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Quercetin and Sesamin Protect Neuronal PC12 Cells from High-Glucose-Induced Oxidation, Nitrosative Stress, and Apoptosis

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Abstract

Complications of diabetes are now well-known to affect sensory, motor, and autonomic nerves. Diabetes is also thought to be involved in neurodegenerative processes characteristic of several neurodegenerative diseases. Indeed, it has been acknowledged recently that hyperglycemia-induced oxidative stress contributes to numerous cellular reactions typical of central nervous system deterioration. The goal of the present study was to evaluate the effects of the polyphenol quercetin and the lignan sesamin on high-glucose (HG)-induced oxidative damage in an *in vitro* model of dopaminergic neurons, neuronal PC12 cells. When incubated with HG (13.5 mg/mL), neuronal PC12 cells showed a significant increase of cellular death. Our results revealed that quercetin and sesamin defend neuronal PC12 cells from HG-induced cellular demise. An elevated level of reactive oxygen and nitrogen species is a consequence of improved oxidative stress after HG administration, and we demonstrated that this production diminishes with quercetin and sesamin treatment. We also found that quercetin and sesamin elicited an increment of superoxide dismutase activity. DNA fragmentation, Bax/Bcl-2 ratio, nuclear translocation of apoptosis-inducing factor, as well as poly(adenosine diphosphate [ADP]-ribose) polymerase cleavage were significantly reduced by quercetin and sesamin administration, affirming their antiapoptotic features. Also, HG treatment impacted caspase-3 cleavage, supporting caspase-3–dependent pathways as mechanisms of apoptotic death. Our results indicate a powerful role for these natural dietary compounds and emphasize preventive or complementary nutritional strategies for diabetes control.

Introduction

Glucose is the principal energy source for the mammalian brain and a substrate that is essential for maintaining normal cerebral function. Reversal of homeostatic glucose parameters may produce considerable nervous system problems in sensory, motor, and autonomic nerves. Type 2 diabetes is a health burden rooted in genetic factors, demographic characteristics, lifestyle-related risk dynamics, and metabolic determinants, such as insulin resistance.[1] Hyperglycemia is indeed the causal link in the evolution of neuropathy and uncontrolled diabetes.[2] In aging, hyperglycemia is also associated with central nervous system damage, a consequence of long-term exposure to glucose.[3] Interestingly, recent data have highlighted the relationship between diabetes and neurodegenerative disorders, such as Parkinson disease (PD).[4] Indeed, epidemiologic studies have implicated a number of exogenous factors in PD causation; for example, the risk of developing PD is double in men and women with prior type 2 diabetes.[4,5]

At the cellular level, mechanisms of high-glucose (HG)-induced cell death are sustained by oxidative[6] and nitrosative stress in many cellular types as well as *in vivo*.[7–9] Excessive formation of reactive oxygen (ROS) and nitrogen species (RNS) as well as insufficient antioxidant capacity may damage cellular components. Free radical attack increases damage to lipids, proteins, and DNA. Furthermore, the effects of glucose on mitochondrial superoxide generation and subsequent nitric oxide synthase (NOS) activation can merge to elicit peroxynitrite, a powerful oxidant.[10] As well, HG-induced ROS and RNS increase apoptosis,[11] a possible mechanism of glucose neurotoxicity.

Quercetin, a flavonoid possessing free radical scavenging properties, may protect from oxidative injury by its ability to modulate intracellular signals and promote cellular survival.[12] Several studies suggest its potential as a cardioprotective, anticarcinogenic, antioxidant, and antiapoptotic molecule.[13–16] Quercetin sulfate/glucuronide, the quercetin metabolite in blood, has also been evaluated for the prevention of HG-induced apoptosis of human umbilical vein endothelial cells.[17]

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Quercetin may have a protective action in human monocytes against HG-induced proinflammatory cytokines.[18] On the other hand, sesamin, a phytonutrient of the lignan class and a lipophilic compound found in sesame seeds and sesame seed oil, is also known for its antioxidant role.[19,20] Sesamin as well as sesamol and sesaminol, the two other primary compounds in sesame, are likely responsible for the increased stability of sesame oil against autooxidation and rancidity caused by free radicals.[21] Certainly, sesamin is recognized to have several positive physiological effects, such as hypocholesterolemic and antihypertensive outcomes.[22,23] It is also recognized to protect against oxidative stress in neuronal PC12 cells.[24]

The present study was designed to examine the neuroprotective effects of the polyphenol quercetin and the lignan sesamin in a cellular model of PD—neuronally differentiated PC12 cells (neuronal PC12)[25] maintained under HG concentration. In this comprehensive investigation, we outline the roles of quercetin and sesamin in preventing neural parameters of cellular stress and apoptosis induced by HG exposure in a cellular dopaminergic system. Our results may open the way to natural dietary compounds as preventive or complementary nutritional strategies for diabetes control.

Materials and Methods

Drugs and chemicals

All reagents and chemicals were purchased from Sigma (St. Louis, MO) unless noted otherwise.

Cell culture and treatments

PC12 cells, obtained from the American Type Culture Collection (ATTC, Rockville, MD), were maintained in a humidified environment at 37°C and 5% CO_2 atmosphere. They were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% (vol/vol) heat-inactivated horse serum, 5% (vol/vol) heat-inactivated fetal bovine serum (FBS), and gentamicin (50 μg/mL). PC12 cell neuronal differentiation was evoked by nerve growth factor-7S (NGF, 50 ng/mL) in DMEM supplemented with 1% FBS for 5 days, as already described.[13] According to the manufacturer's instructions, the glucose concentration in DMEM was 4.5 mg/mL (Sigma D6046) and was called the control condition (CTRL). To produce hyperglycemia, DMEM was supplemented with 13.5 mg/mL D-glucose and called HG medium (Sigma D7777), as already reported.[26–28] PC12 neuronal cells were incubated with CTRL or HG medium with or without quercetin (0.1 μM) or sesamin (1 pM) for 24 or 96 hr. Quercetin and sesamin concentrations were chosen after dose–response and kinetic studies (data not included). To exclude the role of osmotic toxicity for the HG condition, we refer to Koshimura[28] et al. and Tie et al.,[29] who showed that neurotoxicity of glucose is not related to osmolarity in PC12 cells. Charcoal-stripped serum was used in all experiments to remove steroids from the medium.[30] For each experiment, cellular density was 30,000 cells/cm^2.

Cytotoxicity measurements

Cytotoxicity was evaluated in control and hyperglycemic conditions by colorimetric assay, based on the measurement of lactate dehydrogenase (LDH) activity released from damaged cells into supernatant, as already described.[30] LDH is a stable cytoplasmic enzyme present in all cells. It is released rapidly into cell culture supernatant upon damage of the plasma membrane. The amount of enzyme activity detected in culture supernatant correlates with the portion of lysed cells.[31] Briefly, 50 μL of cell-free supernatant was taken to quantify LDH activity by measuring absorbance at 490-nm wavelength in a microplate reader (Thermolab System, Franklin, MA). Total cellular LDH was determined by lysing the cells with 1% Triton X-100 (high control). The assay medium served as a low control and ws subtracted from all absorbance measurements.

$$\text{Cytotoxicity}\,(\%) = \frac{(\text{Experimental value} - \text{Low control})}{(\text{High Control} - \text{Low control})} \times 100$$

DNA fragmentation analysis

DNA fragmentation was assessed with single-stranded DNA (ssDNA) apoptosis enzyme-linked immunosorbent assay (ELISA) kit (Chemicon International, Temecula, CA). This procedure is based on the selective denaturation of DNA by formamide in apoptotic cells but not in necrotic cells. Denatured DNA was detected with a monoclonal antibody to ssDNA. ssDNA staining in early apoptotic cells was achieved with a mixture of antibody and peroxidase-labeled secondary antibody. The reaction was stopped, and ssDNA fragmentation was quantified by measuring absorbance at 405-nm wavelength in a microplate reader (Thermolab System). ssDNA was calculated with reference to control conditions. Absorbance of positive and negative control was also considered as quality control of ELISA.

Detection of mitochondrial superoxide radical ($^{\bullet}O_2^-$)

Neuronal cells were grown and treated on collagen-coated circular glass coverslips. MitoSOX Red estimated intracellular superoxide anion ($^{\bullet}O_2^-$) production in neuronal cells. This superoxide indicator is a fluorogenic dye for the highly selective detection of superoxide in the mitochondria of live cells. After treating neuronal cells with CTRL medium or HG with or without quercetin or sesamin for 24 hr, the medium was removed and the cells were incubated with MitoSOX Red (5 mM) for 10 min at 37°C (Invitrogen, Burlington, ON, Canada). MitoSOX Red is rapidly and selectively targeted to the mitochondria. Once in the mitochondria, it is oxidized by superoxide and exhibits red fluorescence. Cells were washed with Hanks' buffered salt solution (HBSS, Invitrogen), and 4′,6′-diamidino-2-phenylindole (DAPI) was used to counterstain all nuclei. Then, the cells were fixed in 4% paraformaldehyde for 6 min at 37°C. Coverslips were mounted with the Molecular Probes ProLong Antifade Kit (Invitrogen). Images were acquired using a Leitz inverted microscope with a high-pressure mercury burner and necessary filter cubes, and analyzed with NIS-Element 2.2 software (Nikon, Mississauga, ON, Canada). To demonstrate MitoSOX Red selectivity, a positive control was performed using sodium diethyldithiocarbamate (DDC), an inhibitor of superoxide dismutase (SOD), in CRTL medium.

Detection of SOD activity

After 96 hr of treatment, neuronal cells were harvested mechanically and collected by centrifugation at 2,000 × g for

10 min at 4°C. The pellets were homogenized in 1 mL of cold 20 mM HEPES buffer, pH 7.2 (1 mM EGTA, 210 mM mannitol, and 70 mM sucrose) and sonicated (3 times, 5 sec). The samples were then centrifuged at $1,500 \times g$ for 5 min at 4°C, and the supernatant was assayed according to the manufacturer's protocol (Superoxide Dismutase Assay Kit, Cayman Chemical, Ann Arbor, MI). One unit of SOD activity was defined as the amount of enzyme needed to exhibit 50% dismutation of superoxide radicals. The reaction was monitored at 450 nm in a microplate reader (Thermolab System).

Nitrate (NO_3^-) and nitrite (NO_2^-) assays

Nitric oxide (NO) generated by cells was quantified by measuring nitrate and nitrite in culture medium with Nitrate/Nitrite Colorimetric Assay Kit (LDH method, Cedarlane, Burlington, ON, Canada) after HG treatment. Briefly, NO_3^- in the samples was first reduced to NO_2^- by incubation with 10 μL of nitrate reductase and 10 μL of NADPH for 40 min at room temperature. This reaction uses LDH to oxidize excess NADPH. Finally, nitrite concentration in the samples was quantified by the Griess reaction.[32] Nitrate plus nitrite concentration was calculated by measuring absorbance at 570 nm with a $NaNO_3$ standard in a microplate reader (Thermolab System).

Electrophoresis and immunoblot analysis

NGF-differentiated PC12 cells were grown and treated in collagen-coated six-well plates. Total proteins were extracted with a nuclear extraction kit (Activemotif, Brockville, ON, Canada). Proteins were assessed by bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology Inc., Rockford, IL), and equal amounts were loaded onto a 12% sodium dodecyl sulfate polyacrylamide gel. After electrophoretic separation, the gels were transferred to polyvinylidene difluoride (PVDF) membranes (0.22-μm pore size, BioRad, Hercules, CA). The blots were blocked for 1 hr at room temperature in 5% nonfat powdered milk. Dilutions of primary anti-Bax (1:50, Cedarlane), anti-Bcl-2, anti-poly(adenosine diphosphate [ADP]-ribose) polymerase (PARP), and antiapoptosis-inducing factor (AIF) (1:50; 1:100; 1:50, respectively, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were prepared in Tris-buffered solution + Tween (TBS-T) with 0.5 gram of bovine serum albumin (BSA) and 25 mg of sodium azide. Blots were washed and then incubated with peroxidase-conjugated secondary antibody (1:10,000) for 2 hr at room temperature, and finally developed with enhanced chemiluminescence substrate solution.[33]

Immunofluorescence

Neuronal PC12 cells were grown and treated on collagen-coated circular glass coverslips (Fischer Scientific, Ottawa, ON, Canada). Then, they were fixed in 4% paraformaldehyde for 15 min at 37°C, washed, and further incubated in blocking and permeabilizing solution (1% BSA, 0.18% fish skin gelatin, 0.1% Triton-X, and 0.02% sodium azide) for 30 min at room temperature. Fixed cells were incubated with polyclonal anti-cleaved caspase-3 antibody (1:500, New England Biolabs, Pickering, ON, Canada). The slides were transferred to Cy3-conjugated antibody (Medicorp, Montreal, QC, Canada) diluted 1:500 for 1 hr at 4°C. The cells were then rinsed with phosphate-buffered saline (PBS); nuclei were counterstained with 5 μg/mL of DAPI. Coverslips were mounted with the Molecular Probes ProLong Antifade Kit (Invitrogen). Images were acquired by Leitz inverted microscope with a high-pressure mercury burner and necessary filter cubes. The number of apoptotic neuronal cells among 300 randomly-chosen neuronal cells was counted on 10 different optical fields from three slides per group, as already reported,[13] with NIS Elements 2.2 software (Nikon). In each experiment 50 mM Z-DEVD-FMK (Bachem, Torrance, CA), a cell-permeable caspase-3 inhibitor, was used in specific wells of neuronal PC12 cells as internal control for caspase-3 activation.[13]

Statistical analysis

Significant differences between groups were ascertained by one-way analysis of variance (ANOVA), followed by Tukey *post hoc* analysis with the GraphPad InStat program, version 3.06 for Windows (San Diego, CA; www.graphpad.com). All data, analyzed at the 95% confidence interval (CI), are expressed as means ± standard error of the mean (SEM) from three independent experiments. Asterisks indicate statistical differences between the treatment and control condition (***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$), full circles show statistical differences between the treatment and HG condition (•••$p < 0.001$, ••$p < 0.01$, and •$p < 0.05$), diamonds represent statistical differences between the treatment and quercetin condition (◇◇◇$p < 0.001$, ◇◇$p < 0.01$, and ◇$p < 0.05$), and squares identify statistical differences between the treatment and sesamin condition (□□□$p < 0.001$, □□$p < 0.01$, and □$p < 0.05$).

Results

Quercetin and sesamin reduce HG-induced cytotoxicity

Neuronal PC12 cells were grown and differentiated in 4.5 mg/L of glucose, then washed and exposed to HG medium containing 13.5 mg/L glucose, to simulate hyperglycemia. HG administration had a maximal effect, reaching 33% of cytotoxicity after 96 hr of exposure, as illustrated in Fig. 1A. This time period was chosen to study the apoptotic process in the remaining 67% of still living cells.

Figure 1B shows that quercetin or sesamin treatment for 96 hr effectively decreased HG-induced neuronal cell death. Specifically, quercetin and sesamin protected neuronal PC12 cells against hyperglycemia by lowering HG-evoked cellular death to 23% and 20%, respectively (Fig. 1B).

Quercetin and sesamin rescue HG production of $^{\bullet}O_2$, NO_3^-, and NO_2^-

To study the mechanism underlying the neuroprotective effects of quercetin and sesamin against HG, we measured the production of superoxide anion ($^{\bullet}O_2^-$) with a derivative of ethidium bromide, MitoSOX Red, after administration of HG with or without quercetin or sesamin for 24 hr. This time period was considered because free radical generation and eventually oxidative stress are early events in the causative process of cellular death. Figure 2A shows low fluorescence levels in CTRL neuronal cells as well as in cells treated with quercetin or sesamin in CTRL

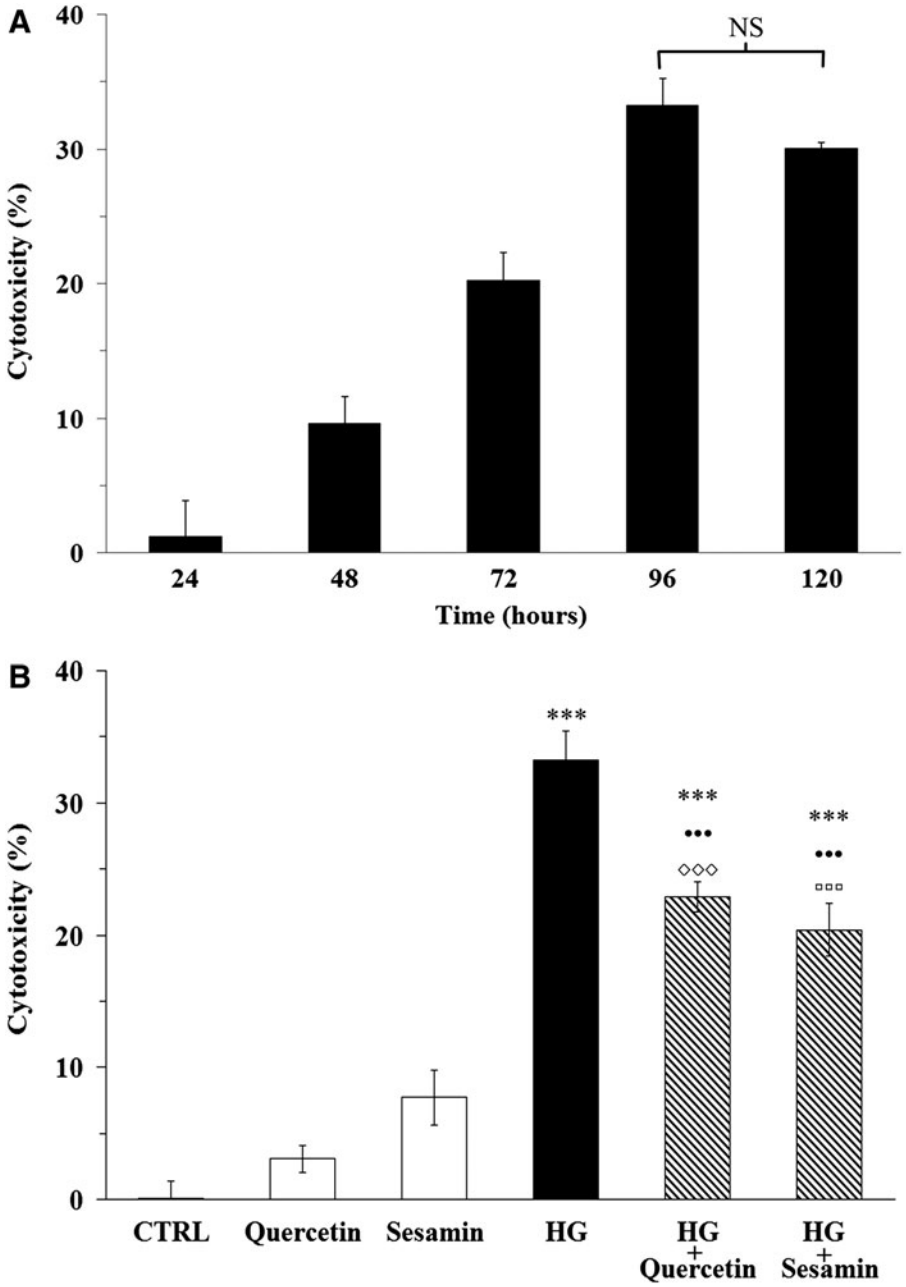

FIG. 1. (**A**) Histograms showing the effect of HG administration for 24, 48, 72, and 120 hr in neuronal PC12 cells. (**B**) Histograms depicting the effect of high glucose (HG) with or without quercitin or sesamin, in neuronal PC12 cells, as revealed by measuring lactate dehydrogenase (LDH) activity. CTRL, Cells were treated with control medium; quercetin + sesamin, cells were treated with quercetin (0.1 μM) or sesamin (1 pM) in control medium; HG, cells were treated with HG medium; HG + quercetin or HG + sesamin, cells were treated with quercetin or sesamin plus HG medium. HG administration increased cell cytotoxicity to 33%. Quercetin or sesamin could reduce HG-induced toxicity (HG + quercetin; HG + sesamin). (***) $p < 0.001$ compared with CTRL; (•••) $p < 0.001$ compared with HG; (◇ ◇ ◇) $p < 0.001$ compared with quercetin; (□ □ □) $p < 0.001$ compared with sesamin, as determined by one-way analysis of variance (ANOVA), followed by the Tukey multiple-comparison test. Values are the average of three independent experiments. NS, Not significant.

medium, after 24 hr (Fig. 2A, CTRL, quercetin and sesamin, respectively), whereas a marked signal was detected in HG- and DDC-treated neuronal cells (Fig. 2A, HG and DDC). When quercetin and sesamin were added to HG medium, fluorescence was reduced (Fig. 2A, HG + quercetin, HG + sesamin). Figure 2B also reports the semiquantitative analysis of mitochondrial superoxide presented in Fig. 2A, revealing high fluorescence levels with HG and DDC and a very significant reduction ($p < 0.001$) when neuronal cells were treated with HG + quercetin or HG + sesamin (Fig. 2B, histogram).

We also examined whether quercetin and sesamin modulated nitrite and nitrate accumulation. As illustrated in Fig. 3, nitrite and nitrate levels were increased three-fold in

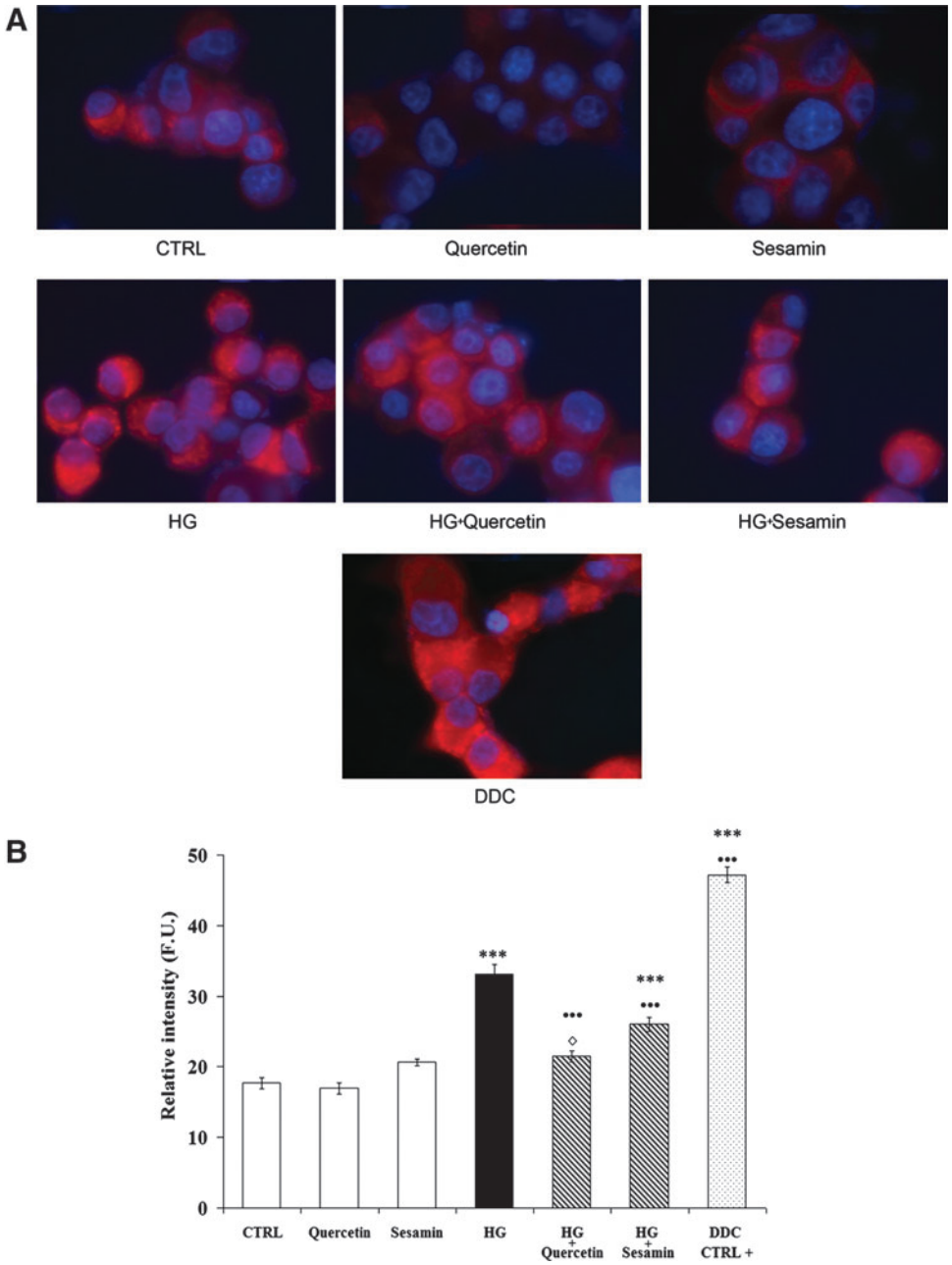

FIG. 2. Effect of quercetin and sesamin on high glucose (HG)-induced superoxide anion ($^{\bullet}O_2^-$) in neuronal PC12 cells. (**A**) Fluorescence microphotographs. CTRL, Cells treated with control medium; quercetin + sesamin, cells treated with quercetin (0.1 μM) or sesamin (1 pM) in control medium; HG, cells treated with HG medium; HG + quercetin or HG + sesamin, cells treated with quercetin or sesamin plus HG medium; DDC, cells treated with diethyldithiocarbamate (DDC), a superoxide dismutase (SOD) inhibitor. A marked red signal is evident only in neuronal PC12 cells treated with HG or DDC. Red fluorescence is less intense in cells treated with control medium (CTRL) or when quercetin or sesamin was added in HG medium (HG + quercetin or HG + sesamin, respectively). (**B**) Semiquantitative image analysis. Fluorescent units (F.U.). Magnification, 400×. $n = 3$. (***) $p < 0.001$ compared with CTRL; (•••) $p < 0.001$ compared with HG; (◇) $p < 0.05$ compared to quercetin, as determined by one-way analysis of variance (ANOVA), followed by the Tukey multiple-comparison test. Values are the average of three independent experiments.

neuronal cells treated with HG for 24 hr in comparison to CTRL (Fig. 3, HG). Quercetin and sesamin decreased nitrite and nitrate concentrations when these natural molecules were administered in neuronal cells exposed to HG medium (Fig. 3, HG + quercetin, HG + sesamin).

Effect of quercetin and sesamin on HG-induced reduction of SOD activity

When SOD is present in the sample, superoxide radicals should be dismutated by the enzyme. Figure 4 illustrates

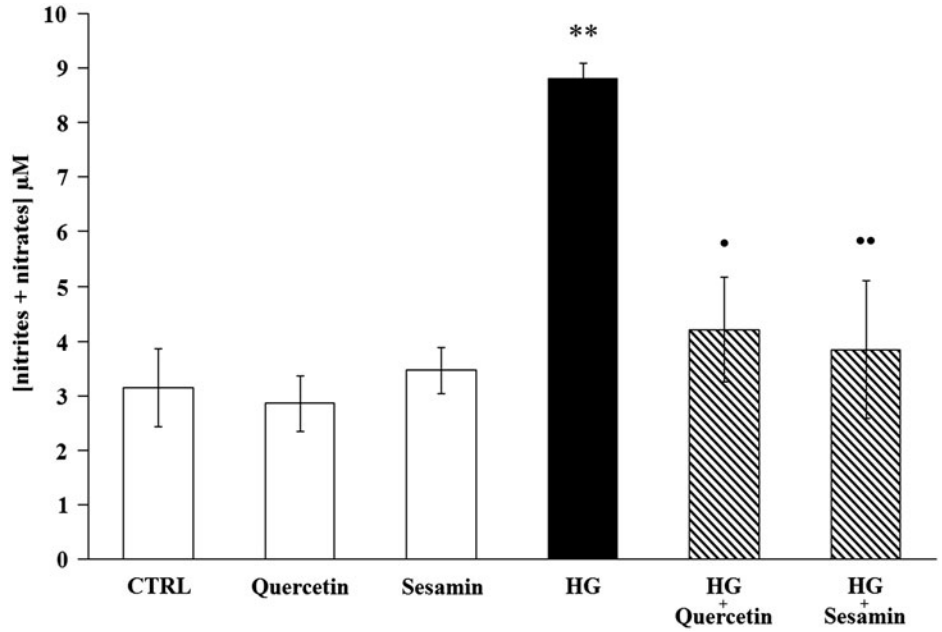

FIG. 3. Histogram showing the effect of high glucose (HG) administration in neuronal PC12 cells, as determined by measurement of nitric oxide synthase (NOS) activity in supernatants after 24 hr of treatment. Quercetin and sesamin reduced HG-induced nitrate/nitrite production (HG + quercetin or HG + sesamin, respectively). (**) $p < 0.01$ compared to CTRL; (••) $p < 0.01$; and (•) $p < 0.05$ compared to HG, as determined by one-way analysis of variance (ANOVA), followed by the Tukey multiple-comparison test. Values are the average of three independent experiments.

that quercetin alone increased SOD activity significantly ($p < 0.05$), whereas sesamin did not (Fig. 4). Our results also show that the HG condition strongly decreased SOD activity compared to the controls after 96 hr. This relatively long time period was necessary to induce a clear and significant decrease of SOD activity for HG-treated cells. When quercetin and sesamin were administered in HG medium, we still detected a significant increment of SOD activity compared to the HG condition ($p < 0.01$ for each molecule).

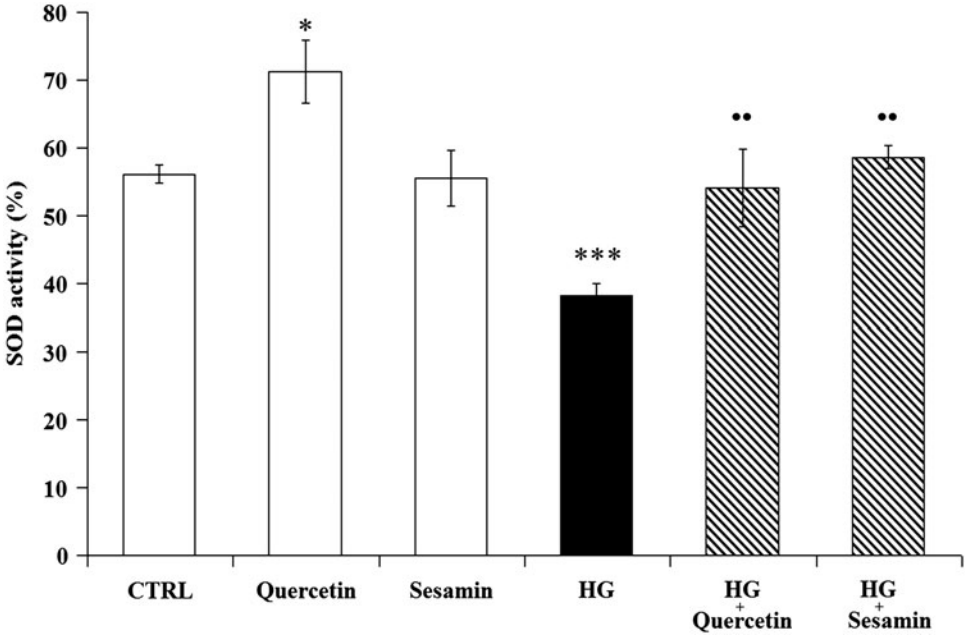

FIG. 4. Superoxide dismutase (SOD) activity. Quercetin markedly augmented SOD activity in our experimental conditions. High glucose (HG) downregulated SOD activity. SOD activity levels were increased over HG values when quercetin and sesamin was administered in HG medium ((HG + quercetin or HG + sesamin, respectively). (***) $p < 0.001$ versus CTRL; (••) $p < 0.01$ compared to HG. Values are the average of three independent experiments.

Quercetin and sesamin reduce HG-induced apoptosis

To determine whether quercetin and sesamin protect neuronal DA cells from HG-induced apoptosis, we measured ssDNA fragmentation, a marker of late apoptosis (Fig. 5A). Neuronal cells treated with 13.5 mg/mL of glucose for 96 hr showed a 52% increase in DNA fragmentation in comparison to control cells (Fig. 5A, HG vs. CTRL). This increment was strongly prevented by quercetin and sesamin treatment in HG medium (Fig. 5A, HG + quercetin, HG + sesamin).

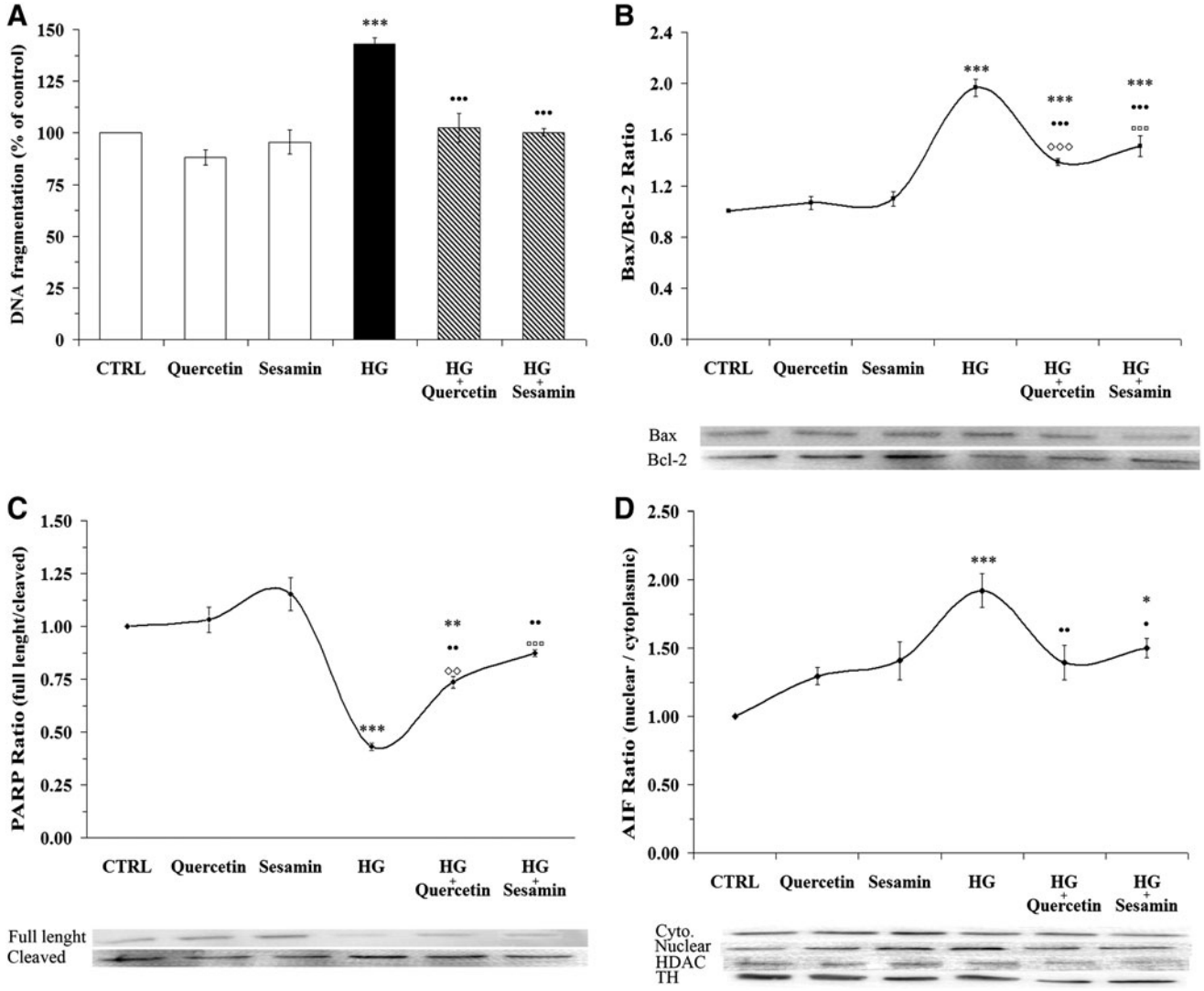

FIG. 5. Apoptosis detection. **(A)** Histogram of DNA fragmentation neuronal cells detected with a monoclonal antibody to single-stranded DNA (ssDNA). Treatment of high glucose (HG)-exposed cells with quercetin or sesamin elicited a significant decrease in DNA fragmentation (HG + quercetin; HG + sesamin). (**B**) Effect of quercetin or sesamin on the Bax/Bcl-2 ratio in neuronal cells. (*Bottom*) Bax and Bcl-2 bands, as revealed by western blots. Quercetin or sesamin alone did not modulate the Bax/Bcl-2 ratio. HG increased the Bax/Bcl-2 ratio, and the addition of quercetin or sesamin to HG medium strongly prevented this increment (HG + quercetin or HG + sesamin, respectively). (**C**) Analysis of poly(adenosine diphosphate [ADP]-ribose) polymerase (PARP) protein expression after each treatment. (*Bottom*) Western blots of full-length and cleaved PARP. These results are presented as the ratio of PARP full-length/PARP cleaved. Quercetin or sesamin did not modulate the PARP ratio when used alone in neuronal cells. A strong decrease of PARP ratio expression was apparent when HG was administered. When quercetin or sesamin was delivered in HG conditions, a significant increase of PARP full-length/PARP cleaved was evident (HG + quercetin; HG + sesamin). (**D**) Effect of quercetin or sesamin on the antiapoptosis-inducing factor (AIF) (nuclear/cytoplasmic) ratio in neuronal cells. (*Bottom*) Western blot bands of cytoplasmic AIF (Cyto), nuclear AIF (Nucl). Anti-histone-deacetylase (HDAC) and anti-tyrosine hydroxylase (TH) are exclusively nuclear and cytoplasmic, respectively. Nuclear AIF and cytoplasmic AIF protein expression levels were analyzed, and the AIF nuclear/AIF cytoplasmic ratio was determined for each treatment. Quercetin or sesamin alone did not modulate the AIF (nuclear/cytoplasmic) ratio. HG increased the AIF (nuclear/cytoplasmic) ratio, and the addition of quercetin or sesamin strongly prevented this increment (HG + quercetin or HG + sesamin, respectively). (***) $p < 0.001$, (**) $p < 0.01$, and (*) $p < 0.05$ compared to CRTL; (•••) $p < 0.001$, (••) $p < 0.01$, and (•) $p < 0.05$ compared to HG; (◇ ◇ ◇) $p < 0.001$, (◇ ◇) $p < 0.01$, and (◇) $p < 0.05$ compared to quercetin; (□ □ □) $p < 0.001$ compared to sesamin, as determined by one-way analysis of variance (ANOVA), followed by the Tukey multiple-comparison test. Values are the average of three independent experiments.

To further investigate the antiapoptotic effect of quercetin and sesamin, we analyzed whether these two natural compounds modulate the Bax and Bcl-2 protein ratio (Fig. 5B). The ratio of proapoptotic Bax to antiapoptotic Bcl-2 has been reported to be correlated with apoptosis.[34] Western blotting (Fig. 5B, bottom) was performed in neuronal cells treated with HG with or without quercetin or sesamin. Our results demonstrate that administration of HG medium for 96 hr significantly increased the Bax/Bcl-2 ratio (Fig. 5B, HG), indicating that HG-induced apoptosis in neuronal PC12 cells is possibly mediated by the mitochondrial pathway. Then, HG-induced increment of the Bax/Bcl-2 ratio was markedly reduced in neuronal cells treated with HG + quercetin or HG + sesamin (Fig. 5B).

Quercetin and sesamin prevent HG-induced PARP cleavage, AIF redistribution, and caspase-3 activation

PARP is a major player in programmed cell death, and its cleavage by cleaved caspase-3 is a hallmark of apoptosis. To investigate the effects of HG on PARP protein levels, western blotting was performed in neuronal PC12 cells. HG treatment markedly activated PARP cleavage (Fig. 5C, HG), which was suppressed by quercetin and sesamin administered in HG medium (Fig. 5C, HG + quercetin and HG + sesamin). Quercetin and sesamin in CTRL medium did not significantly modulate PARP cleavage, and the ratio was similar to the control condition (Fig. 5C, quercetin, sesamin).

AIF is an apoptotic factor released by the mitochondria during early apoptosis events. It is then transported to the nucleus where it provokes chromosomal DNA damage.[35] We investigated AIF translocation from the mitochondria to the nucleus during HG-induced apoptosis (Fig. 5D). When HG was administered, we observed a significant rise of AIF in the nuclear fraction at 22 hr (Fig. 5D, HG), as demonstrated previously in 1-methyl-4-phenylpyridinium ion (MPP^{+}) -treated neuronal cells.[13] We examined whether quercetin and sesamin modulate HG-induced AIF translocation. We detected a decrease of AIF expression in cytosolic and nuclear fractions when quercetin and sesamin were administered prior to HG medium (Fig. 5D, HG + quercetin and HG + sesamin). Anti-histone-deacetylase (HDAC) and anti-tyrosine hydroxylase (TH) antibodies served to control the purity of nuclear and cytosolic fractions, respectively. HDAC was present exclusively in nuclear fractions, whereas TH was found exclusively in cytosolic fractions (Fig. 5D, bottom).

We also assessed the appearance of cleaved caspase-3 at 17 kD by immunofluorescence. HG activated caspase-3 cleavage on cells treated for 96 hr (Fig. 6A,B), and quercetin and sesamin reduced the red immunofluorescence signal, indicating their ability to preserve caspase-3. To show that caspase-3 activation is a key step in the HG-induced apoptotic pathway, PC12 neuronal cells were pretreated with 50 mM Z-DEVD-FMK, a cell-permeable selective caspase-3 inhibitor, followed by treatment with HG (Fig.6A,B).

Discussion

Previously, we reported that several natural polyphenols as well as the lignan sesamin exert powerful neuroprotective activity in dopaminergic neurons against the oxidative burden provoked by administration of the potent Parkinsonian toxin MPP^{+} *in vitro*, or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) *in vivo*.[13,30,36] The present study focuses on the neuroprotective influence of the polyphenol quercetin and the lignan sesamin on HG-induced oxidative stress and apoptosis.

Indeed, it has been determined that stimulation of oxidative stress is critical to the evolution of metabolic syndrome, diabetes, diabetic neuropathy, and several neurodegenerative disorders, such as PD and Alzheimer disease.[37–41] In parallel, ample current literature shows that oxidative and nitrosative stress initiates apoptosis in many cell types and animal models.[7,8,13]

HG has a number of known cellular and molecular mechanisms of neurotoxicity.[3] One pathway of glucose neurotoxicity is oxidative and nitrosative stress, which results in excess free-radical generation[3] and apoptotic cell death as a consequence. In this study, we focused on apoptosis as the best-documented death pathway in our cellular system; however, we cannot exclude the intervention of other less-known mechanisms of cellular death. We have demonstrated the defensive role of quercetin and sesamin in counteracting cellular distress parameters evoked by HG in neuronal PC12 cells. We tested NGF-differentiated PC12 cells, a known, reliable, and efficient model for the investigation of oxidative stress and neuroprotection of dopaminergic neurons.[30] After NGF administration, PC12 cells differentiated into a neuronal-like phenotype that secreted high dopamine (DA) levels, expressed TH, high-affinity DA transporter (DAT), neurofilaments, as well as estrogen receptor-α and -β.[30,42,43] In this cellular paradigm, HG was delivered as 13.5 mg/mL glucose for 96 or 24 hr. In general, neuronal PC12 cells were grown and differentiated in medium containing 4.5 mg/mL glucose[13,28,36]; thus, 13.5 mg/mL represents a three-fold elevation of glucose levels for this cell line.

The present study reveals that HG evokes $^{\bullet}O_2^-$, NO_3^-, and NO_2^- generation in PC12 neuronal cells and leads to neuronal apoptotic cell death. We then demonstrated that quercetin and sesamin administration effectively diminished intracellular $^{\bullet}O_2^-$, NO_3^-, and NO_2^- generation induced by exposure to HG. We also found that treatment of neuronal cells with HG plus quercetin or sesamin reduced HG-elicited neuronal cellular death and apoptosis, as detected by LDH assay and DNA fragmentation.

Oxidative and nitrosative stress has long been implicated in normal aging as well as in age-associated disorders, such as neurodegenerative diseases, diabetic neuropathy, and diabetes (for review, see refs. 44–47). PD, in particular, has been linked with elevated levels of oxidative and nitrosative stress in the mesencephalic substantia nigra pars compacta (SNpc).[48] At the cellular level, the antioxidant enzyme SOD is the primary defense mechanism of protection against $^{\bullet}O_2^-$ damage produced by mitochondrial dysfunction.[49] Recent data show heightened SOD activity in blood of PD patients.[50] In the present study, we noted an increase of $^{\bullet}O_2^-$ production and a decrease of SOD activity in the HG condition in neuronal PC12 cells. When quercetin and sesamin were administered together with HG, we observed a decline of $^{\bullet}O_2^-$ production and an increment of SOD activity, indicating that these two natural molecules may reduce, in part, oxidative stress generated by mitochondrial malfunction. We also saw that quercetin alone augmented SOD activity significantly ($p < 0.05$), which suggested its ability to eliminate $^{\bullet}O_2^-$ formed during

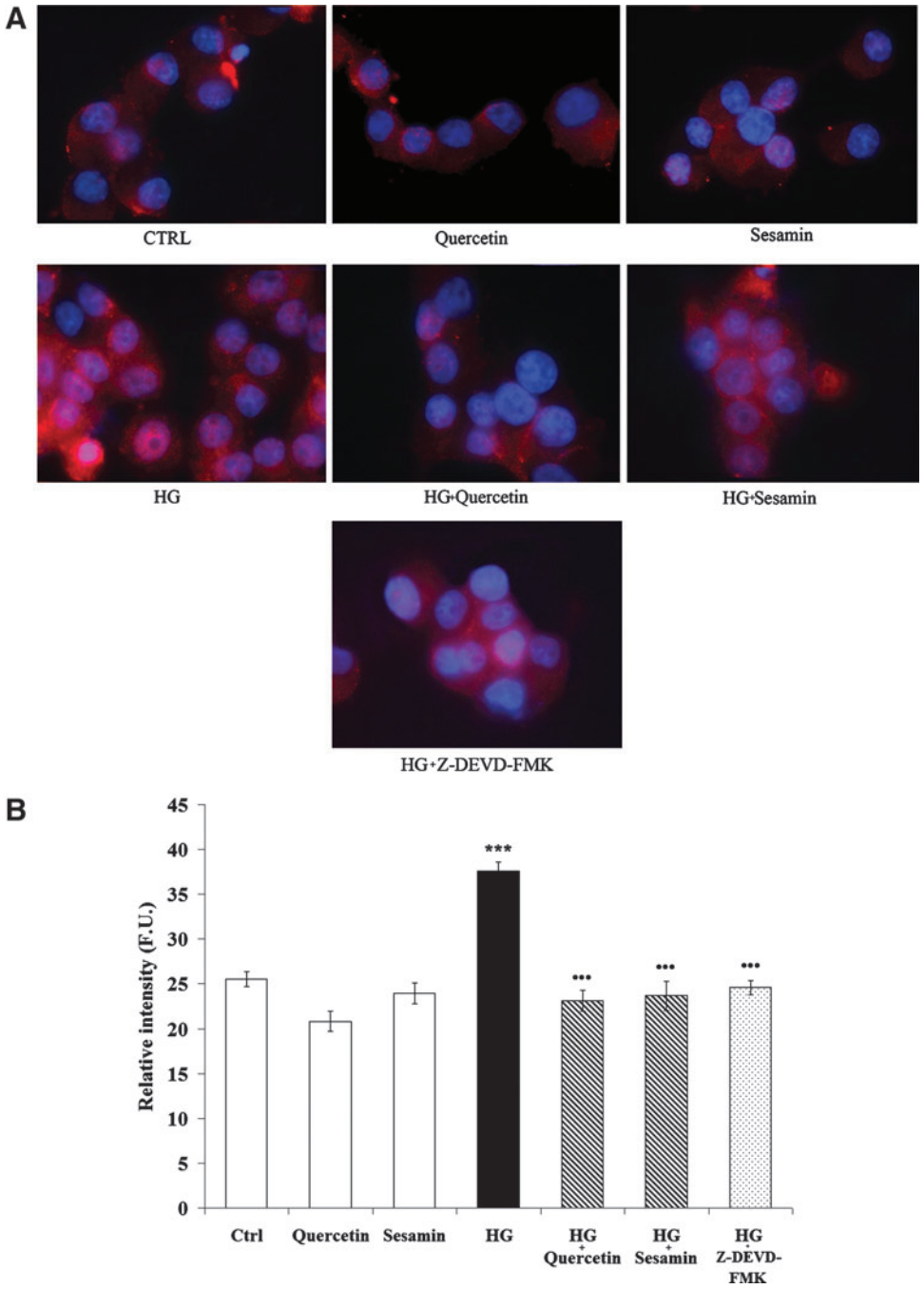

FIG. 6. **(A)** Immunofluorescence detection of apoptotic neuronal PC12 cells, as described in Materials and Methods. (Blue) Neuronal PC12 nuclei were counterstained blue with 4′,6′-diamidino-2-phenylindole (DAPI); (red) anti-cleaved caspase-3 antibody. Double-staining clearly reveals several apoptotic cells, on slides treated with HG and fewer apoptotic cells when quercetin or sesamin was administered in high-glucose (HG) conditions (HG + quercetin or HG + sesamin, respectively). To show that caspase-3 activation is a key step in the HG-induced apoptotic pathway, PC12 neuronal cells were pretreated with 50 mM Z-DEVD-FMK, a cell-permeable caspase-3 inhibitor, followed by treatment with HG. (**B**) The number of apoptotic neuronal cells among 300 randomly chosen neuronal cells was counted on 10 different optical fields from three slides per group. (***) $p < 0.001$ compared to CTRL; (•••) $p < 0.001$ compared to HG, as determined by one-way analysis of variance (ANOVA), followed by the Tukey multiple-comparison test. Values are the average of three independent experiments. F.U., Fluorescent units.

Fe^{2+}-induced oxidative stress, as reported in the SNpc of PD patients.[51,52] On the other hand, sesamin administered for 96 hr does not seem to increase SOD; however, it could reverse the HG-reduced SOD activity. We do not have a clear explanation for this result at this time. Our hypothesis is that sesamin does not act directly on SOD but rather as a direct scavenger of superoxide anion when in presence of SOD. Indeed, we have already demonstrated that sesamin can increase SOD activity after 24 hr in a normal medium.[24]

NO, an important signaling molecule, is another major contributor to oxidative stress, but can easily react with other ROS to form highly toxic RNS. Nitrosative stress is reported to trigger diabetes in rats and humans.[45] For example, NO can react with $^{\bullet}O_2^-$ to form peroxynitrite ($ONOO^-$), a powerful oxidant shown to play an important role in protein aggregation pertinent to PD.[53] We have demonstrated that quercetin and sesamin, when administered to HG medium, can lower HG-induced nitrite and nitrate production, pointing to an antioxidative role for these natural compounds in the prevention of oxidative damage in dopaminergic neurons.

Moreover, the relationship between HG-induced oxidative stress and programmed cell death is complex. In several cell types, ROS generation increases nuclear factor-κB activity[54,55] and causes Bax activation with cytochrome *c* release. As well, in a mouse model of diabetic neuropathy, markers of oxidative stress and apoptosis could be reduced by administration of *N*-acetylcysteine.[56] In this study, we evaluated the Bax/Bcl-2 ratio, full-length versus cleaved PARP, caspase-3 activation, and DNA fragmentation as markers of apoptosis. Late apoptosis is well-characterized by DNA fragmentation. In our experiments, administration of HG medium induced an increase of about 52% in DNA fragmentation with respect to the controls. Thus, HG-induced breakup of DNA was strongly prevented by treatment with quercetin and sesamin.

At the protein level, the rise in the Bax to Bcl-2 ratio indicates cellular pathways resulting in apoptosis.[13] Bcl-2 is a key member of the antiapoptotic Bcl-2 family and is a potent inhibitor of apoptotic cell death.[57] On the other hand, Bax is also a member of the Bcl-2 protein family. It accelerates the rate of apoptosis by contributing to permeabilization of the outer mitochondrial membrane, either by forming channels by itself or by interacting with components of the outer mitochondrial membrane pore, such as voltage-dependant anion channel.[58] Indeed, our data reveal that the Bax/Bcl-2 protein ratio is increased after HG administration and decreased by quercetin and sesamin treatments in the HG condition. These results suggest that quercetin and sesamin can actually diminish HG-induced apoptotic cell death by affecting the mitochondrial pathway. Moreover, disruption of mitochondrial transmembrane potential is reported to be a key event for cytochrome *c* release and caspase-3 activation,[59] and caspase cleavage of various substrates is responsible for the typical morphological features of apoptosis. To determine whether the apoptotic pathway initiated by HG corresponds to classical and widely studied caspase-dependent apoptosis, we performed immunofluorescence experiments with cleaved caspase-3 antibody. Indeed, after 96 hr of HG treatments, cleaved caspase-3 was detected. Immunofluorescence also revealed that quercetin and sesamin can reduce caspase-3 activation, suggesting that neuronal apoptotic cell death is a caspase-dependent pathway and that quercetin and sesamin act on these same pathways.

As well, DNA fragmentation directed by AIF is the main mediator of caspase-independent apoptosis.[60] AIF is an apoptotic factor released by the mitochondria during early apoptosis events. It is then transported to the nucleus where it provokes DNA fragmentation. Our data disclose a significant increase of the nuclear/cytoplasmic AIF ratio when neuronal cells are treated with HG and a significant decrease when quercetin or sesamin is added to HG medium, indicating that in neuronal cells, apoptosis may be directed by caspase-dependent as well as caspase-independent pathways.

PARP cleavage inactivates this enzyme to participate in DNA repair. In endothelial cells, HG-induced superoxide generation causes DNA strand breaks and PARP activation.[61] Our findings in neuronal PC12 cells reveal that the PARP protein ratio was decreased after HG treatment, and was then improved by quercetin or sesamin administration.

Altogether, our results demonstrate that HG-induced apoptosis of PC12 neuronal cells can be reverted, in part, by quercetin or sesamin, sustaining an important role of these two natural compounds in diabetes treatment. Recent reports mention that chronic administration of quercetin reduces insulin resistance, dyslipidemia, and hypertension *in vivo*.[62] Quercetin also exerts powerful antioxidant effects, supporting a beneficial outcome against diabetes-associated vascular dysfunction.[17] In humans, flavonoids, such as quercetin, as well as the lignan sesamin, are absorbed in the gut, and their bioavailability is much greater than previously believed.[63–65] Systolic blood pressure and plasma-oxidized, low-density lipoprotein concentrations are reduced on quercetin intake in overweight subjects.[66]

Recent studies have reported the powerful properties of various natural polyphenols against oxidative stress in several cellular and *in vivo* paradigms of neurodegenerative disease.[67–70] Finally, because of their potential beneficial effects on human health, more cellular and molecular studies are needed to unravel the powerful role of these dietary compounds, which are becoming increasingly important to reinforce nutritional recommendations for diabetes treatment.

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Author Disclosure Statement

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