

Neuroprotective effects of hydrogen sulfide on sodium azide-induced oxidative stress in PC12 cells

CHENG GAO^{1,2*}, PAN CHANG^{3*}, LIJUN YANG^{2*}, YI WANG², SHAOHUA ZHU²,
HAIYAN SHAN^{4,5}, MINGYANG ZHANG^{1,2,5} and LUYANG TAO²

¹Key Laboratory of Evidence Science, China University of Political Science and Law, Ministry of Education, Beijing 100088; ²Institute of Forensic Sciences, Soochow University, Suzhou, Jiangsu 215123; ³Central Laboratory, The Second Affiliated Hospital of Xi'an Medical College, Xi'an, Shaanxi 710038; ⁴Department of Obstetrics and Gynecology, North District of Suzhou Municipal Hospital, Suzhou, Jiangsu 215000, P.R. China; ⁵Department of Cell Biology and Neuroscience, Rutgers, The State University of New Jersey, Piscataway, NJ 08854, USA

Received April 16, 2017; Accepted October 19, 2017

DOI: 10.3892/ijmm.2017.3227

Abstract. Alzheimer's disease (AD) is the most common neurodegenerative disorder, responsible for >50% of all dementia cases. Sodium azide (NaN_3) inhibits cytochrome oxidase by irreversibly binding to the heme cofactor and selectively reducing the complex IV activity, which is present in post-mortem AD brains. Previous data demonstrated that hydrogen sulfide (H_2S), the third endogenous gaseous mediator, exerted protective effects against neuronal damage. Therefore, it was hypothesized that H_2S may be able to scavenge excess reactive oxygen species (ROS), thereby protecting against oxidative stress and cell death. In the present study, it was observed that cell viability decreased in a concentration-dependent manner 12 h after NaN_3 treatment (20, 30 and 50 mmol/l). A decrease in cell viability (to $51 \pm 3\%$) was observed 12 h after treatment with 30 mM NaN_3 . NaN_3 treatment also led to decreased mitochondrial membrane potential, increased lipid peroxidation (excessive production of malondialdehyde), and increased the protein expression levels of caspase-3. Pretreatment with H_2S (200 $\mu\text{mol/l}$) attenuated NaN_3 -mediated apoptosis, and the anti-apoptotic action of H_2S was partially dependent on suppressing the production

of ROS. The findings of the present study suggested that H_2S exerted a neuroprotective effect against NaN_3 -induced neurotoxicity through mechanisms related to anti-oxidation and anti-apoptosis. Therefore, the findings of the present study suggest there may be a promising future for H_2S -based preventions and therapies for neuronal damage following exposure to NaN_3 .

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder, accounting for 50-70% of all dementia cases and affecting >12 million individuals worldwide. AD is an age-related neurodegenerative illness characterized by a progressive decline in cognitive functions, and histopathologically by the presence of neuritic plaques containing amyloid- β ($\text{A}\beta$) peptide and neurofibrillary tangles, mostly consisting of hyperphosphorylated Tau protein (1). AD exists in familial (early- and late-onset) forms, as well as a sporadic form, which is very common, accounting for ~90% of all cases. AD has a complex multietiological origin; however, its exact genetic and environmental causative factors are unknown. Increasing evidence indicates that different processes contribute to neurodegeneration, including mitochondrial dysfunction, oxidative stress and impairment of the autophagic/lysosomal/endosomal system (2,3). Numerous studies have demonstrated that oxidative stress is one of the initiating events and one of the molecular changes underlying the pathogenesis of AD (4,5).

Oxidative stress may result from suppression of mitochondrial function. Cytochrome oxidase (COX) is a mitochondrial enzyme that plays an important role in aerobic energy metabolism and mitochondrial function. Mitochondrial abnormalities, particularly of COX, are found in the brains of subjects with AD (6). The principal toxic action of sodium azide (NaN_3) is inhibiting the function of COX in the mitochondrial electron transport chain (7). The model of brain mitochondrial COX inhibition by NaN_3 was described in rats in an attempt to mimic the morphological and behavioral pathology of AD (8). The involvement of mitochondrial dysfunction and the consequent overproduction of reactive oxygen species (ROS) is

Correspondence to: Dr Mingyang Zhang, Institute of Forensic Sciences, Soochow University, 178 East Ganjiang Road, Suzhou, Jiangsu 215123, P.R. China
E-mail: mingyangzhang@suda.edu.cn

Dr Haiyan Shan, Department of Obstetrics and Gynecology, North District of Suzhou Municipal Hospital, 242 South Guangji Road, Suzhou, Jiangsu 215000, P.R. China
E-mail: gghostqth@163.com

*Contributed equally

Key words: hydrogen sulfide, sodium azide, oxidative stress, PC12 cells

increasingly recognized and widely accepted as an etiopathological factor of AD. The tissue-specific inhibition of COX by NaN_3 may serve as a useful tool for the evaluation of AD *in vivo* and *in vitro*.

Hydrogen sulfide (H_2S), is considered as the third most abundant endogenous signaling gasotransmitter following nitric oxide (NO) and carbon monoxide, which affects physiological and pathophysiological processes in a wide range of biological systems (9). H_2S is produced endogenously in mammals, including humans. In particular, cystathionine- β -synthase (CBS) in the central nervous system (CNS) and cystathionine- γ -lyase in the cardiovascular system are the key enzymes that are mainly responsible for the endogenous generation of H_2S (10). 3-Mercaptopyruvate sulfurtransferase (3-MST) is also known to be a significant producer of endogenous H_2S in the brain (11). Increasing evidence demonstrates that H_2S is associated with AD pathogenesis (12). The dysfunction of CBS in the transsulfuration pathway may lead to a decrease in H_2S production in AD (13). Interestingly, the levels of H_2S are severely decreased in AD patients; moreover, plasma H_2S levels are negatively correlated with the severity of the disease in AD patients (14). Moreover, our previous research demonstrated downregulation of the expression and activity of CBS and 3-MST in neuron-like rat pheochromocytoma (PC12) cells induced by NaN_3 (data not shown). However, it is not known whether H_2S has any therapeutic benefits in AD. Therefore, the present study was undertaken to assess the beneficial effects of sodium hydrosulfide (NaHS), which is an exogenous H_2S donor, on the underlying cellular and molecular mechanisms in neuronal cells treated with NaN_3 .

The characteristics of neuronal damage induced by NaN_3 treatment were first investigated. The neuroprotective activity of H_2S and its effect on oxidative stress were also investigated in PC12 cells with neuronal damage induced by NaN_3 . To the best of our knowledge, this is the first study to demonstrate that H_2S can suppress NaN_3 -induced oxidative stress and apoptosis in PC12 cells. This study was conducted to gain better insight into the physiological functions of H_2S under normal and injury conditions, and its association with the cellular and molecular mechanisms underlying nervous system disease.

Materials and methods

Cell culture. Rat pheochromocytoma PC12 cells were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). PC12 cells were grown on polystyrene tissue culture dishes in DMEM containing 10% horse serum and 5% fetal bovine serum (FBS; Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, China), supplemented with 2 mmol/l glutamine, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 100 U/ml penicillin (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C with 95% air, 5% CO_2 . Prior to differentiation, the medium was exchanged twice a week and the cultures were subcultured at a ratio of 1:4 once a week. For differentiation, the cells were washed and incubated in a fresh medium containing nerve growth factor (NGF; final concentration, 50 ng/ml) for 48 h at 37°C in a cell incubator. The concentration of NGF was maintained throughout all

experiments. All the experiments were performed on cells between passages 3-8.

Cell injury model. The injury model was constructed as follows: briefly, the DMEM was removed, PC12 cells were washed twice with glucose-free Earle's balanced salt solution (EBSS) at pH 7.5, and maintained in glucose-free DMEM without FBS. Subsequently, neurotoxic damage was induced by adding the indicated concentration of NaN_3 to the cultured cells for different periods of time. The cells were preincubated with the indicated concentrations of NaHS (donor of H_2S) for 30 min prior to NaN_3 treatment and maintained throughout the entire experiment. NaHS was dissolved in saline and was freshly prepared immediately prior to use. The stock solutions were directly added into the bath solution to achieve the final concentration. Control cultures were maintained in DMEM for the same duration under normoxic conditions. The concentrations of all the reagents were maintained throughout the injury period.

Determination of cell viability. The viability of PC12 cells was determined using the Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan), according to the manufacturer's instructions. PC12 cells were cultured in 96-well plates at 37°C under an atmosphere of 5% CO_2 and 95% air. At the end of treatment, CCK-8 reagent (10 μl) was added to each well and the plates were then incubated at 37°C for 3 to 4 h in the incubator. Absorbance at a wavelength of 450 nm was measured with a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). The means optical density (OD) from 6 wells in the indicated groups were used to calculate the cell viability, which was expressed as a percentage of cell survival rate compared with the control. All the experiments were performed in triplicate and repeated three independent times.

Determination of mitochondrial membrane potential (MMP). MMP was examined by staining PC12 cells with JC-1. Staining was performed using 2.5 mg/ml JC-1 at 37°C for 15 min. After staining, cells were rinsed three times with phosphate-buffered saline (PBS). A confocal laser scanning microscope was used to measure MMP using the JC-1 assay kit (C2006; Beyotime Institute of Biotechnology, Haimen, China). Under the microscope, images of different color were obtained. Green fluorescence indicated cells with low MMP ($\Delta\psi\text{m}$), revealing that JC-1 maintains (or reacquires) monomeric form, while red fluorescence indicated cells with high $\Delta\psi\text{m}$. The relative proportions of red and green fluorescence were used to measure the extent of mitochondrial depolarization.

Intracellular ROS measurement. Production of intracellular ROS was determined using the fluorescent probe dichlorofluorescein diacetate (DCFH-DA), which can cross cell membranes and is subsequently hydrolyzed by intracellular esterase to non-fluorescent DCFH (Beyotime Institute of Biotechnology). Following treatment with NaN_3 for 12 h in the presence or absence of 200 mM H_2S , the culture medium was changed to fresh DMEM containing 10 μM DCFH-DA for 30 min in an incubator at 37°C in the dark. After washing three times

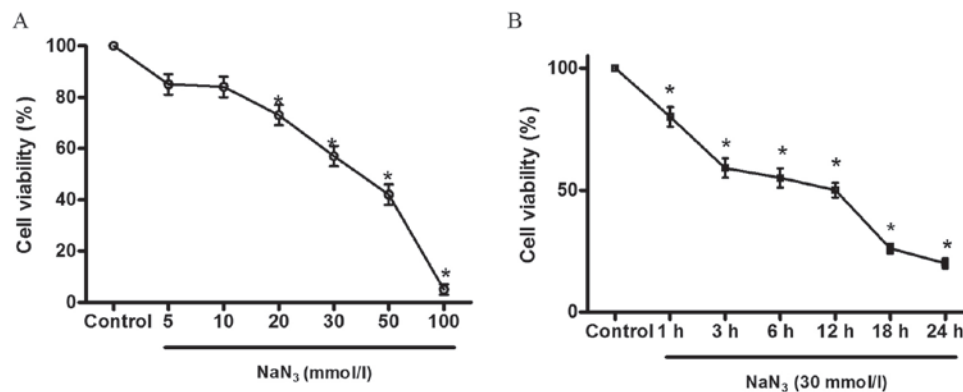


Figure 1. Concentration- and time-dependent effect of sodium azide (NaN₃) on cell viability in PC12 cells. (A) PC12 cells were treated with 5, 10, 20, 30, 50 and 100 mmol/l NaN₃ for 12 h, and cell viability was determined by the Cell Counting Kit-8 (CCK-8) assay. Exposure of PC12 cells to NaN₃ for 12 h induced a decrease in cell viability in a concentration-dependent manner. (B) PC12 cells were treated for 1, 3, 6, 12, 18 and 24 h with 30 mmol/l NaN₃, and cell viability was determined by the CCK-8 assay. Exposure of PC12 cells to 30 mmol/l NaN₃ induced a decrease in cell viability in a time-dependent manner. Columns represent means $\pm$ standard error of the mean of viable cells (n=6), and expressed as percentage of control values. *P<0.05 vs. control group.

with PBS, the cells were observed under a fluorescence spectrophotometer with an excitation wavelength of 488 nm and an emission wavelength of 535 nm.

Measurement of lipid peroxidation. Malondialdehyde (MDA), a terminal product of lipid peroxidation, was measured to estimate the extent of lipid peroxidation in PC12 cells. The cells were then homogenized in lysis buffer [1% NP-40, 50 mmol/l Tris, pH 7.5, 5 mmol/l EDTA, 1% sodium dodecyl sulphate (SDS), 1% sodium deoxycholate, 1% Triton X-100, 1 mmol/l phenylmethanesulfonylfluoride, 10 μ g/ml aprotinin, and 1 μ g/ml leupeptin] and clarified by centrifuging for 20 min in a microcentrifuge at 4°C. MDA concentration in cell homogenates was determined with an MDA assay kit (S0131; Beyotime Institute of Biotechnology), using the thiobarbituric acid method. The assay was based on the ability of MDA to form a conjugate with thiobarbituric acid and create a red product, which has maximum absorbance at 532 nm.

Western blot analysis. The cells were then homogenized in lysis buffer (1% NP-40, 50 mmol/l Tris, pH 7.5, 5 mmol/l EDTA, 1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 1 mmol/l phenylmethanesulfonylfluoride, 10 μ g/ml aprotinin, and 1 μ g/ml leupeptin) and clarified by centrifuging for 20 min in a microcentrifuge at 4°C. Following determination of its protein concentration with the Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA), the resulting supernatant (50 μ g of protein) was subjected to SDS polyacrylamide gel electrophoresis. The separated proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) by a transfer apparatus at 90V for 1 h. The membrane was then blocked with 5% non-fat milk and incubated with primary antibody against caspase-3 (1:500, cat. no. BS1518) and Bcl-2 (1:500, cat. no. BZ00479) (both from Bioworld Technology, Inc., St. Louis Park, MN, USA) or GAPDH (1:1,000, cat. no. G8795; Sigma-Aldrich; Merck KGaA, St. Louis, MO, USA). After incubating with an anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody, protein was visualized using an enhanced chemiluminescence system (ECL; cat. no. 32106; Pierce; Thermo Fisher Scientific, Inc., Bellefonte, PA, USA).

Immunofluorescence analysis. Immunofluorescence analysis was performed as follows: the PC12 cell in a 24-well plate were fixed with 4% paraformaldehyde for 15 min at room temperature and washed with PBS three times, for 10 min each time. After the cells were prepared, they were blocked with 5% donkey serum (Gibco; Thermo Fisher Scientific, Inc.) with 0.3% Triton X-100 and 5% bovine serum albumin (BSA) for 2 h at room temperature and incubated with rabbit polyclonal primary anti-caspase-3 antibodies (1:50, cat. no. ab13847; Abcam, Cambridge, UK). Briefly, the cells were incubated with the primary antibodies overnight at 4°C, followed by a mixture of fluorescein isothiocyanate-conjugated secondary antibodies for 2 h at room temperature, then washed with PBS three times, for 10 min each time. Finally, the samples were mounted with coverslips using antifade mounting medium (Beyotime Institute of Biotechnology) and observed under a fluorescence microscope (Eclipse Ti-S; Nikon, Tokyo, Japan). At least three random slides from each group were examined.

Statistical analysis. All statistical analyses were conducted with SPSS statistical software 16.0 (SPSS, Inc., Chicago, IL, USA). All the values are expressed as means $\pm$ standard error of the mean. The statistical significance of differences between groups was determined by one-way analysis of variance followed by Tukey's post hoc multiple comparison tests or Student's t-test (two means comparison). P<0.05 was considered to indicate statistically significant differences. Each experiment consisted of at least three replicates per condition.

Results

NaN₃ is cytotoxic to PC12 cells. Cell cytotoxicity was evaluated by the CCK-8 assay following incubation of PC12 cells with increasing concentrations of NaN₃ from 5 to 100 mmol/l at 1, 3, 6, 12, 18 and 24 h. As shown in Fig. 1A, at concentrations of 5-100 mmol/l, treatment of PC12 cells with NaN₃ for 12 h led to a concentration-dependent reduction in cell viability. The results demonstrated that the general trend of cell survival rate decreased with increasing treatment time (Fig. 1B).

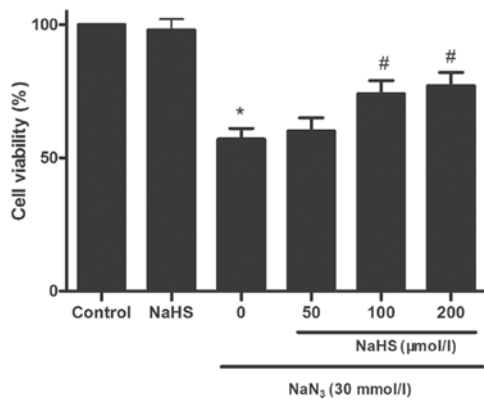


Figure 2. Protective effect of hydrogen sulfide (H_2S) against sodium azide (NaN_3)-induced cytotoxicity in PC12 cells. PC12 cells were treated with NaN_3 (30 mmol/l) in the absence or presence of the H_2S donor sodium hydrosulfide (NaHS; 50, 100 and 200 μ mol/l) for 12 h, and cell viability was then determined by the Cell Counting Kit-8 (CCK-8) assay. Exposure of PC12 cells to NaN_3 induced a decrease in cell viability. There was no effect on cell viability with NaHS pretreatment at a concentration of 50 mmol/l. With NaHS pretreatment at a concentration of 100 and 200 μ mol/l, cell viability following injury increased in a concentration-dependent manner. Values are expressed as mean $\pm$ standard error of the mean ($n=6$). * $P<0.05$ vs. control group; # $P<0.05$ vs. NaN_3 alone group.

H_2S protects PC12 cells against NaN_3 -induced cytotoxicity. To investigate the effect of H_2S on NaN_3 -induced cytotoxicity, cell viability was analyzed by determining the percentage of CCK-8 reduction. As shown in Fig. 2, treatment with NaN_3 at concentrations of 30 mmol/l for 12 h attenuated cell viability. The cytotoxic effects of NaN_3 on PC12 cells were significantly prevented by pretreatment with NaHS at 100–200 μ mol/l for 30 min. At 200 μ mol/l, NaHS alone did not measurably affect the viability of PC12 cells.

H_2S exerts a protective effect against NaN_3 -induced dissipation of the mitochondrial membrane potential. To confirm that NaN_3 induced a mitochondrial membrane potential reduction in PC12 cells, laser microscopy was used to visualize the fluorescence dye-stained mitochondria. When PC12 cells were exposed to NaN_3 , the mitochondrial membrane rapidly depolarized, as shown by the increase in green fluorescence (Fig. 3C). Pretreatment with NaHS reduced the changes in mitochondrial membrane potential, as indicated by repression of green fluorescence and restoration of red fluorescence (Fig. 3D). The quantitative analysis of the red/green ratios also demonstrated the protective role of H_2S in NaN_3 -induced a mitochondrial membrane potential reduction (Fig. 3E).

H_2S reduces NaN_3 -induced intracellular ROS accumulation in PC12 cells. The mitochondrion is considered to be the main site of ROS production, and an increased ROS level within the cell reflects mitochondrial dysfunction. Therefore, the effect of H_2S on ROS levels was investigated. *N*-acetyl-L-cysteine (NAC) is commonly used to identify and test ROS inducers and to inhibit ROS as a positive control. Intracellular ROS accumulation may be measured by the use of DCF-DA, which freely crosses the cell membrane. Once inside the cells, the compound is hydrolyzed by cellular esterase to DCF, which interacts with peroxides forming fluorescent 2',7'-dichlorofluorescein. PC12 cells treated with

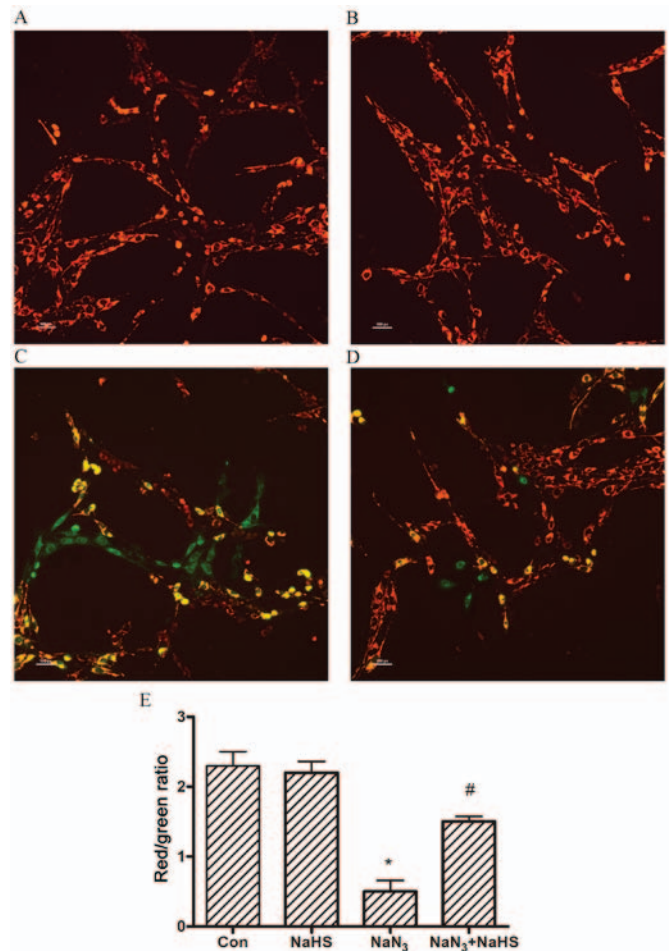


Figure 3. Effect of hydrogen sulfide (H_2S) on the mitochondrial membrane potential. Laser microscope images of JC-1 fluorescence (as an indicator of mitochondrial membrane potential) in PC12 cells after a 12-h exposure to NaN_3 alone or in combination with 200 μ mol/l sodium hydrosulfide (NaHS). (A) Control, (B) 200 μ mol/l NaHS, (C) 30 mmol/l NaN_3 , (D) 30 mmol/l NaN_3 + 200 μ mol/l NaHS, and (E) the bar chart demonstrates the quantitative analysis of the red/green ratio. The data are expressed as means $\pm$ standard error of the mean ($n=6$). * $P<0.05$, significantly different from the control group; # $P<0.05$, significantly different from the NaN_3 group. Scale bars, 30 μ m.

NaN_3 displayed intense fluorescence after staining with DCF dye (Fig. 4C). Intracellular ROS accumulation resulting from NaN_3 treatment was significantly reduced when H_2S (Fig. 4D) or NAC (Fig. 4E) was present in the medium.

H_2S attenuates NaN_3 -induced lipid peroxidation increase in PC12 cells. When PC12 cells were exposed to NaN_3 (30 mmol/l) for 12 h, an increase in the lipid peroxidation level, as indicated by the excessive formation of MDA in PC12 cells, was observed to 150% of control values (Fig. 5). Pretreatment with H_2S at concentrations of 200 μ mol/l significantly decreased lipid peroxidation (decrease in the formation of MDA) compared with the levels observed in the NaN_3 group.

H_2S suppresses NaN_3 -induced apoptosis in PC12 cells. To certify the effects of H_2S on NaN_3 -induced apoptosis, the following studies were performed: caspase-3 is a critical marker of apoptosis. To determine the effects of NaN_3 on

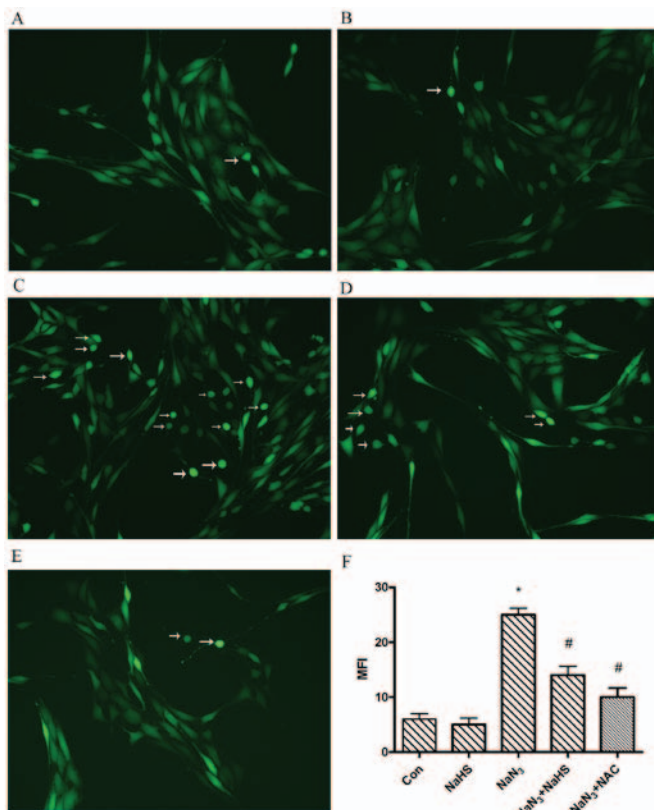


Figure 4. Protective effect of hydrogen sulfide (H_2S) on sodium azide (NaN_3)-induced intracellular reactive oxygen species (ROS) accumulation. Intracellular peroxide levels were determined based on the dichlorofluorescein (DCF) fluorescence, as described in Materials and methods. Cells were plated and grown for 24 h and were then exposed to NaN_3 in the presence or absence of NaHS. (A) Control, (B) 200 μ mol/l NaHS, (C) 30 mmol/l NaN_3 , (D) 30 mmol/l NaN_3 + 200 μ mol/l NaHS, (E) 30 mmol/l NaN_3 + N-acetyl-L-cysteine (NAC), and (F) the bar chart demonstrates the quantitative analysis of median fluorescence intensity (MFI). NAC is commonly used to identify and test ROS inducers, and to inhibit ROS. The data are expressed as means $\pm$ standard error of the mean (n=6). *P<0.05, significantly different from the control group; #P<0.05, significantly different from the NaN_3 group. Arrows indicate DCF-positive cells. Scale bars, 50 μ m.

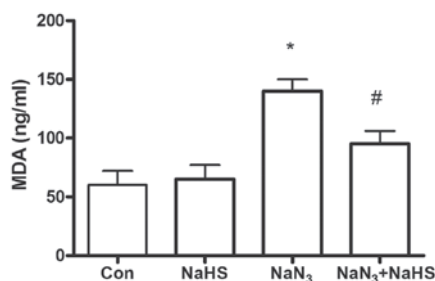


Figure 5. Effect of hydrogen sulfide (H_2S) on malondialdehyde (MDA) formation induced by sodium azide (NaN_3) in PC12 cells. MDA is a by-product of a free radical attack on lipids. PC12 cells were treated with NaN_3 (30 mmol/l) in the absence or presence of sodium hydrosulfide (NaHS; 200 μ mol/l) for 12 h, and MDA was then determined by the thiobarbituric acid assay. Values are expressed as means $\pm$ standard error of the mean (n=6). *P<0.05 vs. control group; #P<0.05 vs. NaN_3 alone group.

cell apoptosis and the response of H_2S to the effects of NaN_3 , the levels of caspase-3 and Bcl-2 expression were measured by western blot analysis. As illustrated in Fig. 6, 12-h treatment with NaN_3 (30 mmol/l) significantly increased the

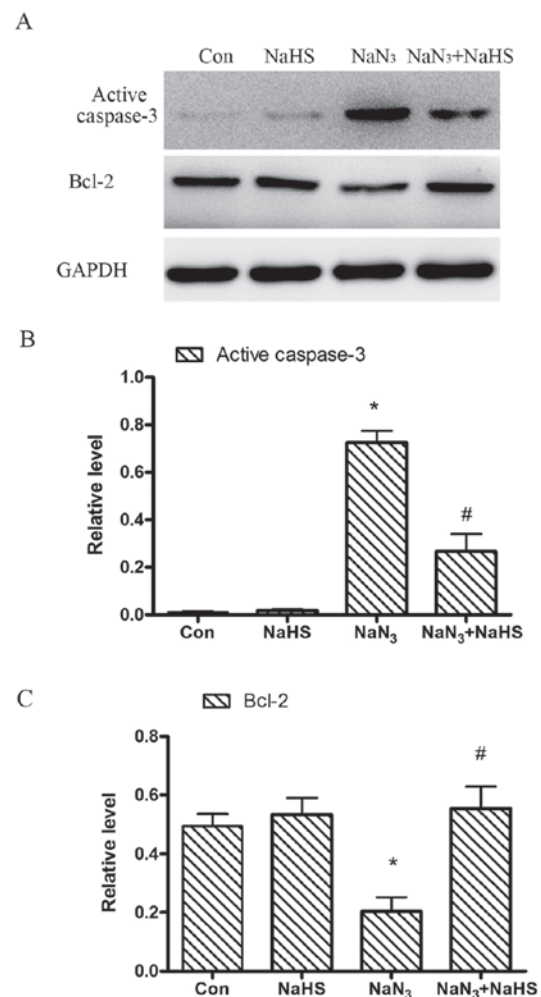


Figure 6. Effect of hydrogen sulfide (H_2S) on sodium azide (NaN_3)-induced apoptosis-associated protein expression in PC12 cells. (A) Sample immunological probe for caspase-3, Bcl-2 and GAPDH. The bar chart demonstrates the ratio of caspase-3 or Bcl-2 relative to β -actin for different concentrations of NaN_3 . (B) A 12-h treatment with NaN_3 (30 mmol/l) significantly increased the amount of caspase-3 expression, but (C) decreased the Bcl-2 expression. However, co-treatment with 200 μ mol/l sodium hydrosulfide (NaHS) for 12 h significantly abolished the NaN_3 -induced increase in caspase-3 expression and decrease in Bcl-2 expression. Con, control group. Semi-quantitative analysis (relative optical density) of the intensity of staining of caspase-3 or Bcl-2 to β -actin for different concentrations of NaN_3 . The data are expressed as means $\pm$ standard error of the mean (n=3). *P<0.05, significantly different from the control group; #P<0.05 vs. NaN_3 alone group.

amount of caspase-3 expression (Fig. 6B), but decreased Bcl-2 expression (Fig. 6C). However, co-treatment with 200 μ mol/l NaHS for 12 h significantly abolished the NaN_3 -induced decrease in Bcl-2 expression and increase in caspase-3 expression. These results indicate that H_2S is able to block the NaN_3 -elicited downregulation of Bcl-2 expression and upregulation of caspase-3 expression. In addition, we observed the effect of NaHS on autophagic cell death in PC12 cells treated with NaN_3 by caspase-3 staining. As shown in Fig. 7, at 12 h after treatment with NaN_3 (30 mmol/l), the number of caspase-3-positive cells observably increased. Co-treatment with NaHS (200 μ mol/l) significantly ameliorated the NaN_3 -induced increase of caspase-3-positive cells. The control and NaHS-treated groups displayed few caspase-3-positive cells.

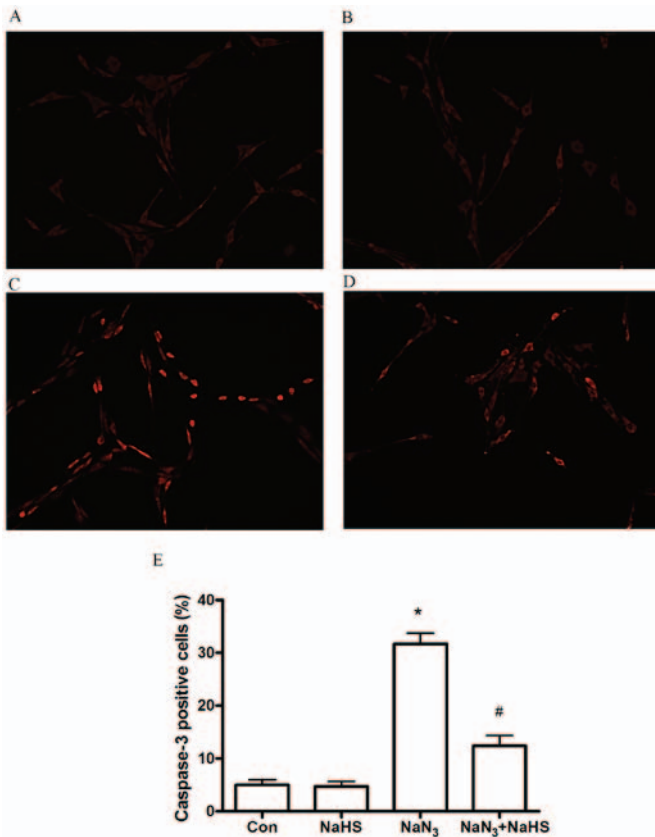


Figure 7. Effect of hydrogen sulfide (H_2S) on sodium azide (NaN_3)-induced apoptosis in PC12 cells. Apoptosis was visualized as staining for caspase-3 in PC12 cells. (A) Control group, (B) sodium hydrosulfide (NaHS) group, (C) NaN_3 group, (D) NaN_3 + NaHS group and (E) the bar chart demonstrates the quantitative analysis of caspase-3-positive cells among the groups. The data are expressed as means $\pm$ standard error of the mean (n=3). * $P < 0.05$, significantly different from the control group; # $P < 0.05$ vs. injury group. Scale bars, 50 μm .

Discussion

Endogenous H_2S may have multiple physiological functions in the brain. Our previous study demonstrated that H_2S improved spatial memory impairment and alleviated cerebral edema in traumatic brain injury (TBI) mice (15,16). A number of epidemiological studies provided compelling evidence that sustaining a TBI is associated with increased risk for degenerative conditions that may result in dementia, including AD; however, several of the underlying mechanisms have yet to be fully elucidated. A complex disease such as AD involves multiple interwoven pathways leading to neuronal damage, whereas increasing evidence suggests that oxidative stress is one of the initiating events (17,18). The aim of the present study was to examine the cell damage occurring in cultured PC12 cells when exposed to NaN_3 . Due to its ability to induce oxidative stress through inhibition of the electron transfer between COX and oxygen, this model represents an interesting tool in neurotoxicity studies. However, to the best of our knowledge, no study to date has investigated whether H_2S can prevent cytotoxicity induced by NaN_3 in PC12 cells, and whether oxidative stress plays an important role in NaN_3 -induced apoptosis. In the present study, the possible molecular mechanisms underlying the neuroprotective effects of H_2S against NaN_3 -induced neuron

cell injury were investigated. Furthermore, we investigated the connection between the generation of ROS and the activity of caspase-3 in NaN_3 -induced apoptosis in PC12 cells and demonstrated a concentration- and time-dependent reduction of cell viability induced by NaN_3 . Our findings demonstrated that NaHS, a H_2S donor, attenuated NaN_3 -mediated apoptosis, and the anti-apoptotic action of H_2S was partly dependent on suppressing the production of ROS and inhibition of caspase-3 activity, and was associated with increasing the expression of the anti-apoptotic protein Bcl-2.

AD is a multifactorial neurodegenerative disorder. Among the numerous contributing factors, cellular stress and, in particular, oxidative stress, have attracted considerable attention, as several studies reported its involvement in AD pathogenesis (19,20). The evidence of oxidative damage in the postmortem AD brain is quite compelling, with significant accumulation of markers of oxidative damage of lipids, proteins and DNA, increased accumulation of transition metals, such as Fe, Cu and Zn, as well as impaired antioxidant defense (21). The recent redox proteomics analysis of the postmortem AD brain has demonstrated oxidative damage to key enzymes involved in energy metabolism, neurotransmitter-related proteins, mitochondrial proteins and proteasomal components (22). Multiple lines of evidence indicate that oxidative stress is an early event in AD, occurring prior to cytopathological changes, and may therefore play a key pathogenic role in the disease (20,23). Oxidative stress is the production of ROS in amounts exceeding the ability of the body's antioxidant systems to counteract their effects (24). These free radical species, which contain one or more unpaired electrons, act as electron donors, causing oxidation that potentially leads to damage of body macromolecular polymers, such as lipids, proteins and nucleic acids (25). The most important of all cell targets of ROS are nervous system cells, particularly neurons, which are highly susceptible to the harmful effects of ROS (26).

NaN_3 , as a COX inhibitor, has been extensively considered as a useful tool to study different pathological conditions. Mitochondrial energy metabolism is suggested to be a determining element for interpreting impaired neuron function, reduced molecular turnover, and enhanced cell death (27,28). Inhibition of mitochondrial COX has been found to induce cell death in a variety of cells. Sato *et al* reported that SCC131 cells died 48–72 h after NaN_3 treatment at concentrations more than 5 mM (29). Lutton *et al* reported that NaN_3 treatment at a concentration of 1 mM induced necrosis in rat osteoclasts (30). In those studies, the longest treatment time required to induce cell death was more than 24 h. The reason for this finding may be differences between the types of cells, specifically differential sensitivity of the excretory function or the detoxification function, and the quantity of the mitochondria of the target cells. PC12 cells, which are generally considered to display neuronal-like characteristics, appear to be more sensitive to NaN_3 . To induce oxidative stress in PC12 cells, NaN_3 concentrations ranged from 1 to 10 mM in several experiments (31,32). Wang *et al* reported that the viability of PC12 cells treated with 64 mM NaN_3 for 4 h decreased by 47.8% (33). Zhang *et al* reported that cultured PC12 cells was incubated with NaN_3 20 mM for 3–24 h to induce apoptosis (34). Increased autophagy was also observed in multiple and distinct experimental injury models (35,36). We tested the 5-mM concentration of NaN_3 at

36 h. Although the result of the cell viability assay revealed that NaN_3 induced cell death, autophagic cell death was not observed under these conditions. However, it is not known whether the role of autophagy is protective or detrimental for neural cell injury. It is possible that the role of autophagy after cell injury is dependent upon the cell's capacity to respond to the cumulative burden of damaged or dysfunctional macromolecules and organelles. If the increase in autophagic capacity is insufficient, augmenting autophagy would likely be beneficial. When there is excessive increase in autophagic capacity, inhibiting autophagy may be beneficial. Thus, the role of autophagy may be dictated by whether it is able to meet intracellular demands. The cell viability data were important in order to evaluate whether cells were still physiologically responsive, or if they were likely to be entering the cell death process. Therefore, the overall toxic effects of NaN_3 was evaluated by monitoring cell viability in PC12 cells. In order to induce cell death in PC12 cells, high concentrations of NaN_3 (30 mM) were applied in our experiments. Under these more severe stress conditions, when PC12 cell viability is already severely hampered, an accumulation of autophagic cell death was observed (37). A future study is planned to focus mainly on autophagic cell death in PC12 cells induced by NaN_3 .

Mitochondrial dysfunction induced by NaN_3 provides a common platform for investigating the mechanisms of neuronal injury, which may prove useful for screening potential protective agents against neuronal death (38). Hyperoside has the neuroprotective capacity to attenuate NaN_3 -induced apoptosis in PC12 cells (34). Wang *et al* reported that aloe vera extract exerted a protective effect against mitochondrial functional impairment induced by NaN_3 in PC12 cells (33). H_2S has increasingly been recognized as a gasotransmitter of comparable importance to nitric oxide and carbon monoxide in mammalian systems. Evidence suggests that these gasotransmitters are involved in the origin of life and play key roles in the endosymbiotic events that contribute to the biogenesis and development of mitochondria. In addition to its function as a signaling molecule, H_2S also acts as a cytoprotectant in neurons and cardiac muscle (11). The neuroprotective properties of H_2S have long been observed, leading to extensive research that has been widely reported and continues to attract interest (39). In a rat model, it was demonstrated that H_2S exerts a protective effect and diminishes oxidative stress and homocysteine-induced toxicity by its antioxidant properties in the adrenal medulla and smooth muscle cells of the vesicles (40). This raises the possibility of H_2S being a possible therapeutic strategy in the treatment of neurodegenerative disorders. To investigate whether ROS are involved in NaN_3 -induced injury, PC12 cells were pretreated with NAC (a ROS scavenger) prior to exposure to NaN_3 . The cell viability data were important in order to evaluate if cells remained physiologically responsive, or if they were likely to be entering cell death. We observed that NaN_3 induced not only ROS production, but also initiated injury of PC12 cells, including a decrease in cell viability, loss of MMP and caspase-3 activation, as well as an increase in the number of apoptotic cells. These effects were significantly prevented by NAC pretreatment, indicating that NaN_3 -induced neuronal injury is due to its induction of ROS. Exogenously applied free H_2S is immediately absorbed in a sulfur store as bound sulfane sulfur (41). H_2S may be transiently stored and

then released when the cells are stimulated. H_2S is absorbed in brain homogenates more slowly compared with liver and heart homogenates, and the release from brain homogenates is also slower compared with that from liver and heart homogenates (41,42). Once H_2S is released from bound sulfane sulfur or from H_2S -producing enzymes, free H_2S may remain longer in the brain compared with the liver and the heart. Therefore, considering the slow absorption of H_2S in the brain, pretreatment with NaHS was selected. Interestingly, it was observed that NaHS (a donor of H_2S) shared similar neuroprotective properties with NAC with a comparable potency in this experimental model. This may support the ability of H_2S in: i) inhibiting NaN_3 -mediated protein cytotoxicity; ii) inhibiting NaN_3 -mediated oxidative damage; and iii) inhibiting generation of ROS induced by NaN_3 .

H_2S is synthesized in a number of different cell types and can easily diffuse without involvement of any transporters. H_2S is involved in a number of organ-specific functions, such as thermoregulation, modulating myocardial activity and bronchodilation (43). H_2S also exerts organ-protective effects in ischaemia, acting as a vasodilator and negative inotrope to reduce blood pressure (44). A number of studies have investigated the possible benefit of H_2S in hypertension, and found that H_2S donor administration significantly reduced blood pressure and oxidative stress in hypertensive mice (45,46). H_2S has also been found to play a role in the pathology and treatment of chronic obstructive pulmonary disease. Exogenously supplied H_2S may counteract the oxidative stress-mediated lung damage that occurs in allergic mice (47). Low H_2S levels have been observed in a number of different diseases, while there is evidence that H_2S may be beneficial in a number of chronic organ degenerative conditions. Chronic kidney disease is associated with a significant reduction in plasma H_2S concentration, and H_2S may ameliorate adenine-induced chronic renal failure in rats by inhibiting apoptosis through ROS signaling pathways (48). Diabetes is a chronic metabolic disease affecting the metabolism of carbohydrates and other nutrients. H_2S protects against the development of hyperglycemia-induced endothelial dysfunction by attenuating the hyperglycemia-induced enhancement of ROS formation (49). As regards degenerative diseases of the CNS, H_2S treatment can specifically inhibit 6-OHDA-evoked NADPH oxidase activation and oxygen consumption in Parkinson's disease (50). Moreover, H_2S protects neurons against oxidative stress, which is responsible for neuronal damage and degeneration in AD (51). In conclusion, H_2S donors have consistently been shown to be beneficial in acute organ injury and in chronic organ pathology through ROS signaling pathways. More specifically, data available thus far strongly suggest that H_2S may be a potent preventive and therapeutic agent used for the prevention and improvement of the symptoms of oxidative stress-associated diseases, which is worthy of further investigation in future studies.

Although H_2S exhibited promising efficacy in NaN_3 -mediated cell injury, research is still underway to identify selective oxidative stress regulators as potential treatment drugs. In summary, the evidence presented herein indicates an actively protective role for H_2S against oxidative stress and apoptosis induced by NaN_3 . Our data may provide a novel pathway to elucidate the underlying molecular and cellular mechanisms

in the CNS following inhibition of COX, and a novel strategy for the treatment of CNS diseases. Future studies attempting to characterize the functional consequences of H₂S under conditions of oxidative stress and the identification of substrates and downstream signaling targets are now possible. Other H₂S donors, such as drug-like H₂S donor ATB-346 or orally active H₂S donor SG-1002, may be used to evaluate the protective effect of H₂S in injury models in the future. Testing these drug-like H₂S donors will not only consolidate the protective effect of H₂S, but also shed light on the clinical application of H₂S as a therapeutic agent.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (grant nos. 81601306, 81301039 and 81530062); the China Postdoctoral Science Foundation Funded Project (grant no. 2015M570476); the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD); the Jiangsu Talent Youth Medical Program (grant no. QNRC2016245); the Key Laboratory of Evidence Science (China University of Political Science and Law), Ministry of Education (grant no. 2016KFKT05); the Shanghai Key Laboratory of Forensic Medicine (grant no. KF1502); and the Suzhou Science and Technology Development Project (grant no. SYSD2015119).

References

1. Takahashi RH, Nagao T and Gouras GK: Plaque formation and the intraneuronal accumulation of β -amyloid in Alzheimer's disease. *Pathol Int* 67: 185-193, 2017.
2. Martinez-Vicente M: Neuronal mitophagy in neurodegenerative diseases. *Front Mol Neurosci* 10: 64, 2017.
3. Menzies FM, Fleming A, Caricasole A, Bento CF, Andrews SP, Ashkenazi A, Füllgrabe J, Jackson A, Jimenez Sanchez M, Karabiyik C, *et al*: Autophagy and neurodegeneration: pathogenic mechanisms and therapeutic opportunities. *Neuron* 93: 1015-1034, 2017.
4. Cai Q and Tammineni P: Alterations in mitochondrial quality control in Alzheimer's disease. *Front Cell Neurosci* 10: 24, 2016.
5. Cai Q and Tammineni P: Mitochondrial aspects of synaptic dysfunction in Alzheimer's disease. *J Alzheimers Dis* 57: 1087-1103, 2017.
6. Cadonic C, Sabbir MG and Albensi BC: Mechanisms of mitochondrial dysfunction in Alzheimer's disease. *Mol Neurobiol* 53: 6078-6090, 2016.
7. Bennett MC, Mlady GW, Kwon YH and Rose GM: Chronic in vivo sodium azide infusion induces selective and stable inhibition of cytochrome *c* oxidase. *J Neurochem* 66: 2606-2611, 1996.
8. Weinstock M and Shoham S: Rat models of dementia based on reductions in regional glucose metabolism, cerebral blood flow and cytochrome oxidase activity. *J Neural Transm (Vienna)* 111: 347-366, 2004.
9. Huang CW and Moore PK: H₂S synthesizing enzymes: biochemistry and molecular aspects. *Handb Exp Pharmacol* 230: 3-25, 2015.
10. Kimura H: Hydrogen sulfide and polysulfides as biological mediators. *Molecules* 19: 16146-16157, 2014.
11. Kimura H: Hydrogen sulfide: its production, release and functions. *Amino Acids* 41: 113-121, 2011.
12. Liu Y, Deng Y, Liu H, Yin C, Li X and Gong Q: Hydrogen sulfide ameliorates learning memory impairment in APP/PS1 transgenic mice: a novel mechanism mediated by the activation of Nrf2. *Pharmacol Biochem Behav* 150-151: 207-216, 2016.
13. Dwyer BE, Raina AK, Perry G and Smith MA: Homocysteine and Alzheimer's disease: a modifiable risk? *Free Radic Biol Med* 36: 1471-1475, 2004.
14. Wei HJ, Li X and Tang XQ: Therapeutic benefits of H₂S in Alzheimer's disease. *J Clin Neurosci* 21: 1665-1669, 2014.
15. Zhang M, Shan H, Chang P, Wang T, Dong W, Chen X and Tao L: Hydrogen sulfide offers neuroprotection on traumatic brain injury in parallel with reduced apoptosis and autophagy in mice. *PLoS One* 9: e87241, 2014.
16. Zhang M, Shan H, Wang T, Liu W, Wang Y, Wang L, Zhang L, Chang P, Dong W, Chen X, *et al*: Dynamic change of hydrogen sulfide after traumatic brain injury and its effect in mice. *Neurochem Res* 38: 714-725, 2013.
17. Tayler H, Fraser T, Miners JS, Kehoe PG and Love S: Oxidative balance in Alzheimer's disease: relationship to APOE, Braak tangle stage, and the concentrations of soluble and insoluble amyloid- β . *J Alzheimers Dis* 22: 1363-1373, 2010.
18. Chauhan V and Chauhan A: Oxidative stress in Alzheimer's disease. *Pathophysiology* 13: 195-208, 2006.
19. Henriques AG, Domingues SC, Fardilha M, da Cruz e Silva EF and da Cruz e Silva OA: Sodium azide and 2-deoxy-D-glucose-induced cellular stress affects phosphorylation-dependent AbetaPP processing. *J Alzheimers Dis* 7: 201-212, 2005.
20. Zhu X, Raina AK, Lee HG, Casadesus G, Smith MA and Perry G: Oxidative stress signalling in Alzheimer's disease. *Brain Res* 1000: 32-39, 2004.
21. Butterfield DA, Swomley AM and Sultana R: Amyloid β -peptide (1-42)-induced oxidative stress in Alzheimer disease: importance in disease pathogenesis and progression. *Antioxid Redox Signal* 19: 823-835, 2013.
22. Butterfield DA, Di Domenico F, Swomley AM, Head E and Perluigi M: Redox proteomics analysis to decipher the neurobiology of Alzheimer-like neurodegeneration: overlaps in Down's syndrome and Alzheimer's disease brain. *Biochem J* 463: 177-189, 2014.
23. Petersen RB, Nunomura A, Lee HG, Casadesus G, Perry G, Smith MA and Zhu X: Signal transduction cascades associated with oxidative stress in Alzheimer's disease. *J Alzheimers Dis* 11: 143-152, 2007.
24. Dringen R: Metabolism and functions of glutathione in brain. *Prog Neurobiol* 62: 649-671, 2000.
25. Gadalla MM and Snyder SH: Hydrogen sulfide as a gasotransmitter. *J Neurochem* 113: 14-26, 2010.
26. Dumont M and Beal MF: Neuroprotective strategies involving ROS in Alzheimer disease. *Free Radic Biol Med* 51: 1014-1026, 2011.
27. Ott M, Gogvadze V, Orrenius S and Zhivotovsky B: Mitochondria, oxidative stress and cell death. *Apoptosis* 12: 913-922, 2007.
28. Yin F, Boveris A and Cadenas E: Mitochondrial energy metabolism and redox signaling in brain aging and neurodegeneration. *Antioxid Redox Signal* 20: 353-371, 2014.
29. Sato E, Suzuki T, Hoshi N, Sugino T and Hasegawa H: Sodium azide induces necrotic cell death in rat squamous cell carcinoma SCC131. *Med Mol Morphol* 41: 211-220, 2008.
30. Lutton JD, Moonga BS and Dempster DW: Osteoclast demise in the rat: physiological versus degenerative cell death. *Exp Physiol* 81: 251-260, 1996.
31. Amador FC, Henriques AG, da Cruz E Silva OA and da Cruz E Silva EF: Monitoring protein phosphatase 1 isoform levels as a marker for cellular stress. *Neurotoxicol Teratol* 26: 387-395, 2004.
32. Satpute R, Bhattacharya R, S Kashyap R, J Purohit H, Y Deopujari J, M Taori G and F Dagainawala H: Antioxidant potential of *Fagonia arabica* against the chemical ischemia-induced in PC12 cells. *Iran J Pharm Res* 11: 303-313, 2012.
33. Wang Y, Cao L and Du G: Protective effects of Aloe vera extract on mitochondria of neuronal cells and rat brain. *Zhongguo Zhong Yao Za Zhi* 35: 364-368, 2010 (In Chinese).
34. Zhang L, Cheng X and Hu J: Neuroprotective effects of hypoxanthine on sodium azide-induced apoptosis in PC12 cells. *Chin J Nat Med* 9: 450-455, 2011.
35. Smith CM, Chen Y, Sullivan ML, Kochanek PM and Clark RS: Autophagy in acute brain injury: feast, famine, or folly? *Neurobiol Dis* 43: 52-59, 2011.
36. Liu L, Sun T, Xin F, Cui W, Guo J and Hu J: Nerve growth factor protects against alcohol-induced neurotoxicity in PC12 cells via PI3K/Akt/mTOR pathway. *Alcohol Alcohol* 52: 12-18, 2017.
37. Shan H, Chu Y, Chang P, Yang L, Wang Y, Zhu S, Zhang M and Tao L: Neuroprotective effects of hydrogen sulfide on sodium azide-induced autophagic cell death in PC12 cells. *Mol Med Rep*: Aug 25, 2017 (Epub ahead of print).
38. Selvatici R, Previati M, Marino S, Marani L, Falzarano S, Lanzoni I and Siniscalchi A: Sodium azide induced neuronal damage in vitro: evidence for non-apoptotic cell death. *Neurochem Res* 34: 909-916, 2009.

39. Kimura H: Hydrogen sulfide: production, release, and functions. *Nippon Yakurigaku Zasshi* 139: 6-8, 2012 (In Japanese).
40. Zhang Y, Tang ZH, Ren Z, Qu SL, Liu MH, Liu LS and Jiang ZS: Hydrogen sulfide, the next potent preventive and therapeutic agent in aging and age-associated diseases. *Mol Cell Biol* 33: 1104-1113, 2013.
41. Ishigami M, Hiraki K, Umemura K, Ogasawara Y, Ishii K and Kimura H: A source of hydrogen sulfide and a mechanism of its release in the brain. *Antioxid Redox Signal* 11: 205-214, 2009.
42. Kimura H: Hydrogen sulfide: from brain to gut. *Antioxid Redox Signal* 12: 1111-1123, 2010.
43. Yu XH, Cui LB, Wu K, Zheng XL, Cayabyab FS, Chen ZW and Tang CK: Hydrogen sulfide as a potent cardiovascular protective agent. *Clin Chim Acta* 437: 78-87, 2014.
44. Jin Z, Chan H, Ning J, Lu K and Ma D: The role of hydrogen sulfide in pathologies of the vital organs and its clinical application. *J Physiol Pharmacol* 66: 169-179, 2015.
45. Al-Magableh MR, Kemp-Harper BK and Hart JL: Hydrogen sulfide treatment reduces blood pressure and oxidative stress in angiotensin II-induced hypertensive mice. *Hypertens Res* 38: 13-20, 2015.
46. Meng G, Ma Y, Xie L, Ferro A and Ji Y: Emerging role of hydrogen sulfide in hypertension and related cardiovascular diseases. *Br J Pharmacol* 172: 5501-5511, 2015.
47. Benetti LR, Campos D, Gurgueira SA, Vercesi AE, Guedes CE, Santos KL, Wallace JL, Teixeira SA, Florenzano J, Costa SK, *et al*: Hydrogen sulfide inhibits oxidative stress in lungs from allergic mice in vivo. *Eur J Pharmacol* 698: 463-469, 2013.
48. Wu D, Luo N, Wang L, Zhao Z, Bu H, Xu G, Yan Y, Che X, Jiao Z, Zhao T, *et al*: Hydrogen sulfide ameliorates chronic renal failure in rats by inhibiting apoptosis and inflammation through ROS/MAPK and NF- κ B signaling pathways. *Sci Rep* 7: 455, 2017.
49. Suzuki K, Olah G, Modis K, Coletta C, Kulp G, Gerö D, Szoleczky P, Chang T, Zhou Z, Wu L, *et al*: Hydrogen sulfide replacement therapy protects the vascular endothelium in hyperglycemia by preserving mitochondrial function. *Proc Natl Acad Sci USA* 108: 13829-13834, 2011.
50. Hu LF, Lu M, Tiong CX, Dawe GS, Hu G and Bian JS: Neuroprotective effects of hydrogen sulfide on Parkinson's disease rat models. *Aging Cell* 9: 135-146, 2010.
51. Kimura Y and Kimura H: Hydrogen sulfide protects neurons from oxidative stress. *FASEB J* 18: 1165-1167, 2004.



This work is licensed under a Creative Commons Attribution 4.0 International (CC BY 4.0) License.