

Toxicity of Hydrogen Sulfide on Rat Brain Neurons

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ABSTRACT

Hydrogen sulfide (H₂S) is a toxic compound known as a member of the gasotransmitter family. H₂S has the ability to inhibit the cytochrome c oxidase enzyme in the mitochondrial respiratory chain. Mitochondria play an important role in energy production and the brain needs energy for normal function. Mitochondrial dysfunction is associated with neurodegenerative diseases. This study investigated the mechanisms of cytotoxicity induced by H₂S in brain neurons. thioacetamide has been used to produce H₂S in water solutions. The results of the study showed that thioacetamide at concentrations of 116, 232 and 464 µg/ml was able to increase the level of reactive oxygen species (ROS), collapse in mitochondrial membrane potential (MMP), damage to the lysosomal membrane, increase in the level of oxidized glutathione (GSSG) and decrease in the level of reduced glutathione (GSH) in brain neurons. The results of the study suggested that H₂S causes damage to mitochondria and lysosomes in brain neurons that could be associated with neurodegenerative diseases.

Introduction

Hydrogen sulphide (H₂S) has attracted attention due to its important physiological and pathological roles in the central nervous system (CNS). H₂S is known as the third member of the gaso-transmitter family. This compound has roles in the CNS including neuro-protection and modulation of neurotransmission [1, 2]. Furthermore, H₂S is involved in regulating several pathological or physiological cellular functions. It has been identified in various tissues including the nervous system, brain, lungs, kidneys and heart [3, 4]. This compound is easily distributed in biological membranes and exposure of organisms to its low concentrations can lead to acute toxicity [5]. In workplaces such as textile and paper industries, natural gas extraction plants and the oil refinery there is a possibility of exposure to

H₂S [6]. In addition, research has shown that H₂S as a toxic gas has the ability to inhibit the mitochondrial cytochrome c oxidase. Cytochrome c oxidase is one of the important enzymes in the electron transfer chain in mitochondria and the binding of H₂S to this enzyme is associated with inhibition of oxidative phosphorylation [3, 5].

Mitochondria are very important organelles that play a role in different cellular processes. These organelles are known as cellular powerhouses and are vital for cell bioenergetics. It is also one of the main sources of adenosine triphosphate (ATP) production [7–9]. Its location in the cell varies between cell types. However, they mitochondria most often localized near sites of high ATP utilization as their main role is to produce and supply ATP to the cells via the enzyme complexes in the mitochondrial respiratory chain [10]. Mitochondria

are involved in other processes such as cell death [11, 12]. The CNS needs high ATP to function properly. Oxidative phosphorylation in mitochondria is the most important source of ATP production in the CNS. Therefore, brain tissue is highly dependent on mitochondria [10, 13, 14]. Furthermore, this tissue utilizes 1/4 of total body glucose and 1/5 of body oxygen consumption [15].

Mitochondrial dysfunction is associated with dysfunction of the nervous system and neurodegenerative diseases. Therefore, the normal function of brain tissue depends on mitochondria [15, 16]. In this study, the mechanism of cytotoxicity induced by H₂S in brain neurons was investigated.

Materials and Methods

Chemicals

2',7'-dichlorofluorescein diacetate (DCFH-DA) (CAS Number: 4091-99-0), Rhodamine 123 (Rh 123) (CAS Number: 62669-70-9), 5,5'-dithiobis 2-nitrobenzoic acid (DTNB) (CAS Number: 69-78-3), acridine orange (CAS Number: 65-61-2) and dimethyl sulfoxide (DMSO) (CAS Number: 67-68-5) were purchased from Sigma (St. Louis, MO USA) (Cambridge, UK). In addition, other chemicals were selected with the analytical grade.

Animals

The Wistar rat (200–250 g) were purchased from the Institute Pasteur (Tehran, Iran). There were five (n = 5) rats in each group for experiments. All animal were kept in individual cages under controlled room temperature (20–25 °C) and humidity (50–60%), and exposed to 12 h light/dark cycles. All experiments were done according to the guidelines of ethical standards and Institutional Animal Care and Use Committee of Shahid Beheshti University of Medical Sciences in Tehran, Iran.

Rat neurons isolation

Rat neurons were isolated using the method of Brewer et al. (2007) [17]. Briefly, the hippocampus, cortex, and other parts were dissected. Then, 0.5 mm slices were created, and were digested with papain for 30 minutes at 30 °C. In the next step, the cells were triturated to release. Neurons were purified using a density gradient. In the following, the cells were concentrated and re-suspended in the desired medium. Then, the neurons were in the Neurobasal/B27 plus growth factors, and plated on poly-Lys-coated glass substrate. Finally, they were incubated with 9% oxygen and 5% carbon dioxide (CO₂) at 37 °C.

Brain neurons exposure

In this study, brain neurons were exposed to different concentrations of thioacetamide (0, 50, 100, 200, 300 and 400 µg/ml). Thioacetamide has been used to produce H₂S. At first, the brain neurons were incubated with thioacetamide (0, 50, 100, 200, 300 and 400 µg/ml) to evaluate cell viability. Then, the brain neurons were incubated with thioacetamide at concentrations of 116, 232 and 464 µg/ml to assess the reactive oxygen species (ROS) level, mitochondrial membrane potential (MMP) collapse, lysosomal membrane damage, and finally reduced glutathione (GSH) and oxidized glutathione (GSSG) levels. Levels of ROS and collapse in the MMP

were assessed at 15, 30 and 60 minutes incubation times. Furthermore, lysosomal damage and GSH and GSSG levels were assessed at 30, 60 and 120 min incubation times.

Viability assay

Briefly, Trypan blue dye (0.4% w/v) was used to evaluate brain neurons viability. To perform the test, neurons were plated onto 96 well plate (1 × 10⁴ cells/ml) and incubated with thioacetamide at concentrations of 0, 50, 100, 200, 300 and 400 µg/ml. In the next step, brain neurons viability was performed by trypan blue (0.4% w/v) staining [18].

Brain neurons ROS level

In the first step, neurons were exposed to concentrations of 116, 232 and 464 µg/ml of thioacetamide for 15, 30 and 60 min. Next, the neurons were washed with PBS. In the following, DCFH-DA (10 µM) probe was used to assess the level of ROS. In the final stage, the fluorescence intensity (DCF) was measured at λ Ex = 495 nm, and λ Em = 530 nm. Fluorescence intensity (DCF) is directly related to the level of ROS [18, 19].

Brain neurons MMP collapse

In the first step, neurons were exposed to concentrations of 116, 232 and 464 µg/ml of thioacetamide for 15, 30 and 60 min. Next, the neurons were washed with PBS. In the following, Rh 123 (10 µM) probe was used to assess the MMP collapse. In the final stage, the fluorescence intensity (Rh 123) was measured at λ Ex = 470 nm, and λ Em = 540 nm. Fluorescence intensity (Rh 123) is directly related to the collapse in MMP [18, 20].

Brain neurons lysosomal membrane damage

To perform this test, neurons were exposed to concentrations of 116, 232 and 464 µg/ml of thioacetamide for 30, 60 and 120 min. In the following, the neurons were washed with PBS. After incubation, damage to the lysosomal membrane was assessed by acridine orange (5 µM) probe. Then, the fluorescence intensity (acridine orange) was measured at λ Ex = 495 nm, and λ Em = 530 nm [21, 22]. An increase in fluorescence intensity (acridine orange) indicates damage to the lysosome membrane of neurons.

Brain neurons GSH and GSSG level

At first, neurons were exposed to concentrations of 116, 232 and 464 µg/ml of thioacetamide for 30, 60 and 120 min. After these times, TCA 10% (0.5 ml) was added to the cells and centrifugation was performed for 2 min at 11000 rpm. To measure GSH and GSSG level, phosphate-EDTA buffer (4.5 ml) was used to dilute the supernatant. Then, diluted supernatant (100 µl) was added to phosphate-EDTA buffer (2.8 ml) and OPT solution (100 µl). After incubation (15 min) at room temperature, in each sample GSH and GSSG level were measured in quartz cuvettes at λ Ex = 350 nm, and λ Em = 420 nm [23].

Statistical Tests

Data are shown as Mean ± SD (n = 5). All statistical tests were carry out using SPSS (version 22) and GraphPad Prism (GraphPad Prism software, version 6). Statistical significance was determined using the one-way ANOVA test followed by the post hoc Tukey for evaluation of cell viability and the two-way ANOVA test followed by the post

hoc Bonferroni for evaluation of ROS level, MMP collapse, lysosomal membrane damage and GSH and GSSG level. Statistical significance was set at $P < 0.05$.

Results

Thioacetamide and cell viability

The effects of thioacetamide on cell viability were evaluated at different concentrations of 50 to 400 $\mu\text{g/ml}$ and the concentration of 232 $\mu\text{g/ml}$ of thioacetamide was IC_{50} . In ► **Fig. 1**, the results showed that thioacetamide at concentrations of 116 ($1/2 \text{IC}_{50}$), 232 (IC_{50}), and 464 (2IC_{50}) $\mu\text{g/ml}$ was able to reduce cell viability ($p < 0.001$).

Thioacetamide and ROS level

Over-generation of ROS is associated with damage to macromolecules (DNA, proteins and lipids). As shown in ► **Fig. 2**, thioacetamide at concentrations of 116, 232, and 464 $\mu\text{g/ml}$ and at incubation times of 15, 30 and 60 minutes was able to increase the level of ROS generation in neurons ($p < 0.0001$). There is a direct relationship between fluorescence intensity (DCF) and the level of ROS.

Thioacetamide and MMP collapse

Collapse in the MMP is associated with an increase in mitochondrial membrane permeability and the release of pro-apoptotic proteins (such as cytochrome c) and ultimately activation of apoptotic signaling. In ► **Fig. 3**, the results showed that exposure of neurons to thioacetamide at all concentrations (116, 232, and 464 $\mu\text{g/ml}$) and incubation times (15, 30 and 60 minutes) caused a collapse in the MMP ($p < 0.0001$).

Thioacetamide and lysosomal damage

As shown in ► **Fig. 4**, thioacetamide at concentrations of 116, 232, and 464 $\mu\text{g/ml}$ and at incubation times of 30, 60 and 120 minutes was able to damage the lysosome ($p < 0.05$). There is a direct rela-

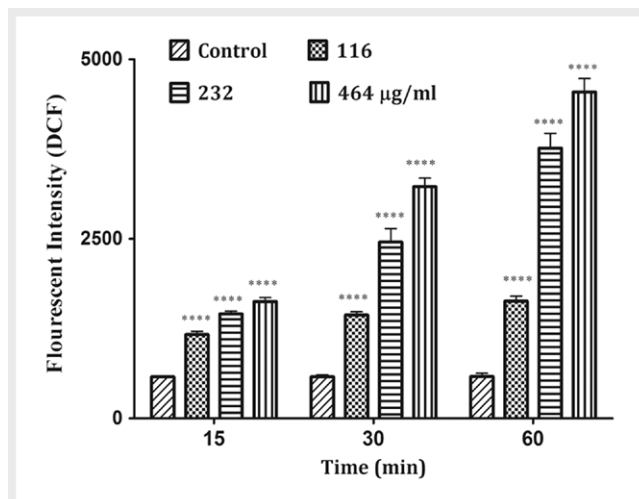
tionship between fluorescence intensity (acridine orange redistribution) and the lysosomal damage.

Thioacetamide and GSH and GSSG level

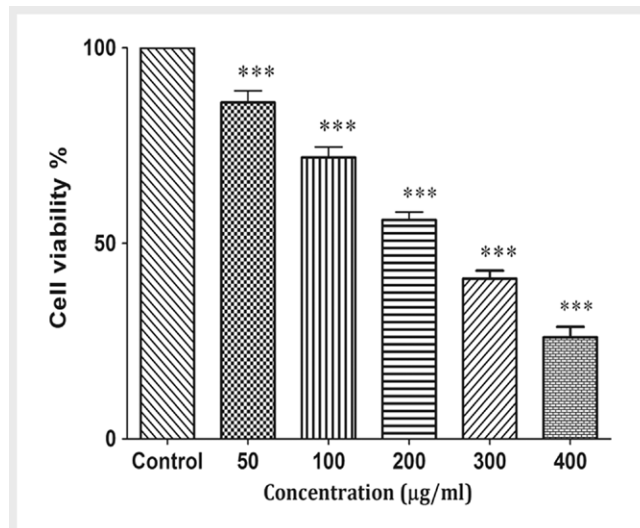
In ► **Fig. 5a, b**, the results showed that exposure of neurons to thioacetamide at all concentrations (116, 232, and 464 $\mu\text{g/ml}$) and incubation times (30, 60 and 120 minutes) caused a decrease in GSH level ($p < 0.05$) and an increase in GSSG level ($p < 0.05$).

Discussion

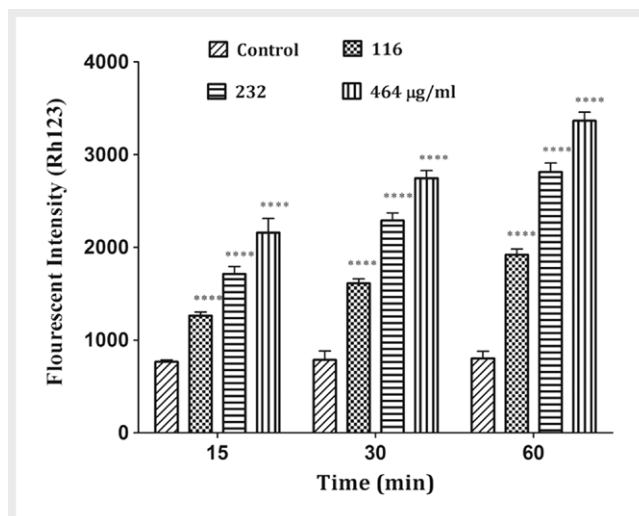
The aim of this research was to evaluate cell viability, ROS levels, collapse in the MMP, lysosomal membrane damage, intracellular



► **Fig. 2 ROS generation assay.** The effect of thioacetamide (116, 232, and 464 $\mu\text{g/ml}$) on ROS generation. Data are shown as mean \pm SD ($n = 5$). **** $p < 0.0001$ vs the corresponding control group.



► **Fig. 1 Cell viability assay.** The effect of thioacetamide (50, 100, 200, 300 and 400 $\mu\text{g/ml}$) on cell viability. Data are shown as mean \pm SD ($n = 5$). *** $p < 0.001$ vs the corresponding control group.



► **Fig. 3 MMP collapse assay.** The effect of thioacetamide (116, 232, and 464 $\mu\text{g/ml}$) on MMP collapse. Data are shown as mean \pm SD ($n = 5$). **** $p < 0.0001$ vs the corresponding control group.

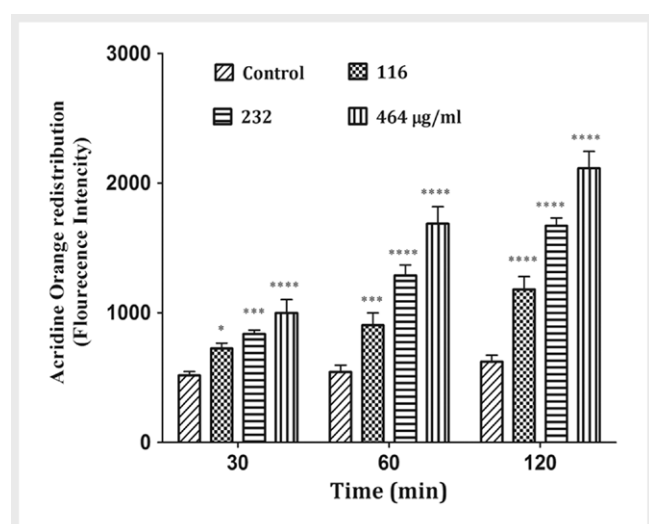
GSH and GSSG levels in brain neurons after exposure to H₂S. H₂S along with carbon monoxide (CO) and nitric oxide (NO) is known as one of the gasotransmitters. This compound has been identified in many tissues of the body, including the brain, and has been shown to play a protective role in this tissue [24, 25]. In contrast, studies have shown that hydrogen sulfide is a toxic compound and exposure to it can have side effects. H₂S has the ability to inhibit the enzyme cytochrome c oxidase in the mitochondrial respiratory chain and subsequently inhibit oxidative phosphorylation [2, 5, 6]. Accordingly, this study investigated the cellular mechanism of H₂S toxicity on rat neurons.

Mitochondria are known as one of the vital organelles in eukaryotic organisms. This organelle is involved in various physiological processes including energy (ATP) and ROS production and cell death

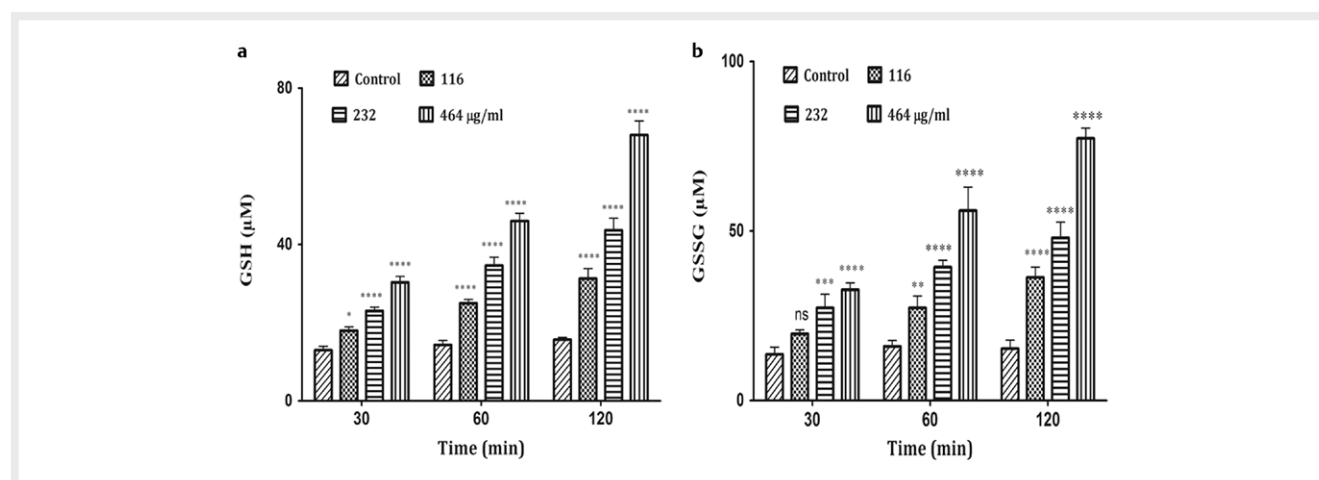
[26, 27]. The brain needs this organelle to perform its normal functions [28–30]. Compared to other tissues, the brain consumes higher ATP. Mitochondria are known as an important source of ATP production in cells and therefore each single neuron has a high number of mitochondria (hundreds to thousands). This indicates the critical dependence of nerve cells on mitochondria for ATP production [14, 15]. Therefore, a compound that can impair mitochondrial function can also damage the brain and cause neurodegenerative diseases.

Initially, the results showed that thioacetamide, the source of H₂S production, reduced cell viability in a concentration-dependent pattern. The IC₅₀ concentration for thioacetamide was 232 µg/ml. Next, the study of ROS levels showed that thioacetamide caused an increase in ROS levels in brain neurons. In eukaryotic organisms, mitochondrial respiratory chain complexes are known to produce ROS. Electron emission from mitochondrial respiratory chain complexes (especially complexes I and III) and their reaction with oxygen is accompanied by the formation of ROS (such as superoxide anion) [10, 31, 32]. ROS in different concentrations have a variety of physiologic roles. ROS in high concentrations have the ability to damage different tissues and are involved in the etiology of various diseases [33]. Since the nervous system is dependent on mitochondria for ATP, damage to mitochondria is associated with a disturbance of the nervous system functions [15, 16]. The results showed that thioacetamide was probably able to increase the level of ROS through mitochondria respiratory chain in the brain neurons, which could lead to irreversible consequences.

Furthermore, the results showed that thioacetamide could cause a collapse in the MMP of brain neurons. Previous studies have shown that there is a direct relationship between an increase in the level of ROS and a collapse in the MMP. Collapse in the MMP is known as an irreversible event that can lead to the induction of cell death signaling [33]. It is possible that thioacetamide collapsed the MMP through ROS production. As a result, an increase in the level of free radicals and the consequent collapse of the mitochondrial membrane potential may be associated with the death of brain neurons. Lysosomes are organelles that contain ROS and can increase the level of ROS [34, 35]. In patients with neurodegenerative



► **Fig. 4 Lysosomal damage assay.** The effect of thioacetamide (116, 232, and 464 µg/ml) on lysosomal damage. Data are shown as mean ± SD (n=5). *p<0.05, ***p<0.001, and ****p<0.0001 vs the corresponding control group.



► **Fig. 5 GSH and GSSG level assay.** The effect of thioacetamide (116, 232, and 464 µg/ml) on GSH (a) and GSSG (b). Data are shown as mean ± SD (n=5). *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001 vs the corresponding control group.

disorders, changes in mitochondria and lysosomes occur simultaneously, indicating a close functional relationship between the two organelles [36]. Exposure of brain neurons to thioacetamide is associated with damage to the lysosomal membrane, which can be accompanied by leakage of ROS from the lysosomes.

Subsequently, Intracellular GSH and GSSG levels were evaluated. In brain neurons, thioacetamide was able to decrease GSH level and increase GSSG level. GSH in the cell plays a protective role against ROS. Reduction in intracellular GSH content leads to the vulnerability of the defense system of cells against ROS. On the other hand, an increase in the level of ROS is associated with damage to small and large biomolecules (RNA, DNA and proteins) [37, 38].

Conclusion

The results of this study showed that thioacetamide as the potent generator of H₂S in aqueous media can disrupt the function of mitochondria and lysosomes, along with an increase in the level of ROS and collapses in the MMP in rat brain neurons. These events are also associated with a reduction in GSH levels as one of the most important intracellular antioxidants.

Conflict of Interest

The authors declare that they have no conflict of interest.

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