Nitric Oxide 90 (2019) 15–28

Contents lists available at ScienceDirect

Nitric Oxide

journal homepage: www.elsevier.com/locate/yniox

Hydrogen sulfide suppresses homocysteine-induced glial activation and inflammatory response

Mohit Kumar, Rajat Sandhir*

Department of Biochemistry, Basic Medical Science Block-II, Panjab University, Chandigarh, 160014, India

ARTICLE INFO

Keywords:
Astrocytes
Glia
Homocysteine
Hydrogen sulfide
Inflammation
Memory

ABSTRACT

Neuro-inflammation plays a critical role in hyperhomocysteinemia (HHcy)-associated neurodegenerative disorders. Hydrogen sulfide (H_2S) has been suggested as an endogenous neuromodulator and potent anti-inflammatory molecule. In present study, we have investigated the effect of NaHS supplementation (a H_2S source) on inflammatory response in animals subjected to HHcy. NaHS adminstration restored the decreased levels of H_2S and polysulfides with a concomitant increase in the activity of cystathionase (CSE) and cystathionine β-synthase (CBS) in the brain regions of HHcy animals. NaHS supplementation reduced the expression of glial fibrillary acidic protein (GFAP) and ionized calcium binding adaptor molecule 1 (Iba1) suggesting attenuation of astrocyte and microglia activation in HHcy animals. Tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) were decreased in the cortex and hippocampus of HHcy animals following NaHS supplementation. Moreover, NaHS supplementation also decreased the TNF-α, IL-6 and MCP-1 in the serum of HHcy animals. NaHS supplementation reduced nitrite levels, 3-nitrotyrosine (3-NT) modified proteins and inducible nitric oxide synthase (iNOS) in the cortex and hippocampus of HHcy animals. However, NaHS administration increased endothelial nitric oxide synthase (eNOS) expression in brain regions of Hcy treated animals. Expression of platelet endothelial cell adhesion molecule (PECAM) was decreased in the microvessels from HHcy animals supplemented with NaHS. Furthermore, HHcy-induced memory deficits assessed by Morris water maze and novel object recognition test were reversed by NaHS administration. Taken together, the findings suggest that NaHS supplementation ameliorates Hcy-induced glia mediated inflammatory response and cognitive deficits. Therefore, H_2S may be a novel therapeutic molecule to treat HHcy associated neurological disorders and neuro-inflammatory conditions.

1. Introduction

Hyperhomocysteinemia (HHcy) is a condition that results from elevated levels of plasma homocysteine (Hcy), a sulfur containing amino acid. The major factors contributing to HHcy are defects in Hcy metabolizing enzymes or deficiency of dietary folate [1]. HHcy is an independent risk factor in cerebrovascular and neuro-inflammatory diseases [2,3]. Additionally, data from epidemiological studies have shown that Hcy is associated with neuropsychological and neurodegenerative disorders like Alzheimer's disease (AD), Parkinson's disease (PD), depression and schizophrenia [3,4]. HHcy has been associated with neurodegeneration and impairment in learning, memory and exploratory behaviour of rats [5]. Hcy has been reported to promote production of pro-inflammatory cytokines from endothelial cells and monocytes. Hcy has also been shown to increase cytokine levels in serum and brain of HHcy animals [6,7]. Moreover, in a study by Zou et al. [8] has shown that Hcy promotes activation and proliferation of microglial cells. Chronic mild HHcy induces inflammatory response and causes protein and DNA damage, as well as ultrastructural changes in cerebral cortex [9]. Hcy has been shown to increase microglia activation and neuro-inflammation via activation of STAT3 following ischemic stroke [10].

Hydrogen sulfide (H_2S) was considered as a toxic gas for decades until its presence was discovered in the brain and other tissues [11]. Endogenous H_2S is synthesized via *trans*-sulfuration pathway by cystathionine-β-synthase (CBS) [12]. CBS is predominantly expressed in astrocytes and microglia [13]. In earlier studies, H_2S has been reported to act as a pro-inflammatory mediator by up-regulating the production of cytokines and chemokine via NF-κB pathway [14]. The first report on anti-inflammatory action of H_2S by Hu et al. [15] suggested that, H_2S attenuates LPS-induced inflammatory response in cultured microglia by inhibiting tumor necrosis factor α production. A study performed using

* Corresponding author. Department of Biochemistry, Basic Medical Science Block-II, Sector-25, Panjab University, Chandigarh, 160014, India.
E-mail address: sandhir@pu.ac.in (R. Sandhir).

https://doi.org/10.1016/j.niox.2019.05.008
Received 17 December 2018; Received in revised form 30 April 2019; Accepted 26 May 2019
Available online 28 May 2019
1089-8603/ © 2019 Elsevier Inc. All rights reserved.

Abbreviations

AD	Alzheimer's disease
APTS	3-Aminopropyl-triethoxysilane
AzMC	7-Azido-4-methylcoumarin
BBB	Blood brain barrier
CBS	Cystathionine-β-synthase
CCR2	C–C chemokine receptor type 2
CSE	Cystathionase
eNOS	Endothelial nitric oxide synthase
FITC	Fluorescein isothiocyanate
GFAP	Glial fibrillary acidic protein
Hcy	Homocysteine
H_2S	Hydrogen sulfide
Iba-1	Ionized calcium binding adaptor molecule-1
IL	Interleukin
iNOS	Inducible nitric oxide synthase
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MWM	Morris water maze
NaHS	Sodium hydrogen sulfide
NEDA	N-(1-naphtyl) ethylenediamine dihydrochloride
NF-κB	Nuclear factor κB
NO	Nitric oxide
NOR	Novel object recognition
3-NT	3-nitrotyrosine
PD	Parkinson's disease
PECAM	Platelet endothelial cell adhesion molecule
RRID	Research Resource Identifiers
STAT-3	Signal transducer and activator of transcription-3
TNF-α	Tumor necrosis factor-alpha

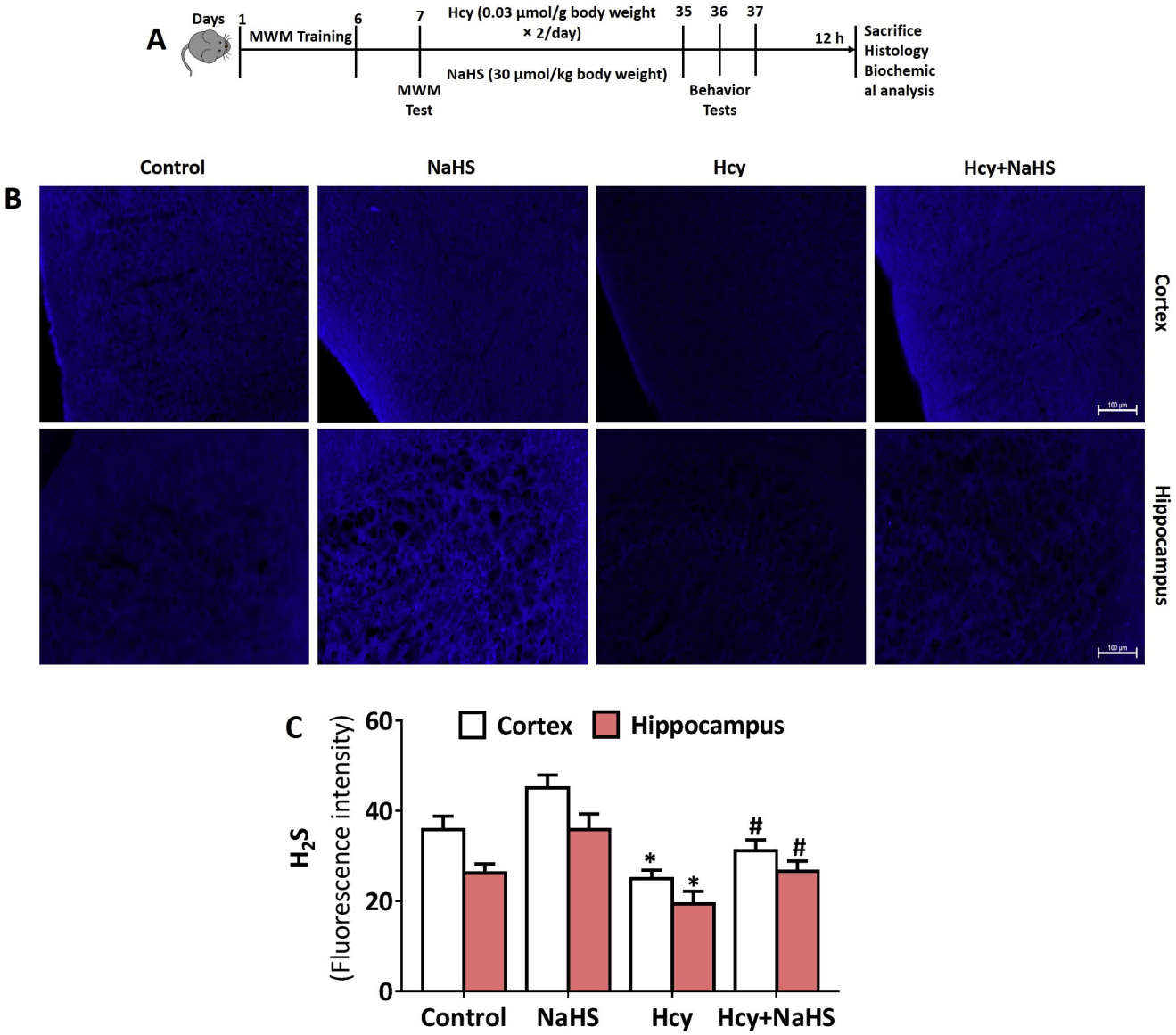

Fig. 1. Effect of H_2S administration on endogenous H_2S production assessed by *in situ* staining using AzMC fluorescent probe: Treatment paradigm followed for the study (A); Representative photographs of the cortex and hippocampus stained with H_2S specific AzMC fluorescence probe (B); Histogram representing fluorescence intensity of H_2S in the cortex and hippocampus quantified with imageJ (C). Scale bar represents 100 µm. Values are expressed as mean ± SD; n = 3. *significantly different from control group ($p < 0.05$), #significantly different from Hcy treated group ($p < 0.05$).

cultured microglia by using specific mitogen-activated protein kinase (MAPK) inhibitor suggested that, MAPK pathway may regulate H_2S mediated neuro-inflammation [16]. Another study on cultured astrocytes has shown that H_2S acts as an endogenous anti-inflammatory molecule [17]. H_2S has been reported to inhibit pro-inflammatory response in amyloid-β exposed microglia as well as in murine models of AD and in traumatic brain injury [18–20]. Hcy has been reported to trigger inflammation by inhibiting cystathionase (CSE)-H_2S signaling through epigenetic regulation of CSE transcription in macrophages [21]. CBS and CSE double gene therapy has been shown to prevent Hcy mediated mesangial inflammation [22]. Moreover, impaired H_2S levels and interleukin-10 signaling has been observed in Hcy associated colitis [23]. However, the role of H_2S as an anti-inflammatory molecule in HHcy-induced neuroinflammation has not been investigated. Therefore, this study has been planned to investigate the role of H_2S (NaHS) as an anti-inflammatory molecule in terms of activation of microglia and astrocytes along with its effect in improving Hcy-induced cognitive deficits.

2. Material and methods

2.1. Chemicals

All chemicals used in this study were of analytical grade and purchased from Merck India (Mumbai, India), Himedia Laboratories (Mumbai, India) and Sisco Research Laboratories Pvt. Ltd. (Mumbai, India). Homocysteine (Hcy; #H4628), 3-Aminopropyl-triethoxysilane (APTS; #440140), RNA later (#R0901), SYBR green (#S4438), sodium hydrogen sulfide (NaHS, #161527), primary antibodies against glial fibrillary acidic protein (GFAP, #G9269, RRID: AB_477035) were purchased from Sigma-Aldrich Co. (St. Louis, Mo, USA). Anti-rabbit IgG Alexa Fluor® 647 conjugate (#4414, RRID: AB_10693544) was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). RevertAid H minus first strand cDNA synthesis kit was obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA, #K1622). Primers were procured from Integrated DNA Technologies (IDT) Inc. (Coralville, IA, USA). Enzyme linked immunosorbent assay (ELISA) kits (OptEIA™ set with specific antibody) for interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) and monocytes chemoattractant protein-1 (MCP-1) were obtained from BD Biosciences, (San Diego, CA, USA, #550534). Pre-stained protein marker, polyvinylidene difluoride (PVDF) membrane (#1620177), stain free polyacrylamide gel (#1610183) and chemiluminescence kit (#1705060) were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Primary antibodies against ionized calcium binding adaptor molecule-1 (Iba-1, #sc32725, RRID: AB_667733), endothelial nitric oxide synthase (eNOS, #sc8311, RRID: AB_647997), inducible nitric oxide synthase (iNOS, #sc8310, RRID: AB_2152867), platelet endothelial cell adhesion molecule (PECAM, #sc376764), Horseradish peroxidase (HRP) linked anti-rabbit IgG (#sc2005, RRID: AB_631736) and 7-Azido-4-methylcoumarin (#sc396668) were procured from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Primary antibody against 3-nitrotyrosine (3-NT, #N1085) was purchased from A.G. Scientific, Inc. (San Diego, CA, USA). HRP linked anti-mouse IgG were obtained from Merck India (Mumbai, India, #621140480011730).

2.2. Animals and their treatments

Male *Sprague Dawley* rats (8–10 weeks) weighing between 180 and 200 g were procured from the Central Animal House of Panjab University, Chandigarh. The animals were housed in clean hygienic polypropylene cages at room temperature with 12-h light and dark cycle. Animals were given free access to clean drinking water and standard animal pellet diet (Ashirwad Industries, Kharar, Punjab, India) throughout the study. Animals were acclimatized to laboratory conditions before the start of the study. The animals were handled with utmost care by trained individuals with regular changing of bedding material throughout the experiment. The study procedures performed were approved by the Institutional Animal Ethics Committee of Panjab University **(PU/IAEC/S/15/107)** and were according to the guidelines for the use and care of experimental animals. Exclusion criteria included sign of illness and behavioural defect in the animals during start of the study. Animals were randomly segregated into the following experimental groups with 6–8 animals in each group:

Control: Animals received normal saline subcutaneously and intraperitoneally throughout the study.

Hcy treated: Animals received Hcy (dissolved in normal saline) subcutaneously at a dose of 0.03 μmol/g of body weight, twice a day at 8 h interval for 30 days and normal saline intraperitoneally.

NaHS treated: Animals received NaHS (dissolved in normal saline) intraperitoneally at a dose of 30 μmol/kg/day for 30 days and normal saline subcutaneously.

Hcy + NaHS treated: Animals received NaHS and Hcy at doses mentioned above.

The treatment paradigm followed for the study is illustrated in Fig. 1 A.

The body weight, food and water intake of the animals were monitored on daily basis. The doses of Hcy and NaHS used in this study were based on the previous reports [24–26] and were evaluated in the laboratory.

2.3. Tissue preparation

On the last day of treatment blood was collected from the orbital sinus of rats under light ether anesthesia. Depth of anesthesia was checked by toe-pinch response to pain. After that, animals were sacrificed by cervical dislocation and brain regions (cortex and hippocampus) were dissected, rinsed in ice-cold isotonic saline and stored at −80 °C for further analysis. Cortex and hippocampus were weighed and a 10% (w/v) homogenate was prepared in ice-cold phosphate buffer saline (PBS; 50mM, pH 7.4) by using a Potter-Elvehjem-type glass homogenizer.

2.4. Estimation of H_2S

The production of H_2S in the tissues was assessed by using AzMC fluorescence probe as described previously [27]. Animals were anesthetized and perfused transcardially with 0.1 M phosphate buffer saline (PBS, pH 7.4) followed by 2% (w/v) paraformaldehyde in 0.1 M PBS (pH 7.4). The brains were removed and post-fixed in 2% (w/v) paraformaldehyde followed by immersion in series of sucrose gradients (10%, 20%, 30%, w/v) prepared in PBS for cryoprotection. The sections (20 μm thickness) were cut with the help of Lieca Cryotome (CM1900; Germany) and collected on APTS coated slides. The sections were air dried and washed with PBS (3 times) followed by treatment with AzMC (50 μM) in PBS for 1 h. The slides were again washed three times in PBS and mounted with suitable mounting media. The slides were observed under fluorescence microscope (Nikon Eclipse 80i, Japan) and fluorescence intensity was measured using ImageJ software (NIH, Bethesda, MD, USA).

The H_2S levels were also assessed in brain regions using AzMC probe as described previously [28]. Briefly, homogenate (20 μl) was incubated with 190 μl of activity buffer (200 mM Tris HCl pH 8.0, 5 μM pyridoxal 5'-phosphate, 10 mM glutathione, 0.5 mg/mL BSA and 10 μM AzMC probe) for 1 h at 37 °C. The fluorescence intensity was measured with excitation at 365 nm and emission at 450 nm using multiplate reader (BioTek Instruments, Inc., Winooski, VT, USA). The results were expressed as μmoles of H_2S produced/mg protein using NaHS as standard.

2.5. CBS and CSE activity assays

CBS and CSE activity was assessed by the method described

previously [28]. Briefly, homogenate sample (20 µl) was incubated with 160 µl of activity buffer [200 mM Tris HCl pH 8.0, 5 µM pyridoxal 5'-phosphate, 10 mM glutathione, 0.5 mg/mL BSA, 2.5 mM cysteine, 2.5 mM Hcy] and 10 µl of DMSO. Then, AzMC (10 µM) was added to the final volume of 200 µl and incubated for 1 h at 37 °C. The fluorescence intensity was measured with excitation at 365 nm and emission at 450 nm using multiplate reader (BioTek Instruments, Inc., Winooski, VT, USA). The results were expressed as fluorescence intensity/mg protein.

CBS activity was measured by pre-incubating the sample (20 µl) with 10 µl of propargylglycine (300 mM), an inhibitor of CSE. Then, 150 µl of activity buffer [200 mM Tris HCl pH 8.0, 5 µM pyridoxal 5'-phosphate, 10 mM glutathione, 0.5 mg/mL BSA, 2.5 mM cysteine, 2.5 mM Hcy] and 10 µl of DMSO were added to the sample. Then, 10 µM of AzMC was added to a final volume of 200 µl and incubated for 1 h at 37 °C. The fluorescence intensity was measured with excitation at 365 nm and emission at 450 nm using a multiplate reader. The results were expressed as fluorescence intensity/mg protein. The activity of CSE was calculated by using the formula [(fluorescence intensity of CBS + CSE) - (fluorescence intensity of CBS)].

2.6. Polysulfide assay

Polysulfide levels were estimated in the homogenate by cold cyanolysis method as described previously [29]. Briefly, the reactions of CBS and CSE were terminated by incubating the samples at 25 °C for 30 min with 40 µL each of ammonium hydroxide (2 M) and potassium cyanide (1.25 M) to a final volume of 900 µL with ddH_2O. Then, 20 µL of 38% (v/v) formaldehyde and 200 µL of Goldstein's reagent [25 g ferric nitrate and 262 mL nitric acid in 1L of ddH_2O] were added to the reaction mixture. The content was centrifuged at 10,000 g for 5 min and supernatant was collected. The absorbance was measured at 460 nm and the results were expressed as nmoles of polysulfides/mg protein using sodium thiocyanate as standard.

2.7. Nitrite assay

Nitrite levels were estimated in homogenate by the method of Green et al. [30]. Homogenate was incubated with an equal volume of Griess's reagent (0.1%, w/v N-(1-naphtyl) ethylenediamine dihydrochloride (NEDA) and (1%, w/v sulfanilamide in 5%, v/v phosphoric acid)) for 10 min at room temperature. The absorbance was measured at 548 nm and the results were expressed as nmoles of nitrite/mg protein using sodium nitrite as standard.

2.8. Enzyme-linked immunosorbent assay (ELISA)

Cytokine (TNF-α and IL-6) and chemokine (MCP-1) levels were measured in serum, cortex and hippocampus using ELISA kits as per instructions provided by the manufacturer. Briefly, ELISA plates were coated with specific antibodies and incubated overnight at 4 °C. After overnight incubation, specific antibodies coated wells were incubated with respective standards and samples for 2 h at RT. After immobilization of antigens, detection antibody (HRP labeled) was added. The wells were washed with washing buffer at every step. After the last wash, enzyme reagent (streptavidin-HRP) was added and incubated for 30 min at RT. The wells were washed again and substrate solution (stabilized chromogen) was added to each well followed by incubation for 20 min. The stop solution was added to terminate the reaction and the absorbance was read at 450 nm using automated microplate reader (ELx800, BioTek instruments, Winooski, USA). The concentrations of cytokine and chemokine were determined from the regression line using the respective standards in a concentration range of 7.8–10,000 pg/mL. The results were expressed as pg/mg protein for cortex and hippocampus and pg/ml for serum.

2.9. Estimation of protein

The protein content was estimated according to the method of Lowry et al. [31] using BSA as a standard.

2.10. Real time RT-PCR

Animals were anesthetized and decapitated; cortex and hippocampus were dissected and stored in RNA later. Total RNA was isolated using TRIzol Reagent as per manufacturer's instructions. Thereafter, RNA samples were treated with DNase to eliminate genomic DNA contamination. cDNA was prepared from 1 µg of total RNA from each sample using RevertAid First Strand cDNA Synthesis Kit. Real-time PCR was performed using LightCycler® 96 System (Roche Diagnostics, Mannheim, Germany). 10 ng cDNA and gene-specific primers (Table 1) were added to SYBR Green PCR Master Mix and subjected to PCR amplification. The data was collected and analyzed on the LightCycler® 96 software (Roche Diagnostics, Mannheim, Germany). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene. Relative gene expression was determined using the corrected delta-delta Ct method [32].

2.11. Western blotting

Western blot was performed by the method of Towbin et al. [33]. Homogenate was prepared in lysis buffer [pH 7.4, KCl (50 mM), Na_3VO_4 (1 mM), DTT (125 mM), PMSF (1 mM) and protease inhibitor cocktail (10µl/10 ml)] followed by centrifugation at 20,000 g for 20 min. The supernatant containing 50 µg of protein was separated on 10% SDS-PAGE along with pre-stained protein marker. The protein from gel was transferred to the PVDF membrane in an ice-cold buffer containing Tris-HCl (25 mM), glycine (192 mM) and 20% (v/v) methanol for 2 h. Blocking was done by incubating the membrane with 5% (w/v) non-fat skimmed milk in PBS for 2 h at room temperature. After blocking, the membranes were incubated overnight with respective primary antibodies (1:1000) in 2.5% (w/v) skimmed milk in PBS with gentle shaking. This was followed by incubation with respective horseradish peroxidase-conjugated secondary antibody (1:1000) for 2 h. The bands for specific protein were visualized using chemiluminescence kit and analyzed by AlphaEaseFC™ software (Alpha Innotech,

Table 1
Sequence of primers used for real time RT-PCR analysis.

Gene	Accession number	Forward primer (5′-3′)	Reverse primer (5′-3′)	Amplicon size (bp)
GFAP	(NM_017009)	TCCTGGAACAGCAAAACAAG	CAGCCTCAGGTTGGTTTCAT	224
Iba1	(NM_017196)	GTCCTTGAAGCGAATGCTGG	CATTCTCAAGATGGCAGATC	157
IL-6	(NM_012589)	CCTACCCCAACTTCCAATGCTC	TTGGATGGTCTTGGTCCTTAGCC	78
TNF-α	(NM_012675)	CCCAGACCCTCACACTCAGAT	TTGTCCCTTGAAGAGAACCTG	215
MCP-1	(NM_031530)	AGCACCTTTGAATGTGAACT	AGAAGTGCTTGAGGTGGTTG	82
eNOS	(NM_021838)	AAGACAAGGCAGCTGTGGAA	GCTGGGGACAGGAAATAGTT	232
iNOS	(NM_012611)	TTGTGCGCAGTGTCAGTGG	TCCTTTGAGCCCTGTGTGC	143
GAPDH	(NM_017008)	CAACTCCCTCAAGATTGTCAGCAA	GGCATGGACTGTGGTCATGA	118

San Leandro, CA, USA). Stain-free gel was used as total protein loading control [34].

2.12. Immunostaining

Immunohistochemistry was performed for GFAP and Iba-1 in brain sections to study activation of astrocytes and microglia as described by Sandhir et al. [95]. Animals were anesthetized and perfused transcardially with 0.1 M phosphate buffer saline (PBS, pH 7.4) followed by 2% (w/v) paraformaldehyde in 0.1 M PBS (pH 7.4). The brains were removed and post-fixed in 2% (w/v) paraformaldehyde followed by immersion in sucrose gradient (10%, 20%, 30%, w/v) prepared in PBS for cryoprotection. The sections (20 μm thickness) were cut with the help of Lieca Cryotome (CM1900; Germany) and collected on APTS coated slides. The sections were air dried and washed with PBS (3 times) followed by treatment with 1% (v/v) Triton X-100 in PBS for 30 min. The non-specific binding was blocked with 1% (w/v) BSA. After blocking the sections were incubated with respective antibodies (1:100) overnight at 4 °C in a humid chamber. The sections were rinsed with PBS and incubated with horseradish peroxidase (HRP) labeled secondary antibody (1:200) for 2 h at room temperature. Slides were rinsed with PBS and colour was developed by using 3,3-diaminobenzidine-hydrogenperoxide (DAB-H_2O_2) solution and examined under microscope (Nikon Eclipse 80i, Japan). The immunopositive cells were calculated using ImageJ software (NIH, Bethesda, MD, USA). Control for immunolabeling was performed by following the same procedure but without incubating with primary antibodies.

2.13. Immunofluorescence

eNOS and PECAM localization was studied in isolated microvessels from the cortex region of brain by immunofluorescence. Microvessels were isolated by the method as described previously [35]. Briefly, cortex tissue was homogenized in microvessel isolation buffer containing [NaCl (100 mM), KCl (4.7 mM), $CaCl_2$ (2.5 mM), KH_2PO_4 (1.2 mM), $MgSO_4$ (1.2 mM), HEPES (15 mM), $NaHCO_3$ (2.5 mM), D-glucose (10 mM), and sodium pyruvate (1 mM) with 10 g/l dextran, pH 7.4] and equal amount of 26% (w/v) dextran was added. The mixture was centrifuged at 5600 g for 10 min and pellets obtained were suspended in microvessel isolation buffer and passed through a 70 μm filter (BD Biosciences). The filtered homogenates were then centrifuged at 3000 g for 10 min at 4 °C and the pellet was suspended in PBS and allowed to attach to APTS coated slides for 30 min at 37 °C. The attached microvessels were washed thrice with PBS for 10 min before staining. The slides were incubated with blocking buffer (5%, w/v BSA in PBS containing 0.1%, v/v Triton X-100) for 2 h followed by overnight incubation with respective primary antibodies (1:200) at 4 °C. The slides were then washed thrice with PBS and incubated with respective secondary antibody (1:200) for 2 h at 4 °C. The slides were again washed three times in PBS and mounted using suitable mounting media. The slides were observed under fluorescence microscope (Nikon Eclipse 80i, Japan) and fluorescence intensity was measured using ImageJ software (NIH, Bethesda, MD, USA).

2.14. Neurobehavioral studies

The animals in respective groups were assessed for cognitive functions using Morris water maze and Novel object recognition tests.

2.14.1. Morris water maze

Morris water maze (MWM) is a widely used method to study spatial learning and memory of rodents that relies on distal cues to navigate from start location to the submerged escape platform [36]. The apparatus consisted of a water tank having diameter of 140 cm. The target platform (10 cm^2) is typically submerged 1–2 cm below the surface of the water. All animals were acclimatized to the MWM apparatus 5 min each for 3 days. This was followed by 6 days of training to reach on a hidden platform. On day 7, data was acquired and treatment initiated and continued for next 30 days. At the end of the treatment, acquisition phase data was recorded again under exactly the identical experimental conditions. The distance travelled, time taken to reach the escape platform and average speed was analyzed using ANY-maze™ tracking software (Stoelting Co., Wooddale, IL, USA).

At the end of the study, the extent of memory retrieval was assessed by probe trial as described earlier [37]. In the probe trial, hidden platform was removed from the tank and animals were allowed to explore the maze for 180 s. Various parameters including time spent in quadrant containing platform, time spend in opposite quadrant and number of target crossings were recorded. These parameters provide assessment of degree of memory retrieval after learning.

2.14.2. Novel object recognition (NOR)

NOR test was used to assess spatial memory that is primarily based on the tendency of the rats to explore a novel object more than the familiar one in the absence of any reinforcement or externally applied rules [96]. The test consisted of an acclimatization phase and the acquisition phase. In acclimatization phase, rats were habituated to a rectangular shaped open field box. On next day, each animal was allowed to explore the two identical objects for 3 min inside the apparatus. The acclimatization session was for a period of two days for the animals in all groups. In the acquisition phase (24 h after the training session), animals were allowed to explore two different objects for 3 min and the activity of the rats was recorded using ANY-maze™ video tracking software (Stoelting, Wood Dale, IL, USA). During the acquisition, one of the familiar object was exchanged with a novel object while the other was the familiar object. The familiar and the novel objects were different in shape, size, colour, appearance and texture. Various parameters like memory score, number of entries to the novel object zone and discrimination index were assesed. The memory score was calculated by using the formula (time spent in exploring the novel object/total time exploring both objects)*100. The discrimination index was calculated by the formula (time spent in exploring the novel object - time spent in exploring the familiar object/time spent in exploring both the objects) *100.

2.15. Statistical analysis

All values are expressed as mean ± standard deviation (SD). The normality of the data was tested by using Shapiro-Wilk normality test. Data was analyzed using one-way analysis of variance (ANOVA) followed by post-hoc Newman-Keuls test and Kruskal-Wallis H test followed by post-hoc Mann Whitney test for comparison between various groups using GraphPad Prism 6 software (La Jolla, CA, USA). Values with $p < 0.05$ were considered as statistically significant.

3. Results

3.1. Effect of H_2S on body weight, food and water intake of the animals

After treating the animals with Hcy and NaHS for 30 consecutive days, no change in body weight, food and water intake was observed in the animals of each group (Table 2).

3.2. Effect of H_2S on endogenous H_2S production and polysulfide levels

A significant decrease in the endogenous H_2S levels were observed in the cortex (30.1%) and hippocampus (26.3%) of HHcy animals as compared to control animals (Fig. 1 B). NaHS supplementation increased the endogenous H_2S levels in the cortex (24.8%) and hippocampus (37.2%) of Hcy treated animals, which were restored near to the control levels. A similar decrease in H_2S levels was observed in the cortex (22.7%) and hippocampus (31.1%) of HHcy animals in the

Table 2
Effect of H_2S administration on body weight, food and water intake of HHcy animals.

	Body weight (g)			
Group	Initial	Final	Water intake (ml/animal/day)	Food intake (g/animal/day)
Control	188.7 ± 6.29	215 ± 12.9	27.33 ± 4.02	33.66 ± 4.71
NaHS	187.4 ± 4.87	212 ± 8.3	27.66 ± 3.54	35.91 ± 4.90
HHcy	187.5 ± 8.7	211.3 ± 14.3	27.85 ± 2.69	40.11 ± 7.21
HHcy + NaHS	193.7 ± 11.0	216.2 ± 13.1	28.08 ± 3.47	34.50 ± 6.13

Values are expressed as mean ± SD; n = 6.

homogenates from cortex and hippocampus (Fig. 2 A). The decrease in H_2S levels were restored to normal in HHcy animals supplemented with NaHS. In addition, the polysulfide levels were also decreased in the cortex (25.4%) whereas, no significant change was observed in the hippocampus of Hcy treated animals (Fig. 2 B). Interestingly, NaHS administration increased polysulfide levels in the cortex (36.4%) of HHcy animals.

3.3. Effect of H_2S on CBS and CSE activity

The activity of CBS and CSE was assessed to evaluate the effect of exogenous H_2S on H_2S metabolism. CBS activity was significantly decreased in the cortex (21.6%) whereas, no significant difference was observed in the hippocampus of Hcy treated animals (Fig. 2C). NaHS supplementation, on the other hand, increased the CBS activity significantly in the cortex (25.7%) of HHcy animals. The activity of CSE was significantly reduced in the cortex (31%) and hippocampus (23.8%) of Hcy treated animals (Fig. 2 D). Interestingly, CSE activity was increased in the cortex (26.6%) and hippocampus (58.6%) following NaHS supplementation.

3.4. Effect of H_2S on Hcy-induced glial activation

Ionized calcium-binding adapter molecule 1 (Iba1) is a marker for activation of microglia. Iba1 mRNA expression was significantly increased in the cortex (52.25%) and hippocampus (142.63%) of HHcy animals (Fig. 3 A). Western blot analysis of Iba-1 protein correlated with Iba1 mRNA expression (Fig. 3 F). Immunohistochemical staining revealed a significant increase in iba1 positive cells with hyper ramified morphology in the cortex and hippocampus of Hcy treated animals (Fig. 3H, J). However, NaHS treatment decreased the mRNA and protein expression of Iba1 in the hippocampus of Hcy treated animals. Moreover, following NaHS supplementation, morphology of microglia changed from hyper ramified to ramified shape along with decrease in Iba1 positive cells. Ramified cells have small cell body and more number of projections. In case of immune response the number of projections and projection length progressively decrease with increased somal area converting ramified microglia to hyper ramified or amoeboid stage.

Glial fibrillary acidic protein (GFAP) is a marker of astrocyte activation and used to study neuroinflammatory response. GFAP mRNA expression was significantly increased in hippocampus (1.09 fold) whereas, no change was observed in cortex of Hcy treated animals (Fig. 3 B). The protein levels of GFAP were significantly increased in the hippocampus (2.7 fold) whereas; no significant change was observed in the cortex of HHcy animals as compared to controls (Fig. 3 E). Immunohistochemical analysis revealed significant increase in GFAP positive cells in the hippocampus of the Hcy treated animals as compared to control animals (Fig. 3 G, I). However, NaHS treatment decreased the mRNA and protein expression of GFAP in the hippocampus of Hcy treated animals. Moreover, NaHS supplementation reduced the number of GFAP positive cells and morphological changes observed in the cortex and hippocampus of Hcy treated animals. Astocytes in Hcy treated animals had shorter projection and darkly stained large cell body as compared to control animals suggesting activated astrocytes. NaHS supplementation reversed the activated astrocytes towards resting stage having longer and thin projections with reduced somal area.

3.5. Effect of H_2S on Hcy-induced expression of IL-6, TNF-α and MCP-1

The mRNA expression of pro-inflammatory cytokines and chemokine is depicted in Fig. 4. The expression of IL-6 mRNA was significantly increased in the cortex (4.09 fold) and hippocampus (5.87 fold) of Hcy treated animals as compared to the controls. Relative mRNA expression of TNF-α was increased in the cortex and hippocampus by 2.42 fold and 3.13 fold respectively. The expression of MCP-1 mRNA was increased by 1.26 fold in the cortex and 1.19 fold in the hippocampus of Hcy treated animals as compared to controls. However, NaHS supplementation to HHcy animals showed a significant decrease in the mRNA expression of IL-6, TNF-α and MCP-1 as compared to Hcy treated animals.

ELISA was performed to estimate the protein levels of cytokine and chemokine in the cortex, hippocampus and serum of the HHcy animals (Fig. 4). A significant increase in IL-6 protein levels were observed in the cortex (40.99%) and hippocampus (101.91%) of Hcy treated animals. The levels of TNF-α protein were increased by 46.61% in the cortex and 34.33% in the hippocampus of HHcy animals. The levels of

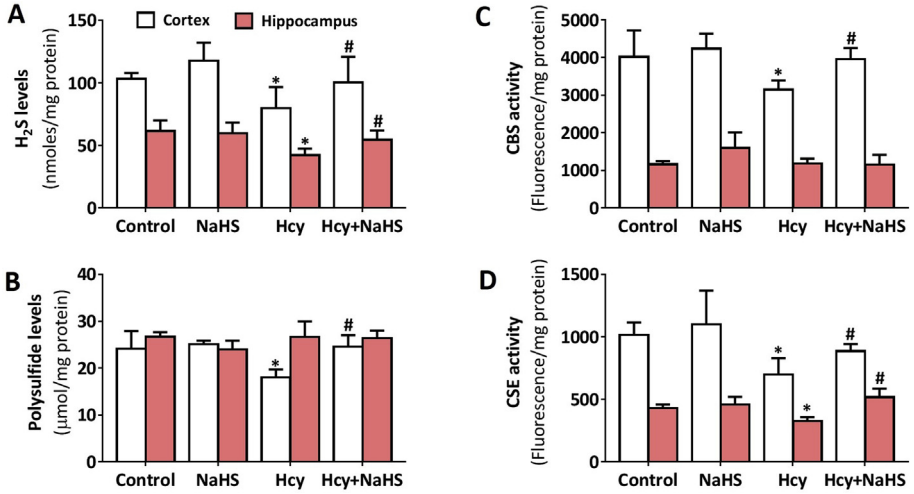

Fig. 2. Effect of H_2S administration on endogenous H_2S levels, polysulfide levels, CBS and CSE enzyme activity in HHcy animals: H_2S levels assessed by AzMC probe (A); Polysulfide levels (B); CBS (C) and CSE activity (D) in the cortex and hippocampus of HHcy animals. Values are expressed as mean ± SD; n = 6. *significantly different from control group ($p < 0.05$), #significantly different from Hcy treated group ($p < 0.05$).

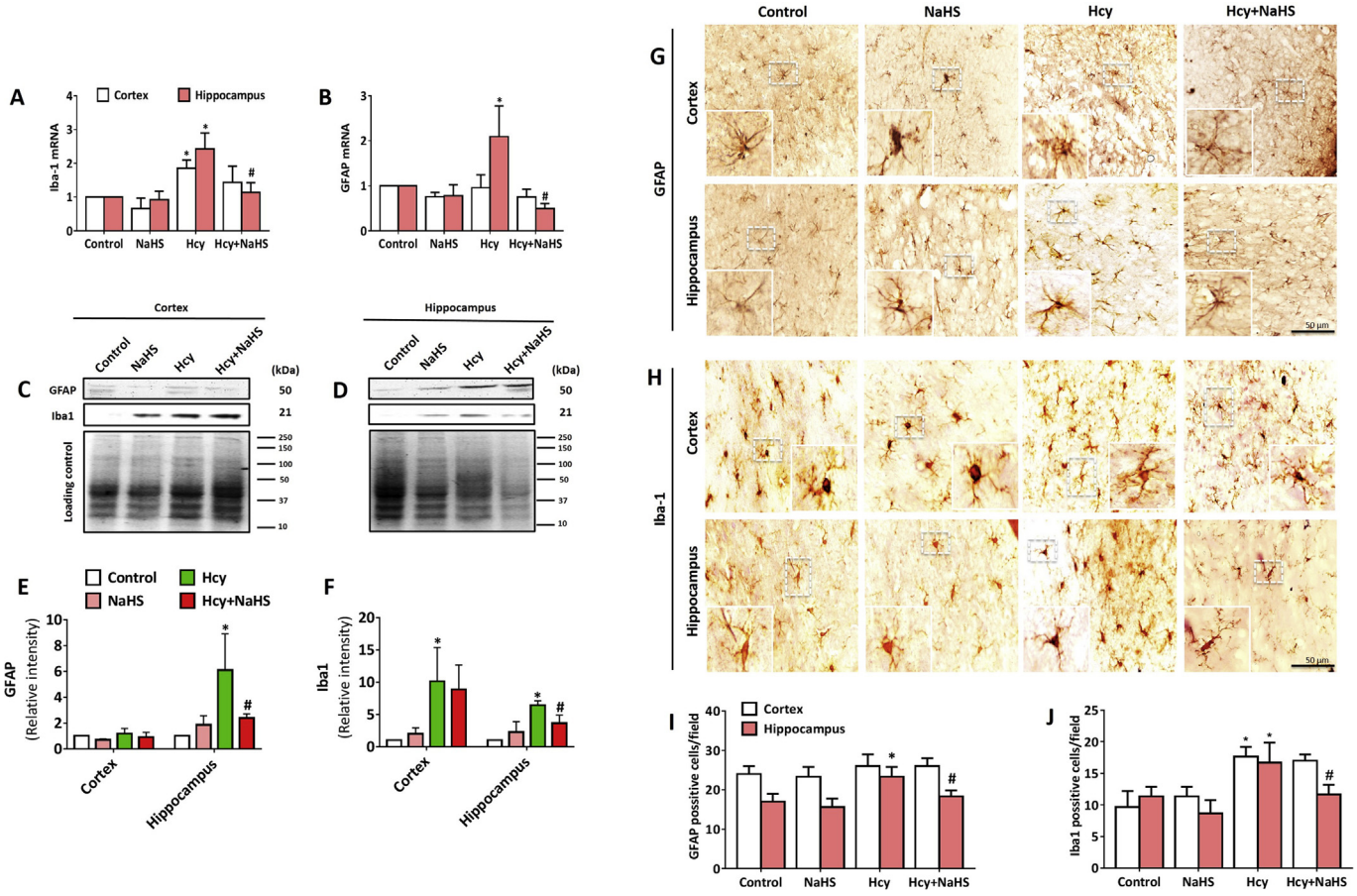

Fig. 3. Effect of H_2S administration on Iba-1 and GFAP expression and immunohistochemistry in HHcy animals: Relative mRNA expression of Iba-1 (A); Relative mRNA expression of GFAP (B); Immunoblots of GFAP and Iba-1 (C, D); Densitometry of GFAP and Iba-1 (E, F); Representative photographs of GFAP stained cells (G); Representative photographs of Iba-1 stained cells (H); Histogram showing number of GFAP and Iba-1 positive cells (I, J) in the cortex and hippocampus of HHcy animals. Scale bar represents 50 µm. Values are expressed as mean ± SD; n = 6 for RT-PCR; n = 3 for Western blot and immunohistochemistry. *significantly different from control group ($p < 0.05$), #significantly different from Hcy treated group ($p < 0.05$).

MCP-1 protein were also increased in the cortex (31.56%) and hippocampus (48.39%) of Hcy treated animals as compared to controls.

Moreover, a significant increase in the levels of IL-6 (45.07%), TNF-α (21.01%) and MCP-1 (2.39 fold) were also observed in serum of Hcy treated animals as compared to control animals. Moreover, supplementation with NaHS showed a significant reduction in the levels of cytokines and chemokine in the cortex, hippocampus and serum of HHcy animals suggesting anti-inflammatory effect of H_2S.

3.6. Effect of H_2S on Hcy-induced alterations in nitric oxide synthases

In order to assess the effect of H_2S on vascular functions, the expression of endothelial nitric oxide synthase (eNOS) was studied in HHcy animals. eNOS mRNA expression was significantly decreased in the cortex (75.57%) whereas, no change was observed in the hippocampus of Hcy treated animals as compared to control. NaHS administration, significantly increased eNOS mRNA expression in the cortex (4.6 fold) of HHcy animals (Fig. 5 A). Similarly, protein levels of eNOS were significantly decreased in the cortex (27.52%) whereas, no change was observed in the hippocampus of HHcy animals as compared to control animals (Fig. 5 D). Moreover, immunofluorescence analysis of eNOS in the isolated microvessels showed a significant decrease (36.70%) in fluorescence intensity in Hcy treated animals as compared to control animals (Fig. 5). Interestingly, NaHS supplementation significantly increased the eNOS protein in cortex (191.55%) and hippocampus (38.29%) of Hcy treated animals. Similarly, NaHS administration increased the fluorescence intensity of eNOS in isolated microvessels by 67.17%.

Inducible nitric oxide synthase (iNOS) is one of the direct markers of an inflammatory response. The expression of iNOS was studied to assess the effect of H_2S on Hcy-induced inflammation. A significant increase in iNOS mRNA expression was observed in the cortex (63.09%) and hippocampus (184.02%) of Hcy treated animals as compared to control animals (Fig. 5 B). iNOS protein was significantly elevated in the cortex (24.93%) and hippocampus (121.91%) of HHcy animals (Fig. 5 D). NaHS supplementation significantly decreased the iNOS mRNA expression in cortex and hippocampus by 61.81% and 37.01% respectively. The protein levels of iNOS were decreased in the cortex and hippocampus of HHcy animals following NaHS supplementation.

3.7. Effect of H_2S on Hcy-induced nitrite and 3-nitrotyrosine (3-NT) levels

The data for nitrite levels and 3-NT modified proteins is depicted in Fig. 5C and Fig. 5E respectively. Nitrite levels were significantly increased in the hippocampus (86.37%) whereas; no change was observed in the cortex of the Hcy treated animals. NaHS supplementation significantly decreased nitrite levels in the hippocampus by 76.29% as compared to the HHcy animals.

3-NT modification of proteins was significantly increased in the cortex (40.01%) and hippocampus (42.12%) of Hcy treated animals as compared to control animals suggesting nitrative stress in the brain of HHcy animals. However, NaHS supplementation significantly reduced 3-NT protein modification in the cortex and hippocampus of Hcy treated animals.

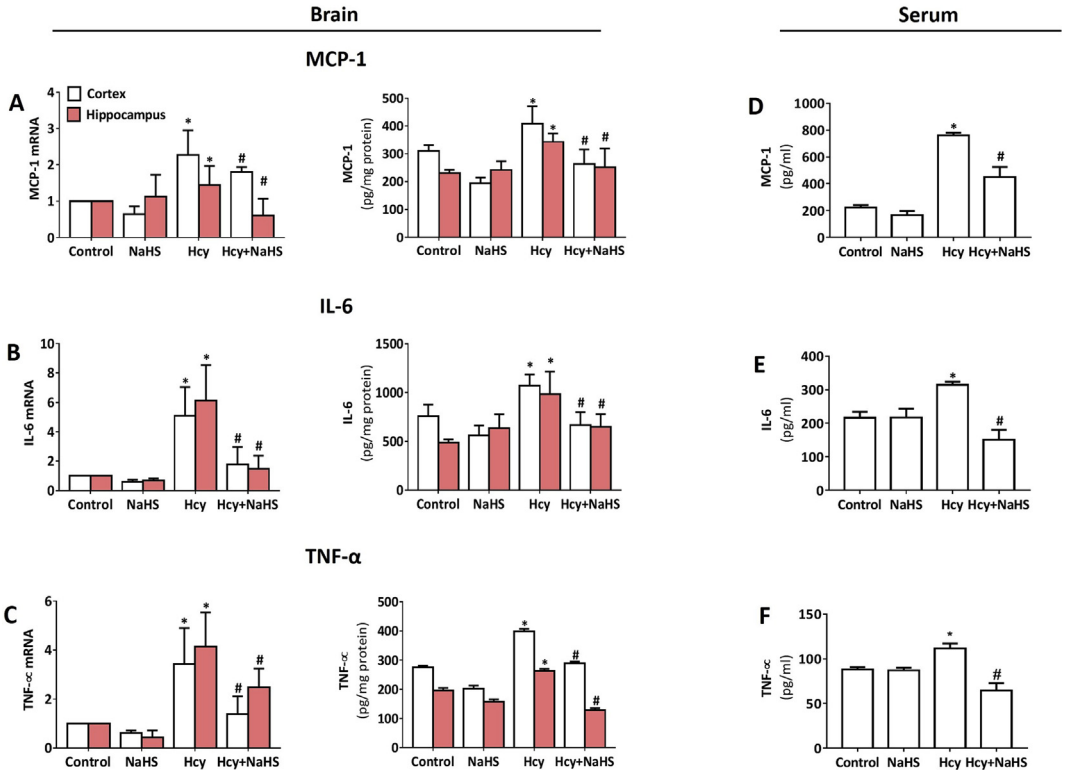

Fig. 4. Effect of H_2S administration on cytokines and chemokine expression in HHcy animals: Relative mRNA expression and protein levels of MCP-1 (A); IL-6 (B) and TNF-∝ (C) in the cortex and hippocampus of HHcy animals. Levels of MCP-1 (D); IL-6 (E) and TNF-∝ (F) in the serum of HHcy animals. Values are expressed as mean ± SD; n = 6. *significantly different from control group ($p < 0.05$), #significantly different from Hcy treated group ($p < 0.05$).

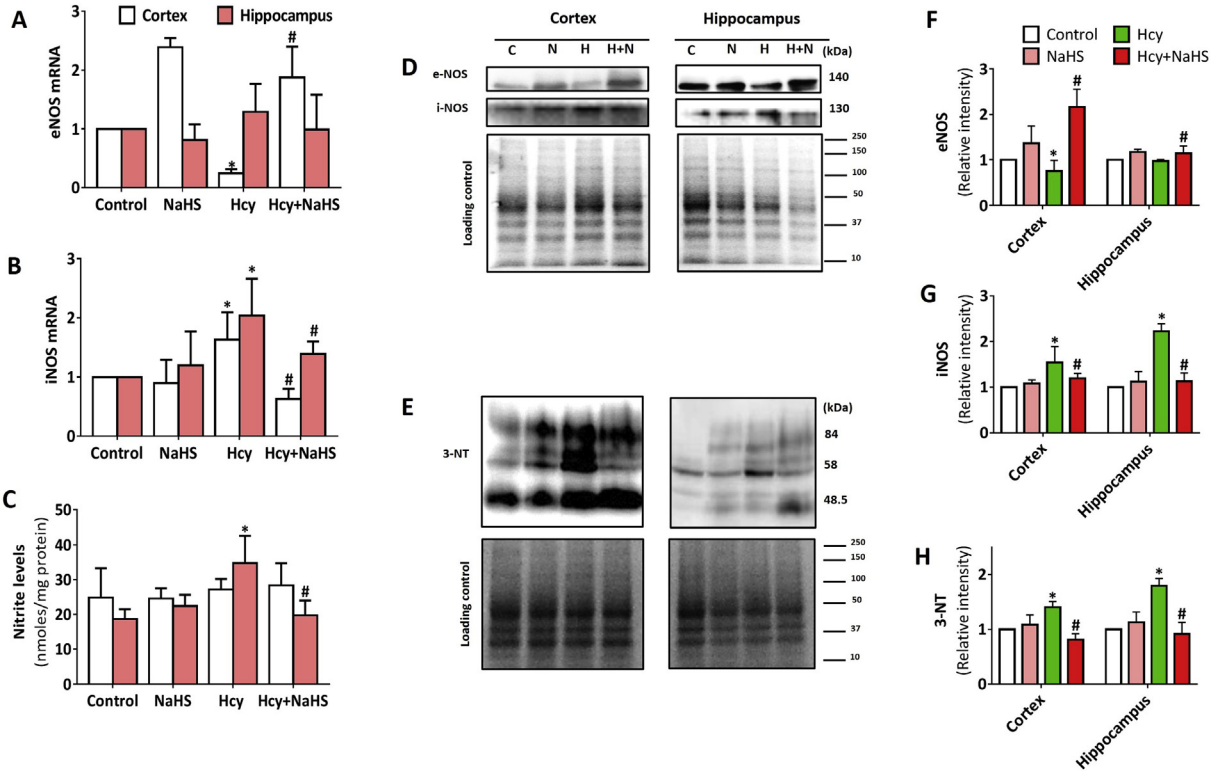

Fig. 5. Effect of H_2S administration on relative mRNA and protein expression of nitric oxide synthases in HHcy animals: eNOS mRNA (A); iNOS mRNA (B); Nitrite levels (C); Immunoblots of eNOS and iNOS (D); Immunoblots of 3-NT modified proteins (E); Densitometry of eNOS (F); iNOS (G) and 3-NT modified proteins (H) in the cortex and hippocampus of HHcy animals. Values are expressed as mean ± SD; n = 6 for RT-PCR; n = 3 for Western blot. *significantly different from control group ($p < 0.05$), #significantly different from Hcy treated group ($p < 0.05$).

3.8. Effect of H_2S on platelet endothelial cell adhesion molecule (PECAM) levels

To assess the effect of H_2S on systemic inflammation, protein expression of platelet endothelial cell adhesion molecule (PECAM) was studied in HHcy animals. PECAM is present on endothelial cells, which mediates the migration of systemic neutrophils at the site of inflammation. The immunofluorescence shows that expression of PECAM was significantly increased by 4.3 fold in the isolated microvessel from the cortex of Hcy treated animals (Fig. 6). However, NaHS supplementation significantly decreased (33.75%) the expression of PECAM.

3.9. Effect of H_2S on HHcy-induced cognitive deficits

3.9.1. Morris water maze

A significant increase in the latency (179.16%) and total distance travelled (180.76%) to reach the hidden platform was observed in the Hcy treated animals as compared to control animals (Fig. 7). NaHS administration significantly decreased the latency (115.37%) and distance travelled (146.11%) in HHcy animals. However, no effect was observed on the average velocity of the animals. The results of the probe trial (Fig. 7 E, F) showed a significant decrease in the time spent in quadrant having platform (19.44%). Similarly, a significant increase in time spent in the opposite quadrant (53.76%) was observed in Hcy treated animals. In addition to this, a significant decrease was observed in number of entries to the target platform (47.61%) in Hcy treated animals as compared to control animals. Interestingly, NaHS supplementation significantly reversed memory deficits in the Hcy treated animals.

3.9.2. Novel object recognition

Novel object recognition (NOR) test was performed to assess memory functions in HHcy animals. The representative heat maps of the animals are shown in Fig. 8 A. The performance in NOR task was impaired in HHcy animals as compared to control animals (Fig. 8). However, no change in memory score was observed in the studied groups. A significant decrease in number of entries to the novel object zone (68%) was observed in Hcy treated animals as compared to control animals. Similarly, there was a significant decrease in the discrimination index (56.07%) in HHcy animals as compared to control animals. Interestingly, NaHS supplementation increased the number of entries to the novel object zone (2.28 folds) and discrimination index (64.35%) in Hcy treated animals suggesting beneficial effect on memory.

4. Discussion

Elevated Hcy levels is an independent risk factor for developing Alzheimer's disease (AD), Parkinson's disease (PD), coronary artery disease, peripheral vascular disease, stroke and age-related dementia [38–40]. Hcy has also been reported to promote proliferation and activation of microglia in a dose and time dependent manner [41]. High methionine-supplementation increases the levels of pro-inflammatory cytokines and induce T cell activation by switching Akt S-nitrosylation to phosphorylation via elevated Hcy levels [42].

Earlier report from our lab has shown that elevated Hcy levels have a detrimental impact on cognitive functions through decreased endogenous H_2S levels and CBS activity in the brain regions, which were reversed by supplementation of NaHS [43]. However, the underlying mechanisms of H_2S mediated neuroprotection against Hcy-induced neurotoxicity and cognitive deficits are still not fully understood. Similar results have been observed in the present study wherein NaHS supplementation increased the H_2S levels with a concomitant increase in the CBS and CSE activity in the brain regions of HHcy animals. Moreover, NaHS supplementation also increased the polysulfide levels in the cortex of Hcy treated animals. Polysulfide and persulfide are synthesized by CBS and CSE in physiological conditions and are involved in redox signaling [31]. The decreased polysulfide levels might be due to reduced activity of CBS and CSE in the brain regions of HHcy animals, which were increased following NaHS administration. The polysulfides produced by H_2S might be responsible for its neuroprotective potential in HHcy animals. However, more comprehensive study is required to understand the role of persulfide and polysulfide in H_2S mediated neuroprotective effects.

A significant increase in the Iba-1 mRNA and protein levels were observed in the cortex and hippocampus of Hcy treated animals. Iba-1 is a calcium binding cytosolic protein and a reliable marker of activated microglia [44]. The results from immunohistochemistry of Iba-1 were in accordance with trends for Iba-1 mRNA and protein expression. Increased expression of Iba-1 has been reported in several neurodegenerative diseases via production of cytotoxic molecules, free radicals, pro-inflammatory cytokines and prostaglandins [45,46]. Our results support the study that Hcy promotes proliferation and activation of microglia [41]. A recent study has shown that Hcy treatment increased viability of BV2 microglial cells in a dose dependent manner [47]. Impaired CBS-H_2S pathway has been shown to contribute microglia-mediated neuro-inflammation following stroke and MPTP-induced neurodegeneration [48,49]. According to the study by Du et al. (2014),

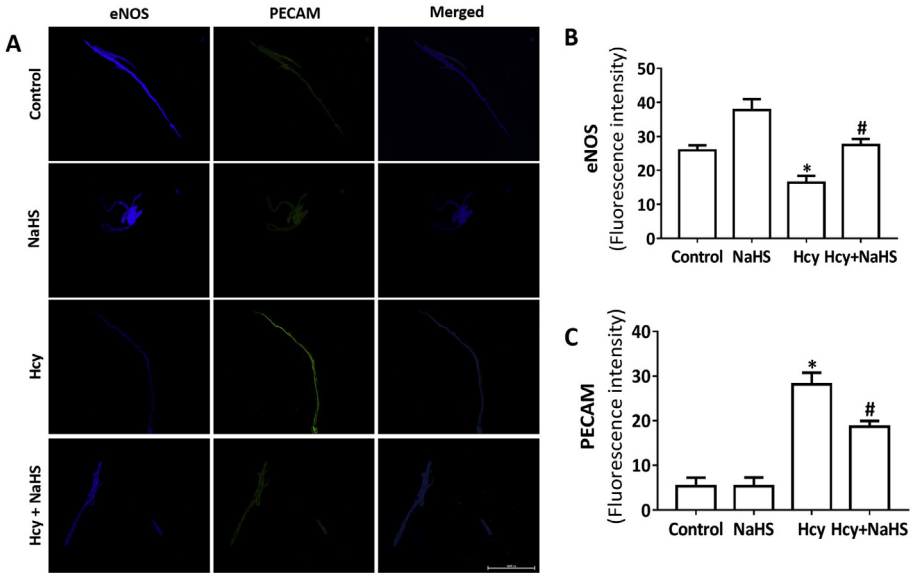

Fig. 6. Effect of H_2S administration on platelet endothelial cell adhesion molecule (PECAM) and eNOS immunofluorescence in microvessel isolated from the cortex of HHcy animals: Representative photographs of microvessels immunostained with anti-PECAM and anti-eNOS antibody (A); Histogram representing fluorescence intensity of eNOS (B) and PECAM (C) quantified with imageJ. Scale bar represents 100 μm. Values are expressed as mean ± SD; n = 3. *significantly different from control group ($p < 0.05$), #significantly different from Hcy treated group ($p < 0.05$).

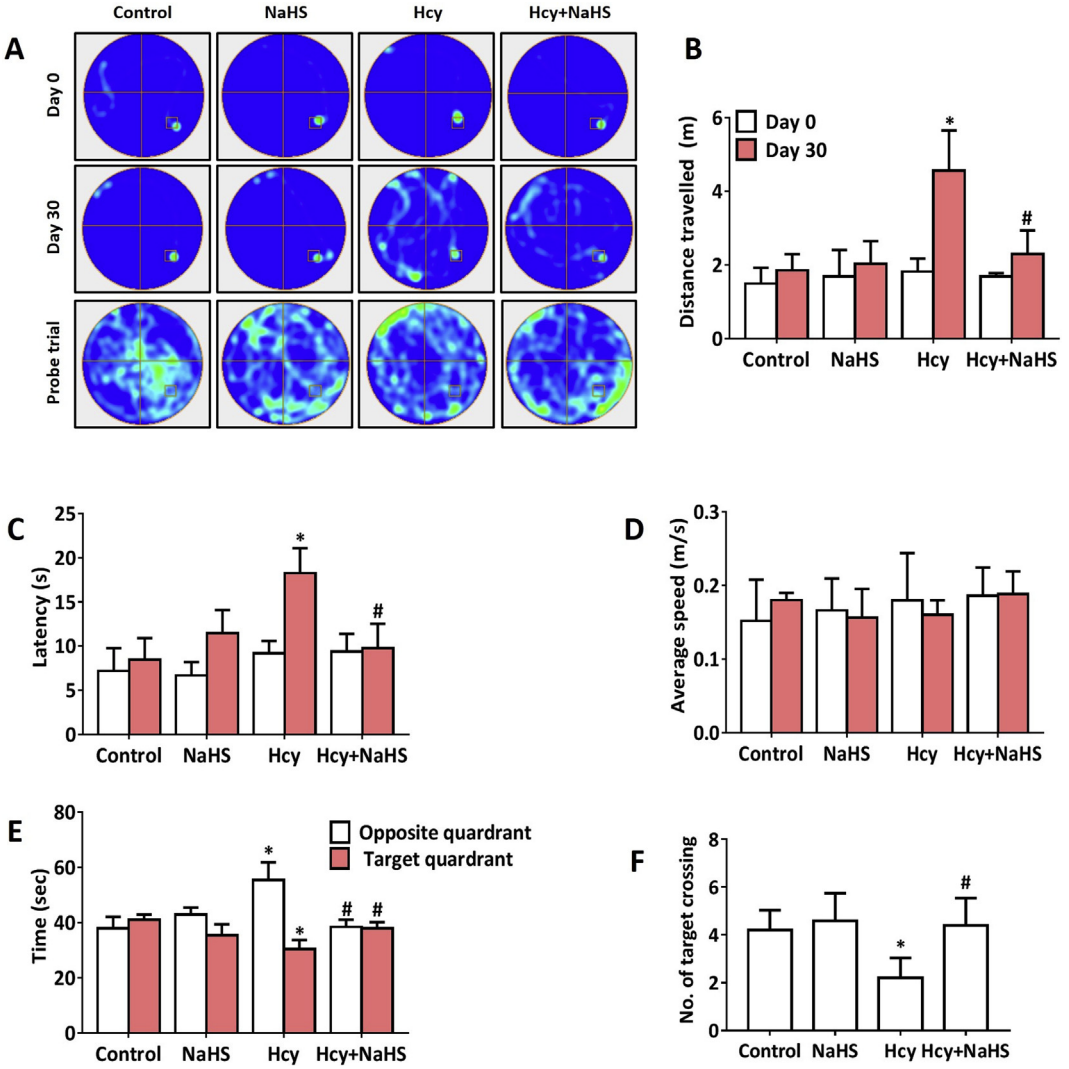

Fig. 7. Effect of H_2S administration on spatial learning and memory assessed by Morris water maze (MWM) in HHcy animals: Representative heat maps of path followed by the animals in various groups using video tracking software ANY-maze™ (A); Total distance travelled to reach the hidden platform (B); Latency to reach the hidden platform i(C); Average speed of the animals (D); Time spent in quadrant having platform and in opposite quadrant (E); Number of target crossing during the probe trial (F). Values are expressed as mean ± SD; n = 6. *significantly different from control group ($p < 0.05$), #significantly different from Hcy treated group ($p < 0.05$).

downregulation of CBS/H_2S axis contributes to microglia polarization toward M1 pro-inflammatory phenotype [50]. Recently, H_2S has been reported to attenuate ischemic-induced neuroinflammation by modulating microglia polarization [51]. In current study, NaHS supplementation decreased Iba-1 mRNA and protein expression and maintained microglia in quiescent state. Moreover, NaHS supplementation also improved the Hcy-induced morphological changes in microglia. Hcy altered the morphology of microglia with less number of projections and bigger somal area suggesting activation of microglia. However, NaHS treatment increased the length and number of projections with reduced somal area suggesting microglia in resting phase. The current study suggests that anti-inflammatory effect of NaHS may involve suppression of glia activation through elevation in H_2S levels. Hcy has been reported to exaggerate microglia activation via STAT3 mechanism [10] however, H_2S mediated inhibition of glial activation may involve the ability of H_2S to suppress STAT3 pathway [52]. Furthermore, our results corroborate the findings that H_2S promotes learning and memory and suppresses microglial response in repetitive febrile seizures [53].

Activation of glial cells is a characteristic feature of nerve tissue injury or stress, characterized by change in morphology and number of glial cells following up-regulation of intermediate filament proteins within the affected tissue [54]. GFAP is an astrocyte specific protein and considered as a reliable marker for reactive astrogliosis [55]. GFAP mRNA and protein levels were significantly increased in the hippocampus, whereas no change was observed in the cortex of Hcy treated animals. The results of our study are in accordance to the earlier reports wherein increased GFAP mRNA and protein expression has been observed in HHcy [47,56]. The transcription and translational changes of GFAP can affect the morphology of astrocytes in brain [57]. In present study, morphological changes were observed in astrocytes with decreased projection number and length with increased cell body following HHcy. A recent study has shown that Hcy can induce glial reactivity in cultured astrocytes [58] and enhance reactive gliosis in animal model of HHcy [59]. Inflammation targeted therapies can be beneficial in Hcy associated disorders and can prevent neuronal damage and maintain neuronal functions. Our results are in agreement with previous report that H_2S administration decreases mRNA and protein levels of GFAP in rodent model of AD [60]. Moreover, NaHS supplementation also reversed the Hcy-induced morphological changes in astrocytes towards resting phase. H_2S supplementation has been reported to attenuate Hcy-induced neuronal loss and prevent astrocyte

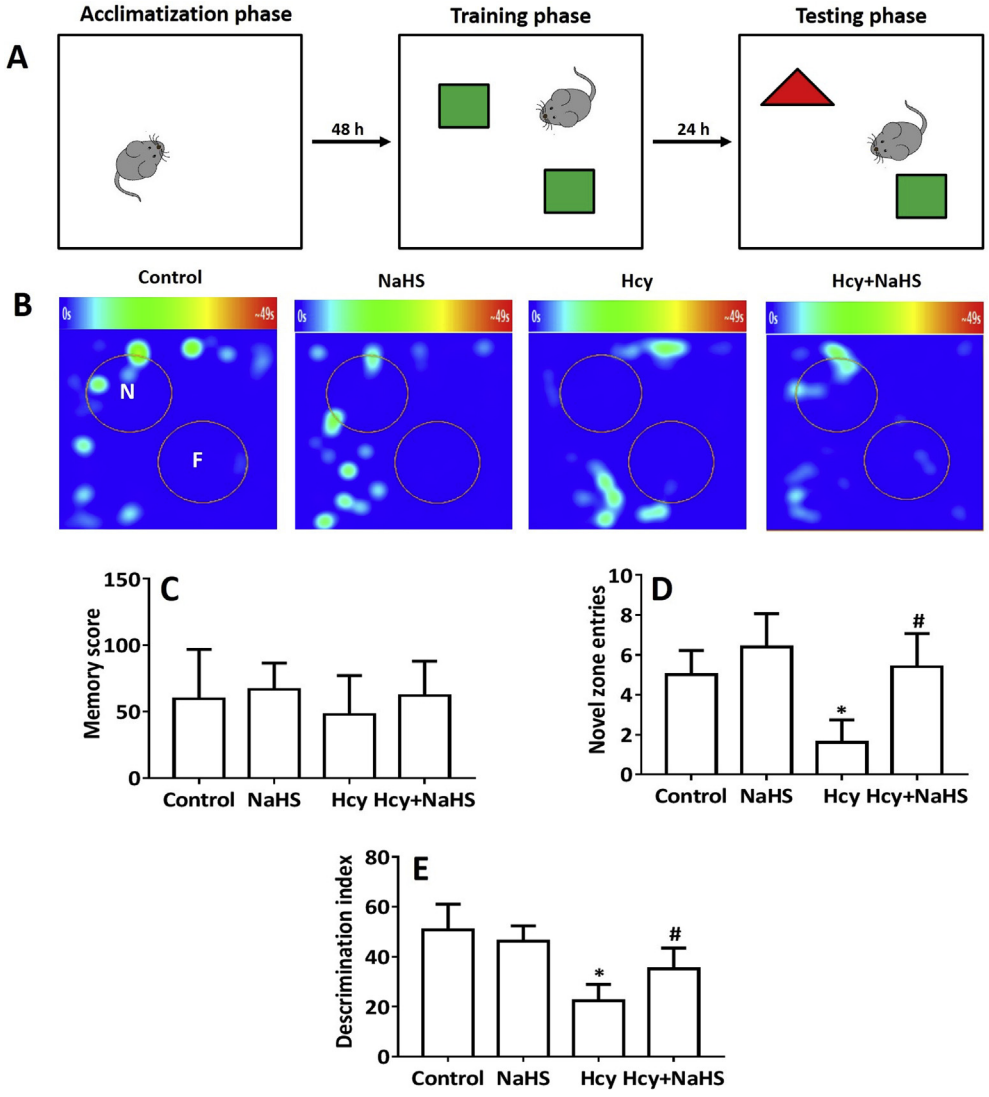

Fig. 8. Effect of H_2S administration on spatial memory assessed by novel object recognition (NOR) task in HHcy animals: Schematic representation of three phases of the test (A), green rectangle represent familiar object and red triangle represent novel object in the testing phase; Representative track plots of animals using video tracking software ANY-maze™ (B), N = novel object zone; F = familiar object zone; Memory score (C); Number of entries in the novel object zone (D) and Discrimination index (E). Values are expressed as mean ± SD; n = 6. *significantly different from control group ($p < 0.05$), #significantly different from Hcy treated group ($p < 0.05$).

activation in traumatic brain injury [19,61].

The pro-inflammatory cytokines released by glial cells as part of the early acute phase reactionhave unique and specific actions on neurons and circuits within the brain [62,63]. TNF-α is a marker of microglia with M1 phenotype [50] that synergistically acts with IL-1β to induce expression of IL-6 and is the key initiator of immune-mediated inflammation in brain [64]. In addition to the immune response, these cytokines are suggested to affect memory, modulate animal behaviour, neurotransmission and glucocorticoid functions [63]. Studies on monocytes and endothelial cells have suggested the participation of inflammation in the pathogenesis of HHcy [65,66]. The results of present study have shown that Hcy administration increased IL-6, TNF-α and MCP-1 mRNA and protein levels in the cortex and hippocampus. Elevated Hcy levels have been reported to increase the production of IL-1β in macrophages through nuclear factor Kappa β (NF-κB) mediated response [67]. Studies have shown that Hcy treatment stimulates MCP-1 expression in several cell types and pre-treatment of cells with NF-κβ inhibitors can suppress the stimulatory effect of Hcy on MCP-1 expression [68,69]. Hence, increased expression of cytokines and chemokine in HHcy might be mediated via NF-κB activation. Our data is in agreement with the report that Hcy induced mRNA and protein expression of inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8, and IL-12) [70]. NaHS supplementation decreased the levels of TNF-α, IL-6 and MCP-1 in HHcy animals. Reports suggest beneficial effect of H_2S and sulfides in neuro-inflammation through inhibition of NF-κB [71]. However, in present study the anti-inflammatory effect of H_2S on Hcy-induced neuro-inflammation might be due to inhibition of NF-κB by H_2S following improvement in memory deficits. Our data corroborate with the previous findings where H_2S is reported to improve cognitive deficits and suppress release of pro-inflammatory cytokines in repetitive febrile seizures and glia-mediated neuro-inflammation [72–74].

In order to verify the effect of Hcy and NaHS on systemic inflammation, we measured the levels of TNF-α, IL-6 and MCP-1 in serum of rats. The results reveal significant increase in the levels of cytokines (TNF-α, IL-6) and chemokine (MCP-1) in serum of the animals subjected to HHcy. The local release of TNF-α can stimulate neutrophils and endothelial cells to induce the expression of adhesion molecules for migration across the blood brain barrier [75]. The infiltration of monocytes across the vascular endothelium plays a key role at the site of inflammation. MCP-1 is believed to be the major chemotactic molecule for monocytes. In the present study we found a significant increase in systemic levels of MCP-1, which is in line with the report that Hcy induces the expression on MCP-1 in endothelial cells and promotes chemotaxis [69]. Moreover, Hcy also increases the expression of MCP-1 receptor (CCR2) in monocytes [76]. However, increased production of MCP-1 might be responsible for increased neutrophil infiltration and chemotaxis of monocytes in brain, which contributes to neuro-inflammation.

Elevated nitrite levels were observed in the hippocampus of Hcy

treated animals. A few studies support that Hcy promote the nitrite production [26] whereas, other supported the idea of negative correlation between Hcy and nitrite levels [77]. Our data in line with the report that Hcy induces nitrite production and increases 3-NT modification in proteins in the brain regions of HHcy animals indicating nitrative stress [2]. Additionally, decrease in expression of eNOS and increased iNOS expression have also been observed in the brain regions of Hcy treated animals. However, eNOS mediated endothelial dysfunction might be responsible for Hcy-induced neurotoxicity. The possible explanation for increased nitrite levels in the brain might be due to induction of iNOS by cytokines [54]. NaHS supplementation significantly decreased 3-NT modified proteins in brain regions and nitrite levels in hippocampus of HHcy animals suggesting H_2S as an anti-oxidant. Studies suggest that H_2S acts as anti-oxidant by preventing oxidative damage to brain and neurons [61,78] NaHS was also able to restore the eNOS and iNOS levels (mRNA and protein) in the cortex and hippocampus of Hcy treated animals. These results are in line with the previous report that H_2S restored the altered eNOS [79] and iNOS [26] levels in the brain. Moreover, a recent study has shown that, over-expression of CBS reduced the 3-NT modifications of proteins in striatal region of MPTP-induced model of PD [49].

Expression of platelet endothelial cell adhesion molecule (PECAM) was significantly increased in the microvessels of the HHcy rats. The increase in PECAM-1 protein in microvessels results in increase in transmigration of lymphocytes and neutrophils [80]. Our results corroborate with the finding that Hcy exposure increases the expression of endothelial adhesion molecules following increased leukocyte: endothelial interactions [81]. However, therapies targeting the pro-inflammatory cytokines, chemokines and cell adhesion molecules could be beneficial in Hcy associated neurodegenerative disorders. Interestingly, in our results H_2S supplementation reduced the levels of TNF-α, IL-6, MCP-1 in the serum and PECAM in the microvessels of HHcy animals, which is in line with earlier report [82]. H_2S inhalation has been reported to reduce serum cytokine and chemokine levels in hyperdynamic murine septic shock [83]. Peripheral inflammation has also been reported to induce microglia activation [84,85]. Therefore, H_2S may also prevent peripheral inflammation associated microglia activation in HHcy-induced inflammatory response. The data suggests H_2S to be potent anti-inflammatory molecule against Hcy-induced inflammation by reducing cytokine and chemokine levels along with reduced expression of PECAM in endothelial cells.

The release of inflammatory cytokines accompanied with activation of glia leads to enhanced neuro-inflammation and cognitive impairments [86]. Cortex and hippocampus are thought to play a central role in memory formation and retrieval [87]. Any kind of stress to cortex and hippocampus leads to impairment in spatial learning and memory [88]. There are various reports suggesting neuro-inflammation to play role in development of memory impairments [89,90]. Evidence suggests the role of neuro-inflammation in progression of chronic degenerative disorders and psychiatric diseases [64,91]. However, increase in pro-inflammatory cytokines may contribute to cognitive impairment in HHcy. The results from current study showed a progressive decline in spatial memory in animals following Hcy administration as assessed by Morris water maze and novel object recognition task. These findings are in agreement with the previous reports suggesting the role of Hcy in memory dysfunctions [92]. Interestingly, NaHS administration to HHcy animals showed a significant improvement in spatial memory and cognitive behaviour which is in accordance with the report by Li et al. [93]. A recent study has also shown that H_2S supplementation improves cognitive functions in streptozotocin-induced diabetes in rats [94].

5. Conclusion

In conclusion, our study demonstrates H_2S as a potent anti-inflammatory molecule in HHcy-induced inflammation. NaHS supplementation suppressed Hcy-induced glial activation and production of pro-inflammatory cytokines via increased endogenous H_2S production that subsequently improved cognitive deficits. H_2S pathway in glial cells may represent an important therapeutic target for preventing HHcy associated neurological disorders. However, the precise mechanism and role of H_2S/CBS axis in glial activation requires further clarification.

Author contributions

M.K. and R.S. contributed equally. M.K. and R.S. designed the study. M.K. performed the experiments. M.K. and R.S. analyzed the data and prepared the manuscript. All authors have read and approved the final version of the manuscript.

Conflicts of interest

The authors state no conflict of interest.

Acknowledgements

The financial assistance provided by the Department of Bio-Technology (DBT), Govt. of India, (grant number BT/361/NE/TBP/2012) is acknowledged. The fellowship to MK from the University Grants Commission (UGC), New Delhi [F.17-7(J)/2004(SA-1)] is also acknowledged. The authors also acknowledge financial assistance provided under the promotion of University Research and Scientific Excellence (PURSE) by Department of Science and Technology New Delhi and under Special Assistance Program (SAP) of UGC (DRS Phase-II).

References

[1] M. Kumar, S. Goudihalli, K. Mukherjee, S. Dhandapani, R. Sandhir, Methylenetetrahydrofolate reductase C677T variant and hyperhomocysteinemia in subarachnoid hemorrhage patients from India, Metab. Brain Dis. 33 (2018) 1617–1624.
[2] A.A. da Cunha, A.G.K. Ferreira, A.T.S. Wyse, Increased inflammatory markers in brain and blood of rats subjected to acute homocysteine administration, Metab. Brain Dis. 25 (2010) 199–206, https://doi.org/10.1007/s11011-010-9188-8.
[3] C.-G. Zou, R. Banerjee, Homocysteine and redox signaling, Antioxidants Redox Signal. 7 (2005) 547–559, https://doi.org/10.1089/ars.2005.7.547.
[4] M.P. Mattson, T.B. Shea, Folate and homocysteine metabolism in neural plasticity and neurodegenerative disorders, Trends Neurosci. 26 (2003) 137–146, https://doi.org/10.1016/S0166-2236(03)00032-8.
[5] S.A. Blaise, E. Nédélec, H. Schroeder, J.-M. Alberto, C. Bossenmeyer-Pourié, J.-L. Guéant, J.-L. Daval, Gestational vitamin B deficiency leads to homocysteine-associated brain apoptosis and alters neurobehavioral development in rats, Am. J. Pathol. 170 (2007) 667–679, https://doi.org/10.2353/ajpath.2007.060339.
[6] S. Dalal, S. Parkin, S. Homer-Vanniasinkam, A. Nicolaou, Effect of homocysteine on cytokine production by human endothelial cells and monocytes, Ann. Clin. Biochem. 40 (2003) 534–541, https://doi.org/10.1258/000456303322326452.
[7] E.B.S. Scherer, S.O. Loureiro, F.C. Vuaden, A.A. Cunha, Mild hyperhomocysteinemia increases brain acetylcholinesterase and proinflammatory cytokine levels in different tissues, Mol. Neurobiol. 50 (2014) 589–596, https://doi.org/10.1007/s12035-014-8660-6.
[8] C.-G. Zou, Y.-S. Zhao, S.-Y. Gao, S.-D. Li, X.-Z. Cao, M. Zhang, K.-Q. Zhang, Homocysteine promotes proliferation and activation of microglia, Neurobiol. Aging 31 (2010) 2069–2079, https://doi.org/10.1016/J.NEUROBIOLAGING.2008.11.007.
[9] D.D.S. Moreira, P.W. Figueiró, C. Siebert, C.A. Prezzi, F. Rohden, F.C.R. Guma, V. Manfredini, A.T.S. Wyse, Chronic mild hyperhomocysteinemia alters inflammatory and oxidative/nitrative status and causes protein/DNA damage , as well as ultrastructural changes in cerebral Cortex : is acetylsalicylic acid Neuroprotective ? Neurotox. Res. 33 (2017) 580–592.
[10] S. Chen, Z. Dong, M. Cheng, Y. Zhao, M. Wang, N. Sai, X. Wang, H. Liu, G. Huang, X. Zhang, Homocysteine exaggerates microglia activation and neuroinflammation through microglia localized STAT3 overactivation following ischemic stroke, J. Neuroinflammation 14 (2017) 187, https://doi.org/10.1186/s12974-017-0963-x.
[11] M.W. Warenycia, L.R. Goodwin, C.G. Benishin, R.J. Reiffenstein, D.M. Francom, J.D. Taylor, F.P. Dieken, Acute hydrogen sulfide poisoning. Demonstration of selective uptake of sulfide by the brainstem by measurement of brain sulfide levels, Biochem. Pharmacol. 38 (1989) 973–981.
[12] M. Kumar, R. Sandhir, Hydrogen sulfide in physiological and pathological mechanisms in brain, CNS Neurol. Disord. - Drug Targets 17 (2018) 654–670, https://doi.org/10.2174/1871527317666180605072018.
[13] Y. Enokido, E. Suzuki, K. Iwasawa, K. Namekata, H. Okazawa, H. Kimura,

Cystathionine β-synthase, a key enzyme for homocysteine metabolism, is preferentially expressed in the radial glia/astrocyte lineage of developing mouse CNS, FASEB J. 19 (2005) 1854–1856, https://doi.org/10.1096/fj.05-3724fje.
[14] H. Zhang, L. Zhi, S. Moochhala, P.K. Moore, M. Bhatia, Hydrogen sulfide acts as an inflammatory mediator in cecal ligation and puncture-induced sepsis in mice by upregulating the production of cytokines and chemokines via NF-κB, Am. J. Physiol. Cell. Mol. Physiol. 292 (2007) L960–L971, https://doi.org/10.1152/ajplung.00388.2006.
[15] L.F. Hu, P.T. Wong, P.K. Moore, J.S. Bian, Hydrogen sulfide attenuates lipopolysaccharide-induced inflammation by inhibition of p38 mitogen-activated protein kinase in microglia, J. Neurochem. 100 (2007) 1121–1128, https://doi.org/10.1111/j.1471-4159.2006.04283.x JNC4283.
[16] L.-F. Hu, P.T.-H. Wong, P.K. Moore, J.-S. Bian, Hydrogen sulfide attenuates lipopolysaccharide-induced inflammation by inhibition of p38 mitogen-activated protein kinase in microglia, J. Neurochem. 100 (2007) 1121–1128, https://doi.org/10.1111/j.1471-4159.2006.04283.x.
[17] M. Lee, C. Schwab, S. Yu, E. McGeer, P.L. McGeer, Astrocytes produce the antiinflammatory and neuroprotective agent hydrogen sulfide, Neurobiol. Aging 30 (2009) 1523–1534, https://doi.org/10.1016/j.neurobiolaging.2009.06.001.
[18] Y.-Y. Liu, J.-S. Bian, Hydrogen sulfide protects amyloid-β induced cell toxicity in microglia, J. Alzheimer's Dis. 22 (2011) 1189–1200, https://doi.org/10.3233/JAD-2010-101002.
[19] M. Campolo, E. Esposito, A. Ahmad, R. Di Paola, I. Paterniti, M. Cordaro, G. Bruschetta, J.L. Wallace, S. Cuzzocrea, Hydrogen sulfide-releasing cyclooxygenase inhibitor ATB-346 enhances motor function and reduces cortical lesion volume following traumatic brain injury in mice, J. Neuroinflammation 11 (2014) 196, https://doi.org/10.1186/s12974-014-0196-1.
[20] D. Giuliani, A. Ottani, D. Zaffe, M. Galantucci, F. Strinati, R. Lodi, S. Guarini, Hydrogen sulfide slows down progression of experimental Alzheimer's disease by targeting multiple pathophysiological mechanisms, Neurobiol. Learn. Mem. 104 (2013) 82–91, https://doi.org/10.1016/j.nlm.2013.05.006.
[21] J. Li, Q. Li, H. Du, Y. Wang, S. You, Homocysteine triggers inflammatory responses in macrophages through inhibiting CSE-H 2 S signaling via DNA hypermethylation of, CSE Promoter 16 (2015) 12560–12577, https://doi.org/10.3390/ijms160612560.
[22] U. Sen, S. Givvimani, O.A. Abe, E.D. Lederer, S.C. Tyagi, Cystathionine β-synthase and Cystathionine γ-lyase Double Gene Transfer Ameliorate Homocysteine-Mediated Mesangial Inflammation through Hydrogen Sulfide Generation vol. 300, (2018), pp. 155–163, https://doi.org/10.1152/ajpcell.00143.2010.
[23] K.L. Flannigan, T.A. Agbor, R.W. Blackler, J.J. Kim, W.I. Khan, E.F. Verdu, Impaired hydrogen sulfide synthesis and IL-10 signaling underlie hyperhomocysteinemia-associated exacerbation of colitis, Proc. Natl. Acad. Sci. U. S. A. 111 (2014) 13559–13564, https://doi.org/10.1073/pnas.1413390111.
[24] H. Wei, J. Xu, M. Li, J. Tang, W. Zou, P. Zhang, L. Wang, C. Wang, X. Tang, Hydrogen sulfide inhibits homocysteine-induced endoplasmic reticulum stress and neuronal apoptosis in rat hippocampus via upregulation of the BDNF-TrkB pathway, Acta Pharmacol. Sin. 35 (2014) 707–715, https://doi.org/10.1038/aps.2013.197.
[25] E.B.S. Scherer, A.A. da Cunha, J. Kolling, M.J. da Cunha, F. Schmitz, A. Sitta, D.D. Lima, D. Delwing, C.R. Vargas, A.T.S. Wyse, Development of an animal model for chronic mild hyperhomocysteinemia and its response to oxidative damage, Int. J. Dev. Neurosci. 29 (2011) 693–699, https://doi.org/10.1016/j.ijdevneu.2011.06.004.
[26] P.K. Kamat, A. Kalani, S. Givvimani, P.B. Sathnur, S.C. Tyagi, N. Tyagi, Hydrogen sulfide attenuates neurodegeneration and neurovascular dysfunction induced by intracerebral-administered homocysteine in mice, Neuroscience 252 (2013) 302–319, https://doi.org/10.1016/j.neuroscience.2013.07.051.
[27] B. Chen, W. Li, C. Lv, M. Zhao, H. Jin, H. Jin, J. Du, L. Zhang, X. Tang, Fluorescent probe for highly selective and sensitive detection of hydrogen sulfide in living cells and cardiac tissues, Analyst 138 (2013) 946–951, https://doi.org/10.1039/C2AN36113B.
[28] M.K. Thorson, T. Majtan, J.P. Kraus, A.M. Barrios, Identification of cystathionine β-synthase inhibitors using a hydrogen sulfide selective probe, Angew. Chem. Int. Ed. 52 (2013) 4641–4644, https://doi.org/10.1002/anie.201300841.
[29] J.L. Wood, Sulfane sulfur, Methods Enzymol. 143 (1987) 25–29.
[30] L.C. Green, D.A. Wagner, J. Glogowski, P.L. Skipper, J.S. Wishnok, S.R. Tannenbaum, Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids, Anal. Biochem. 126 (1982) 131–138.
[31] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[32] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR, Nucleic Acids Res. 29 (2001) e45.
[33] H. Towbin, T. Staehelint, J. Gordon, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, Proc. Natl. Acad. Sci. U. S. A. 76 (1979) 4350–4354.
[34] B. Rivero-Gutiérrez, A. Anzola, O. Martínez-Augustin, F.S. De Medina, Stain-free detection as loading control alternative to Ponceau and housekeeping protein immunodetection in Western blotting, Anal. Biochem. 467 (2014) 1–3, https://doi.org/10.1016/j.ab.2014.08.027.
[35] T.A. Brooks, B.T. Hawkins, J.D. Huber, R.D. Egleton, T.P. Davis, Chronic inflammatory pain leads to increased blood-brain barrier permeability and tight junction protein alterations, AJP Hear. Circ. Physiol. 289 (2005) H738–H743, https://doi.org/10.1152/ajpheart.01288.2004.
[36] R. Morris, Developments of a water-maze procedure for studying spatial learning in the rat, J. Neurosci. Methods 11 (1984) 47–60.
[37] C. V Vorhees, M.T. Williams, Morris water maze: procedures for assessing spatial and related forms of learning and memory, Nat. Protoc. 1 (2006) 848–858, https://doi.org/10.1038/nprot.2006.116.
[38] S.A. Blaise, E. Nédélec, H. Schroeder, J.-M. Alberto, C. Bossenmeyer-Pourié, J.-L. Guéant, J.-L. Daval, Gestational vitamin B deficiency leads to homocysteine-associated brain apoptosis and alters neurobehavioral development in rats, Am. J. Pathol. 170 (2007) 667–679, https://doi.org/10.2353/ajpath.2007.060339.
[39] B.N. Manolescu, E. Oprea, I.C. Farcasanu, M. Berteanu, C. Cercasov, Homocysteine and vitamin therapy in stroke prevention and treatment: a review, Acta Biochim. Pol. 57 (2010) 467–477.
[40] R. Obeid, W. Herrmann, Mechanisms of homocysteine neurotoxicity in neurodegenerative diseases with special reference to dementia, FEBS Lett. 580 (2006) 2994–3005, https://doi.org/10.1016/j.febslet.2006.04.088.
[41] C.-G. Zou, Y.-S. Zhao, S.-Y. Gao, S.-D. Li, X.-Z. Cao, M. Zhang, K.-Q. Zhang, Homocysteine promotes proliferation and activation of microglia, Neurobiol. Aging 31 (2010) 2069–2079, https://doi.org/10.1016/j.neurobiolaging.2008.11.007.
[42] A.F. Aissa, C. Lira, V.P. Venancio, S. Machado, L.C. Hernandes, P. Wellington, R. Curi, M. De Lourdes, P. Bianchi, L.M. Greggi, A. Ferro, C. Lira, V.P. Venancio, S. Machado, L.C. Hernandes, P. Wellington, R. Curi, Methionine-supplemented diet affects the expression of cardiovascular disease-related genes and increases inflammatory cytokines in mice heart and liver, J. Toxicol. Environ. Health Part A. 00 (2017) 1–13, https://doi.org/10.1080/15287394.2017.1357366.
[43] M. Kumar, M. Modi, R. Sandhir, Hydrogen sulfide attenuates homocysteine-induced cognitive deficits and neurochemical alterations by improving endogenous hydrogen sulfide levels, Biofactors 43 (2017) 434–450, https://doi.org/10.1002/biof.1354.
[44] F. Drago, P.-E. Sautière, F. Le Marrec-Croq, A. Accorsi, C. Van Camp, M. Salzet, C. Lefebvre, J. Vizioli, Microglia of medicinal leech (*Hirudo medicinalis*) express a specific activation marker homologous to vertebrate ionized calcium-binding adapter molecule 1 (Iba1/alias aif-1), Dev. Neurobiol. 74 (2014) 987–1001, https://doi.org/10.1002/dneu.22179.
[45] J.A. Smith, A. Das, S.K. Ray, N.L. Banik, Role of pro-inflammatory cytokines released from microglia in neurodegenerative diseases, Brain Res. Bull. 87 (2012) 10–20, https://doi.org/10.1016/j.brainresbull.2011.10.004.
[46] X.-G. Luo, S.-D. Chen, The changing phenotype of microglia from homeostasis to disease, Transl. Neurodegener. 1 (2012) 9, https://doi.org/10.1186/2047-9158-1-9.
[47] E.M. Weekman, A.E. Woolums, T.L. Sudduth, D.M. Wilcock, Hyperhomocysteinemia-induced gene expression changes in the cell types of the brain, ASN Neuro 9 (2017), https://doi.org/10.1177/1759091417742296 175909141774229.
[48] M. Zhang, X. Wu, Y. Xu, M. He, J. Yang, J. Li, Y. Li, G. Ao, J. Cheng, J. Jia, The cystathionine β-synthase/hydrogen sulfide pathway contributes to microglia-mediated neuroinflammation following cerebral ischemia, Brain Behav. Immun. 66 (2017) 332–346, https://doi.org/10.1016/j.bbi.2017.07.156.
[49] Y.-Q. Yuan, Y.-L. Wang, B.-S. Yuan, X. Yuan, X.-O. Hou, J.-S. Bian, C.-F. Liu, L.-F. Hu, Impaired CBS-H 2 S signaling axis contributes to MPTP-induced neurodegeneration in a mouse model of Parkinson's disease, Brain Behav. Immun. 67 (2018) 77–90, https://doi.org/10.1016/j.bbi.2017.07.159.
[50] C. Du, M. Jin, Y. Hong, Q. Li, X.H. Wang, J.M. Xu, F. Wang, Y. Zhang, J. Jia, C.F. Liu, L.F. Hu, Downregulation of cystathionine β-synthase/hydrogen sulfide contributes to rotenone-induced microglia polarization toward M1 type, Biochem. Biophys. Res. Commun. 451 (2014) 239–245, https://doi.org/10.1016/j.bbrc.2014.07.107.
[51] J. Ji, P. Xiang, T. Li, L. Lan, X. Xu, G. Lu, H. Ji, Y. Zhang, Y. Li, NOSH-NBP, a novel nitric oxide and hydrogen sulfide- releasing hybrid, attenuates ischemic stroke-induced neuroinflammatory injury by modulating microglia polarization, Front. Cell. Neurosci. 11 (2017), https://doi.org/10.3389/fncel.2017.00154.
[52] L. Cao, X. Cao, Y. Zhou, B.V. Nagpure, Z.-Y. Wu, L.F. Hu, Y. Yang, G. Sethi, P.K. Moore, J.-S. Bian, Hydrogen sulfide inhibits ATP-induced neuroinflammation and Aβ1–42 synthesis by suppressing the activation of STAT3 and cathepsin S, Brain, Behav. Immun. 73 (2018) 603–614, https://doi.org/10.1016/j.bbi.2018.07.005.
[53] F. Zhuang, X. Zhou, H. Li, X. Yang, Z. Dong, W. Zhou, J. Chen, Hydrogen sulfide promotes learning and memory and suppresses proinflammatory cytokines in repetitive febrile seizures, Neuroimmunomodulation 23 (2016) 271–277, https://doi.org/10.1159/000449504.
[54] M. Aschner, Astrocytic functions and physiological reactions to injury: the potential to induce and/or exacerbate neuronal dysfunction–a forum position paper, Neurotoxicology 19 (1998) 7–17.
[55] W. Kamphuis, C. Mamber, M. Moeton, L. Kooijman, J.A. Sluijs, A.H.P. Jansen, M. Verveer, L.R. de Groot, V.D. Smith, S. Rangarajan, J.J. Rodríguez, M. Orre, E.M. Hol, GFAP isoforms in adult mouse brain with a focus on neurogenic astrocytes and reactive astrogliosis in mouse models of alzheimer disease, PLoS One 7 (2012) e42823, , https://doi.org/10.1371/journal.pone.0042823.
[56] A. Di Meco, J.-G. Li, C. Barrero, S. Merali, D. Praticò, Elevated levels of brain homocysteine directly modulate the pathological phenotype of a mouse model of tauopathy, Mol. Psychiatry (2018), https://doi.org/10.1038/s41380-018-0062-0.
[57] T.E. Morgan, I. Rozovsky, S.K. Goldsmith, D.J. Stone, T. Yoshida, C.E. Finch, Increased transcription of the astrocyte gene GFAP during middle-age is attenuated by food restriction: implications for the role of oxidative stress, Free Radic. Biol. Med. 23 (1997) 524–528.
[58] A. Longoni, B. Bellaver, L.D. Bobermin, C.L. Santos, Y. Nonose, J. Kolling, T.M. dos Santos, A.M. de Assis, A. Quincozes-Santos, A.T.S. Wyse, Homocysteine induces glial reactivity in adult rat astrocyte cultures, Mol. Neurobiol. 55 (2017) 1966–1977, https://doi.org/10.1007/s12035-017-0463-0.
[59] G. Baydas, M. Ozer, A. Yasar, S.T. Koz, M. Tuzcu, Melatonin prevents oxidative

stress and inhibits reactive gliosis induced by hyperhomocysteinemia in rats, Biochemistry. (Mosc). 71 (Suppl 1) (2006) S91–S95.
[60] P.K. Kamat, P. Kyles, A. Kalani, N. Tyagi, Hydrogen sulfide ameliorates homocysteine-induced Alzheimer's disease-like pathology, blood–brain barrier disruption, and synaptic disorder, Mol. Neurobiol. 53 (2016) 2451–2467, https://doi.org/10.1007/s12035-015-9212-4.
[61] M. Kumar, R.S. Ray, R. Sandhir, Hydrogen sulfide attenuates homocysteine-induced neurotoxicity by preventing mitochondrial dysfunctions and oxidative damage: in vitro and in vivo studies, Neurochem. Int. 120 (2018) 87–98, https://doi.org/10.1016/j.neuint.2018.07.010.
[62] C.A. Dinarello, Immunological and inflammatory functions of the interleukin-1 family, Annu. Rev. Immunol. 27 (2009) 519–550, https://doi.org/10.1146/annurev.immunol.021908.132612.
[63] K.A. Jones, C. Thomsen, The role of the innate immune system in psychiatric disorders, Mol. Cell. Neurosci. 53 (2013) 52–62, https://doi.org/10.1016/j.mcn.2012.10.002.
[64] S.-M. Lucas, N.J. Rothwell, R.M. Gibson, The role of inflammation in CNS injury and disease, Br. J. Pharmacol. 147 (2009) S232–S240, https://doi.org/10.1038/sj.bjp.0706400.
[65] R. Poddar, N. Sivasubramanian, P.M. DiBello, K. Robinson, D.W. Jacobsen, Homocysteine induces expression and secretion of monocyte chemoattractant protein-1 and interleukin-8 in human aortic endothelial cells: implications for vascular disease, Circulation 103 (2001) 2717–2723.
[66] S. Dalal, S. Parkin, S. Homer-Vanniasinkam, A. Nicolaou, Effect of homocysteine on cytokine production by human endothelial cells and monocytes, Ann. Clin. Biochem. 40 (2003) 534–541, https://doi.org/10.1258/000456303322326452.
[67] R.F. Zanin, L.S. Bergamin, F.B. Morrone, R. Coutinho-Silva, A.T. de Souza Wyse, A.M.O. Battastini, Pathological concentrations of homocysteine increases IL-1β production in macrophages in a P2X7, NF-κB, and erk-dependent manner, Purinergic Signal. 11 (2015) 463–470, https://doi.org/10.1007/s11302-015-9464-5.
[68] G. Wang, C.W.H. Woo, F.L. Sung, Y.L. Siow, K.O, Increased monocyte adhesion to aortic endothelium in rats with hyperhomocysteinemia: role of chemokine and adhesion molecules, Arterioscler. Thromb. Vasc. Biol. 22 (2002) 1777–1783.
[69] F.L. Sung, Y.L. Slow, G. Wang, E.G. Lynn, K.O, Homocysteine stimulates the expression of monocyte chemoattractant protein-1 in endothelial cells leading to enhanced monocyte chemotaxis, Mol. Cell. Biochem. 216 (2001) 121–128.
[70] S.-J. Su, L.-W. Huang, L.-S. Pai, H.-W. Liu, K.-L. Chang, Homocysteine at pathophysiologic concentrations activates human monocyte and induces cytokine expression and inhibits macrophage migration inhibitory factor expression, Nutrition 21 (2005) 994–1002, https://doi.org/10.1016/j.nut.2005.01.011.
[71] J. Ji, P. Xiang, T. Li, L. Lan, X. Xu, G. Lu, H. Ji, Y. Zhang, Y. Li, NOSH-NBP, a novel nitric oxide and hydrogen sulfide- releasing hybrid, attenuates ischemic stroke-induced neuroinflammatory injury by modulating microglia polarization, Front. Cell. Neurosci. 11 (2017) 154, https://doi.org/10.3389/fncel.2017.00154.
[72] D.K. Mostafa, N.M. El Azhary, R.A. Nasra, The hydrogen sulfide releasing compounds ATB-346 and diallyl trisulfide attenuate streptozotocin-induced cognitive impairment, neuroinflammation, and oxidative stress in rats: involvement of asymmetric dimethylarginine, Can. J. Physiol. Pharmacol. 94 (2016) 699–708, https://doi.org/10.1139/cjpp-2015-0316.
[73] H. Liu, Y. Deng, J. Gao, Y. Liu, W. Li, J. Shi, Q. Gong, Sodium hydrosulfide attenuates beta-amyloid-induced cognitive deficits and neuroinflammation via modulation of MAPK/NF-κB pathway in rats, Curr. Alzheimer Res. 12 (2015) 673–683.
[74] M. Lee, E.G. McGeer, P.L. McGeer, Sodium thiosulfate attenuates glial-mediated neuroinflammation in degenerative neurological diseases, J. Neuroinflammation 13 (2016) 32, https://doi.org/10.1186/s12974-016-0488-8.
[75] L.W. Argenbright, R.W. Barton, Interactions of leukocyte integrins with intercellular adhesion molecule 1 in the production of inflammatory vascular injury in vivo. The Shwartzman reaction revisited, J. Clin. Investig. 89 (1992) 259–272, https://doi.org/10.1172/JCI115570.
[76] G. Wang, K.O, Homocysteine stimulates the expression of monocyte chemoattractant protein-1 receptor (CCR2) in human monocytes: possible involvement of oxygen free radicals, Biochem. J. 357 (2001) 233–240.
[77] N. Weiss, L. Papatheodorou, N. Morihara, R. Hilge, N. Ide, Aged garlic extract restores nitric oxide bioavailability in cultured human endothelial cells even under conditions of homocysteine elevation, J. Ethnopharmacol. 145 (2013) 162–167, https://doi.org/10.1016/j.jep.2012.10.045.
[78] M. Kumar, R. Sandhir, Neuroprotective effect of hydrogen sulfide in hyperhomocysteinemia is mediated through antioxidant action involving Nrf2, NeuroMolecular Med. 20 (2018) 475–490, https://doi.org/10.1007/s12017-018-8505-y.
[79] P.K. Kamat, A. Kalani, S.C. Tyagi, N. Tyagi, Hydrogen sulfide epigenetically attenuates homocysteine-induced mitochondrial toxicity mediated through NMDA receptor in mouse brain endothelial (bEnd3) cells, J. Cell. Physiol. 230 (2015) 378–394, https://doi.org/10.1002/jcp.24722.
[80] C. Michiels, T. Arnould, J. Remacle, Role of PECAM-1 in the adherence of PMN to hypoxic endothelial cells, Cell Adhes. Commun. 5 (1998) 367–374.
[81] K. Alkhoury, S.M. Parkin, S. Homer-Vanniasinkam, A.M. Graham, Chronic homocysteine exposure upregulates endothelial adhesion molecules and mediates leukocyte: endothelial cell interactions under flow conditions, Eur. J. Vasc. Endovasc. Surg. 41 (2011) 429–435, https://doi.org/10.1016/j.ejvs.2010.11.012.
[82] E.C.S. Rios, F.G. Soriano, G. Olah, D. Gerö, B. Szczesny, C. Szabo, Hydrogen sulfide modulates chromatin remodeling and inflammatory mediator production in response to endotoxin, but does not play a role in the development of endotoxin tolerance, J. Inflamm. (Lond). 13 (2016) 10, https://doi.org/10.1186/s12950-016-0119-2.
[83] F. Wagner, K. Wagner, S. Weber, B. Stahl, M.W. Knöferl, M. Huber-Lang, D.H. Seitz, P. Asfar, E. Calzia, U. Senftleben, F. Gebhard, M. Georgieff, P. Radermacher, V. Hysa, Inflammatory effects of hypothermia and inhaled H2S during resuscitated, hyperdynamic murine septic shock, Shock 35 (2011) 396–402, https://doi.org/10.1097/SHK.0b013e3181ffff0e.
[84] I.C.M. Hoogland, C. Houbolt, D.J. van Westerloo, W.A. van Gool, D. van de Beek, Systemic inflammation and microglial activation: systematic review of animal experiments, J. Neuroinflammation 12 (2015) 114, https://doi.org/10.1186/s12974-015-0332-6.
[85] K. Riazi, M.A. Galic, J.B. Kuzmiski, W. Ho, K.A. Sharkey, Q.J. Pittman, Microglial activation and TNF production mediate altered CNS excitability following peripheral inflammation, Proc. Natl. Acad. Sci. Unit. States Am. 105 (2008) 17151–17156, https://doi.org/10.1073/pnas.0806682105.
[86] S.G. Levin, O.V. Godukhin, Modulating effect of cytokines on mechanisms of synaptic plasticity in the brain, Biochem 82 (2017) 264–274, https://doi.org/10.1134/S000629791703004X.
[87] A.R. Preston, H. Eichenbaum, Interplay of hippocampus and prefrontal cortex in memory, Curr. Biol. 23 (2013) R764–R773, https://doi.org/10.1016/j.cub.2013.05.041.
[88] L.M. Veng, A.-C. Granholm, G.M. Rose, Age-related sex differences in spatial learning and basal forebrain cholinergic neurons in F344 rats, Physiol. Behav. 80 (2003) 27–36.
[89] M.A. Alam, V.P. Subramanyam Rallabandi, P.K. Roy, Systems biology of immunomodulation for post-stroke neuroplasticity: multimodal implications of pharmacotherapy and neurorehabilitation, Front. Neurol. 7 (2016) 94, https://doi.org/10.3389/fneur.2016.00094.
[90] W. Lee, M. Moon, H.G. Kim, T.H. Lee, M.S. Oh, Heat stress-induced memory impairment is associated with neuroinflammation in mice, J. Neuroinflammation 12 (2015) 102, https://doi.org/10.1186/s12974-015-0324-6.
[91] M.K. Jha, J.-H. Kim, G.J. Song, W.-H. Lee, I.-K. Lee, H.-W. Lee, S.S.A. An, S. Kim, K. Suk, Functional dissection of astrocyte-secreted proteins: implications in brain health and diseases, Prog. Neurobiol. 162 (2017) 37–69, https://doi.org/10.1016/j.pneurobio.2017.12.003.
[92] J. Kolling, A. Longoni, C. Siebert, T.M. dos Santos, E.P. Marques, J. Carletti, L.O. Pereira, A.T.S. Wyse, Severe hyperhomocysteinemia decreases creatine kinase activity and causes memory impairment: neuroprotective role of creatine, Neurotox. Res. 32 (2017) 585–593, https://doi.org/10.1007/s12640-017-9767-0.
[93] M. Li, P. Zhang, H.J. Wei, M.H. Li, W. Zou, X. Li, H.F. Gu, X.Q. Tang, Hydrogen sulfide ameliorates homocysteine-induced cognitive dysfunction by inhibition of reactive aldehydes involving upregulation of ALDH2, Int. J. Neuropsychopharmacol. (2016), https://doi.org/10.1093/ijnp/pyw103 pyw103.
[94] W. Zou, J. Yuan, Z.-J. Tang, H.-J. Wei, W.-W. Zhu, P. Zhang, H.-F. Gu, C.-Y. Wang, X.-Q. Tang, Hydrogen sulfide ameliorates cognitive dysfunction in streptozotocin-induced diabetic rats: involving suppression in hippocampal endoplasmic reticulum stress, Oncotarget 8 (2017) 64203–64216, https://doi.org/10.18632/oncotarget.19448.
[95] R. Sandhir, G. Onyszchuk, N.E. Berman, Exacerbated glial response in the aged mouse hippocampus following controlled cortical impact injury, Exp. Neurol. 2 (2008) 372–380, https://doi.org/10.1016/j.expneurol.2008.06.013.
[96] M. Antunes, G. Biala, The novel object recognition memory: neurobiology, test procedure, and its modifications, Cogn Process. 2 (2012) 93–110.