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Aqueous extract of *Zizyphus jujuba* fruit attenuates glucose induced neurotoxicity in an *in vitro* model of diabetic neuropathy

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ABSTRACT

Objective(s): The neuroprotective effect of fruit aqueous extract of *Ziziphus jujuba* Lam on glucose-induced neurotoxicity in PC12 cells as an appropriate *in vitro* model of diabetic neuropathy was investigated.

Materials and Methods: Cell viability was determined by the MTT assay. Cellular reactive oxygen species (ROS) generation was measured by DCFH-DA analysis. Cleaved caspase-3, a biochemical parameter of cellular apoptosis, was measured by western blot analysis.

Results: Our data showed that a 4-fold elevation in glucose levels within the medium significantly reduced cell viability, increased intracellular ROS and caspase-3 activation in PC12 cells after 24 hr. Incubation of the high glucose medium cells with 300-µg/ml *Z. jujuba* fruit (ZJF) extract decreased the high glucose-induced cell toxicity and prevented caspase-3 activation and excited ROS generation.

Conclusion: Thus, we concluded that the aqueous extract of *Z. jujuba* protects against hyperglycaemia-induced cellular toxicity. This could be associated with the prevention of ROS generation and neural apoptosis. Moreover, the results suggest that the ZJF has a therapeutic potential to attenuate diabetes complications such as neuropathy.

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Introduction

Diabetes mellitus is one of the most frequent metabolic disorders that cause various central and peripheral nervous system complications such as neuropathy (1, 2). However, the exact mechanisms for the pathogenesis of glucose neurotoxicity remain unclear. Current therapeutic approaches are unable to completely relieve the neuropathic complications such as loss of sensation, pain and motor weakness (2). Incurable diabetic side effects are typically related to sustained high glucose concentrations; consequently, chronic hyperglycaemia is considered to be a key pathogenic factor of diabetic neuropathy and tissue damage (3, 4). Both basic and clinical studies suggest that the oxidative stress caused by hyperglycaemia plays an important role in the pathogenesis of neurotoxicity (2). Oxidative stress is stimulated by glucose through a combination of free radical production and decreased free radical scavenging. Hydrogen peroxide is produced by the action of superoxide dismutase on superoxide, and it is produced in the mitochondria by elevated oxidative metabolism of glucose (5). Elevated cellular oxidative stress induced by hyperglycaemia stimulates several glucose metabolic pathways that are associated with neuropathy progression. These include protein kinase C (PKC) activation, NADPH redox imbalances, sorbitol and fructose accumulation, activation of nuclear enzyme poly (ADP-ribose) polymerase, increased hexosamine pathway activity and superoxide overproduction and reduced levels of crucial anti-oxidative enzymes (2, 6).

Apoptosis is suggested as a potential mechanism for high glucose-induced neural cell death by both *in vitro* and *in vivo* studies (7, 8). Several forms of chemical and physiological inducers of oxidative stress can promote apoptotic cell death. For example, hydrogen peroxide can induce apoptosis in various cell types, and this effect can be inhibited by antioxidants (9). Studies have shown remedial properties of natural products or their active components, and antioxidant therapy in the prevention and/or treatment of chronic diseases such as neurodegenerative and cardiovascular disorders (10).

Ziziphus jujuba is broadly distributed in Iran, and the fruit of this plant has gained wide attention in

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folk herbal medicine for the treatment of a wide range of disorders. Chemical analysis of the *Z. jujuba* fruit revealed that it contains flavonoids (quercetin and kaempferol) and phloretin derivatives (11). In our pervious study, we demonstrated the anti-oxidant activity of *Z. jujube* in the central nervous system and improvements on spatial memory deficits induced by ethanol toxicity in rats (12).

In the present study, we evaluated the effect of the *Z. jujuba* fruit (ZJF) aqueous extract on glucose induced neurotoxicity, and the potential anti-apoptotic effect of this extract on high glucose-treated pheochromocytoma (PC12) cells as an *in vitro* model of diabetic neuropathy. PC12 cells, which are derived from catecholamine-secreting adrenal chromaffin tumour in rats, are appropriate model for the assessment of neuronal cell death.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), heat-inactivated horse serum (HS), penicillin–streptomycin solution and trypsin EDTA were purchased from Gibco BRL (Grand Island, NY, USA). Culture dishes were obtained from SPL Life sciences Inc. (Gyeonggi-Do, South Korea). 3-[4,5-Dimethyl-2-thiazolyl]-2,5-diphenyl-2-tetrazolium bromide (MTT) and dichlorofluorescein diacetate (DCFH-DA) were acquired from Sigma (St. Louis, MO, USA). D-(+)-glucose powder was acquired from Merck Chemicals (Darmstadt, Germany). Primary monoclonal anti-β-actin and primary polyclonal anti-caspase 3 antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA).

Preparation of extract

Fresh ripened fruits of *Z. jujuba* were obtained from local herbal shops of Khoramabad, Iran during the October to November, 2012. Identification of the plant was certified at the botany department of Lorestan University, Lorestan, Iran. Seeds were separated from fruits and about seven hundred grams of the pulp material were ground into fine powder. The powder was extracted three times with distilled water (1500 ml totally). It was centrifuged at 4°C for 20 min at 4000 g, and the supernatant was collected, lyophilized and stored at -20°C until use. The extract was weighed and dissolved in phosphate buffered saline (PBS) to give 10 mg/ml extract as stock aliquot.

Cell culture

PC12 cells were obtained from Pasteur Institute (Tehran, Iran). PC12 cells were cultured in high glucose DMEM supplemented with 10% fetal bovine serum, 5% heat-inactivated horse serum, penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37 °C in a humidified atmosphere (90%) containing 5% CO_2. Growth medium was renewed three times a week. Differentiated PC12 cells were plated at the density of 5000 per well in a 96 micro plate well for the cell viability assay. Control cells were grown in DMEM with 25 mM glucose, and the other cells (high glucose-treated) grown in DMEM with 100 mM glucose. For protein extraction, cells were grown in a 6 well plate and allowed to attach and grow for 24 hr. Then the cells were incubated in medium containing 100 mM glucose and different concentration of ZJF extract for 24 hr.

Cell viability assay

The cell viability was explored with MTT assay. This method is based on reduction of 2-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. MTT (0.5 mg/ml) was added to the 96-well plates and the cells were incubated for 2 hr at 37°C. After removing medium, the resulting formazan was solubilized in 100 µl Dimethyl sulfoxide (DMSO) per well. The optical density was determined at 570 nm by an automatic microplate reader (Eliza MAT 2000, DRG Instruments, GmbH). Results were expressed as percentages of control.

Intracellular ROS generation assay

Generation of intracellular reactive oxygen species (ROS) was assessed by the oxidative-sensitive fluorescent probe DCFH-DA. After treatment of cells with high-glucose media and ZJF extract, attached cells were washed twice with PBS. PC12 Cells were loaded with 10 µM DCFH-DA in culture media and incubated at 37 °C for 30 min. Extra DCFH-DA was excluded by washing with fresh PBS tree times. One hundred micro liter of phosphate buffered saline was added to each well. Fluorescence was evaluated with a micro-plate reader (Perkin Elmer Victor 2) at 485 nm for excitation and at 530 nm for emission.

Western blot analysis

Western blot analysis was used to detect caspase-3 activation in PC12 cells. In brief, cultured cells were collected by trypsin-EDTA (0.5%), followed by two washes with cold PBS and lysed in cold lysis buffer [10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 0.1% sodium dodecyl sulphate (SDS), 0.1% Na deoxycholate, 1% NP-40; 2 µg each of the protease inhibitors aprotinin, leupeptin, and pepstatin A; and 0.5 µmol/l PMSF] and incubated on ice for 30 min. The homogenate was centrifuged twice at 14000 rpm at 4°C for 20 min. The resulting supernatant was removed to clean tubes as the whole cell fraction. Protein concentrations of each fraction were measured with the Bradford method (Bio-Rad Laboratories, Muenchen, Germany). The protein samples from each fraction were separated via 10% sodium dodecyl sulphate- poly acrylamide gel

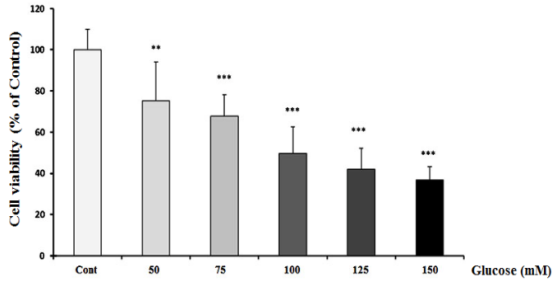

Figure 1. Effect of glucose on PC12 cell viability. PC12 cells exposed to increasing levels of glucose. Cell viability was determinate by MTT assay. Data are expressed as mean ± SD; n = 5 to 6 wells for each group; ** *P*<0.01 and *** *P*<0.001 compared to control cells

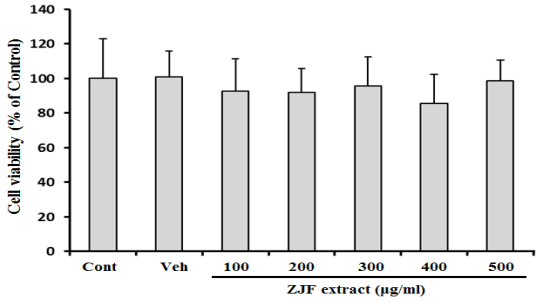

Figure 2. Effects of various doses of *Ziziphus jujuba* fruit (ZJF) extract for 24 hr on PC12 cells viability. Cell viability was determinate by MTT assay. Data are expressed as mean ± SD; n = 5–6 wells for each group

electrophoresis (SDS/PAGE), and subsequently transferred to PVDF membrane for western blotting. Membranes were probed using rabbit monoclonal antibody to caspase-3 (Cell Signaling Technology, USA, 1:1000 overnight at 4ºC), and subsequently exposed to secondary HRP-conjugated IgG. Antigen-antibody complexes were then visualized by ECL system and exposed to Lumi-Film chemiluminescent detection film (Roch, Germany). Beta-actin immunoblotting (antibody from Cell Signaling Technology, INC. Beverly, MA, USA; 1:1000) was used as control for loading. Photographs were digitized and the band intensity was quantified using Lab Work analyzing software (UVP, UK).

Statistical analyses

All results are presented as the mean±SD The difference in cell viability (mean MTT assay) between groups was determined by one-way ANOVA, followed by the Tukey test. The values of caspase 3, and beta-actin band density were expressed as tested cleaved caspase-3/beta-actin ratio for each sample. A probability level of $P<0.05$ was considered significant.

Results

Analysis of cell viability

Since the optimal glucose concentration for PC12 cell cultures is 25 mM, we simulated *in vitro* hyperglycemia by increasing the medium glucose level at the concentrations of 50, 75, 100, 125 and 150 mM for 24 hr. PC12 cells exposed to increasing levels of glucose at 50, 75, 100, 125 and 150 mM exhibited toxicity, reaching a maximal effect in 100 mM glucose, which resulted in 49.6±12.82% of relative cell viability. The glucose concentration of 100 mM was selected for further study as representative of hyperglycemic conditions that can decrease the viability of PC12 cells for assessing the protective effects of the ZJF extract (Figure 1). In this study, 24 hr treatment of cultured PC12 cells with different doses of ZJF extract did not show toxic effects (Figure 2). As shown in Figure 3, ZJF extract in the dose of 300 µg/ml significantly inhibited high glucose-induced toxicity in PC12 cells after 24 hr; whereas, ZJF extract could not prevent cell damage in other concentrations (Figure 3).

Analysis of ROS generation assay

To examine the intracellular ROS in the hyperglycemic condition, we used the DCFH-DA fluorescent method. As shown in Figure 4, the levels of DCF-DA fluorescence in PC12 cells treated 24 hr with high glucose media were increased markedly compared to control cells. Furthermore, treatment with 300 µg/ml ZJF extract significantly decreased the production of the reactive oxygen spices in hyperglycemic cells (Figure 4).

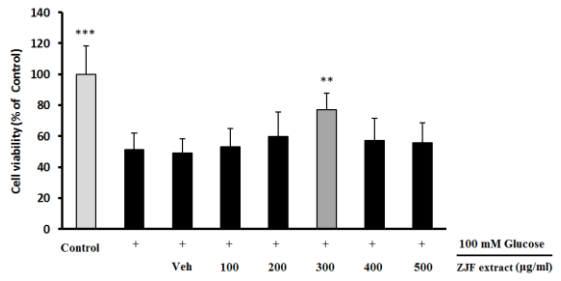

Figure 3. Effects of various doses of *Ziziphus jujuba* fruit (ZJF) extract on high glucose-treated PC12 cell viability. High-glucose medium reduced cell viability and ZJF extract (300 µg/ml) protected the PC12 cells against high-glucose-induced cell death. Data are expressed as mean±SD; n = 5 to 6 wells for each group; ** *P*<0.01 and *** *P*<0.001 compared to high glucose-treated cells

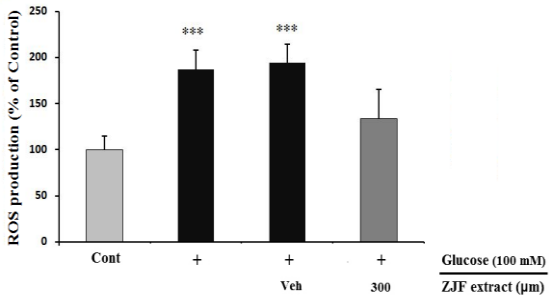

Figure 4. Effects of *Ziziphus jujuba* fruit (ZJF) extract on glucose-induced ROS production in PC12 cells. ROS production was evaluated by fluorescent probe DCFH-DA assay. ZJF extract (300 µg/ml) decreased glucose-induced ROS production. Data are expressed as mean±SD; n= 5 to 6 wells for each group; *** *P*<0.001 compared to control cells

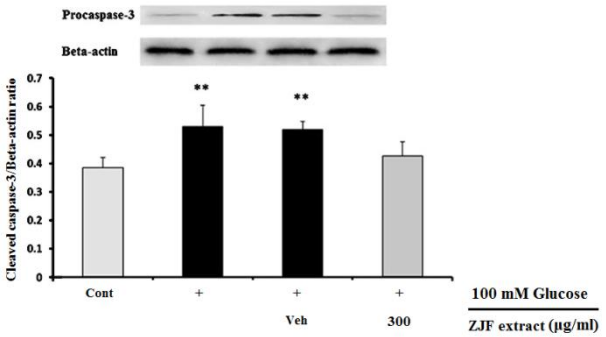

Figure 5. The activation of caspase-3 protein in PC12 cells exposed to high glucose medium and high-glucose plus 300 µg/ml of *Ziziphus jujuba* fruit (ZJF) extract for 24 hr. Caspase 3 activity was determinated by Western blotting. Each value in the graph is the mean ± SD band intensity for each group. Beta-actin was used as an internal control. $^{**}P < 0.01$ compared to control cells

The effect of hyperglycemic condition on the levels of caspase-3 activation

We examined caspase-3 activation by western blot analysis to evaluate the potential activity of ZJF on preventing apoptosis after glucose induced cytotoxicity in PC12 cells. The PC12 cells were categorized to several groups including control, high glucose media and high-glucose media plus different concentrations of ZJF extract. As shown in Figure 5, 24 hr incubation with glucose at 100 mM enhanced expression of procaspase-3 protein compared to the control and vehicle groups. Caspase-3 activation was increased in glucose treated cells after 24 hr. Furthermore, treatment of PC12 cells with 300 µg/ml ZJF significantly antagonized high glucose-induced up-regulation of cleaved caspase-3 (Figure 5).

Discussion

It is well known that diabetes and hyperglycaemic conditions elevate the formation of reactive oxygen species (ROS) and decrease the cellular antioxidant defence capacity (5). Oxidative stress is a main contributor to the development of neuropathy in diabetes. Hyperglycaemia produces oxidative stress, which leads to the production of superoxide and hydroxyl radicals, which in turn have direct toxic effects on nerve tissue (13). Also, caspase (such as caspase-3 and -9) activation reportedly increases in high glucose conditions (13-16). Our data in the present study revealed that glucose-induced toxicity in PC12 cells is mediated through ROS generation and apoptosis. Furthermore, 300 µg/ml ZJF aqueous extract successfully decreased the high glucose-induced ROS generation and suppressed the activation of caspase-3, an apoptosis biomarker.

ROS can cause cell death through apoptosis (17). Chemical and physiological oxidative stress inducers can promote apoptosis by producing additional ROS. For example, hydrogen peroxide can induce apoptotic cell death in several cell types (9). The mechanism by which oxidative stress induces apoptosis is not well understood. Several pathology situations can result from the oxidative stress-induced apoptotic signalling that follows ROS elevation and/or antioxidant diminutions, the disruption of intracellular redox homeostasis and irreversible oxidative reforms of DNA, lipids and proteins (18). Furthermore, caspase-3 activation through oxidative stress is important in the promotion of diabetic neuropathy (6).

In recent years, the demonstration of anti-oxidative and neuroprotective characters of natural herbal products has drawn attention. It is notable that several natural herbal products reportedly protect neuronal cells from death and apoptosis in several neurodegenerative diseases (19, 20). *Zizyphus* species (Rhamnaceae) are broadly used as herbal medicines in different parts of the world, particularly in Asia, for the treatment of various acute and chronic diseases (21). The ZJF has been defined as the 'fruit of life', as it is an excellent source of vital functional components such as flavonoids, polyphenols, polysaccharides and saponins that are responsible for different biological activities including antitumor activity, improvement of central nervous system complaint, regulation of immune function, pain relief and decrease of blood glucose and triglyceride (12, 22-26).

Previous studies have shown that caffeic acid, p-coumaric acid, ferrulic acid and *p*-hydroxybenzoic acid have the highest amount of phenolic components in *Ziziphus* that are responsible for the considerable antioxidant activity (27, 28). As mentioned before, our results revealed that hyperglycaemia leads to a significant increase in caspase-3 activation (as a main biochemical factor of apoptosis), and the ZJF extract had a suppressing effect on its activation and eventually prevented high glucose-induced apoptosis in PC12 (Figure 5). In addition, several studies show that some natural extracts suppress high glucose-induced neuronal cell dysfunction complications (such as diabetic neuropathy) through apoptosis inhibition (14, 29, 30). Among the various natural antioxidants in *Ziziphus*, caffeic acid and ferrulic acid were reported as potent anti-apoptotic flavonoid compounds (31). Additionally, caffeic acid prevents protein tyrosine kinase, lipoxygenase and cyclooxygenase activities (32-34). Some studies show that caffeic acid can block/reduce the activation of tumour necrosis factor-alpha (TNF-α) (35). In addition, TNF-α activates nuclear factor kappa B (NF-κB) (36, 37), which exists in neurons (8, 34). NF-κB activation has been implicated as an important regulator of genes that control apoptosis. Therefore, caffeic acid (one of the main components of *Z. jujuba*) can prevent apoptosis induced by hyperglycaemic conditions.

Normally, the generation of ROS and other free radicals are controlled by several innate scavenger molecules, which are typically found in the cell and quench free radicals. Antioxidant enzymes such as

superoxide dismutase, glutathione peroxidase (GPx) and several non-enzymatic free radical scavengers are involved in the antioxidant defence mechanisms (38). Our previous study demonstrated that the ZJF extract increased GPx activity in the hippocampus of ethanol-treated rats (12). With regard to this, the *Z. Jujuba* extract may prevent hyperglycaemic toxicity by elevating the intracellular antioxidant system (such as GPx) and detoxify the generation of the high glucose-induced mass of free radical and the generation of apoptosis.

Conclusion

This study suggests that the aqueous extract of the ZJF protects PC12 cells against high glucose-induced toxicity. The mechanisms underlying these effects may be due, at least in part, to reduced ROS generation and apoptosis.

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