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ARAŞTIRMA MAKALESİ / RESEARCH ARTICLE

Effects of NO, H_2S and CO on Thiol/Disulfide Balance and Advanced Oxidation Protein Products in Hippocampus and Serum

Hipokampus ve Serumda NO, H_2S ve CO'nun Tiyol/Disülfit Dengesi ve İleri Oksidasyon Protein Ürünleri Üzerindeki Etkileri

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ABSTRACT

Introduction: Nitric oxide (NO), hydrogen sulfide (H_2S), and carbon monoxide (CO) are known as gaseous autocoids. It is not clear how the application of exogenous NO, H_2S and CO alters the thiol/disulfide balance and advanced oxidation protein products (AOPPs) in the hippocampus and serum.

Materials and Methods: In the study, rats were exogenously injected with L-arginine (100 mg/kg) as a NO donor, NaHS (10 mg/kg) as a H_2S donor, and CORM-2 (10 mg/kg) as a CO donor (II) (a tricarbonyldichlororuthenium dimer). Thiol/disulfide balance and advanced protein oxidation products were analyzed in hippocampus and serum samples.

Results: The native thiol level in the hippocampus of the L-arginine group was statistically decreased compared to the native thiol level of the control group ($p \leq 0.0001$). The disulfide level in the hippocampus of the L-arginine group was statistically increased compared to the control group ($p=0.009$). Hippocampal total thiol level of NaHS group and CORM-2 group increased statistically ($p=0.008$,$p=0.0157$, respectively), while serum disulfide level of CORM-2 group decreased ($p=0.0005$). Serum and hippocampus AOPPs levels of the NaHS group were statistically increased compared to the control group (***$p=0.0006$, **$p=0.0047$, respectively). Similarly, the hippocampal AOPP level in the CORM-2 group was found to be statistically increased compared to the AOPP level in the control group ($p=0.0437$).

Conclusion: As NaHS can improve thiol/disulfide balance, new studies are needed for CORM-2 and L-Arginine. This study is the first to report the effects of NO, H_2S and CO on thiol/disulfide balance and AOPPs in the hippocampus and serum.

Keywords: NO, H_2S, CO, AOPPs, Thiol/disulfide

ÖZ

Giriş: Nitrik oksit (NO), hidrojen sülfür (H_2S) ve karbon monoksit (CO) gaz otokoidler olarak bilinir. Eksojen NO, H_2S ve CO uygulamasının hipokampus ve serumda tiyol/disülfit dengesini ve ileri oksidasyon protein ürünlerini (AOPP'ler) nasıl değiştirdiği açık değildir.

Gereç ve Yöntem: Çalışmada, sıçanlara NO donörü olarak L-arginin (100 mg/kg), H_2S donörü olarak NaHS (10 mg/kg) ve CO donörü olarak CORM-2 (10 mg/kg) (trikarbonildiklororutenyum dimer) enjekte edildi. Hipokampus ve serum örneklerinde tiyol/disülfit dengesi ve ileri protein oksidasyon ürünleri (AOPPs) analiz edildi.

Bulgular: L-arginin grubunun hipokampüsündeki serbest tiyol seviyesi, kontrol grubunun serbest tiyol seviyesine göre istatistiksel olarak azaldı ($p \leq 0.0001$). L-arginin grubunun hipokampüsündeki disülfid düzeyi kontrol grubuna göre istatistiksel olarak yüksek bulundu ($p=0,009$). NaHS grubu ve CORM-2 grubunun hipokampal total tiyol düzeyi istatistiksel olarak artarken (sırasıyla $p=0,008$,*$p=0,0157$), CORM-2 grubunun serum disülfit düzeyi azaldı ($p=0,0005$). NaHS grubunun serum ve hipokampus AOPP'leri kontrol grubuna göre istatistiksel olarak yüksekti (sırasıyla $p=0,0006$, $p=0,0047$). Benzer şekilde CORM-2 grubunda hipokampal AOPPs düzeyi kontrol grubundaki AOPPs düzeyine göre istatistiksel olarak yüksek bulundu ($p=0,0437$).

Sonuç: NaHS, tiyol/disülfit dengesini iyileştirebilirken, CORM-2 ve L-Arginin için yeni çalışmalara ihtiyaç vardır. Bu çalışma, NO, H_2S ve CO'nun hipokampus ve serumdaki tiyol/disülfit dengesi ve AOPPs'ler üzerindeki etkilerini bildiren ilk çalışmadır.

Anahtar Sözcükler: NO, H_2S, CO, AOPPs, Tiyol/Disülfit

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Introduction

For many years, the notion that nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H_2S) were simply poisonous gases was prevalent. With the discovery that these gases are actively produced by all mammalian cells, many studies investigating the role of these molecules in cellular physiology and pathophysiology have been reported. The importance of NO on biological processes such as neurotransmission, innate immunity, and vasomotor tone was defined (1,2). CO then emerged as the second bioactive gas and was shown to modulate cell signaling events at physiological concentrations (3).

Similar to NO, CO is extremely important in regulating the immune response, modulating inflammation and tissue damage control in a manner appropriate to the needs of the tissue, while effectively eliminating potentially harmful pathogens (4,5). Similar to NO and CO, H_2S is an important gas molecule in bacteria, plants and mammals. In humans, H_2S functions as a signaling molecule as evidenced in the immune, endocrine, gastrointestinal, reproductive and central nervous systems (6-8).

This trio of gaseous mediators, collectively termed gasifiers, have very different pharmacological properties, including reactivity, diffusion, and molecular targeting. Although they do not have cognate receptors on their own, they interact with a wide variety of proteins and genes, including the related enzymes that make up their endogenous production. Moreover, NO, CO and H_2S have great potential as new therapeutics for various diseases. However, it has not been reported how these gaseous change the thiol disulfide balance and advanced protein oxidation products.

Cerebral ischemia, traumatic brain injury, and neurodegenerative diseases lead to disruption or death of neurons in the CNS (9). Many reports have now shown that exposure to CO and NO has neuroprotective effects depending on its concentration (10-12). The pathophysiological significance of endogenously produced H_2S in various neurological models was investigated. Emerging preclinical evidence has shown that low concentrations of H_2S shed light on the neuroprotective and neuromodulatory role during cognitive decline and brain injury (13, 14).

Oxidative stress occurs when the balance between antioxidant protection and oxidant production is disturbed. Various markers are used to evaluate the balance between oxidants and antioxidants. One of the most recent among them is the thiol/disulfide balance (15). Thiol balances oxidative stress by reducing the formation of reactive oxygen species or accelerating inactivation. Thiol/disulfide balance plays a critical role in antioxidant defense, detoxification, apoptosis, regulation of enzyme activities, and mechanisms of transcription and cellular signal transduction (16, 17). Many studies have been conducted on advanced oxidation protein products (AOPPs), a new marker of protein oxidation (18-21). AOPPs are a sensitive marker for determining the degree of protein oxidation (18, 20, 21). In the study, AOPPs levels were reported to correlate with plasma dityrosine, an indicator of protein oxidation, and pentoside, an advanced glycation end product, but not with substances that react with thiobutyric acid, a marker of lipid peroxidation (20).

Recently, the physiological and pharmacological effects of some gaseous autoacoids have been emphasized. It is thought that the lack or high levels of NO, CO and H_2S cause some pathological events and modulation of their production may open new treatment options (22). In our study, we investigated how NO, CO and H_2S, which are gaseous mediators, change the thiol/disulfide balance and AOPP levels in the hippocampus and serum.

Materials and Methods

Chemical material. For the current experiments, 28 adult male Sprague-Dawley rats (200-250 g, 6-8 weeks old) were used. They were kept in cages in a controlled environment with a 12/12-h light-dark cycle, a temperature of 20-22°C, and a relative humidity of 65-70%. According to the three Rs, (Replace, Reduce, and Refine), the study was conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals approved by the Local Ethics Committee of Eskisehir Osmangazi University (approval number 30.12.2022/924). The rats were divided into 4 groups contained 7 animals in each group. In the experiments, Nitric Oxide (NO) donor L-Arginine (100 mg/kg), Hydrogen Sulfide (H_2S) donor NaHS (10 mg/kg), and Carbon Monoxide donor Tricarbonyldichlororuthenium (II) dimer CORM-2 (10 mg/kg) were used. All drugs were obtained from Sigma company (St. Louis, MO, USA) and dissolved in 0.9% saline and injected intraperitoneally (i.p). Control animals received saline. For anesthesia, ketamine (Alfamine 10%, Alfasan International B.V. Holland) and xylazine (Xylazinbio 2%, Bioveta PLC, Czech Republic) were intraperitoneally administered. 2 ml blood was taken by cardiac puncture and brain was dissected immediately. Then hippocampus was isolated and kept at -80°C until analyses.

Brain tissue homogenization. Each brain size were measured by weight. Tissues were homogenized in 10% (w/v) 50 mM Tris pH 7.4 buffer containing 2 mM EDTA, 0.5% Triton X-100, ultimately containing protease inhibitor cocktail, for 3 x 10 seconds by OMNI Tissue Master 125 (F12520377). All procedures were performed on ice to prevent protein degradation. All solutions used were also kept on ice. Then, homogenates were centrifuged for 10 minutes at 5000 and 10000g at +4°C. After centrifugation, the supernatant was removed and stored at -80°C until used to measure.

Measurement of Total Thiol and Native Thiol.The main principle of the measurement of total thiol: For the measurement of total thiol, 10 µl of R1 and 10 µl of the sample were mixed. Then 110 µl of R2 and 10 µl of R3 reaction medium were added. Principle of native thiol measurement: For the measurement of

native thiol, 10 µl of R1 and 10 µl of the sample were mixed. Then 110 µl of R2 and 10 µl of R3 were added with reaction medium. Then, the first absorbance measurement (A1) was performed spectrophotometrically at a wavelength of 415 nm. The second absorbance measurement (A2) was performed at the same wavelength at the 10th minute when the reaction stopped by determining the A2-A1 absorbance difference was obtained by completing the measurement.(15).

The molar extinction coefficient of 5-thio-2-nitrobenzoic acid (TNB) of 14.100 mol/L^{-1} cm^{-1} was used to calculate the total and native thiol content. The disulfide content was calculated using the formula (total disulfide-native disulfide)/2. All results were expressed in micromoles per liter (µmol/L) (23).

Reagent 1 (for total –SH)

Reagent 1 (R1) was prepared by dissolving 378 mg of sodium borohydrate in 1000 mL of water-methanol solution (50% v/v). The final concentration of sodium borohydrate was 10.0 mM. The reagent was freshly prepared and used daily. This reducing agent solution was used to determine the total thiol content.

Reagent 1 (for native –SH)

Reagent 1' was prepared by dissolving 585 mg of sodium chloride in 1000 mL of water-methanol solution (50% v/v). The final concentration of sodium chloride was 10.0 mM. This reagent is stable at 4°C for at least 6 months. The solution was used for the determination of native thiol content.

Reagent 2

R2 was prepared by dissolving 0.5 mL formaldehyde (final concentration: 6.715 mM) and 3.8 g EDTA (final concentration: 10.0 mM) in 1000 mL TRIS buffer, 100 mM and pH 8.2. This reagent is stable for at least 6 months at 4 °C. It was used for both tubes.

Reagent 3

R3 was prepared by dissolving 3.963 g DTNB in 1000 mL methanol. The final concentration of DTNB was 10.0 mM. The reagent was freshly prepared and used daily. It was used for both vessels.

Measurement of advanced oxidation protein products (AOPPs). The measurement of AOPPs was analyzed using minor modifications of the method developed by Hanasand et al (24) (25). Briefly, 20 µl of plasma and hippocampal homogenates (in three parallel layers) were added to the wells of a 96-well microplate (with transparent bottom) and 180 µl of 0.2 M citric acid was added. Different concentrations of chloramine-T (2-75 µmol/l) diluted in citric acid were used as calibration standards and potassium iodide (10 µl 1.19 M) was added for color development. After 10 min of shaking on a microplate shaker, the absorbance of AOPPs at 340 nm was read. The relative amount of turbidity in the samples was determined at 630 nm. The calibration curve was constructed with chloramine T concentration as one variable and absorbance (340-630 nm) subtracted as the other variable. The study was performed as 2 parallel readings. The results are expressed as mean±SEM.

Statistical analysis. First, the Kolmogorov-Smirnov test was used to examine whether the data were normally distributed in the experimental groups. A non-parametric test was employed for the variables outside the normaldistribution. The comparison of the data between the groups was carried out throughthe independent-samples t test. Statistical significance was based on a value of $p < 0.05$ with a 95% confidence interval. Statistical analyzes were performed using the Graphpad 9.0 program.

Results

L-arginine effects of Thiol/Disulfide Balance. There was no statistically significant difference between serum and hippocampus total thiol levels in the L-arginine group and serum total thiol level in the control group. While no statistically significant difference was found between the serum native thiol levels of the L-Arg group and control group, the hippocampal native thiol level of the L-arg group was statistically decreased as compared to the native thiol level of the control group ($p \leq 0.0001$). There was no statistically significant difference between the serum disulfide level of the L-Arg group and the serum disulfide level of the control group. However, the hippocampus disulfide level of the L-Arg group was statistically increased as compared to the control group ($p=0.009$) (Table 1 and Table 2).

NaHS effects of Thiol/Disulfide Balance. Serum total thiol levels in NaHS group decreased statistically as compared to control group ($p=0.0195$). The hippocampus total thiol levels in the NaHS group increased statistically as compared to the control group ($p=0.008$). While there was no statistically significant difference between serum native thiol level in the NaHS group and control group, the hippocampal native thiol level in the NaHS group increased statistically compared to the control group ($p \leq 0.0001$). There was no statistically significant difference between the serum and hippocampus disulfide levels of the NaHS group and control group (Table 1 and Table 2).

CORM-2 effects of Thiol/Disulfide Balance. When the serum total thiol level of the CORM-2 group was compared with the serum total thiol level of the control group, no statistically significant difference was found. Hippocampus total thiol level in CORM-2 group increased statistically as compared to the control group ($p=0.0157$). There was no statistically significant difference between serum and hippocampus native thiol levels of the CORM-2 group and the control group, the serum disulfide level of the CORM-2 group was statistically decreased as compared to control group ($p=0.0005$). However, there was no statistically significant difference of hippocampus disulfide levels between CORM-2 group and control group (Table 1 and Table 2).

Table 1. Serum total thiol, native thiol, disulfide and AOPPs level of exogenous administration of L-arginine, NaHS, CORM-2

Serum	Control (mean ±sem) p value	L-arginine (100 mg/kg,i.p) (mean ±sem) p value	NaHS (10 mg/kg,i.p) (mean ±sem) p value	CORM-2 (10 mg/kg,i.p) (mean ±sem) p value
Total thiol level	241.7 ±13.69	271.4 ± 24.17 p=0.1234	184.5 ± 20.86 p=0.0195	184.0 ± 15.98 p=0.0644
Native thiol level	156.9 ±25.26	176.0 ± 26.26 p=0.6665	180.2 ± 18.20 p=0.1750	142.0 ± 13.71 p=0.9461
Disulfide level	49.47± 5.872	47.70 ± 9.065 p>0.9999	42.67 ± 1.214 p=0.8182	9.541 ± 9.251 p=0.0005
AOPPs level	8.111 ± 1.885	24.46 ± 8.388 p=0.1014	23.00 ± 1.857 p=0.0006	17.14 ±4.398 p=0.0836

Table 2. Hippocampus total thiol, native thiol, disulfide and AOPPs level of exogenous administration of L-arginine, NaHS, CORM-2

Hippocampus	Control (mean ±sem) p value	L-arginine (100 mg/kg,i.p) (mean ±sem) p value	NaHS (10 mg/kg,i.p) (mean ±sem) p value	CORM-2 (10 mg/kg,i.p) (mean ±sem) p value
Total thiol level	421.9 ± 14.57	500.4 ± 72.76 p=0.5397	682.7 ± 66.95 p=0.0008	549.1 ± 44.28 p=0.0157
Native thiol level	341.3 ± 31.75	161.1 ± 15.15 p<0.0001	633.9 ± 23.67 p<0.0001	343.5 ± 25.79 p=0.8680
Disulfide level	71.73 ± 9.978	227.1 ± 32.73 p=0.0009	71.73 ± 8.136 p=0.8646	102.8 ± 20.48 p=0.4295
AOPPs level	68.02 ± 3.629	57.10 ± 6.355 p=0.2402	108.7 ± 8.237 p=0.0047	56.27 ± 3.383 p=0.0437

L-arginine, NaHS and CORM-2 effects of Advanced Oxidation Protein Products. L-arg group serum AOPPs level did not differ statistically as compared to control group. There was no statistically significant difference between the L-Arg group hippocampus AOPPs level and the control group.

The serum AOPPs level in the NaHS group was statistically increased compared to the serum AOPPs level in the control group (p=0.0006). Likewise, it was found that the hippocampus AOPPs level of the NaHS group was statistically increased as compared to the control group (p=0.0047).

There was no statistically significant difference between the serum AOPPs level of the CORM-2 group and the serum AOPPs level of the control group. It was found that the level of hippocampus AOPPs in the CORM-2 group was statistically decreased as compared to control group (p=0.0437) (Table 1 and Table 2).

Discussion

Recently, the physiological and pharmacological effects of some gaseous autacoids have been emphasized. It is predicted that the deficiency or high level of endogenously produced gaseous autacoids such as nitric oxide, hydrogen sulfide and carbon monoxide cause some pathological events and some new treatment horizons can be opened by modulation of their production (22). In this context, the role of hydrogen sulfide, which is defined as the third endogenous gaseous signaling molecule apart from nitric oxide and carbon monoxide, has received great attention in recent years due to its importance in different signaling pathways (26). Thiol/disulfide balance plays a critical role in antioxidant defense. In the study we completed, nitric oxide donor NO, hydrogen sulfide donor NaHS and carbon monoxide donor Tricarbonyldichlororuthenium (27) dimer CORM-2 were used.

With the exogenous application of nitric oxide donor L-arginine, the hippocampus total native thiol level decreased, while the hippocampus disulfide level increased. Considering the thiol/disulfide balance of oxidative stress, the decrease in the thiol level while the increase in the disulfide level shows an unexpected result of L-arginine against oxidative stress. In a study, it was shown that methionine and arginine supplementation modifies inflammatory and oxidative stress responses by controlling the activity of NF-κB (28). In a different study, it was reported that L-Arg reduced the inflammatory response and oxidative stress (29) but no previous study was reported on how L-Arg supplementation changed the thiol/disulfide balance. Although the reported ethylene studies suggest that L-arginine supplementation reduces oxidative stress, our results suggest that the thiol/disulfide balance increases rather than reduces oxidative stress. In other words, although we expected results for L-Arg to reduce oxidative stress, the results we obtained with the literature could not be confirmed due to the fact that the thiol/disulfide balance we obtained progressed in the direction of increasing oxidative stress. As it is known that Arginase enzyme is another important factor regulating the availability of L-Arg. Interestingly, Scalera et al. found that long-term treatment with L-Arg reduced NO synthesis associated with increased Arginase activity and reduced L-Arg availability (30). Arginases have regulatory effects on NO synthesis, modulate L-Arg availability, and are likely part of a mechanism to limit NO production (31). Moretto et al. showed that chronic treatment

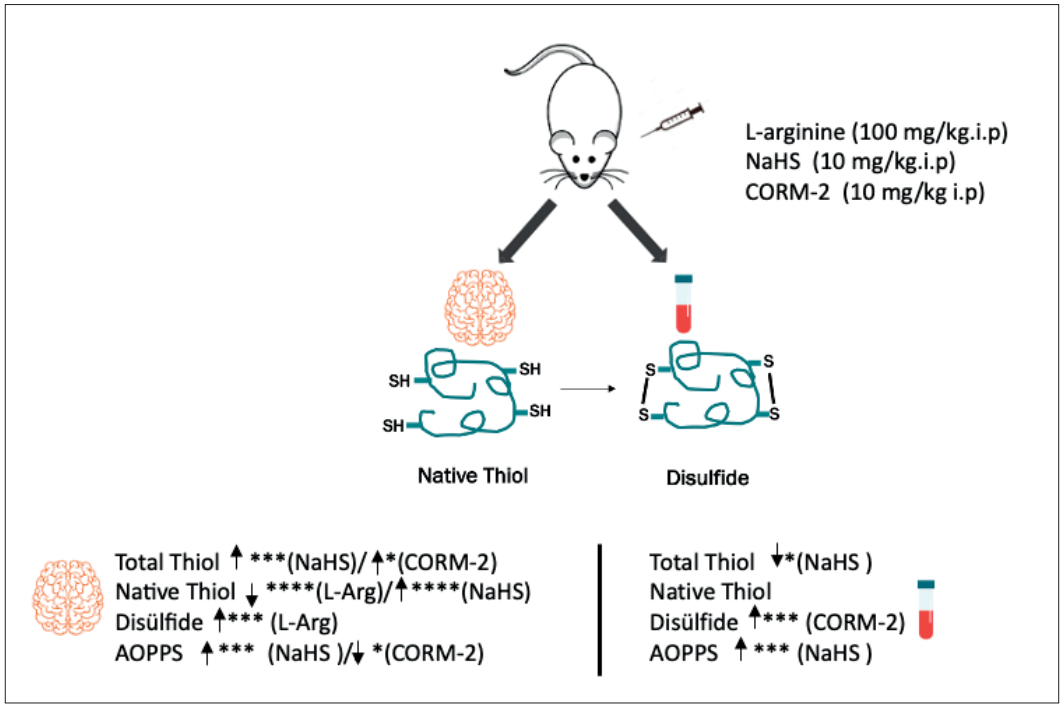

Figure 1. A schematic representation of the effects of NO, H_2S and CO on Thiol/Disulfide Balance and Advanced Oxidation Protein Products in Hippocampus and Serum

with L-Arg increased Arginase activity in rats. However, higher Arginase activity and unbound NOS were thought to lead to an increase in superoxide formation. There is also a recently reported study showing inconsistent oxidative stress response in cell lines treated with L-Arg. The study emphasized that high Arginase activity and unbound NOS can lead to an increase in superoxide formation (32). While the thiol/disulfide balance changed in L-Arg administration, AOPPs levels increased insignificantly in serum but the changes were not significant either in the serum or in the hippocampus.

Exogenous application of hydrogen sulfide donor NaHS increased the hippocampus total thiol level and native thiol level. The hippocampus disulfide level showed a increasing trend when compared with the control group. Considering the thiol/disulfide balance of oxidative stress, the increase in total thiol level and native thiol level indicates that H_2S can protective effect against oxidative stress. It is well known that oxidative stress and inflammation reduce the content of glutathione, an important intracellular antioxidant (33) Consistent with this, Silva-Adaya et al showed that exposure to arsenic resulted in a time- and dose-dependent depletion of GSH and elevated H_2S concentrations in the cortex and cerebellum of mice. However, modulation of cellular redox response targets by S-sulfhydration, such as Nrf2, has been reported to cause overproduction of HS and increased GSH levels in the cortex and cerebellum at 24 hours (34) . H_2S also increased the activity of γ-glutamyl-cysteine synthetase (γGCS), the GSH rate-limiting enzyme that regulates glutathione synthesis (35, 36). H_2S protects neurons from glutamate-induced toxicity (35). It was reported that the thiol balance changed in the fibromyalgia patient group in a study. It was found the total thiol level decreased while the disulfide level increased in patients with fibromyalgia. This information has shown that oxidative stress in the fibromyalgia patient group is affected by thiol balance (37). In our study, we proved that hydrogen sulfide increased hippocampus total thiol level and hippocampus native thiol level. There has been no previous study on how H_2S changes the thiol/disulfide balance. In exogenous H_2S application, while the thiol/disulfide balance changed in a protective way against oxidative stress, an increase in serum AOPPs level was observed. It was concluded that H_2S does not have a lowering effect on AOPPs.

Hippocampal total native thiol level increased with exogenous CO administration. The serum disulfide level, on the other hand, showed a decrease when compared to the control group. Considering the thiol balance of oxidative stress, the increase in total thiol level indicates that CO has a protective effect against oxidative stress. In exogenous CO administration, while the thiol/disulfide balance changed to protect the oxidative stress, the serum AOPPs level decreased, supporting the thiol/disulfide balance.

As a result, the administrations of L-Arg, H_2S and CO shows different findings in terms of thiol/disulfide balance. Treatment with L-Arg caused unexpected responses on the thiol/disulfide balance. Although it was found that L-Arg had an effect on the thiol/disulfide balance by increasing the oxidative stress, it was found that the protective effect of H_2S and CO administration against oxidative stress was by supporting the thiol/disulfide balance. Likewise, it was found that L-Arg administration did not change AOPPs levels as expected, while CO administration

contributed to oxidative defense by reducing the AOPPs level in the hippocampus. It is thought that the inconsistencies of L-Arg administration are due to the differences that may occur in the coupling of NOS and Arginase activity. However, additional studies are still needed to clarify the processes involved in the L-Arg response that produced the unexpected effect.

Currently, the paucity of clinical data using CO, NO and H_2S donors suggests that further efforts are needed to better elucidate the molecular mechanisms underlying both exogenous and endogenous effects of these molecules in different disease conditions. The discovery and availability of new pharmacological donors of CO, NO, and H_2S that can regulate biological functions at physiological levels represent promising strategies as research tools as well as therapeutic enhancements for the treatment of many chronic diseases in humans. In summary, much more research is needed to fully understand the role and critical interaction and interrelationship of these three gases, CO, NO, and H_2S, in biology and medicine so that future human clinical trials can be fully informed. As far as we know, although there are studies in the literature on how these mediators affect oxidative parameters other than thiol/disulfide balance and advanced oxidation protein products, no study has been reported with the perspective of thiol/disulfide balance and advanced oxidation protein products. Our results are the first study to investigate the effects of NO, H_2S and CO on thiol/disulfide balance and AOPPs in hippocampus and serum.

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