

Research Article

Clinical and Experimental Evidences of Hydrogen Sulfide Involvement in Lead-Induced Hypertension

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Lead- (Pb-) induced hypertension has been shown in humans and experimental animals and cardiovascular effects of hydrogen sulfide (H_2S) have been reported previously. However, no studies examined involvement of H_2S in Pb-induced hypertension. We found increases in diastolic blood pressure and mean blood pressure in Pb-intoxicated humans followed by diminished H_2S plasmatic levels. In order to expand our findings, male Wistar rats were divided into four groups: Saline, Pb, NaHS, and Pb + NaHS. Pb-intoxicated animals received intraperitoneally (i.p.) 1st dose of 8 $\mu g/100$ g of Pb acetate and subsequent doses of 0.1 $\mu g/100$ g for seven days and sodium hydrosulfide- (NaHS-) treated animals received i.p. NaHS injections (50 $\mu mol/kg$ /twice daily) for seven days. NaHS treatment blunted increases in systolic blood pressure, increased H_2S plasmatic levels, and diminished whole-blood lead levels. Treatment with NaHS in Pb-induced hypertension seems to induce a protective role in rat aorta which is dependent on endothelium and seems to promote non-NO-mediated relaxation. Pb-intoxication increased oxidative stress in rats, while treatment with NaHS blunted increases in plasmatic MDA levels and increased antioxidant status of plasma. Therefore, H_2S pathway may be involved in Pb-induced hypertension and treatment with NaHS exerts antihypertensive effect, promotes non-NO-mediated relaxation, and decreases oxidative stress in rats with Pb-induced hypertension.

1. Introduction

Lead (Pb) is an important environmental pollutant that presents hazardous effects for human health. Most of the population must have lead in their organisms due to occupational or environmental exposure [1–3]. A variety of harmful effects may arise from lead intoxication, which include alterations in bone density [4, 5] and cognitive [6, 7] and cardiovascular disorders, such as hypertension [8, 9], and these adverse effects may result from both duration of exposure and blood lead levels [10]. The National Institute for Occupational Safety and Health, USA, stated that reference blood lead levels for adults should be $\geq 5 \mu g/dL$ [11] and lead-induced hypertension has been shown in experimental animals with low blood lead levels (9–37 $\mu g/dL$) [9, 12–14]. Multiple mechanisms have been proposed to explain

how lead intoxication impairs the cardiovascular system and leads to hypertension. Although these alterations promoted by lead are not completely understood in the early stages of lead exposure, increases in angiotensin II levels resulting from converting enzyme activation [15], increases in matrix metalloproteinases levels/activity [9, 16], increases in cyclooxygenase-derived contractile prostanoids [12], reductions in nitric oxide (NO) bioavailability [9, 13, 17], and increases in reactive oxygen species (ROS) production [18, 19] have been proposed as mechanisms involved in lead-induced hypertension.

Hydrogen sulfide (H_2S) was formerly known as a pollutant gas with characteristic smell of rotten eggs. Now, there is a large body of literature that indicates H_2S as a gasotransmitter with important physiological functions [20–25]. H_2S is enzymatically produced by cystathionine γ -lyase

(CSE) and cystathionine β -synthase (CBS) [26], though other systems have been described as H_2S producers, such as the 3-mercaptopyruvate sulfurtransferase pathway [27]. H_2S has been described to participate in several physiological processes, such as neurotransmission, inflammation and immune reactions, gastrointestinal function, cancer development, and cardiovascular responses [20, 28, 29]. Some studies report that H_2S may exert a vasodilator effect [30, 31] and thus help in the control of vascular tone. Moreover, H_2S may act as an antioxidant [32, 33]. It has been shown that H_2S displayed an effective preservation of antioxidants enzymes activity [34–36] and also decreased the levels of different biomarkers of oxidative stress *in vivo* and *in vitro* [34–38]. There are evidences that H_2S exerts a vasoprotective effect in hypertension and atherosclerosis [39, 40] and that exogenous donors of H_2S can attenuate vascular dysfunction [41]. Importantly, to our knowledge, no studies have evaluated the role of H_2S in Pb-induced hypertension.

As consistent vasoconstriction and increases in oxidative stress are well-known hallmarks of hypertension and Pb-induced hypertension has been previously reported, we hypothesized that low-lead-level intoxication causes hypertension and reduces H_2S plasmatic levels in humans. In order to advance in the knowledge of H_2S role in Pb-induced hypertension, we suggested that sodium hydrosulfide (NaHS), a donor of H_2S , blunts the increases in systolic blood pressure (SBP) caused by low-lead-level intoxication in rats and that this beneficial effect on blood pressure may be related to H_2S antioxidant capacity.

2. Materials and Methods

2.1. Blood Pressure Measurements and Blood Collection in Human Subjects. Subjects were recruited from the Center for Toxicological Assistance (CEATOX)/UNESP, Botucatu. Written informed consent was obtained from all subjects, and the study was approved by the committee and Institutional Review Board of the Faculdade de Medicina de Botucatu, UNESP (protocol number: 16354513.0.0000.5411). Only male patients were included in this study. Blood pressure measurements were accessed in left arm using an automated device (Z-40, Techline, São Paulo, Brazil). Whole-blood heparin and plasma heparin were collected to perform lead levels analysis and biochemical assays, respectively. Samples were stored at -80°C . Patients with whole-blood lead levels less than $5\ \mu\text{g}/\text{dL}$ were included as patients from control group ($n = 25$) and patients with whole-blood lead levels more than $5\ \mu\text{g}/\text{dL}$ were included as Pb-intoxicated group ($n = 20$).

2.2. Animals and Treatments. Forty male Wistar rats weighing approximately $250 \pm 20\ \text{g}$ were used in this study. Animals were kept in standard rat cages, maintained under controlled temperature (22°C) on a 12-hour light-dark cycle, and given free access to water and rat chow. All procedures for animal experimentation were approved by Ethics Committee, Institute of Biosciences, Sao Paulo State University, Botucatu (protocol number: 458/2013), which complied with international guidelines of the European Community for the use of experimental animals.

The animals were randomly divided into two lead-exposed groups (Pb and Pb + NaHS; $n = 12$ per group) and two control (non-lead-exposed) groups (Saline and NaHS; $n = 8$ per group) for eight days. Animals exposed to lead were injected intraperitoneally (i.p.) with a first dose of $8\ \mu\text{g}/100\ \text{g}$ of lead acetate ($\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2 + 3\text{H}_2\text{O}$, 100% purity, Merck, USA) and subsequent daily doses of $0.1\ \mu\text{g}/100\ \text{g}$ to cover daily loss and treatment with NaHS ($50\ \mu\text{mol}/\text{Kg}/\text{twice}$ daily; *Pb + NaHS group*) or vehicle (water; *Pb group*) i.p. for seven days. Animals from control groups (non-lead-exposed) were injected i.p. with a first dose of $8\ \mu\text{g}/100\ \text{g}$ of sodium acetate ($\text{Na}(\text{C}_2\text{H}_3\text{O}_2) + 3\text{H}_2\text{O}$, 99% purity, J.T.Baker, Canada), a subsequent dose of $0.1\ \mu\text{g}/100\ \text{g}$ to cover daily loss, and treatment with NaHS ($50\ \mu\text{mol}/\text{Kg}/\text{twice}$ daily; *NaHS group*) or vehicle (water; *Saline group*) i.p. for seven days. The protocol of intoxication with lead acetate used in this study was based on previous studies [9, 13, 17].

On the eighth day (i.e., 24 hours after each respective treatment), animals were anesthetized with isoflurane (2–4%) and killed by exsanguination. After thoracotomy, the descending thoracic aortas were removed for vascular experiments studies. Whole-blood samples were collected in tubes containing lyophilized heparin (*Vacutainer* BD, trace metal-free) to access lead concentrations. Blood was also collected in tubes containing ethylenediaminetetraacetic acid (EDTA) (*Vacutainer* Becton-Dickinson, Oxford, UK) and sodium citrate (*Vacutainer* Becton-Dickinson, Oxford, UK) for plasma separation. Plasma was stored at -80°C until biochemical analyses.

2.3. Blood Pressure Measurements. SBP (mmHg) was measured using tail-cuff plethysmography (Insight, Ribeirao Preto, Sao Paulo, Brazil). Briefly, conscious rats were first acclimated in a quiet room, conditioned, and restrained for 5–10 minutes in a warm box (Insight, Ribeirao Preto, Sao Paulo, Brazil). Animals were “trained” to the measurements process for 3 days before the beginning of the treatments (data were discarded). SBP was measured, and the mean of three measurements was recorded from day one to day seven of the experimental protocol, as previously described [9, 13]. Blood pressure measurements were performed one hour before the injections of sodium acetate/Pb/NaHS.

2.4. Determination of Lead Concentrations in Whole Blood. Pb concentration in whole blood from human patients and rats was determined by graphite furnace atomic absorption spectrometry (GF-AAS; Varian SpectrAA 220) as previously described [9, 13]. Briefly, blood samples were diluted 1 + 49 with a diluent solution containing 0.5% (v/v) double-distilled HNO_3 $25\ \mu\text{g}/\text{l}$ Rh and 0.005% (v/v) Triton® X-100. Calibration was performed against matrix matching. The detection limit of the method was $0.5\ \mu\text{g}/\text{L}$. The whole-blood lead concentrations were expressed in $\mu\text{g}/\text{dL}$.

2.5. Determination of H_2S in Plasma. Plasma obtained from human patients and experimental animals was used for the determination of H_2S as described previously [42, 43]. In brief, $75\ \mu\text{L}$ of plasma was mixed with $250\ \mu\text{L}$ of zinc acetate 1% (wt/vol) and $425\ \mu\text{L}$ of water. To the mixture, $150\ \mu\text{L}$

of 20 mmol/L of *N*-dimethyl-*p*-phenylenediamine sulfate in HCl 7.2 M and 150 μ L of 30 mmol/L of FeCl₃ in HCl 1.2 M were added. After 10 minutes of incubation at room temperature, 250 μ L of 10% trichloroacetic acid was added to remove proteins and the reaction mixture was centrifuged at 12000g for 15 minutes. The absorbance of the resulting supernatant (200 μ L) was measured at 670 nm with a spectrophotometer (Synergy 4, BioTek, Winooski, VT, USA) in a 96-well plate. The concentration of H₂S in the solution was calculated against a calibration curve of NaHS (100–3.13 μ M).

2.6. Vascular Reactivity. Rats' thoracic aortas were dissected in 3-4 mm segments (two rings). One of the rings had its endothelium mechanically removed. The rings were mounted into a 10 mL organ chamber containing Krebs-Henseleit solution (NaCl 130; KCl 4.7; CaCl₂ 1.6; KH₂PO₄ 1.2; MgSO₄ 1.2; NaHCO₃ 15; glucose 11.1; in mmol/L) and suspended between two wire hooks; one hook was fixed to a stationary support, and the other hook connected to an isometric force transducer. The Krebs-Henseleit solution was kept at pH 7.4 and 37°C and bubbled continuously with a mixture of 95% O₂ and 5% CO₂. Arterial rings were stretched under 1.5 g basal tension and were allowed to equilibrate for 45 min. Changes in aorta tension were recorded using FORT10 isometric force transducers (WPI, USA) connected to Transbridge 4M Transducer Amplifier (WPI, USA) connected to a PC-based MP100 System and analyzed offline using AcqKnowledge version 3.5.7 software (Biopac Systems Inc., USA).

After tissue equilibration, a control contraction to 96 mM of KCl was elicited. Once KCl maximum contraction was reached, tissue was rinsed with Krebs 3 times, 15 minutes each. Then, aorta rings were stimulated with increasing concentrations of phenylephrine (PHE, 10⁻¹⁰ to 10⁻⁴ M). In order to investigate endothelial function, vascular tissues were precontracted with PHE (10⁻⁶ M for intact rings and 3 × 10⁻⁷ M for denuded rings); increasing concentrations of acetylcholine (ACh, 10⁻⁹ to 10⁻⁵ M) were added to the bath. To evaluate the participation of endothelium-dependent NO in aortic rings relaxation, concentration-response curves to ACh were obtained in the presence of *N* ω -nitro-L-arginine-methyl ester (L-NAME, 3 × 10⁻⁴ M), added in the last 30-minute stabilization period [44, 45]. Concentration-contraction curves were constructed, and the maximal response to PHE was measured. Concentration-effect curves to ACh, with or without L-NAME, were expressed as the % relaxation to PHE contraction. Nonlinear regression (variable slope) of the obtained concentration-effect curves revealed R_{max} (maximal response) and pEC50 (negative logarithm of the concentration that evoked 50% of the maximal response).

2.7. Cell Culture and Plasma Incubation. Human umbilical vein endothelial cell (HUVEC) line (CRL 2873) was obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). HUVECs were cultured in DMEM medium (Gibco, CA, USA) supplemented with 10% (v/v) fetal calf serum (FCS) (Gibco), 50 μ g/ml penicillin, 50 μ g/ml streptomycin, and 0.5 μ g/ml amphotericin B (Gibco) at 37°C in 5% CO₂ incubator. After reaching 80% confluence, HUVECs were resuspended in DMEM medium and replated in 96-well

tissue culture plates (Corning), where they were grown to 80% confluence for incubation experiments. Then, the medium was removed and cells were washed twice in PBS. Cells were incubated in medium, without FCS, with 5% (v/v) plasma from rats treated with Saline, NaHS, Pb, and Pb + NaHS for 24 h. Cell viability was performed by MTT assay as described previously [46]. Viability was compared to control (untreated cells, 100% viability). Cell culture supernatant (CCS) was then stored and kept at -80°C for posterior analysis.

2.8. Determination of Nitrite in Culture Supernatant and Plasmatic Nitrite/Nitrate (Total NOx). Nitrite levels were assessed in HUVECs culture supernatant in duplicate using Griess reagents [47]. Briefly, 50 μ L of samples was incubated with 50 μ L of 1% sulfanilamide solution in 5% phosphoric acid for 10 minutes protected from light. Then, 50 μ L of 0.1% *N*-(1-Naphthyl)-ethylenediamine dihydrochloride solution was added followed by 10-minute incubation. Plate was read in spectrophotometer (Synergy 4, BioTek, Winooski, VT) at 540 nm. A standard curve was generated by incubation of nitrite solutions (0.46–29.5 μ mol/L) with the previous reagents.

Plasma total NOx concentrations were determined using Griess reagents followed by reduction of nitrous species with vanadium chloride III [48]. Briefly, before addition of Griess reagents, plasma was incubated with 100 μ L of saturated solution of vanadium chloride III for 3 hours at 37°C with agitation. Absorbance at 535 nm was read at spectrophotometer (Synergy 4, BioTek, Winooski, VT) and NOx concentrations in plasma were calculated using a standard curve of sodium nitrite (1.56–100 μ M). Nitrite levels in HUVECs supernatant and total NOx levels in plasma were expressed in μ mol/L.

2.9. Determination of Lipid Peroxidation. Lipid peroxidation was determined in rats' plasma through the formation of malondialdehyde (MDA). MDA reacts with 2-thiobarbituric acid (TBA) and produces a colorimetric reaction that is measured by spectrophotometer at wavelength of 532 nm [49]. In test tubes, a reaction mixture containing 100 μ L of distilled water, 50 μ L of 8.1% sodium dodecyl sulfate (SDS), 100 μ L of plasma samples, 375 μ L of acetic acid 20%, and 375 μ L of TBA 0.8% was incubated in water bath at 95°C for one hour and subsequently centrifuged at 4000 rpm for 10 minutes. Standard curve was prepared in a similar manner, replacing samples with 25 μ L of known concentrations of MDA. Plasmatic TBA reactive species (TBARS) were calculated against a standard curve of MDA (20–320 nmol). Results were expressed as plasma MDA levels in nmol/mL.

2.10. Evaluation of the Plasmatic Antioxidant Status. Direct reductions of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma, St Louis, MO, USA) were assessed to determine the antioxidant status of rats' plasma, as previously described [50]. Briefly, 12.5 μ L of dye solution (5 mg/mL in PBS) was mixed with 100 μ L of plasma and final volume was adjusted to 200 μ L with PBS. Reaction mixture was incubated for 60 min at 37°C and then the reaction was terminated by the addition of 750 μ L of 0.04 M hydrochloric

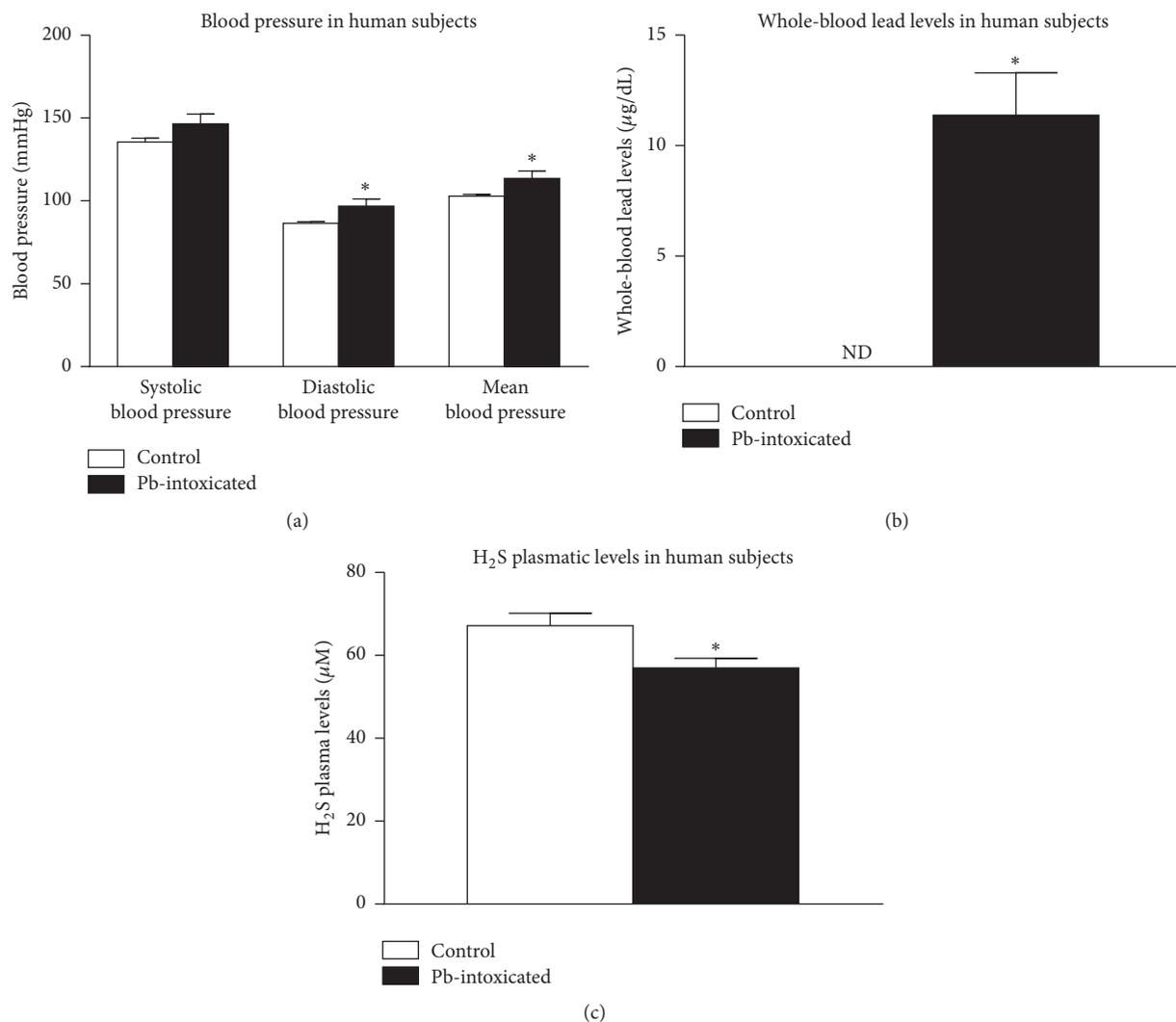


FIGURE 1: Blood pressure parameters and biochemical analysis in humans. (a) Systolic, diastolic, and mean blood pressure of control and Pb-contaminated patients. (b) Whole-blood lead levels of control and Pb-contaminated patients. (c) H₂S plasmatic levels of control and Pb-contaminated patients. Values represent mean \pm SEM. $n = 20-25$. "ND" means nondetectable. * $P < 0.05$ versus control group.

acid in isopropanol. Tubes were centrifuged for 10 min at 1000g and the absorbance of the collected supernatant was measured at 570 nm. Results are expressed as % of control group (Saline group taken as 100% of antioxidant status).

2.11. Data Analysis and Statistics. Statistical analyses were performed using GraphPad Prism® 6.0 software (San Diego, CA). The results are expressed as means \pm SEM. For human parameters, comparisons were made using Student's *t*-test. Comparisons between animal groups were assessed by one-way analysis of variance (ANOVA) followed by Tukey's test. For vascular reactivity experiments, individual concentration-contraction or concentration-relaxation curves were constructed; sigmoidal curves were fitted to the data using the least square method, and the comparisons among R_{max} and pEC_{50} values were determined by ANOVA followed by Tukey's test. A $P < 0.05$ was considered significant.

3. Results

3.1. Pb-Intoxication Is Related to Increased Blood Pressure and Decreased H₂S Levels in Human Subjects. Hypertension has been reported previously as a consequence of Pb-intoxication; however, no linkage has been made with hydrogen sulfide levels. In our study, there were no significant differences in SBP in humans intoxicated with Pb (146 ± 5 mmHg) compared to control subjects (135 ± 3 mmHg) ($P = 0.08$, Figure 1(a)). However, diastolic blood pressure and mean blood pressure from patients intoxicated with Pb (96 ± 4 and 113 ± 4 mmHg, resp.) were higher than diastolic blood pressure and mean blood pressure of control subjects (86 ± 1 and 102 ± 2 mmHg, resp.) ($P = 0.02$ and $P = 0.02$, resp., Figure 1(a)). The values of whole-blood lead levels in Pb-intoxicated subjects were 11.38 ± 1.92 µg/dL and whole-blood lead levels in control group were below the limit of detection of the technique ($P = 0.0002$, Figure 1(b)). Plasmatic H₂S

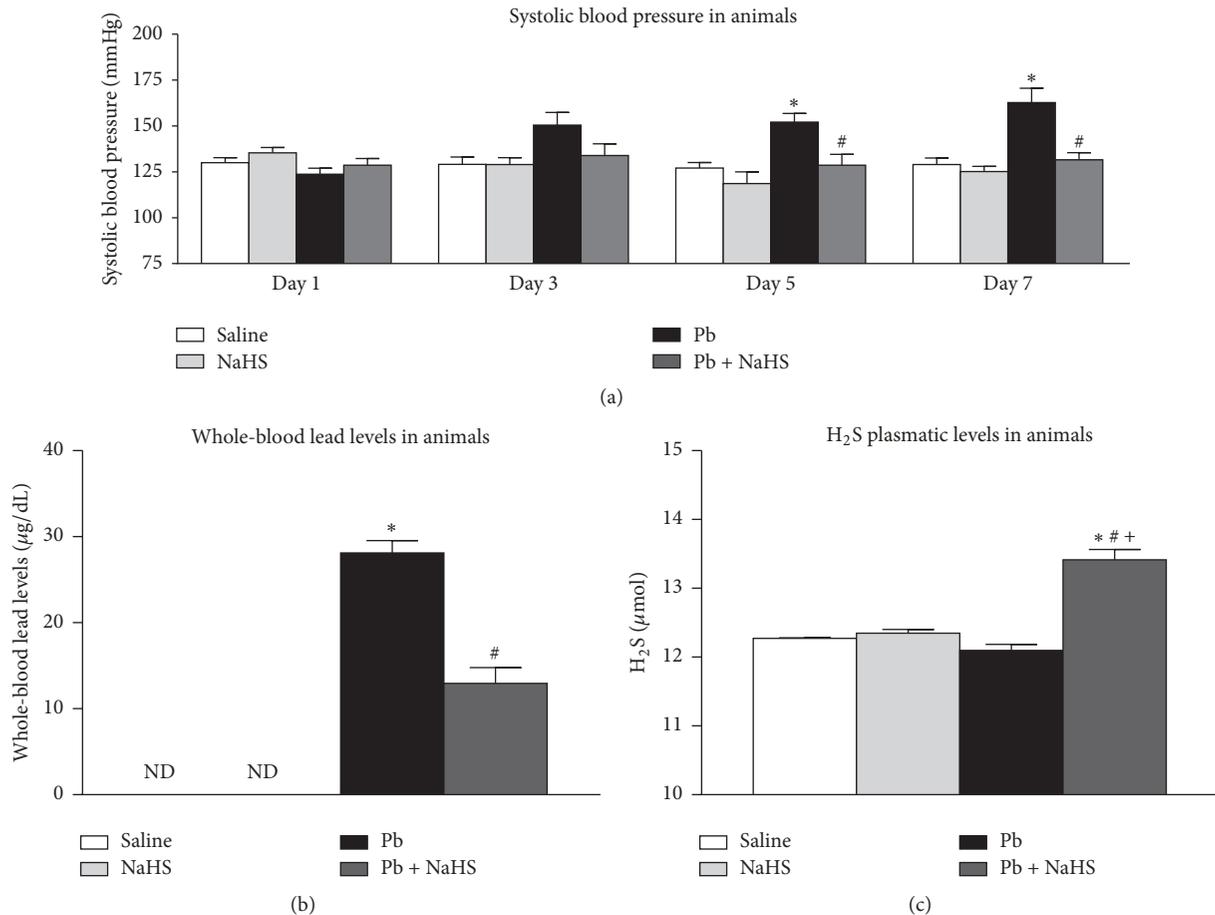


FIGURE 2: Blood pressure parameters and biochemical analysis in rats among four different groups: Saline (non-Pb-exposed), NaHS (non-Pb-exposed + NaHS), Pb (Pb-exposed), and Pb + NaHS (Pb-exposed + NaHS). (a) Systolic blood pressure measured with tail-cuff plethysmography on days 1, 3, 5, and 7 of experimental protocol. (b) Whole-blood lead levels measured after 7 days of Pb exposition. (c) H₂S plasmatic levels. Values represent mean \pm SEM. $n = 6-12$. * $P < 0.05$ versus Saline group; # $P < 0.05$ versus Pb group; + $P < 0.05$ versus NaHS group.

levels in control group were $67.15 \pm 2.99 \mu\text{M}$, whereas subjects from Pb-intoxicated group presented lower H₂S plasmatic levels ($56.99 \pm 2.28 \mu\text{M}$) ($P = 0.01$, Figure 1(c)).

3.2. NaHS Treatment Blunts Pb-Induced Hypertension in Rats and Reduces Whole-Blood Lead Levels. In order to better understand the involvement of H₂S in Pb-induced hypertension, we performed experiments in rats. There were no significant differences in SBP among four groups in day one (123 to 132 ± 3 mmHg, Figure 2(a)) and day three (129 to 150 ± 7 mmHg, Figure 2(a)). Animals from Pb group showed an increase in SBP versus Saline on days five (152 ± 5 versus 127 ± 3 mmHg, $P = 0.007$, Figure 2(a)) and seven (163 ± 8 versus 129 ± 3 mmHg, $P = 0.001$, Figure 2(a)). Moreover, this increase was blunted by treatment with NaHS on days five (128 ± 6 mmHg, $P = 0.007$, Figure 2(a)) and seven (131 ± 4 mmHg, $P = 0.0007$, Figure 2(a)).

No lead was detected in whole blood of animals from groups Saline and NaHS in the end of seven days of experimental protocol; however, rats from Pb group presented

whole-blood lead levels of $28.12 \pm 1.45 \mu\text{g/dL}$ and an approximately 2-fold decrease was found in animals from Pb + NaHS group ($12.96 \pm 1.81 \mu\text{g/dL}$, $P = 0.001$, Figure 2(b)). H₂S levels in plasma were significantly higher in group Pb + NaHS ($13.42 \pm 0.15 \mu\text{mol}$) versus Saline ($P = 0.0001$), NaHS ($P = 0.0001$), and Pb groups ($P = 0.0001$) (12.27 ± 0.01 , 12.35 ± 0.05 , and $12.10 \pm 0.08 \mu\text{mol}$, resp., Figure 2(c)).

3.3. Protective Effect of NaHS Treatment on Vascular PHE-Induced Contraction in Hypertensive Animals Is Dependent on Endothelium. Vascular reactivity experiments were performed to assess direct vascular responses among the four different animal groups. KCl-induced contraction was not different between the experimental groups in aortic rings with (+E) or without (-E) endothelium (Figures 3(a)-3(b), Table 1). No differences in maximum response (R_{max}) to PHE-induced contraction were observed between Saline, Pb, and Pb + NaHS groups in endothelium intact rings; however, greater R_{max} was reached in NaHS versus Pb ($P = 0.0035$) and Pb + NaHS ($P = 0.0077$) groups (Figure 3(c), Table 1).

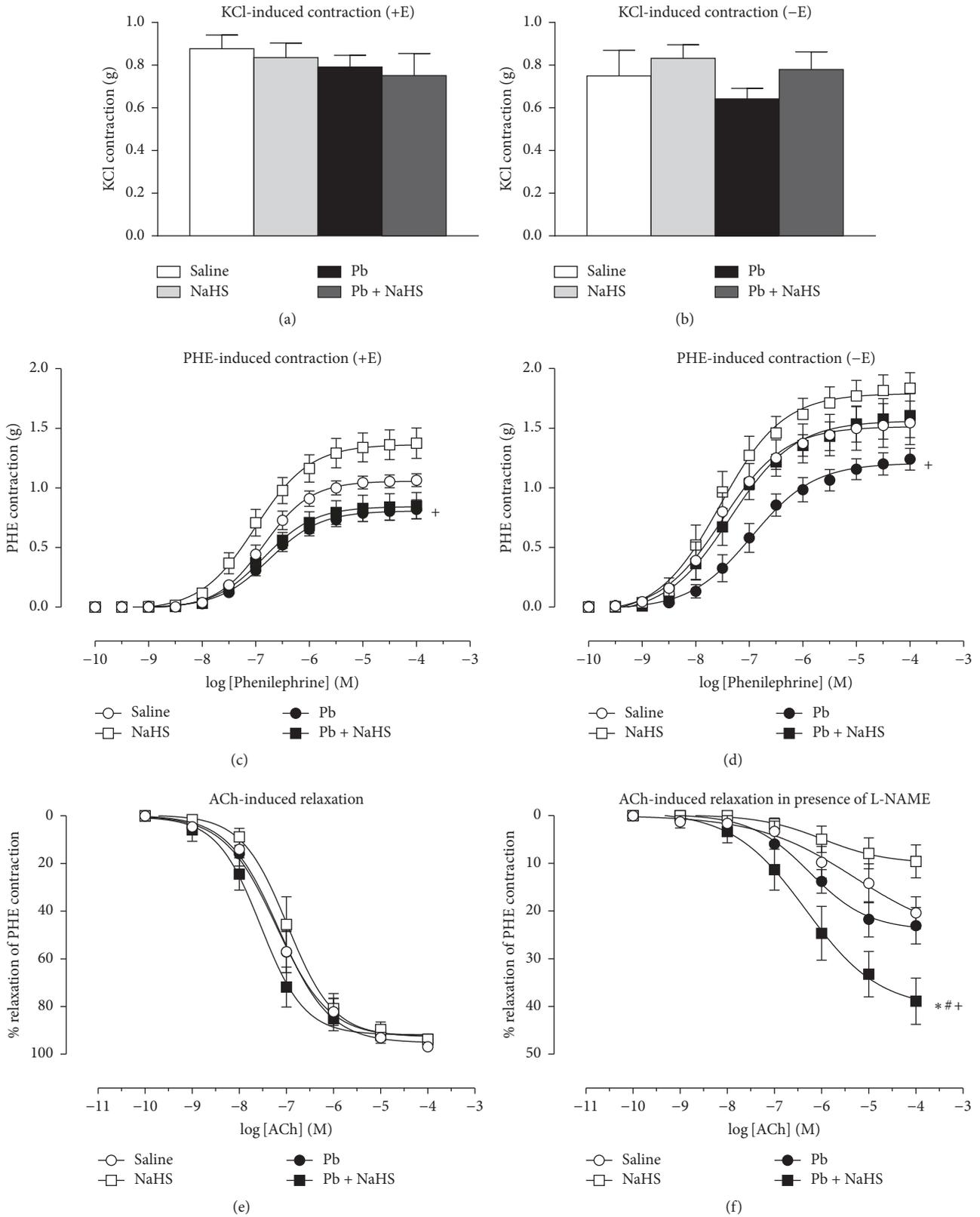


FIGURE 3: Vascular reactivity of thoracic aorta rings with (+E) or without (-E) endothelium from animals of four different groups: Saline (non-Pb-exposed), NaHS (non-Pb-exposed + NaHS); Pb (Pb-exposed), and Pb + NaHS (Pb-exposed + NaHS). ((a) and (b)) KCl-induced contraction. ((c) and (d)) PHE-induced contraction. (e) ACh-induced relaxation. (f) ACh-induced relaxation in presence of L-NAME. Values represent mean \pm SEM. $n = 5-7$. * $P < 0.05$ versus NaHS group; # $P < 0.05$ versus Saline group; + $P < 0.05$ versus Pb group.

TABLE 1: KCl-induced contraction, PHE-induced contraction, and ACh-induced relaxation in thoracic aorta of Saline, NaHS, Pb, and Pb + NaHS rat.

	Aortic rings							
	+E				-E			
	Saline	NaHS	Pb	Pb + NaHS	Saline	NaHS	Pb	Pb + NaHS
KCl R_{max} , g	0.87 ± 0.06	0.84 ± 0.06	0.79 ± 0.05	0.75 ± 0.10	0.75 ± 0.11	0.83 ± 0.06	0.64 ± 0.04	0.78 ± 0.08
PHE R_{max} , g	1.05 ± 0.02	1.36 ± 0.05	0.80 ± 0.02 ⁺	0.84 ± 0.04 ⁺	1.51 ± 0.07 [*]	1.79 ± 0.06 [*]	1.20 ± 0.05 ⁺⁺	1.56 ± 0.06 [*]
PHE pEC ₅₀ , -log M	6.84 ± 0.06	7.00 ± 0.10	6.75 ± 0.08	6.85 ± 0.12	7.50 ± 0.16 [*]	7.54 ± 0.12 [*]	6.95 ± 0.11	7.35 ± 0.12 [*]
ACh R_{max} , %	97.79 ± 1.47	93.76 ± 2.01	93.78 ± 2.10	93.88 ± 2.12	—	—	—	—
ACh pEC ₅₀ , -log M	7.07 ± 0.20	6.99 ± 0.20	7.13 ± 0.22	7.54 ± 0.25	—	—	—	—
ACh + L-NAME R_{max} , %	20.38 ± 3.34	9.60 ± 0.70	23.08 ± 3.80	38.90 ± 4.84 ^{*##}	—	—	—	—

Data represents mean ± SEM ($n = 5-7$). * $P < 0.05$ versus Saline. ⁺ $P < 0.05$ versus NaHS. [#] $P < 0.05$ versus Pb. ^{*} $P < 0.05$ versus intact vessels (+E) of the same animal group.

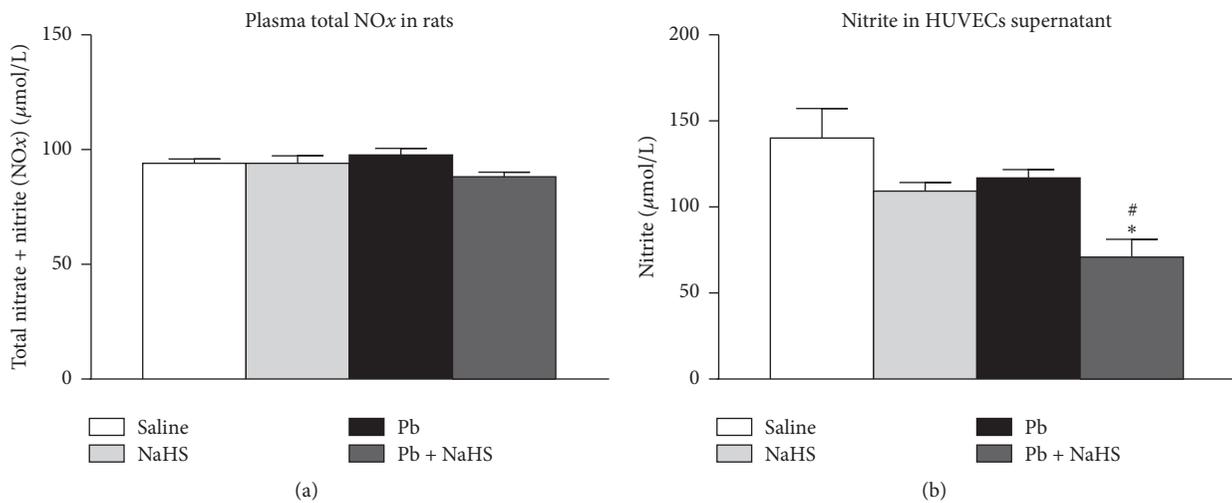


FIGURE 4: Nitric oxide availability in plasma and cell culture supernatant: Saline (non-Pb-exposed), NaHS (non-Pb-exposed + NaHS), Pb (Pb-exposed), and Pb + NaHS (Pb-exposed + NaHS). (a) Total plasmatic NOx. (b) Nitrite levels assessed in supernatant of HUVECs incubated with 5% (v/v) of plasma from animals of four different groups. Values represent mean ± SEM. $n = 6-12$. * $P < 0.05$ versus Saline group; [#] $P < 0.05$ versus Pb group.

No differences were observed in pEC₅₀ among the four experimental groups. Denuded rings (-E) presented higher R_{max} compared to R_{max} of respective group with intact ring (+E) ($P = 0.0001$) and an increase in pEC₅₀ ($P = 0.0001$) was also observed except in Pb group (Table 1). Removal of endothelium caused a higher PHE-induced contraction in NaHS versus Pb group ($P = 0.0374$, Figure 3(d), Table 1) and no differences in R_{max} were observed among the other groups. No differences were observed in pEC₅₀ among the experimental groups with denuded rings (Table 1).

3.4. Treatment with NaHS in Pb-Induced Hypertension Elicits an ACh-Induced Relaxation That Is Non-NO-Mediated. Relaxant responses evoked by ACh (that trigger NO release from endothelial cells) were tested in endothelium intact (+E) rings precontracted with PHE. No differences in R_{max} and pEC₅₀ to ACh-induced relaxation were observed among all four groups (Figure 3(e), Table 1). Blockade of nitric oxide synthase (NOS) using L-NAME caused a decrease in

relaxation in Saline, NaHS, and Pb groups (around 20% of relaxation); however, maximum relaxation was greater in rings from Pb + NaHS group compared to the other groups (around 40% of relaxation) ($P = 0.004$, Figure 3(f), Table 1).

ACh elicits a vasodilatory effect that is dependent on endothelial NO. As we observed relaxation in Pb + NaHS group even in the presence of L-NAME, we sought to investigate the effect of NaHS treatment in NO production. Plasmatic total NOx was not different between animals from experimental groups (94.08 ± 1.82 , 94.04 ± 3.27 , 97.69 ± 2.91 , and 88.18 ± 2.03 µmol/L in groups Saline, NaHS, Pb, and Pb + NaHS, resp., Figure 4(a)). Experiments were performed to evaluate the production of NO directly by endothelial cells; therefore, HUVECs were incubated with plasma from animals of the different groups. Surprisingly, nitrite levels were decreased in supernatant of HUVECs incubated with plasma from Pb + NaHS group (70.96 ± 10.37 µmol/L) compared with Saline (140.10 ± 17.14 µmol/L, $P = 0.0020$) and Pb (116.90 ± 4.92 µmol/L, $P = 0.0261$) groups (Figure 4(b)).

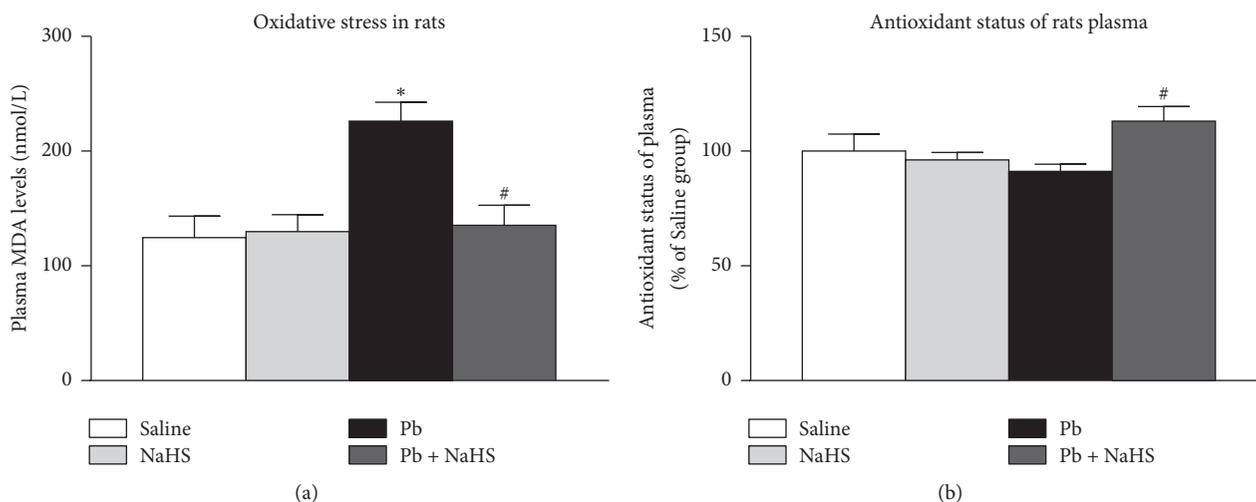


FIGURE 5: Oxidative stress parameters. Saline (non-Pb-exposed), NaHS (non-Pb-exposed + NaHS), Pb (Pb-exposed), and Pb + NaHS (Pb-exposed + NaHS). (a) MDA levels in plasma. (b) Antioxidant status of plasma as determined by MTT assay. Values represent mean \pm SEM. $n = 6-12$. * $P < 0.05$ versus Saline group; # $P < 0.05$ versus Pb group.

3.5. Treatment with NaHS Reduces Oxidative Stress in Pb-Induced Hypertension. Plasmatic MDA levels were elevated in animals exposed to Pb (226.10 ± 16.57 nmol/L) compared with animals from Saline ($P = 0.0014$) and NaHS ($P = 0.0036$) groups (124.70 ± 18.56 and 130.00 ± 14.62 nmol/L, resp.); however, treatment of animals exposed to Pb with NaHS blunted the increase in plasmatic MDA (135.40 ± 17.61 nmol/L in Pb + NaHS group, $P = 0.0025$, Figure 5(a)). Antioxidant status of plasma was not different between Saline and Pb groups (104.60 ± 6.67 and $91.14 \pm 3.22\%$, resp.) (Figure 5(b)). NaHS alone did not alter antioxidant status of plasma ($96.20 \pm 3.25\%$ in NaHS group); however, plasma from animals of Pb + NaHS group showed an increase in the antioxidant status of plasma ($113.10 \pm 6.40\%$) compared to Pb group ($P = 0.020$, Figure 5(b)).

4. Discussion

In our study, we observed an increase in diastolic and mean blood pressure in humans intoxicated with Pb and this was related to decreased plasmatic levels of H_2S . We confirmed that acute exposition to low lead levels promoted an increase in SBP measured by tail cuff plethysmography in awaken rats. Moreover, we showed that treatment with H_2S donor, NaHS, was able to blunt the increases in SBP promoted by the acute exposition to low lead levels and that these effects of NaHS on blood pressure may be related to H_2S actions in vascular responses and H_2S antioxidant capacity.

We observed that patients intoxicated with Pb presented SBP similar to control patients. However, we detected an increase in diastolic and mean blood pressure in Pb-intoxicated patients, which is in accordance with previous reports in literature showing increases in blood pressure following Pb-intoxication in humans [8, 51–55]. As H_2S has been shown to have several actions in cardiovascular system, we investigated the levels of H_2S in plasma from patients and

found decreased plasmatic H_2S levels in patients from Pb-intoxicated group. This result is in accordance with previous studies showing that decreased levels of H_2S are related to hypertension [42, 56–58] and this observation leads us to the interest in understanding the role of this gaseous mediator in Pb-induced hypertension.

In the present study, following intoxication with lead acetate, rats developed higher SBP from exposition day five to day seven, whereas treatment with NaHS blunted this increase in blood pressure. In accordance, we detected the presence of lead in whole blood in the animals from the Pb group. Interestingly, we observed an approximately 2-fold decrease in whole-blood lead levels in animals treated with NaHS (Pb + NaHS group) compared with animals from Pb group. In fact, the reaction of H_2S with lead acetate generates lead (II) sulfide (PbS) [59].

PbS is a solid, dark color, almost insoluble compound. Indeed, PbS is insoluble and is a very stable compound at blood's pH [60]. Thus, the interaction of H_2S with lead acetate and consequent formation of PbS in animals from Pb + NaHS group may explain the lower whole-blood lead levels detected in our study. Accordingly, previous studies in rats fed with different compounds of lead showed that animals receiving PbS presented diminished whole-blood lead levels when compared with rats receiving different Pb-based compounds, such as lead acetate and lead oxide [60].

One may consider that the nonobserved increase in SBP in animals from Pb + NaHS group may be due to the lower whole-blood Pb levels. Although the diminished Pb levels in Pb + NaHS group may partially contribute to the attenuation in the increases of SBP, these animals presented whole-blood Pb levels of 12.96 ± 1.81 $\mu\text{g/dL}$. In addition, it has been shown that whole-blood lead levels below or very close to those found in our study are related to increases in blood pressure. Previous studies from our group showed that animals with 9 ± 1 $\mu\text{g/dL}$ [9] and 11.5 ± 1.2 $\mu\text{g/dL}$ [13] presented an increase

in SBP and other groups also reported an increase in blood pressure of rats with whole-blood lead levels of $12 \pm 1.34 \mu\text{g/dL}$ [12] and $9.98 \pm 1.70 \mu\text{g/dL}$ [18]. Therefore, a mechanistic action of H_2S must be involved in the decreases of blood pressure promoted by NaHS in Pb-induced hypertension.

Interestingly, followed by the reductions in whole-blood lead levels, H_2S in plasma was increased in Pb + NaHS group. As H_2S and lead acetate may interact and the PbS generated is expected to be eliminated from the organism, one would inquire that H_2S should be lower in Pb + NaHS group. However, it has been demonstrated that NaHS may induce the formation of polysulfides (H_2S_n , $n > 1$) [61] and that either endogenous or exogenous H_2S may generate polysulfides through its interaction with ROS, such as superoxide anion [62–64].

Lead-intoxicated animals (Pb group) presented increases in oxidative stress, which was reduced in Pb + NaHS group. Reductions in oxidative stress in animals of Pb + NaHS group may be related to the reaction of exogenous H_2S with ROS. Also, recent studies suggest that effects initially described for H_2S may rely (partially or totally) on polysulfides [65]; therefore, polysulfides generated in Pb + NaHS animals may be responsible for the beneficial effects observed in our study. Importantly, the method used in our study to measure H_2S , the methyl blue formation method, may not distinguish between H_2S itself and polysulfide compounds. Thus, increased levels of H_2S shown in Figure 2(c) may represent polysulfides formation.

In order to understand the vascular effects promoted by lead intoxication and the NaHS treatment, vascular reactivity experiments have been performed. No differences were observed in KCl-induced contraction between groups with (+E) or without (–E) endothelium. However, greater contraction to PHE was reached by aortic rings from animals treated with NaHS when compared to both groups receiving Pb (Pb and Pb + NaHS group). While the findings on KCl-induced contraction do not point to increased vascular Ca^{2+} influx through voltage-gated Ca^{2+} channels [44, 66], PHE may also activate Ca^{2+} influx through receptor and store-operated Ca^{2+} channels [44, 66]; thus a greater influx of Ca^{2+} induced by changes promoted by the NaHS treatment must be considered. Also, the increases in contractile responses in NaHS group could be explained by changes in endothelium-dependent relaxation pathways. It was demonstrated that, in low doses, NaHS elicited a vasoconstriction in rats' aortas and mixing NaHS with NO donors inhibited the vasorelaxant effect of NO, both *in vitro* and *in vivo* [67]. Since the procontractile effects of H_2S were prevented by removing the endothelium or inhibition of eNOS, it has been suggested that H_2S acted to remove the basal vasorelaxatory influence of NO to produce contraction. Further, these authors showed that H_2S and NO may interact to generate an inactive nitrosothiol product [68].

Removal of endothelium promoted increases in R_{max} and pEC_{50} ($P < 0.05$, Table 1); however, no differences were observed in pEC_{50} between intact and denuded rings from Pb group ($P > 0.05$, Table 1). This indicates that presence of endothelium and, consequently, the protective factors released by endothelial cells were not able to counterbalance

the vasoconstrictor effect of PHE in Pb group, which may suggest the installation of some endothelial damage after seven days of exposure to low lead levels.

Rings from Pb and Pb + NaHS groups presented the lower mean values of contraction, and although these values were not significantly different from Saline group, they reached statistical significance compared to NaHS group. Interestingly, Pb + NaHS aortic rings presented decreased R_{max} when compared to NaHS group only in rings with intact endothelium, whereas removal of endothelial cells abolished this diminished contraction. Since removal of endothelium abolished this difference in contraction between Pb + NaHS group and NaHS group, we suggest that NaHS treatment may exert a protective effect that is dependent on endothelium only in the presence of hypertension. Vascular effects of H_2S are not fully comprehended and both contraction [58, 69, 70] and relaxation [58, 67, 71–73] have been shown to occur as an effect of H_2S . It has been shown before that removal of endothelium [73] or inhibition of NO production [74] reduced relaxant responses to H_2S ; this supports our findings in which removal of endothelium abolished this protective effect induced by NaHS treatment in hypertensive animals.

Although relaxation induced by interactions of H_2S with NO has been described previously [31, 71, 75, 76], in our hands, following Pb exposition and NaHS treatment, no differences in endothelial NO-dependent relaxation promoted by ACh were observed. However, when relaxation was triggered by ACh in the presence of NOS inhibitor L-NAME, a relaxation around 40% was reached in aortic rings from animals of Pb + NaHS group. Previous studies showed that H_2S may increase expression and activity of eNOS [77, 78], thus producing more NO, and this excessive NO could be responsible for relaxation observed in the presence of L-NAME. However, we did not observe any alteration in plasmatic total NO_x from animals of different groups; and interesting data was observed with nitrite measured in HUVECs' culture supernatant. Following incubation with plasma from different animal groups, we observed that cells treated with plasma from Pb + NaHS group presented lower nitrite levels compared to cells treated with plasma from Saline and Pb groups. These results support our observation in vascular reactivity experiments where relaxation in Pb + NaHS group may be triggered in an NO-independent way. Since NO-independent relaxation was reached in vessels from Pb + NaHS group and not in vessels from animals treated only with NaHS, we speculate that only in presence of hypertension, which may trigger several defensive mechanisms in the organism, does H_2S promote relaxation independently from NO presence and strongly recommend future studies in the field.

As interaction with NO seems not to be involved in the beneficial effects of H_2S in Pb-induced hypertension, we performed additional experiments to better understand the role of H_2S during Pb-induced hypertension. Increases in oxidative stress contribute to hypertension and have been pointed as a possible mechanism underlying Pb-induced hypertension [18, 19], while H_2S has been shown to exert antioxidant activity [79, 80]. Therefore, we sought to investigate the effects of H_2S in oxidative stress in Pb-induced

hypertension. We found that plasma from Pb group presented higher levels of MDA. As MDA is a metabolite of lipid peroxidation [81], increased MDA levels in these animals indicate higher levels of oxidative stress. In our hands, treatment of hypertensive animals with NaHS (Pb + NaHS group) blunted the increase in oxidative stress; moreover, decreased MDA levels were followed by an increase in the antioxidant status of plasma in Pb + NaHS group, as measured by the MTT assay [50]. Accordingly, previous studies have shown the role of H₂S in reduction of oxidative stress and increases in antioxidant capacity [36, 82–84]. These results suggest that beneficial effects of H₂S in Pb-induced hypertension may be related to H₂S antioxidant capacity and reductions in oxidative stress.

In conclusion, we showed that increases in diastolic and mean blood pressure in human patients intoxicated with Pb may be related to decreased H₂S plasmatic levels. Treatment with H₂S donor blunted increases in SBP in rats and this beneficial effect of NaHS may not be related to NO. Importantly, we showed that treatment with NaHS in hypertensive animals led to vascular relaxation induced by ACh which is non-NO-mediated. Therefore, the antioxidant capacity of H₂S may also be involved in reductions in blood pressure in Pb-induced hypertension.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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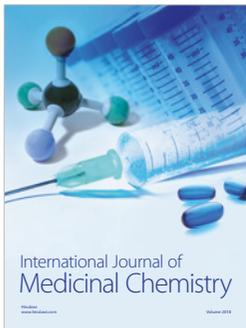
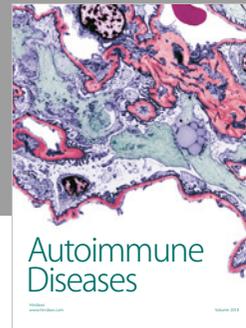
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