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The Neuroprotective Effect of Sodium Nitrite on Ischemic Stroke-Induced Mitochondrial Dysfunction via Downregulation of Intrinsic Apoptosis Pathway

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Abstract

Objective: Ischemic stroke leads to programmed cell death via intrinsic mitochondrial apoptosis pathways. Nitric oxide donors (NODs) are various kinds of drugs with the ability to produce nitric oxide (NO) as a potential bioregulator of apoptosis. Therefore, we aimed to evaluate the effect of sodium nitrite (SN) on ischemic injury-induced mitochondrial damage.

Materials and Methods: A 4-hour oxygen-glucose deprivation (OGD) cellular model was developed to mimic cerebral ischemia injury. Cell viability was determined to demonstrate the efficiency of SN as a NO donor on OGD injured PC12 cells. Immunoblotting was performed to measure the expression of Bcl2, Bax and cleaved caspase 3 proteins. Mito Tracker Green label was used for staining the active mitochondria.

Results: The present study confirmed that nitrite inhibited apoptosis via upregulation of Bcl-2 and downregulation of cleaved caspase-3 in OGD-injured PC12 cells as demonstrated by western blot analyses. In addition, nitrite restored mitochondrial vital activity and cell viability in OGD-injured cells.

Conclusion: Resultant data illustrated the protective effects of nitrite and may suggest the in vivo use of nitrite for further confirmations.

Keywords: Oxygen-glucose deprivation, PC12, Nitrite, Bcl2, Bax, Mitochondria

Introduction

The low oxygen supply and hypoxia create very stressful conditions for cells, especially cells with high energy consumption, such as neurons. Hypoxia triggers a variety of cellular responses in relation to the death or survival (1). This condition is created in various disorders such as carbon monoxide poisoning, myocardial infarct and strokes including hemorrhagic and ischemic strokes. Among the mentioned pathological conditions, stroke is the most important because of the highest incidence (approximately 85% of all strokes) (2).

Acute hypoxia resulted from ischemic stroke leads to early necrotic cell death, followed by late-programmed cell death via intrinsic mitochondrial or extrinsic apoptosis pathways (3-6). Dysfunction of mitochondria that is resulted from ischemia is considered as an initiator of cell death cascade (7). Mitochondria as a main source of adenosine triphosphate (ATP) are also involved in reactive oxygen species (ROS) production, calcium homeostasis and apoptosis (8). Furthermore, mitochondria have recently been a focus of attention for more study due to recognition of apoptosis (9) and activation of apoptosis after brain ischemia (10,11).

A stroke initiated by preliminary necrotic death occurs rapidly in the core area and is followed by secondary apoptotic death in cells located around the ischemic area. At the first step, ATP production is rapidly felled (12), Na^+, Cl^- and Ca^{2+} flowed toward the cell and K^+ was released into the extracellular fluid (13,14). Elevated intracellular Ca^{2+} result in production of nitric oxide (NO) (15). NO has dual opposite roles, neurotoxicity and neuroprotection, in the cytopathology of stroked cells (16). After ischemia, NO level falls due to hypoxia (17). However, upon reperfusion, production of this small molecule is initiated mainly by activation of neuronal nitric oxide synthetase (nNOS), as shown in ischemic mice (18). The rate of NO decreases to normal physiologic level in 1 hour after reperfusion (18-20) and elevates again due to hyperexpression of inducible NOS (iNOS) in 8 days later (21,22). Calorie restriction as another common outcome of ischemic stroke also increases endothelial NOS (eNOS) levels, which, in turn, raises cGMP production in mouse tissues particularly in white adipose tissue. One of the common outcomes of these events is mitochondrial biogenesis triggering.

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Nuclear genes related to mitochondrial biogenesis are peroxisome proliferator-activated receptor gamma, coactivator 1-alpha (PGC-1α), nuclear respiratory factors (Nrfs) and mitochondrial transcription factor A (TFAM). These genes are unregulated upon cell injury, initiation of mitochondrial biogenesis and reduction of fat synthesis and tolerance to stress. Additionally, elevated levels of NO acutely inhibit cell respiration by binding to cytochrome c oxidase and inhibit glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a key enzyme in the glycolysis pathway. Therefore, these antithetic roles of NO in cerebral ischemia, neurotoxic and neuroprotective, have created a great scientific discuss and conflicting findings in experimental stroke models (24,25).

Moreover, NO is a potential bioregulator of apoptosis with controversial effects on B-cell lymphoma 2 (Bcl-2) regulation (26). The BCL-2 family proteins are well-known controllers of apoptosis and are categorized into 3 subfamilies according to the number of BH (BCL-2 Homology) domains they partake. The first subfamily possessing 4 BH domains—BH1–4, includes the anti-apoptotic proteins BCL-2, BCL-w, MCL-1, BCL-xL and A1/BFL-1. The second subfamily is pro-apoptotic group having 3 BH (BH1–3) domains including BAX, BAK and BOK. The third subfamily have only BH3 domain (BH3 only) which senses the message of cell damage and leads to the mitochondrial outer membrane permeability by activating Bax and Bak proteins. The increase in permeability of the mitochondrial outer membrane leads to the formation of the apoptosome, which activates procaspase-9 (27-29), procaspase-3 and DNA fragmentations (30). Nitric oxide donors (NODs) are the various kinds of drugs with the ability to produce NO or NO derived radicals such as the nitrosonium ion (NO+) or the nitroxyl anion (NO–) in vitro or in vivo (31,32).

The biochemical importance of nitrite is related to being a potent source of NO in low pH and hypoxia (3). Evidence show that nitrate is reduced to nitrite by the tongue crypts bacteria having a nitrate reductase enzyme and is concentrated in saliva. When nitrite-rich saliva is mixed with the low pH gastric juice, nitrite reduces first to the nitrous acid (HNO_2) then to NO and after entering the circulation act as vasodilator. A number of nitrite reductase enzymes, possessing multi-haem ingredients, catalyze the reduction of nitrite to NO in blood and tissues. Furthermore, metaloenzymes containing copper enable transferring a single electron to produce NO (30). Therefore, it seems that, some of biological responses such as hypoxic vasodilatation (5), angiogenesis (6,7) and gene expression (5,6) can be regulated by nitrite through generating bioavailable NO (12). There is some evidence to confirm these effects (2,18,31). However, the reduction of nitrite to NO in physiologic condition (pH = 7.4) is unrealistic unless the condition changes. In the last five years, a great deal of interest in the interactions of nitrite with mitochondria has emerged (32).

We aimed to evaluate the effect of sodium nitrite (SN) on ischemic injury-induced mitochondrial damage and investigated the expression of Bcl-2 family proteins and mitochondrial vital activity in oxygen glucose deprivation (OGD) injured PC12 cells as an in vitro stroke-like model.

Material and Methods

Chemicals and Antibodies

Nitrite sodium (sigma; 563218) was a gift from Dr. Amiri. The other antibodies listed below were from Santa Cruz (Santa Cruz Biotechnology Inc., CA, USA): anti-Bcl-2 (C 21) (sc-783), anti-Bax (P-19) (sc-526), anti-B-actin (sc-130656) and HRP-conjugated goat anti-rabbit secondary polyclonal antibody (sc-2030).

Cell Culture

The pheochromocytoma-derived PC12 cell line, purchased from Pasteur institute (Tehran, Iran) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) enriched with 10% horse serum and incubated at 37°C under normoxic condition (95% air, and 5% CO2).

In Vitro Oxygen Glucose Deprivation Model

PC12 cells were deprived of oxygen and glucose for 4 hours to induce in vitro ischemic injury as follows: PC12 cells were cultured in six well plates in NO/NG condition, then they were rinsed with phosphate buffer solution (PBS) once at 80% confluence and incubated in a homemade hypoxia chamber with 95% N2, 5% CO2 and humidity of 60% for 4 hours after adding hanks balanced salt solution (HBSS) in each well and then the cells were harvested after trypsinizing. For SN-induced neuroprotection studies, PC12 cells were cultured in the mentioned media with 100 μM SN.

Western blot

SDS-PAGE and Western blot analyses were performed as previously described (33). Cells were hemogenated in ice-cold RIPA lysis buffer containing protease inhibitors, followed by centrifugation at 12000 g. The supernatant was taken for protein quantification by Bradford method, then the samples were mixed 1:1 by sample loading buffer 2X (sigma) and were boiled for 10 minutes before loading onto 12% polyacrylamide gel for electrophoresis. After transferring proteins onto the PVDF membrane, blocking and washing were performed. Then membrane were incubated with polyclonal anti-Bax, anti-Bcl-2 and anti-B-actin antibodies (1:500 dilution), at 4°C overnight, then with a goat anti-rabbit polyclonal horseradish peroxidase (HRP) conjugated secondary antibody (1:10 000 dilutions in 1% milk/TBS) for 2 hours at room temperature. The blots were established by using a chemiluminescence detection kit (Amersham Biosciences, Piscataway, NJ) and radiographic film (Fuji). The density of bands was measured by ImageJ software. β-actin was used as loading controls.

MTT Assay

PC12 Cells seeded in 96 well plates at a starting density of 10^4 cells/100 µl/well, after 48 hours when cell count reached to 10^5, they were co-treated with drugs and tolerated OGD. Cell viability was determined after 4 hours of treatment using the 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (MTT) assay. A 570 nm filter was used to measure the absorbance by using a kinetic microplate reader (Stat Fax, Awareness, USA).

Mito Tracker Green Stain

The cell-permeable probe, MitoTracker® dye, which contains a mildly thiol-reactive chloromethyl moiety and accumulates in active mitochondria, was applied for mitochondrial labeling. After seeding the cells in six well plates at a starting density of 10^4 cells/2 mL/well and performing the required interventions, the Mito Tracker Green (#9074 cell signaling, USA) was directly added into media at a final concentration between 100 nM and incubated for 15 to 30 minutes at 37°C in hypoxia chamber. Following the trypsinizing and providing cell suspension, the fluorescence intensity of samples was read in Excitation of 490 nm an emission of 516 nm by using a fluorometer (Fluoroskan Ascent, Thermo Scientific, Waltham, MA). Then we calculated the green fluorescence intensity per mg protein.

Statistical Analysis

The data were entered into SPSS software (version 16.0, USA) and the comparisons of means were done by one-way analysis of variance (ANOVA) followed by Tukey test for post hoc comparisons. Values up to $P<0.05$ were considered significant. The analysis revealed the statistical power of 0.8 for this study using $\alpha=0.05$ and $\beta=0.2$.

Results

Effect of SN on the Expression of Bcl-2 and Bax Proteins in OGD-Injured PC12 Cells

Data in Figure 1 showed a significant decrease in the expression of Bcl-2 (chart was not shown) and Bcl-2/Bax ratio in OGD group ($P<0.001$). No significant change was observed in the expression of Bcl-2 or Bcl-2/Bax ratio in presence of 100 µmol SN in NO/NG-SN group vs. NO/NG but in SN pretreated OGD group, higher levels of Bcl-2 (chart was not shown) and Bcl-2/Bax ratio were observed vs. OGD group ($P<0.001$).

Effect of SN on the Cleavage Of Caspase3 Protein in OGD-Injured PC12

Data in Figure 2 shows the results of cleaved caspase 3 and β-actin ratio in 4 experimental groups. After oxygen and glucose deprivation, the ratio increased 4-fold versus NO/NG control group ($P<0.001$) and in the presence of 100 µmol SN, this ratio decreased to near normoxic normoglycemic levels, in NO/NG and NO/NG treated with SN groups.

Effect of SN on Mitochondrial Vital Activity in OGD-Injured PC12

We measured the mitochondrial vital activity in all experimental groups by using the Mito Tracker Green fluorescent label. As shown in Figure 3, the fluorescence intensity decreased to 70% in OGD vs. NO/NG group ($P<0.001$). Pretreatment with SN could significantly elevate the activity of mitochondria vs. OGD group ($P<0.001$). No significant change was observed in NO/NG-SN group vs. NO/NG group ($P=0.120$).

Effect of SN on Cell Viability in OGD-Injured PC12

The MTT assay results showed a significant decrease in the percentage of live cells in OGD group vs. NO/NG group ($P<0.001$). Pretreatment with 100 µM SN in OGD-SN led to a 50% increase in cell viability vs. OGD group. The number of live cells has no difference in NO/NG-SN vs. NO/NG ($P=0.232$) (Figure 4).

Discussion

There is a concept that reperfusion creates a high-risk condition for the tissues after ischemia, particularly in the brain (13). The role of endogenous NO in ischemic-

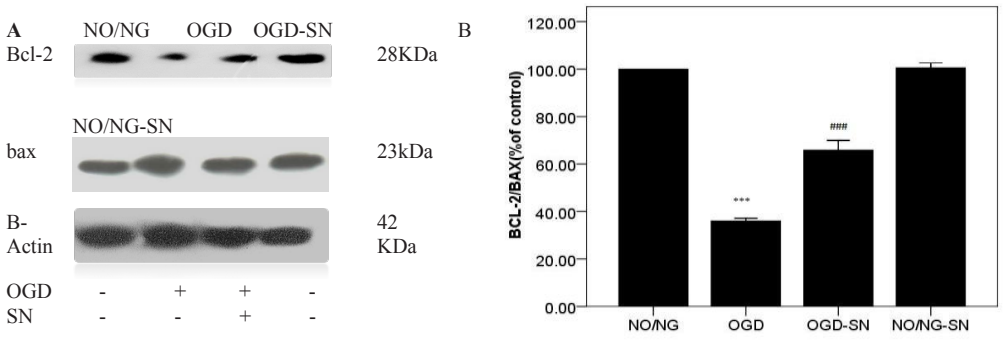

Figure 1. Changes in the Expression of Bcl-2 and Bax in Four Experimental Groups.
(A) Immunoblotting scanned images of the expression of Bcl-2, Bax and B-Actin proteins in 4 experimental groups. (B) The bar chart indicates the quantified protein bands of blot imaging. NO/NG normoxic and normoglycemic group. OGD oxygen and glucose deprivation (4 hours) group, SN pretreated group (100 µm). ***P <0.001 vs. NO/NG control group. ### P < 0.001 vs. OGD group. P < 0.05 considered significant.

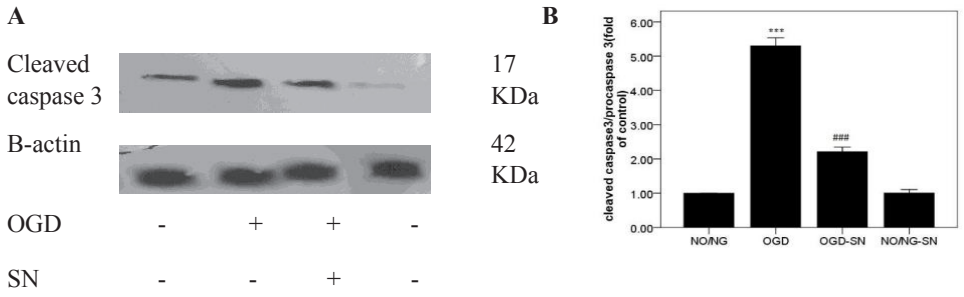

Figure 2. Activation of Caspase 3 in 4 Experimental Groups.
(A) immunoblotting scanned images of cleaved caspase 3 protein expression levels in 4 experimental groups. (B) The bar chart indicates the quantified protein bands of blot imaging. NO/NG normoxic and normoglycemic group. OGD (4 hours) group, SN pretreated group (100 µm). ***P <0.001 vs. NO/NG control group. ### $P < 0.001$ vs. OGD group. $P < 0.05$ considered significant

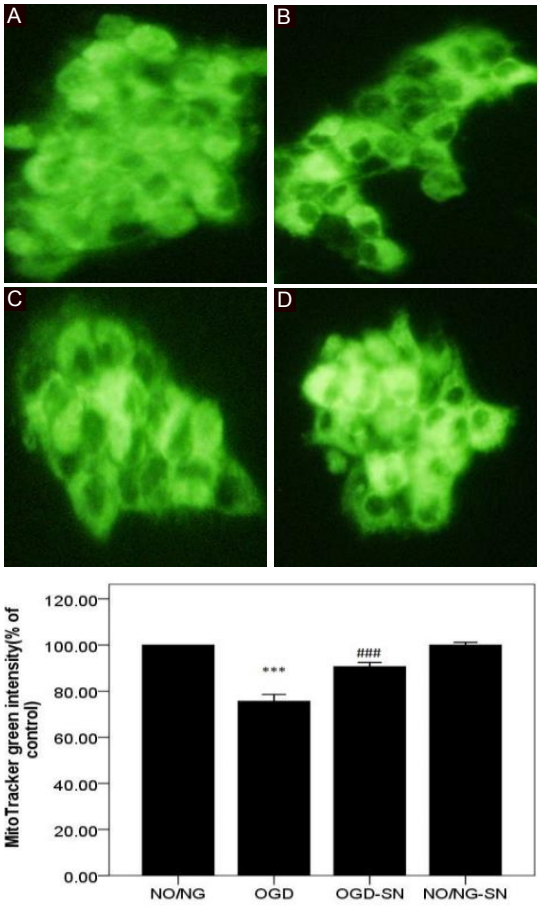

Figure 3. Mitochondrial Vital Activity in 4 Experimental Groups.
(A) NO/NG control PC12 cells show complete uptake of Mito Tracker Green florescence and the full brightness in cells. (B) The same culture as in A with OGD induction for 4 hours. Note the significant loss of brightness in comparison with A. (C) The same culture as in B with 100 µM SN, showing further staining in comparison with B. (D) The same culture as in A, with 100 µM SN. See that the view of cells is similar to the culture A, even more radiant.

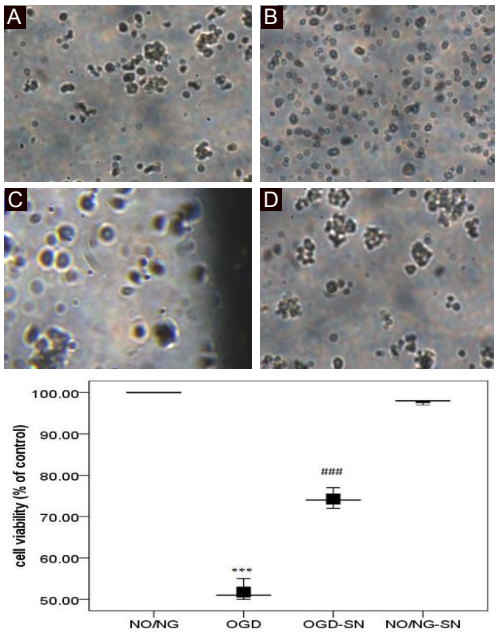

Figure 4. Mitochondrial Vital Activity in 4 Experimental Groups.
(A) Notice that the well developed and attached cells are observed in NO/NG control PC12 cells. (B) The same culture condition as in A with OGD induction for 4 hours. (C) The same culture condition as in B with 100 µM SN, showing lower cell death and more attachment to flask in comparison with B. (D) The same culture condition as in A, with 100 µM SN. Notice that all the cells in this field of view are healthy, developing and all together similar to A.

reperfusion (I/R) models has been well studied (12,18); however, the exact underlying mechanism is still not completely clarified. A recently accomplished study on the role of NO in I/R injury revealed its importance in the management of focal cerebral ischemia (24). In the current study, the neuroprotective effect of SN as an NO donor was investigated in OGD-injured PC12 cells. Data showed an elevated cell viability which, at least partially, resulted from increase in Bcl-2 expression and restoration of mitochondrial vital activity along with a decrease in intracellular caspase 3 activity.

In the past decade, there has been some in vitro and in vivo evidence on the effects of nitrite on cardiac I/R injury (20,35,36). For instance, 0.7-7 mg/L administration of nitrite in the Langendorff apparatus during cardiac ischemia improved left ventricular function and reduced infarct size in rats (20). Furthermore, a similar protection

was reported in another investigation in an in vivo canine model (36) and administration of nitrite with a concentration of 48 nmoL decreased the ischemic area size in mice with cardiac infraction (35). The nitrate intake (1 g/L in drinking water for 7 days) resulted in reduced infarct size in myocardial IR injury (37). However, it remains debatable whether conclusions of one or even several studies on cardiac tissue are extendable to a neuronal cell in stroke-like conditions. Therefore, we provided an in vitro stoke-like model using PC12 cells. OGD is a frequently used stroke-like model for assessment of the effectiveness of a neuroprotective agent. In this study, we defined OGD using a blood glucose cutoff level of 0 mg/dL and 20% PO in isobaric condition because the stroke model of cells was associated with complete deprivation of oxygen and glucose at 1 atmosphere (38). We also showed that pretreatment with low dose of nitrate (100 μM) protected the neuronal PC12 cells from OGD injury. Surprisingly, a vegetable-rich diet in humans could contain similar dose of nitrate.

Based on data, OGD-induced PC12 cells showed lower cell viability in MTT assay test that confirms the stroke induction in cells (39). The degree of cell death in the other in vitro models has some differences. These conflicting results can be in part related to the OGD media and duration (40). Moreover, the OGD induction methods and the cell type can modify the OGD –induced cell death. Cell death happens in two ways, necrosis or apoptosis, and the details of both have been reviewed frequently (44). Our results showed OGD injured cell had the lower Bcl-2 level, unchanged Bax expression and lower Bcl-2 to Bax ratio as well as higher cleaved caspase 3 along with lower mitochondrial vital activity and thus the involvement of programmed cell death in OGD model was confirmed.

Programmed cell death, also called apoptosis, is controlled by many apoptosis-related proteins such as Bcl-2 family proteins consisting of Bcl-2, Bax, Bad and Bcl-xL. Bcl-2 and Bax levels are directly related to the occurrence of apoptosis. Upon the increase in the expression of Bcl-2, Bcl-2 binds to Bax to form heterodimers and inhibits the apoptosis. When Bcl-2/Bax ratio increases, Bax forms hemodimers (7) which act as a potent activator of caspase-3, the executor of apoptosis (23). Activation of Caspase-3 results from proteolysis of procaspase-3 and formation of active fragment of cleaved Caspase-3 (19) directly reflecting cellular apoptosis (20).

In the present study, the effects of OGD in the form of downregulation of Bcl-2 protein, decrease in mitochondrial vital activity and upregulation of cleaved Caspase-3 were significantly reversed by SN treatment, suggesting neuroprotective effect of SN. Moreover, recently a crosstalk between apoptosis and autophagy in PC12 cells has been reported that is mediated by Bcl-2 family (45).

Overall, it seems that the neuroprotective effect of nitrite treatment may be partially related to the mitochondrial protective properties of nitrite (46). All findings rely on the effect of SN on intrinsic apoptosis pathway and thus restoring mitochondrial vital activity as a major energy source needed for cell viability. In the last years, studies in a number of laboratories and in a number of animal models have demonstrated that nitrite is a potent neuroprotective agent during focal ischemia/reperfusion injury of brain (33,46). Previous studies have reported that nitrite intake improves insulin signaling and activates Akt as a classic cell survival pathway.

Since nitrite can be reduced to NO in vitro, it is known as a NO donor (47,48) but regarding the fact that the mentioned event happens at low oxygen and pH conditions (33,36) it could be an unrealistic assumption when considering biological situations (47-50) but recently the measurement of the mitochondrial pH by new probes (SypHer and mito-roGFP1) in myocytes revealed a rapid decrease in pH in the first minutes of ischemia that improved to near-normal levels after 15 minutes of reperfusion. It was revealed that after oxygen and glucose deprivation, the fall in mitochondrial pH probably makes the media suitable for the reduction of nitrite to NO in mitochondria (51). This point requires further elucidation.

On the other hand, in neurons, nitrite-derived NO can inhibit nuclear translocation of NF-κB as an apoptosis related transcription factor by the following mechanisms: 1) It enters the intracellular space by diffusion, where IκB kinase is located and inhibit it (45); This inhibition prevents phospho-Iκb production and its degradation; 2) It stabilizes the Iκb and NF-κB complex (2). 3) It is capable of nitrosylating NF-κB subunites (p65and p50) (31). All of these events downregulates NF-κB target genes that are generally inflammatory and apoptotic genes (44). There is other evidence which shows that the cerebral I/R induced-nNOS inhibits phosphorylation of c-Jun, and pro-apoptotic members of Bcl-2 family and thus downregulates neuronal apoptosis (45). NO is also capable of nitrosylating caspase-3 directly (37). However, all of these effects reduce brain damage after the ischemic reperfusion, suggesting nitrite and its derivatives as neuroprotective agents. This complex network needs additional elucidation, more experimental studies and clinical trials.

Some limitations can affect interpretation of the data, which should be noticed. First, we did not measure the levels of DNA fragmentation to confirm the occurrence of apoptosis, however, cleavage of caspase 3 is counted as a point of no return for apoptosis (52). Second, we have no access to the high-tech instrument for measuring the mitochondrial inner status.

Conflict of Interests

The authors declare that they have no competing interests.

Ethical Issues

Not applicable.

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