic cell line (MN9D), leptin can reduce 6-OHDA-induced apoptotic markers (caspase-3 and caspase-9) activity and increase cell viability [20].

It has been shown that leptin can reduce apoptosis induced by serum depletion through the Bcl-2:Bax ratio in clonal rodent pancreatic beta-cells [53]. Also, Zhang and colleagues have previously shown that peripheral administration of leptin, after brain injury, enhanced Bcl-2 and reduced caspase-3 expression by decreasing oxidative stress and neuronal apoptosis [57]. Finally, several studies revealed that leptin can exert anti-apoptotic properties through the reduction of caspase-3 activity and elevation of the anti-apoptotic to pro-apoptotic factors (Bcl-2 and Bax proteins) [56,58,59].

On the other hand, it has been shown that leptin deficiency leads to insulin resistance which is frequently related to hyperglycemia [60]. Human and animal studies revealed that leptin replacement therapy could reverse the diabetic phenotype [61–63]. In addition, various investigations studied the metreleptin (a synthetic analog of the leptin hormone) on type 2 diabetes mellitus patients [64,65]. For instance, in a study of leptin in type 2 diabetes mellitus, patients just decreased glycated hemoglobin (hemoglobin A1c) marginally, with no circulating markers of inflammation or change in body weight [65]. In other investigations, 14-day administration of leptin in obese people with type 2 diabetes mellitus did not produce any significant effects on insulin sensitivity or body weight [64]. Therefore, in accordance with these studies, leptin has weak antidiabetic potential when administrated in hyperleptinemic obese people with type 2 diabetes mellitus. In contrast to the minor effects produced in type 2 diabetes mellitus, administration of leptin in nonobese type 1 diabetic animals regulated circulation of glucose, hemoglobin A1c, free fatty acids, as well as an extensive range of liver intermediate metabolites levels [66].

It is notable that this study was focused on oxidative stress induced by hyperglycemia but high-glucose concentration by itself can exert detrimental activities though nonenzymatic glycation of cellular proteins. Further studies can be designed to address this question.

In conclusion, this study was carried out to assess the inhibitory effect of leptin on high-glucose-induced neurotoxicity through the oxidative stress in PC12 cells. To our knowledge, this is the first report on the effects of leptin protection on ROS-mediated glucose toxicity in PC12. Our results emphasized that leptin can increase PC12 cells viability, at least in part, through the attenuation of hyperglycemia-induced oxidative stress and apoptosis.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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