

Novel neuroprotective role of hydrogen sulfide in a rat model of stress brain injury

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Abstract. Hydrogen sulfide (H₂S) is a gaseous mediator recognized as important neuromodulator agent in the central nervous system. Since stress is among the most important factors involved in several pathophysiological brain processes. This study aim to investigate the effect of exogenous H₂S on the possible negative effect of stress on the brain of rats and the underlying mechanisms. Rats were divided into 3 groups: control, stressed, H₂S treated + stress. Brain injury markers measured were serum S100 protein and gamma enolase. Stress leads to obvious detrimental effects on the brain tissues; it produced significant increase in serum level of the above mentioned brain injury markers, and significant increase in brain levels of nitric oxide (NO), tumor necrosis factor-alpha (TNF α), and malondialdehyde (MDA) the lipid peroxidation degradative product along with significant decrease in brain glutathione level. H₂S pre-treatment before stress application abolished the above detrimental effects of stress on the brain tissue since it produced significant decreases in the stress-induced expression of brain injury markers, brain TNF α , brain NO and brain MDA, and significant increases in the stress-induced reduction of brain glutathione. H₂S has significant neuroprotective role in the nervous system against stress-induced significant brain injury through its antioxidant and anti-inflammatory effects.

Key words: Hydrogen sulfide — Stress — Brain — Antioxidant — Anti-inflammatory

Introduction

In this modern world, stress is an unavoidable phenomenon. Although stress is an essential mechanism for survival, it is known to induce alterations in numerous physiological responses even leading to pathological states (McEwen 2002). The stress-induced effects are supposed to be an outcome of altered activity of different mechanisms as central neurotransmitters, neurohormonal factors, mainly those linked with the pituitary-adrenal axis, and free radical generation (Jankord and Herman 2008; Zafir and Banu 2009). One of the main systems that were affected by stress is the central nervous system; it was found that stress has profound effects on the structure and function of the brain at the cellular and subcellular levels (McEwen 2012; Miller et al. 2012).

Hydrogen sulfide (H₂S) has been considered as a novel gaseous signaling molecule, similar to nitric oxide (NO) and

carbon monoxide (CO) (Wagner et al. 2009, Hermann et al 2012). H₂S is produced from l-cysteine by two pyridoxal 5'-phosphate (PLP)-dependent enzymes, cystathionine β -synthase (CBS), and cystathionine γ -lyase (CSE) and PLP-independent 3-mercaptopyruvate sulfurtransferase (3MST) (Shibuya et al 2009; Singh et al 2009). The endogenous production of H₂S was initially defined in the brain and attributed to CBS (Abe and Kimura 1996; Boehning and Snyder 2003). In addition, H₂S is produced in the vasculature *via* cystathionine γ -lyase (CGL) enzyme, where it mediates smooth muscle relaxation and subsequent vasodilation (Pryor et al. 2006).

In the past, it was thought that H₂S has been just a toxic gas with an odor of rotten eggs with little or no physiological significance (Ono et al. 2014). However, the past few years have demonstrated its role in many physiological and pathological processes in mammals, such as oxidative stress, inflammation, apoptosis, and angiogenesis (Martelli et al. 2012; Wang 2012). It was established that H₂S exerts important regulatory effects in numerous biological systems such as the central nerv-

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ous system and the cardiovascular system (Kimura 2010; Kashfi and Olson 2013).

Thus, the aim of the current work was to investigate brain lesions following stress exposure, and the potential effect of exogenous H₂S on this type of lesions and the mechanism driving this activity.

Materials and Methods

Ethical approval

The local ethics committee in our university approved this animal experiment protocol, and it was conducted in compliance with the NIH Guide for Care and Use of Laboratory Animals (National Institutes of Health 1992).

Experimental groups and animals

Thirty adults Sprague–Dawley male albino rats (8–10 weeks) weighing between 200 and 250 g were used throughout the present study. Rats were housed at room temperature with a supply of a standard diet of commercial rat chow and water *ad libitum*.

Animals were left to acclimatize to the environment for 2 weeks prior to inclusion in the experiment. The rats were divided into three different groups ($n = 10$): Group 1: control rats (rats were not subjected to stress, but were handled for a few seconds, food and water were removed during the period of time that the stressed rats were kept in the restrainer, and the rats were intraperitoneally injected with vehicle 4 h before scarification); Group 2: acute cold-restraint stress (ACRS) rats (rats were individually restrained by using a well-ventilated plastic rodent restrainer that allowed for a close fit to rats and they were placed in a refrigerator at 4°C for 3 h (Leza et al. 1998), vehicle was given by intraperitoneal injection immediately before placing the animal into the restrainer; Group 3: H₂S-treated + ACRS (rats were pretreated with 60 µmol/kg single intraperitoneal dose of the H₂S donor sodium hydrosulfide (NaHS) 45 min before the stress application. The doses were selected on the basis of our preliminary experiments and on the basis of previous reports which use the same dose or less but on different organs (Distrutti et al. 2006; Matsunami et al. 2009).

Chemicals

The chemicals were obtained from the following sources: NaHS (Cayman chemical company, USA), chemicals and reagents used in assay of catecholamines (epinephrine, norepinephrine and dopamine) (Sigma, St. Louis, USA), chemicals and reagents used in assay of corticosterone

(Sigma, St. Louis, USA), reagents for determination of; serum S100B protein, serum neuron-specific enolase (NSE), brain nitric oxide (NO), brain malondialdehyde (MDA), brain glutathione (GSH), and brain tumor necrosis factor-alpha (TNF-α) (Bio-Diagnostic).

Animal sacrifice, sample collection, and parameters measured

The rats were anesthetized by light ether anesthesia, the stressed rats anesthetized immediately after stress exposure and the control animals anesthetized at the same time, for withdrawal of blood samples from retro-orbital venous plexuses, and then the rats were decapitated. The blood samples were immediately collected in 10 ml tubes, allowed to clot, and then delivered into centrifuge tubes (3,000 rpm for 20 min); serum samples were separated in 2 ml Eppendorf tubes to be used immediately as fresh samples (preferred) or to be stored at -20°C until used. Serum samples were used to determine S100B protein by Enzyme-Linked Immunosorbent assay (ELISA), NSE by ELISA, corticosterone by spectrofluorometric method (Mattingly 1962), and catecholamines (epinephrine, norepinephrine, and dopamine) by spectrofluorometric method (Ciarlone 1978).

The brains of the rats were dissected out, washed with ice cold isotonic saline and weighed. The brain was then minced, and homogenate (10% w/v) was prepared in chilled phosphate buffer (Rabuffetti 2000). The homogenate was used for estimating levels of NO by colorimetric method, MDA by colorimetric method, GSH by ELISA, and TNF-α by ELISA.

Statistical analysis

All values are presented as mean ± SEM. Data were evaluated by use of the SPSS statistical software (v.11.0, SPSS, Chicago, IL, USA), and independent samples *t* test; $p < 0.05$ being considered statistically significant.

Results

Effect of ACRS exposure

ACRS of the rats for 3 h produced a significant ($p < 0.05$) increase in serum corticosterone and catecholamines levels; these parameters were used as an evidence of stress exposure (Table 1).

ACRS exposure lead to obvious detrimental effects on the brain tissues since it produced a significant ($p < 0.05$) increase in serum S100B protein and NSE levels, which are used to quantify the brain cellular injury in our study (Table 2). Moreover, it produced a significant ($p < 0.05$) increase in brain NO level indicating nitrosative stress in stressed group, a significant ($p < 0.05$) increase in brain MDA level

Table 1. Effect of ACRS exposure on serum corticosterone and serum catecholamines (epinephrine, norepinephrine, and dopamine) in male albino rats

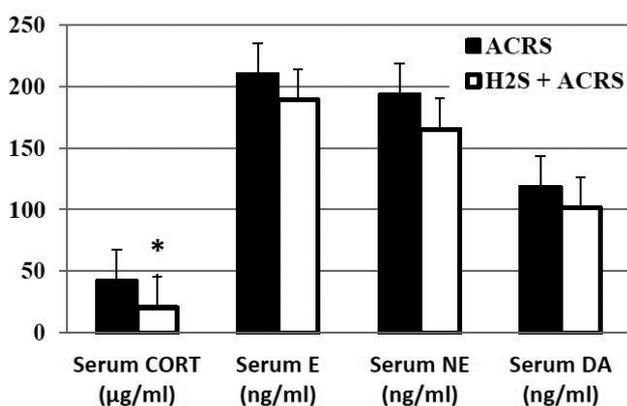
Serum	Control	ACRS
CORT ($\mu\text{g/ml}$)	14.9 ± 0.7	$42.3 \pm 3.8^*$
E (ng/ml)	72.2 ± 2.9	$210.3 \pm 28.5^*$
NE (ng/ml)	65.8 ± 3.6	$193.4 \pm 31.9^*$
DA (ng/ml)	58.6 ± 2.8	$118.5 \pm 22.9^*$

Values are mean \pm SEM ($n = 10$). * $p < 0.05$ versus the control group. ACRS, acute cold-restraint stress. ACRS, acute cold-restraint stress; CORT, corticosterone; E, epinephrine; NE, norepinephrine; DA, dopamine.

along with a significant ($p < 0.05$) decrease in the brain GSH level indicating oxidative stress induced by ACRS, and a significant ($p < 0.05$) increase in the brain pro-inflammatory cytokine TNF α which is indicative of neuro-inflammation during stress (Table 3).

Effect of hydrogen sulfide supplementation in ACRS male albino rats

The second area of our study was aimed at determining the role of H₂S in modulating the severity of brain injury induced by stress exposure in the models used. Rats pretreated with 60 $\mu\text{mol/kg}$ single intraperitoneal dose of the H₂S donor sodium hydrosulfide (NaHS) 45 min before the stress application produced a significant ($p < 0.05$) decrease in serum

**Figure 1.** Effect of hydrogen sulfide supplementation on serum corticosterone and serum catecholamines (epinephrine, norepinephrine, and dopamine) in ACRS male albino rats. H₂S supplementation before the stress application produced a significant ($p < 0.05$) decrease in serum corticosterone but failed to produce significant change in serum catecholamines levels compared to ACRS group. Values are mean \pm SEM; number of samples ($n = 10$); * $p < 0.05$ versus ACRS group. ACRS, acute cold-restraint stress; H₂S, hydrogen sulfide; CORT, corticosterone; E, epinephrine; NE, norepinephrine; DA, dopamine.**Table 2.** Effect of ACRS exposure on some brain injury markers, serum S100B protein and serum NSE, in male albino rats

Serum	Control	ACRS
S100B protein (pg/ml)	70.3 ± 6.9	$305.3 \pm 22.4^*$
NSE (pg/ml)	25.4 ± 2.5	$189.8 \pm 19.9^*$

Values are mean \pm SEM ($n = 10$). * $p < 0.05$ versus the control group. NSE, neuron-specific enolase; ACRS acute cold-restraint stress.

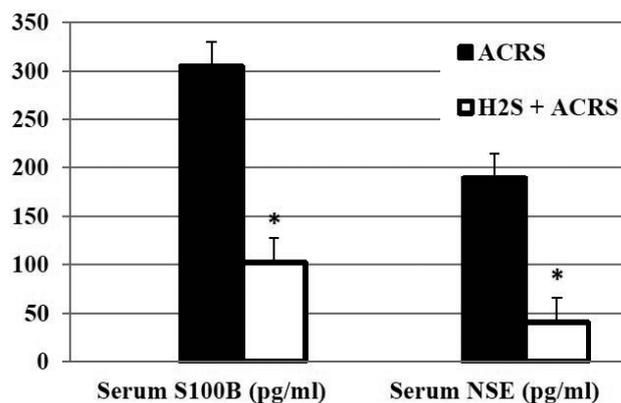
Table 3. Effect of ACRS exposure on brain lipid peroxide, GSH, nitric oxide, and TNF α levels in male albino rats

Brain	Control	ACRS
MDA (nmol/g tissue)	2.7 ± 0.1	$5.9 \pm 0.4^*$
GSH ($\mu\text{mol/g}$ tissue)	3.2 ± 0.3	$1.2 \pm 0.2^*$
NO ($\mu\text{mol/g}$ tissue)	25.4 ± 1.2	$42.5 \pm 3.2^*$
TNF α (pg/mg tissue)	12.7 ± 0.8	$27.6 \pm 1.9^*$

Values are mean \pm SEM ($n = 10$). * $p < 0.05$ versus the control group. ACRS, acute cold-restraint stress; MDA, malondialdehyde; GSH, glutathione; NO, nitric oxide; TNF α , tumor necrosis factor-alpha.

corticosterone but failed to produce significant change in serum catecholamines levels compared to ACRS group (Fig. 1).

NaHS supplementation in ACRS rats in the above dose also abolished several detrimental effects of ACRS on the brain tissue since it produced significant ($p < 0.05$) decreases in the stress-induced expression of serum S100B protein and NSE, indicating good neuroprotective effects of H₂S in stressed animals (Fig. 2).

**Figure 2.** Effect of hydrogen sulfide supplementation on some brain injury markers, serum S100B protein and serum NSE, in ACRS male albino rats. H₂S supplementation before the stress application produced significant ($p < 0.05$) decreases in the stress-induced expression of serum S100B protein and NSE, indicating good neuroprotective effects of H₂S in stressed animals. Values are mean \pm SEM; $n = 10$; * $p < 0.05$ versus ACRS group. ACRS, acute cold-restraint stress; H₂S, hydrogen sulfide; NSE, neuron-specific enolase.

Studying the effect of NaHS supplementation on the brain of the ACRS rats, we found that the significant increased levels of brain TNF α , brain NO and brain MDA observed with ACRS exposure were significantly ($p < 0.05$) decreased by NaHS supplementation, and the significant decreased level of brain GSH observed with ACRS exposure was significantly ($p < 0.05$) elevated by NaHS supplementation, indicating that the neuroprotective effect of H₂S in stressed animals can be referred to its antioxidant and anti-inflammatory effects (Fig. 3).

Discussion

Stress is known to induce alterations in numerous physiological responses even leading to pathological states (Charmandari et al 2005). Experimental stress can be induced using numerous methods such as immobilization,

cold-restraint, and starvation. Each stress model affects different organs to different degrees (Gilgun-Sherki et al. 2002). Immobilization stress considered a convenient and easy method to induce both physical (muscle work) and psychological stress (escape reaction), leading to restricted mobility and aggression (Dhanalakshmi et al. 2007). It was believed to be the most severe type of stress in rodent models and has a comparative effect in humans (Gilgun-Sherki et al. 2002).

On the other hand, cold exposure was also stated to induce lesions such as microhemorrhages, vessel dilatation, pyknosis, necrosis, neuronal shrinkage, and edema (Kamada et al. 1995). Cold stress also induces prominent histopathological changes in several organs such as necrosis, degeneration, vascular congestion, hemorrhage, and dilatation (Ateş et al. 2006). Regarding the above findings ACRS was used in the present study as a strong stress model to investigate the stress effect on the brain.

Stress activates the hypothalamic pituitary adrenocortical (HPA) axis and sympatho-adreno-medullary axes and elevates levels of both glucocorticoids (cortisol in humans; corticosterone in rodents) and catecholamines that are part of the humoral adaptive response to the stressor (Ulrich-Lai and Herman 2009). This explains the significant increase in serum levels of corticosterone, epinephrine, norepinephrine, and dopamine observed in this study with ACRS exposure.

In the present work NaHS supplementation to ACRS rats produced a significant decrease in serum corticosterone but failed to produce significant change in serum catecholamines levels compared to ACRS group may be because H₂S dose used in the present work below the minimum dose that can produce effect on catecholamines levels as reported by Kulkarni et al. (2009).

H₂S also appears to have a role in neuroendocrine function as it shows a significant role in controlling the HPA axis (Dello et al. 2000). Definitely, the increase in H₂S level in hypothalamus either obtained by addition of H₂S precursor or with H₂S-enriched media are associated with inhibition of stimulated release of corticotrophin releasing hormone from the hypothalamus. *In vivo* experiments in rat, under rest and after stress-induced adrenocorticle releasing activation, show that S-adenosyl L-methionine significantly reduces the rise in serum corticosterone level (Dello et al. 2000). In fact, Lou et al. found that, in a rat model of water immersion and restraint stress, exposure to H₂S significantly reversed the stress-induced increases in plasma corticotropin and corticosterone levels (Lou et al. 2008).

Neurochemical and immunohistological studies have confirmed that some specific isoenzymes or isoproteins, e.g. S100B protein, and NSE are specifically scattered in glial cell (S100B) and neuron (NSE) (Schmechel et al. 1978; Marangos and Schmechel 1987). Various clinical investigations have demonstrated the possibility of using these marker proteins

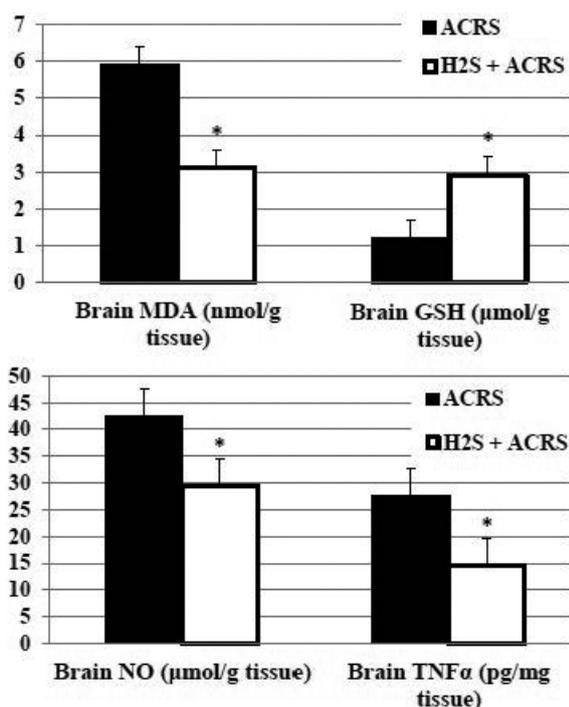


Figure 3. Effect of hydrogen sulfide supplementation on brain MDA, GSH, NO, and TNF α levels in ACRS male albino rats. H₂S supplementation before the stress application produced significant ($p < 0.05$) decrease in the level of brain TNF α , brain NO and brain MDA, and significant ($p < 0.05$) increase in the level of brain GSH, indicating that the neuroprotective effect of H₂S in stressed animals can be referred to its antioxidant and anti-inflammatory effects. Values are mean \pm SEM; $n = 10$; * $p < 0.05$ versus ACRS group. ACRS, acute cold-restraint stress; H₂S, hydrogen sulfide; MDA, malondialdehyde; GSH, glutathione; NO, nitric oxide; TNF- α , tumor necrosis factor-alpha.

for estimating the destructive processes or the pathological changes in the nervous system (Persson et al. 1987; Rothermundt 2003).

In the present study, the levels of serum S100 protein and NSE were significantly ($p < 0.05$) elevated in the ACRS rats indicating the obvious detrimental effects of stress on the brain tissue. This harmful effect of stress on the brain are in line with previous studies which reported that though stress is a necessary mechanism for survival, severe and/or long-term stress disrupts normal brain structure and function (Magarinos et al. 1997; McEwen 2002). The significant increase in corticosterone level with stress exposure can be a cause of the harmful effect of stress on the brain we found, and this come in agreement with previous studies which reported that exposure to persistently high levels of corticosterone or severe and/or prolonged stress causes over activation and dysregulation of the HPA axis and induces negative effects on the brain morphology and chemistry with serious consequences (Elliott et al 1993; Danzer 2012). It was found also that acute, short exposure to glucocorticoids can significantly exacerbate brain postischemic outcome (Payne and Schurr 2001; Payne 2003).

For determining the role of H₂S in modulating the severity of brain injury induced by stress exposure in the models used, biochemical assay of the serum homogenates revealed that stress-induced expression of serum S100B protein and NSE drastically decreased with NaHS pretreatment, indicating good neuroprotective effects of H₂S in stressed animals.

We studied the brain tissue homogenate to explore the mechanism driving the brain injury found with stress exposure and the effect of NaHS supplementation; we found that ACRS caused: a significant increase in brain NO level indicating nitrosative stress in stressed group, a significant increase in brain MDA levels along with a significant decrease in brain GSH levels, indicating oxidative stress induced by ACRS, and a significant increase in the brain level of the pro-inflammatory cytokine TNF α which is indicative of neuro-inflammation during stress.

On studying the effect of NaHS supplementation on the brain of the ACRS rats, we found that the increased brain levels of TNF α , NO and MDA observed with ACRS exposure were suppressed by NaHS supplementation, and the decreased brain level of GSH observed with ACRS exposure was elevated by NaHS supplementation.

Nitric oxide is a free radical gas synthesized from arginine and oxygen by two constitutive enzyme isoforms, nitric oxide synthase neuron (nNOS) and endothelial (eNOS). The third type, inducible (iNOS) is rarely present normally and can be expressed mainly in microglia in the CNS during immunological challenge and stress (Orlando et al. 2008). The increased brain NO level with ACRS exposure observed in this study can be explained by: (1) induced expression of iNOS by stress mediators (O'Connor et al. 2003) which in

turn increased NO production, the iNOS expressing microglia are consistently found in case of neurodegenerative diseases and has been reported as a key mediator of glial induced neuronal death (Singh and Gupta 2011); (2) previous reports found that a longer period of acute restraint lasting 2 h induced changes in gene expression of nNOS and significant increase in nNOS mRNA in brain areas related to stress, the medial parvocellular part of paraventricular nuclei and medial amygdaloid nucleus (De Oliveira et al. 2000; Yamaguchi et al. 2010). The overexpression of NO with stress is one of the major contributors to the formation of reactive nitrogen species (Min et al. 2009), which one of the leading causes of brain injury with stress exposure is found in the present study.

Regarding to H₂S effect, it was reported that it acts as a direct scavenger to neutralize cytotoxic reactive species as peroxynitrite (Muellner et al. 2009), and this explains the decrease in brain NO level we found in stressed rats pretreated with NaHS. Conversely, Altaany et al. have shown that H₂S therapy augmented NO production, they reported that H₂S can increase the coupling of eNOS by inducing S-sulfhydration and inhibiting S-nitrosylation, which leads to an increased activity of eNOS. H₂S also increased eNOS activity through promoting its phosphorylation (Altaany et al. 2014). On the contrary, high concentration of NaHS (300–3000 μ M) significantly inhibited the activity of recombinant bovine eNOS (Kubo et al. 2007). Furthermore, the mechanisms by which H₂S regulates eNOS remain to be clarified.

MDA is a degradative product of lipid peroxidation that involves the chain reaction of free radicals with polyunsaturated fatty acids and a marker of oxidative stress, while GSH is the most abundant non-protein thiol that buffers reactive oxygen species (ROS) in the brain tissue (Dringen et al. 2000). It eliminates H₂O₂ and organic peroxides by glutathione peroxides (GPx). GSH also transports amino acids across the cellular membrane by the γ -glutamyl cycle and detoxifies foreign agents by glutathione S-transferase (GST) (Meister 1988). So, GSH is considered the brain's major antioxidant system and plays a key role against oxidative stress (Dringen et al. 2000). Since oxidative stress is a process due to an imbalance between prooxidant and antioxidant systems, the increased brain MDA and decreased brain GSH levels we found in the present study with ACRS exposure is an indication of the oxidative stress occurred. These results come in agreement with previous reports who found that immobilization stress increases lipid peroxidation but decreases free radical scavenging (Liu et al. 1996), and cold exposure results in a higher degree of oxidative stress since the rate of oxygen consumption is increased during cold stress (Terblance et al. 2000; Şahin and Gümüşlü 2004). Oxidative stress was considered as one of the more important events in cerebrovascular disease such as stroke, Parkinson

and Alzheimer's diseases (Uttara et al. 2009), and earlier studies showed that it is related to neurodegenerative disorder and degeneration of the neuronal membrane (Williams and Chung 2006; Petursdottir et al. 2007).

Concerning to H₂S effect, it appeared that it has a direct antioxidant effect on the brain indicated by the decrease in MDA brain level and the increase in GSH brain level in ACRS rats pretreated with NaHS, these results come in agreement with previous studies (Kimura and Kimura 2004; Rossoni et al. 2007) reported that H₂S is a strong antioxidant and broadly proposed to protect many systems through its antioxidant role. The robust antioxidant actions of H₂S are associated with direct scavenging of ROS and/or increased expressions and functions of antioxidant enzymes. H₂S decreased lipid peroxidation by scavenging hydrogen peroxide and superoxide, and results in upregulated gene expression of definite factors, such as HO-1, glutathione reductase, glutathione S-transferase, thioredoxin, and catalase, which has role in the endogenous antioxidant defense (Szabó 2007). Furthermore, H₂S has an inhibitory effect on phosphodiesterase-5 (PDE-5), which leads to decrease NADPH oxidase formation, and the level of antioxidant enzymes increases (Calvert et al. 2010). This explains the decreased brain MDA level and the increased brain GSH level, we found in stressed rats pretreated with NaHS.

Collectively, these findings suggest that H₂S is capable of preventing the generation of ROS, scavenging ROS, and strengthening the endogenous antioxidant system.

It has earlier been reported that there is a close association of neuroinflammation with the pathogenesis of numerous neurovascular-associated disorders (Mrak and Griffin 2001). The activated microglia release pro-inflammatory cytokines, such as TNF- α and interleukin-beta (IL1- β) trigger neuronal damage and work as mediators of neuroinflammation (Liu et al. 2003; Rai et al. 2013). So, in this study the increased expression of pro-inflammatory cytokine TNF α with ACRS exposure is an indicative of neuro-inflammation during stress, which is one of the leading causes of neuron-degeneration.

Although H₂S has been concerned to play a pro-inflammatory role in systemic inflammation (Zhang et al. 2007; Ang et al. 2010), in the present study, we found that H₂S supplementation significantly decrease the increased TNF α with stress exposure, and this agree with a majority of elegant studies strongly suggest that H₂S is a powerful anti-inflammatory molecule in various models (Lefer 2007; Wang et al. 2009; Taniguchi et al. 2011). Recent evidence suggested that H₂S might exert anti-inflammatory effect *via* numerous mechanisms such as upregulation of antioxidant defense (Kida et al. 2011). The inconsistency between the present study and earlier studies may be a result of the dose of H₂S donor used or a different inflammatory model.

So, the protective effect of H₂S on the brain we found in the present study can be due to its direct effect on the brain

or due to decreasing the stress hormones such as glucocorticoids which trigger the fight-or-flight response that is intended to save human beings when they are confronted by danger. However as reported before, all these important glucocorticoids, which are meant to protect the brain, can also trigger a curious cascading death of the brain cells.

In conclusion, the preliminary results of this study suggest that H₂S exerts significant neuroprotective effects against stress-induced brain injury. The mechanisms of its action can be related to its antioxidant and anti-inflammatory effects. H₂S can exert its neuroprotective effects by acting directly on the brain, or by correcting the marked increase in glucocorticoids occurred with stress exposure. These insights afford the opportunity to design therapeutic approaches targeted to improve the stress-induced brain injury by its anti-inflammatory and antioxidant effect. However, it is clear that further basic mechanistic research is required before any potential therapeutic benefits may be realized.

Conflict of interest: The authors do not have any conflicting interests in this study.

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