EXPERIMENTAL STUDY

Evaluation of the neuroprotective efficiency of sodium hydrosulfide in neonatal rats with the induced hypoxic-ischemic encephalopathy model

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ABSTRACT
AIMS: Hypoxic ischemic encephalopathy is one of the main causes of neonatal deaths. The objective of this study was to evaluate the neuroprotective effect of antioxidant and anti-inflammatory properties of sodium hydrosulfide (NaHS) in neonatal rats with hypoxic ischemic encephalopathy, as well as its effect on neuronal apoptosis through histopathological and biochemical tests.

METHODS: Forty-seven-day-old rats with induced hypoxia-ischemia (HI) were randomly separated into four groups. Half an hour after the induction of hypoxic-ischemia, serum physiological (SF), 50 μmol/kg NaHS, or 100 μmol/kg NaHS were intraperitoneally given to the rats.

RESULTS: Apoptotic cells in the brain tissue of rats in HI + NaHS 50 μmol/kg, and HI + NaHS 100 μmol/kg groups decreased compared to HI group (p = 0.00). While HI + NaHS 50 μmol/kg and HI + NaHS 100 μmol/kg groups yielded no difference in TNF-α, IL-6, and iNOS levels as compared to the HI group, an increase in NGF was detected in the 50 μmol/kg and 100 μmol/kg NaHS groups (p = 0.34, p = 0.24, p = 0.26, p = 0.026, p = 0.017). When TOS, TAS and OSI levels were compared, an increase in TOS and OSI and a decrease in TOS were observed in the treatment groups as compared to HI group.

CONCLUSIONS: NaHS given to hypoxic-ischemic encephalopathy model significantly decreased apoptosis in neurons and had a neuroprotective efficacy with an increase in NGF levels (Tab. 1, Fig. 3, Ref. 25). Text in PDF www.elis.sk

KEY WORDS: hypoxic-ischemic encephalopathy, neonatal, NaHS, NGF, antioxidant.

Introduction

Presenting with impaired consciousness or convulsions and characterized by respiratory depression and hypotonia, neonatal encephalopathy (NE) is defined as a heterogeneous clinical syndrome occurring in the early postnatal period in rats born in and after the 35th gestational week. NE may have many different causes, but birth asphyxia and neonatal hydropic encephalopathy are responsible for most cases (1). The numbers of cases with irreversible damage and death following perinatal asphyxia are high, and thus, neuroprotective strategies preventing damage development are emphasized.

As many studies have proven, therapeutic hypothermia is neuroprotective, and as such, it is the standard treatment for term and late preterm neonates with encephalopathy (2). Additional strategies to increase the neuroprotective effects of hypothermia are targeted in continuing studies (3). To prevent the formation of free oxygen radicals, agents removing free oxygen radicals (selenium, vitamin E, N-acetyl cysteine, and ascorbic acid), stem cell transfer, dexamethasone, calcium channel blockers, magnesium sulfate and antiepileptic drugs can be used (4). Endogenous hydrogen sulfide (H2S) is formed in the brain due to cystathionine b-synthase catalysis of cysteine. H2S, in physiological concentrations, is effective for preventing and removing different oxidizing materials in the nervous system and thus increases neuronal protection, and decreases oxidative-stress-related tissue damage. Sodium hydrosulfide (NaHS) is an H2S transmitter and can directly transform into H2S in serum. Many studies have shown that NaHS can decrease damage which may develop in a target organ during ischemia-reperfusion in the myocardium, kidney, liver, and large intestines. It also has anti-inflammatory, antiapoptotic, and antioxidant characteristics. The neuroprotective and antioxidant efficacies of NaHS in cerebral ischemia damage have been observed but incompletely clarified (5, 6). Therefore, the objective of this study was to evaluate the neuroprotective effect of antioxidant and anti-inflammatory properties of NaHS, in neonatal rats with hypoxic ischemic encephalopathy, as well as its effect on neuronal apoptosis through histopathological and biochemical tests.
Materials and methods

Animals
This study was approved by the Ethics Committee of Animal Care and Usage of Kutahya Health Sciences University, Kutahya, Turkey. Sprague–Dawley rats, at 16–18 days of pregnancy, were kept separately in a barrier facility at a temperature of 25.0 ± 1.0 °C and relative humidity of 60 ± 5 % with food and water ad libitum, under a 12/12 h light/dark cycle. These conditions were designed to prevent exposing the animals to bright light or noise. Pups weighing 13–19 g were selected on postnatal day 7 (P7).

Hypoxic ischemic (HI) brain injury model
The HI brain injury model in neonatal rats was established according to the method detailed by Rice-Vannucci (7). P7 rat pups were anesthetized by isoflurane inhalation. After anesthesia, the necks of the rat pups, using standard sterile techniques, were draped and prepared. In a supine position, the left common carotid artery was ligated with a 5-0 silk suture. Rats were exposed to hypoxia (8 % oxygen concentration and 92 % nitrogen) using standard published protocols, for 2 h at 37 °C. The intraperitoneal (ip) injection in a dose of 50 μmol/kg of sodium hydrosulfide (NaHS) was administrated 30 min after HI brain injury.

RNA extraction and cDNA synthesis
Total RNA was extracted from the frozen brain tissue samples using the GeneJET RNA Purification Kit (Thermo, Cat No: # K0732) according to the manufacturer’s protocol. The purity and concentration of the extracted RNA was determined spectrophotometrically at 260 nm and 280 nm wavelengths (Maestro Nano Micro-Volume spectrophotometer, Maestrogen Inc., Las Vegas, NV). Complementary DNAs (cDNA) were synthesized from 1 μg of the total RNA using an EasyScript™ cDNA Synthesis Kit (abm) according to the manufacturer’s instructions. The cDNAs were stored at -20 °C until used in the quantitative real-time polymerase chain reaction (qRT-PCR).

Quantitative real-time PCR
The mRNA levels of transforming growth factor-alpha (TNF-α), interleukin 6 (IL-6), nerve growth factor (NGF), inducible nitric oxide synthase (iNOS), and the housekeeping gene beta-actin (β-actin) were measured by qRT-PCR using a LightCycler 480 Real-Time PCR System (Roche Diagnostics, Germany). The specific primers for measuring target genes and β-actin were used, and the lengths for amplified products were as follows: β-actin (Sense: 5’-ACC AGA GGC ATA CAG GGA CAA-3’, 100 bp; anti-sense: 5’-ACC AGA GGC ATA CAG GGA CAA-3’). Quantitative results are presented as fold changes relative to the control group (C).

Sample collection
Neonatal rats (n = 9, in each group) were anesthetized with isoflurane inhalation 5 h after NaHS injections and then sacrificed. Brain tissue samples were collected in liquid nitrogen and rapidly dissected. Brain tissue was precisely divided into two hemispheres. The right hemisphere was kept in a 10% formalin solution for histological examination and analyzed for neuronal apoptosis using the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL) method. The left hemisphere was stored at ~80 °C until analyzed biochemically and via molecular biologic studies.

Biochemical analyses
The brain tissue extracted from the sacrificed animals was first placed in a phosphate buffer (pH 7, 4). Briefly, the tissues were disrupted with a homogenizer (WiseTis, HG-15A, Korea). Later, the tissue samples were centrifuged at 7,800 x g for 15 min at +4 °C. Total anti-oxidative status (TAS) and total oxidative status (TOS) levels, which are among the oxidative stress parameters, were measured using kits (Rell Assay, Gaziantep, Turkey) according to the manufacturer’s protocol (9, 10). Changes in the absorbance of samples were measured at 530 nm and 660 nm using an ELISA microplate reader (Thermo Multiscan GO, 1510, Finland), and the results were expressed as mmol Trolox Eq/mg protein. The oxidative stress index (OSI) was defined by formula: OSI (arbitrary unit) = [(TOS, mmol/L)/(TAS, mmol Trolox equivalent/ L)/100].

Table 1. Oxidative stress markers of brain tissue in all groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>C (n=9)</th>
<th>HI (n=9)</th>
<th>HI+NaHS 50 μmol/kg (n=9)</th>
<th>HI+NaHS 100 μmol/kg (n=9)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAS (mmol/L)</td>
<td>0.32±0.07a</td>
<td>0.29±0.05b</td>
<td>0.48±0.07</td>
<td>0.65±0.11</td>
<td>0.04</td>
</tr>
<tr>
<td>TOS (μmol/L)</td>
<td>14.4±0.77bc</td>
<td>25.9±3.28c</td>
<td>20.7±0.80b</td>
<td>17.5±1.77c</td>
<td>0.000</td>
</tr>
<tr>
<td>OSI</td>
<td>4.78±0.97ab</td>
<td>15.9±4.08a</td>
<td>4.63±0.90b</td>
<td>4.28±1.05b</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Note: in each line, the difference between the means with the same letters are significant, p<0.05 (Mann-Whitney U test).
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Fig. 1. Effect of NaHS on mRNA expression of TNF-α, IL-6, NGF and iNOS: A; There were no significant differences in TNF-α gene mRNA expression levels in the brain tissues among C, HI, HI + NaHS 50 μmol/kg, and HI + NaHS 100 μmol/kg groups (p > 0.05). B; * shows significant difference between C and HI groups (p ≤ 0.05; Mann–Whitney U test). C; * shows significant difference between HI and HI + NaHS 50 μmol/kg groups (p ≤ 0.05; Mann–Whitney U test), † shows significant difference between HI and HI + NaHS 100 μmol/kg groups (p ≤ 0.05; Mann–Whitney U test). D; * shows significant difference between C and HI groups (p ≤ 0.05; Mann–Whitney U test).

TNF-α (Sense: 5′-CCA CCA CGC TCT TCT GTC TAC-3′ and anti-sense: 5′-GCT ACG GGC TTG TCA CTC G-3′, 148 bp); IL-6 (Sense: 5′-CTT CCA GCC AGT TGC TTT CTT G-3′ and anti-sense: 5′-TGG TCT GTT GTG GGT GGT ATC C-3′, 109 bp); NGF (Sense: 5′-CCA AGG ACG CAG CTT TCT AT-3′ and anti-sense: 5′-CTC CGG TGA TGT GGG TGT GGT ATC C-3′, 126 bp), and iNOS (Sense: 5′-TGT TGT AGC AGT TGC GTT GGT ATC C-3′ and anti-sense: 5′-CAC CTT CTT GGA GTT CAC CCA GT-3′, 500 bp), All PCR amplifications were performed in duplicate reactions and in the final volume of 20 μl containing 5 μl cDNA, 0.6 μl of specific primers, and 10 μl of master mix SYBR green (EvaGreen Express qPCR MasterMix, abm, Canada) 3.8 μl ddH₂O using the following protocol:
1. Pre-incubation at 95 °C for 30 s to activate DNA Taq polymerase, 2. Forty two-step cycles with denaturation at 95 °C for 15 s, 3. Annealing at 60 °C for 60 s, 4. Extension at 72 °C for 30 s.

In addition, the no-template negative control (H₂O) was routinely run in every PCR. The melting curve was examined at the end of the amplification process to ensure the specificity of PCR products. The expression levels of target genes (TNF-α, IL-6, NGF, iNOS) were normalized against β-actin expression. To determine the relative quantification of gene expression, the comparative cycle of threshold (Ct) method, with arithmetic formula (2−ΔΔCt), was used.

Histopathologic examination
After being fixed with 10 % buffered formalin and embedded in paraffin, brain samples were cut into 5-mm thick sections and then stained with hematoxylin–eosin (H&E). Brain pathological injury was assessed via light microscope (Olympus BX51, Japan).

TUNEL staining
Rat brains were examined by a pathologist blinded to the groups. The TUNEL method (in situ apoptosis detection kit, Bio-
gen, catalog no S7101) was used to identify and quantify apoptotic neuronal cells and to detect DNA fragmentation in neurons. The procedure was as follows:

1. Coronal brain sections, 5-mm thick, were deparaffinized and treated with alcohol.
2. Sections were incubated with protein kinase K at room temperature for 15 min.
3. Sections were fixed in 4% paraformaldehyde phosphate-buffered saline (PBS) at room temperature for 30 min.
4. Sections were washed three times with PBS.
5. The TUNEL reaction mixture was added to the sections and incubated in a humidified chamber with 50 μl of terminal deoxy-nucleotidyl transferase (TdT) buffer in the dark for 1 h at 37 °C.
6. TUNEL labelled slides were counter-stained with 1% methyl green.
7. The reaction was stopped, and coverslips were mounted onto glass slides.
8. Apoptotic cells were counted in the hippocampus, subthalamic nucleus, and parietal cortex of the right hemisphere.
9. In evaluating the numeric density, total TUNEL-positive stained neurons were counted in five high power fields (5 x 400) under the light microscope (Olympus BX51, Japan).

**Statistical analysis**

Statistical analysis was conducted with SPSS (Statistical Package for Social Sciences, Chicago, IL, USA) 16.0 pocket program.

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**Fig. 2. Effect of NaHS on brain histopathologic examinations:** Images are representatives of the H&E-stained sections of the brain tissue from the experimental groups (H&E staining). Original magnification: ×20. A: C group, B: HI group, C: HI + NaHS 50 μmol/kg group, D: HI + NaHS 100 μmol/kg group. E: * shows significant difference between HI and HI + NaHS 50 μmol/kg groups (p ≤ 0.05; Mann-Whitney U test); † shows significant difference between HI and HI + NaHS 100 μmol/kg groups (p ≤ 0.05; Mann-Whitney U test).
All results were given as means ± standard error (SE). Multiple groups were compared via Kruskal-Wallis test, while Mann-Whitney U test was used to compare two groups. Values lower than p ≤ 0.05 were accepted as statistically significant.

**Results**

**Effect of NaHS on tissue TAS, TOS and OSI levels**

There were statistically significant differences in tissue TAS, TOS, and OSI levels among all the groups (p = 0.04, p = 0.000 and p = 0.03, respectively) (Tab. 1). TAS levels were significantly increased in the HI + NaHS 100 μmol/kg group as compared to C and HI groups (p = 0.014, p = 0.04, respectively). Tissue TOS levels of HI and HI + NaHS 50 μmol/kg groups were significantly higher than those measured in the C group (p = 0.000 and p = 0.000). In addition, 100 μmol/kg NaHS treatment showed significantly decreased TOS levels in the HI + NaHS 100 μmol/kg group as compared to the HI group (p = 0.021). High levels of OSI were found in the HI group as compared with C, HI + NaHS 50 μmol/kg, and HI + NaHS 100 μmol/kg groups (p = 0.027, p = 0.024 and p = 0.011, respectively).

**Effect of NaHS on mRNA expression of TNF-α, IL-6, NGF and iNOS**

There were no significant differences in TNF-α, IL-6, NGF, and iNOS gene mRNA expression levels in the brain tissues among C, HI, HI + NaHS 50 μmol/kg, and HI + NaHS 100 μmol/kg groups (p > 0.05). Compared with the C group, plasma iNOS levels were
decreased in HI + NaHS 50 μmol/kg and HI + NaHS 100 μmol/kg groups, but this was not statistically significant. Expression levels of IL-6 and iNOS mRNA were increased in the HI group as compared to the C group (p = 0.017, p = 0.05). Low levels of NGF gene mRNA expression were significant in the HI group as compared with HI + NaHS 50 μmol/kg and HI + NaHS 100 μmol/kg groups (p = 0.026 and p = 0.017) (Fig. 1).

Effect of NaHS on brain histopathologic examinations

Based on histopathological analyses of the brains, normal histology was observed in the C group. There were no hemorrhages, neutrophils, leukocyte infiltrations, or neuron degenerations in this group. Numerous small hemorrhages, neutrophil leukocyte infiltrations, and increased numbers of degenerated neurons were observed in the HI group (Fig. 2). With routine hematoxylin eosin staining, apoptotic neurons showed round bounding, nuclear condensation, and cytoplasmic shrinkage as compared to neurons of normal morphology. Normal-looking neurons did not show positive staining with TUNEL, and most apoptotic cells were negative-positive with TUNEL. Apoptotic cell numbers were statistically significantly different among C (2.5 ± 0.16), HI (10 ± 0.21), HI + NaHS 50 μmol/kg (5.9 ± 0.45), and HI + NaHS 100 μmol/kg (6.9 ± 0.34) (p = 0.000) groups with hematoxylin eosin staining. Compared with HI + NaHS 50 μmol/kg and HI + NaHS 100 μmol/kg groups, the apoptotic cell numbers were increased significantly in the HI group (p = 0.000 and p = 0.000) (Fig. 2E).

Effect of NaHS on the number of TUNEL-positive apoptotic neurons

There were significantly more apoptotic cells in C (2.6 ± 0.16), HI (13.2 ± 0.72), HI + NaHS 50 μmol/kg (5.7 ± 0.64), and HI + NaHS 100 μmol/kg (6.9 ± 0.43) groups with TUNEL staining (p = 0.000) (Fig. 3). The HI + NaHS 50 μmol/kg and HI + NaHS 100 μmol/kg groups did not differ significantly in terms of apoptotic cell numbers (p = 0.43). However, the apoptotic cell numbers in HI + NaHS 50 μmol/kg and HI + NaHS 100 μmol/kg groups were significantly lower than in the HI group (p = 0.000 and p = 0.000, respectively) (Fig. 3E).

Discussion

Hypoxic ischemic encephalopathy is one of the main causes of neonatal deaths, cerebral palsy, mental retardation, and epilepsy after the neonatal period. H2S is a molecule known to play a protective role against ischemia reperfusion in cardiac, liver, kidney, and brain tissue through angiogenesis induction, vasodilatation, and regulation of neuronal activity and glucose metabolism (11, 14). NaHS is an H2S transmitter and can directly transform into H2S through intraperitoneal NaHS application at doses of 50 μmol/kg and 100 μmol/kg. The studies detected that inflammatory cytokines such as TNF-α, are intensively released during the reperfusion period following hypoxia-ischemia. Yin Jun et al. examined the neuroprotective effects of NaHS (15), and a significant decrease in TNF-α levels was detected in the 100 μmol/kg group as compared to the 50 μmol/kg group in ischemia-reperfusion damage among sampled rat brains. Again, in a study investigating the effects of NaHS on lipopolysaccharide-induced inflammation in microglial cell cultures, it was concluded that NaHS decreased TNF-α expression (16). Parallel to this study, the results acquired by the present researchers show that a decrease was observed in TNF-α mRNA gene expression level in the NaHS groups, but this was not statistically significant. Astrocytes are also the main inflammatory mediators producing IL-6. A model studying astrocyte efficiency against neuroinflammation in degenerative neurological diseases in cell cultures has shown that exogenous applications of H2S decreased the IL-6 expression (17). In the present study, a statistically insignificant decrease was detected in the IL-6 level compared to HI, HI + NaHS 50 μmol/kg, and HI + NaHS 100 μmol/kg groups.

Although in previous studies, many mechanisms of anti-inflammatory and antioxidant efficiency were not clearly identified while investigating the effects of NaHS, its antioxidant effects were emphasized. Such studies have also shown that this effect was provided by decreasing iNOS, SOD, MDA, and hydrogen peroxide (H2O2) levels. In neonatal rats, it was shown that in the hypoxia-induced encephalopathy model, NaHS decreased both neuronal and inducible nitric oxide synthase mRNA expressions (18). Again, the neuroprotective effect of NaHS was checked; its antioxidant character was emphasized by decreasing oxidative stress; it was shown that NaHS given to rats with neuron damage related to oxygen-glucose-deficiency (OGD) inhibited MDA, NO, and H2O2 (19). Two studies conducted on rats with prenatal smoke exposure found that NaHS treatment healed neuronal chromatosis, functional disorder in central chemoreception, and Phox2b expression in the parafacial respiratory group (pFRG) region. They also detected that NaHS treatment stopped the decrease in glutathione levels occurring due to this effect in the medulla oblongata of neonatal rats. It also stopped the increase in malondialdehyde content and superoxide dismutase activity (20, 21). In another study, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) activity and antioxidant efficiency were evaluated in rats with medium cerebral artery occlusion and cerebral ischemia induction. They illustrated the antioxidant efficacy of NaHS by showing that it suppressed the enzyme activity of NOX, which is a reactive oxygen species (ROS) derivative, and thus strongly preserved the unity of the blood-brain barrier (22). Based on the antioxidant efficiency of NAHS in the present study, a significant increase in TAS levels, and a decrease in TOS and OSI levels were detected, especially in the 100 μmol/kg NaHS group as compared to the HI group.

Although the antiapoptotic efficacy of NaHS has not yet been completely explained, a study of cell cultures showed that when caspase-3 was inhibited in the mitochondrial pathway of cell apoptosis, H2S mediated the antiapoptotic effects (23). In another study examining the neuroprotective effects of NaHS against neurotoxicity induced by β- amyloid peptide, numbers of apoptotic neurons were compared through the TUNEL method, and a significant decrease was detected in TUNEL-positive stained
apoptotic neuron counts in the NaHS group (24). In the present study, the authors detected a decrease in apoptotic cell counts, both in low- and high-dose groups, and showed the anti-apoptotic efficacy of NaHS. A limited number of studies evaluating NGF levels to investigate the known neuroprotective effect of NaHS reported that NGF may provide this effect through angiogenesis, and NGF-stimulated retinal cells induced the multiplication of retinal neurons (25). The present authors considered that the significant increase in NGF gene mRNA expression levels in treated groups, as compared to the HI group, could be a demonstrator of the neuroprotective efficacy of NaHS.

**Conclusion**

This study has found that NaHS has an antioxidant effect by increasing TAS levels and decreasing TOS levels in the brain tissue of HIBH-induced neonatal rats. It has also shown some of the neuroprotective effects of NaHS by inducing NGF expression. This effect may be demonstrated through the antioxidant characteristics of NaHS and its ability to decrease apoptosis. Although some advancements have been achieved through this animal research concerning the molecular mechanisms of NaHS, which is an H2S transmitter, more research should be conducted, and clinical proof is required to better understand the role of H2S in brain damage, especially during the reperfusion period.

**References**


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