



OPEN Dissolving sodium hydrosulfide in drinking water is not a good source of hydrogen sulfide for animal studies

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Hydrogen sulfide (H_2S) has many physiological and pathological roles in the human body. Sodium hydrosulfide (NaHS) is widely used as a pharmacological tool for assessing H_2S effects in biological experiments. Although H_2S loss from NaHS solution is a matter of minutes, some animal studies use NaHS in solution as an H_2S -donating compound in drinking water. This study addresses whether 30 μM NaHS in drinking water prepared in rat/mouse water bottles remains stable for at least 12–24 h, as presumed by some authors. NaHS solutions (30 μM) were prepared in drinking water and immediately transferred to rat/mice water bottles. Samples were obtained from the tip of water bottles and from inside of the bottles at 0, 1, 2, 3, 4, 5, 6, 12, and 24 h for sulfide measurement using the methylene blue method. Furthermore, NaHS (30 μM) was administered to male and female rats for two weeks, and serum sulfide concentrations were measured every other day in the first week and at the end of the second week. NaHS solution was unstable in the samples obtained from the tip of water bottles; it declined by 72% and 75% after 12 and 24 h, respectively. In the samples obtained from the inside of the water bottles, the decline in the NaHS was not significant until 2 h; however, it decreased by 47% and 72% after 12 and 24 h, respectively. NaHS administration did not affect serum sulfide levels in male and female rats. In conclusion, NaHS solution prepared in drinking water can not be used for H_2S donation as the solution is unstable. This route of administration exposes animals to variable and lower-than-expected amounts of NaHS.

The history of hydrogen sulfide (H_2S) as a toxin dates back to 1700¹; however, its possible role as an endogenously-produced biological signaling molecule was reported in 1996 by Abe and Kimura². In the past three decades, many functions of the H_2S in various human systems were elucidated^{1,3}, leading to the recognition that H_2S -donating molecules might have clinical utility in the treatment or management of some diseases^{3,4}; see Cirino et al.³ for a recent review.

In many cell culture and animal studies, sodium hydrosulfide (NaHS) is widely used as a pharmacological tool for assessing H_2S effects^{5–8}. However, NaHS is not an ideal H_2S donor because of its fast conversion to H_2S/HS^- , contamination with polysulfides, oxidation, and volatilization when in solution^{4,9}. In many biological experiments, NaHS is dissolved in water, resulting in passive volatilization and H_2S loss^{10–12}, spontaneous H_2S oxidation^{11–13}, and photodecomposition¹⁴. Sulfide loss from stock solutions via H_2S volatilization is fast¹¹, and in open chambers, H_2S is lost with a $t_{1/2}$ of about 5 min, where its concentration falls by approximately 13% per minute¹⁰. Although H_2S loss from a NaHS solution is a matter of minutes, some animal studies have used NaHS solutions in drinking water as an H_2S source for 1–21 weeks, changing the NaHS-containing solution every 12–24 h^{15–26}. This practice is incompatible with scientific research principles as the drug dose should be defined to translate it to other species, particularly humans²⁷.

Preclinical studies in biomedicine aim to improve patient care or health. However, the results of most animal studies are not translated to humans^{28–30}. One cause for this failure in translation is paying less attention to the

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methodological quality of animal studies³⁰. Therefore, this study addresses whether 30 μM NaHS solutions in drinking water prepared in rat/mouse water bottles remain stable for 12–24 h, as claimed or presupposed in some studies.

Materials and methods

Ethical approval

All experiments of the current study were affirmed by the published guidelines for the care and use of laboratory animals in Iran³¹. All experiments of the current study were also reported following ARRIVE guidelines³². The ethics committee of the Research Institute for Endocrine Sciences, affiliated with the Shahid Beheshti University of Medical Sciences, confirmed and approved all experimental procedures of the current study.

Materials

Zinc acetate dihydrate (CAS: 5970-45-6) and ferric chloride anhydrous (CAS: 7705-08-0) were purchased from Biochem, Chemopahrama (Cosne Sur Loire, France). Sodium hydrosulfide hydrate (CAS: 207683-19-0) and N,N-dimethyl-p-phenylenediamine (DMPD) (CAS: 535-47-0) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Isoflurane was purchased from Piramal (Bethlehem, PA, USA). Hydrochloric acid (HCl) was obtained from Merck (Darmstadt, Germany).

Preparation of NaHS solutions

NaHS solutions (30 μM) was prepared in drinking water and immediately transferred to rat/mice water bottle. This concentration was chosen based on many publications that use NaHS as an H_2S source in their studies; please see the discussion section. NaHS is a hydrated molecule and may have variable numbers of waters of hydration (i.e., $\text{NaHS} \cdot x\text{H}_2\text{O}$); according to the manufacturer, the percent of NaHS used in our study was 70.7% (i.e., $\text{NaHS} \cdot 1.3 \text{H}_2\text{O}$) and this value was considered in our calculations, where we used a molecular weight of 56.06 g/mol, which is for anhydrous NaHS. Waters of hydration (also called waters of crystallization) are water molecules integral to the crystal structure³³. Hydrates have different physical and thermodynamic properties compared to anhydrides³⁴.

The solvent's pH and temperature were measured before adding the NaHS to the drinking water. NaHS solution was immediately transferred to the water bottles of rats/mice in the animal cages. Samples were obtained from the tip of water bottles and from inside of the bottles at 0, 1, 2, 3, 4, 5, 6, 12, and 24 h for sulfide measurement. Sulfide measurement was done immediately after each sampling. We obtained samples from the tip of the bottles as some studies have stated that the small aperture of water bottles minimizes H_2S evaporation^{15,19}. This issue seems to be true for the solution inside the bottle. However, this is not the case for solution in the tip of water bottles with a higher evaporation rate and auto-oxidation; indeed, animal drinks this water first.

Experiments in rats

In this study male and female Wistar rats were used. Rats were housed in polypropylene cages (2–3 rats/cage) under standard conditions (temperature 21–26 °C and humidity 32–40%) of 12-h light (7 am to 7 pm) and 12-h dark (7 pm to 7 am). They had free access to tap water and the regular chow diet (Khorak Dam Pars, Co., Tehran, Iran). Age-matched (6-month old) female ($n = 10$, body weight: 190–230 g) and male ($n = 10$, body weight: 320–370 g) Wistar rats were randomly assigned to control and NaHS (30 μM)-treated groups ($n = 5$ /group). We used the KISS (Keep It Simple, Stupid) approach to determine sample size, which combines past experience with power analysis³⁵. We first performed a pilot study with 3 rats and determined the mean total serum sulfide levels and standard deviation ($8.1 \pm 0.81 \mu\text{M}$). Then, considering 80% power and assuming a two-sided 5% significance level, we determined our provisional sample size ($n = 5$ based on previous literature) that corresponded to a standardized effect size of 2.02 from predefined values presented by Festing for sample size calculation in laboratory animals³⁵. After multiplying this value by SD (2.02×0.81), the predicted detectable effect size (1.6 μM) was 20%, which is acceptable. It means that $n = 5$ /each group is sufficient to detect a mean change of 20% between groups. Randomization of rats to control and NaHS-treated groups was done using the random function of the Excel software³⁶ (Supplementary Fig. 1). Blinding was done at the outcome level, and the researcher who performed biochemical measurements was blinded to the groups.

NaHS groups of both sexes were treated with 30 μM of NaHS solution prepared in drinking water for 2 weeks; fresh solutions were provided every 24 h, at which time body weights were measured. Blood samples were obtained from the tail tips of all rats under isoflurane anesthesia every other day in the first week and at the end of the second week. Blood samples were centrifuged at 3000 g for 10 min, and sera were separated and maintained at -80°C for subsequent measurement of serum urea, creatinine (Cr), and total sulfide. Serum urea was measured by the enzymatic urease method and serum Cr by the photometric Jaffe method using commercially available kits (Man Company, Tehran, Iran) and an auto-analyzer (Selectra E, serial number 0–2124, Netherlands). Intra- and inter-assay coefficient of variation for both urea and Cr were $<2.5\%$.

Total sulfide measurement

The methylene blue (MB) method was used to measure total sulfide in NaHS-containing drinking water and serum; MB is the most commonly used method for sulfide measurement in stock solutions and biological samples^{11,37}. The MB method is useful for estimating total sulfide pools³⁸ and measures inorganic sulfide present as H_2S , HS^- , and S^{2-} in the aqueous phase³⁹. In this method, sulfur is precipitated as zinc sulfide (ZnS) in the presence of zinc acetate^{11,38}. Precipitation by zinc acetate is the most widely used technique for separating sulfide from other chromophores¹¹. ZnS is redissolved under highly acidic conditions using HCl ¹¹. The sulfide reacts with DMPD in ferric chloride (Fe^{3+} acts as an oxidizing agent) catalyzed reaction with 1:2 stoichiometric ratio to

give the MB dye, which is detected spectrophotometrically at 670 nm^{40,41}. The detection limit of the MB method is around 1 μM ¹¹.

In the current study, 100 μL of each sample (solution or serum) was added to a test tube; then, 200 μL of zinc acetate (1% w/v in distilled water), 100 μL of DMPD (20 mM in HCl 7.2 M), and 133 μL of FeCl_3 (30 mM in HCl 1.2 M) were added. The mixture was incubated in a dark environment at 37°C for 30 min. The solution was centrifuged at 10,000 g for 10 min, and the absorbance of the supernatant was read at 670 nm using a microplate reader (BioTek, MQX2000R2, Winooski, VT, USA). A NaHS (0–100 μM) calibration curve in ddH₂O was used to determine sulfide concentrations (Supplementary Fig. 2). All solutions used for measurement were prepared freshly. Intra- and inter-assay coefficients of variation of sulfide measurement were 2.8% and 3.4%, respectively. Using the spiked samples method, we also determined the total sulfide recovered for NaHS-containing drinking water and serum samples⁴². Recovery values for NaHS-containing drinking water and serum samples were $91 \pm 1.1\%$ ($n=6$) and $93 \pm 2.4\%$ ($n=6$), respectively.

Statistical analyses

GraphPad Prism version 8.0.2 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com) was used for statistical analyses. The temperature and pH of drinking water before and after adding NaHS were compared using a paired t-test. Loss of H_2S from NaHS-containing solutions was calculated as the percent decrease from baseline absorbance, and to assess whether this loss is statistically significant, one-way repeated measure ANOVA followed by the Dunnett multiple comparison test was used. Two-way mixed (between-within) ANOVA with the Bonferroni posthoc test was used for comparing body weight, serum urea, serum Cr, and serum total sulfide between control and NaHS-treated rats over time in each sex. Two-sided P-values < 0.05 were considered statistically significant.

Results

Stability of NaHS solution

The pH of the drinking water was 7.60 ± 0.01 before the addition of the NaHS and 7.71 ± 0.03 ($n=13$, $p=0.0029$) after adding NaHS. The drinking water temperature was 26.5 ± 0.2 , which decreased to 26.2 ± 0.2 ($n=13$, $p=0.0128$) after adding NaHS. 30 μM solutions of NaHS were prepared in drinking water and maintained in the water bottles. The NaHS solution was unstable, and its concentration declined with time. When samples were obtained from the tip of the water bottles, a large decrease (68.0%) was observed during the first hour, and the amount of NaHS in the solution declined by 72% and 75% after 12 and 24 h, respectively. In the samples obtained from the inside of the water bottles, the decline in the NaHS was not significant until 2 h; however, it decreased by 47% and 72% after 12 and 24 h, respectively. These data indicate that regardless of the sampling location, the percent of NaHS in a 30 μM solution prepared in drinking water is decreased to about one-fourth of the initial value after 24 h (Fig. 1).

Changes in NaHS concentration of drinking water

The concentration of NaHS in freshly prepared 30 μM solutions was $30.3 \pm 0.4 \mu\text{M}$ (range: 28.7–31.9 μM , $n=12$). However, it declined to low values (mean: $3.0 \pm 0.6 \mu\text{M}$) after 24 h. As shown in Fig. 2, rats were not exposed to a stable concentration of NaHS during the study.

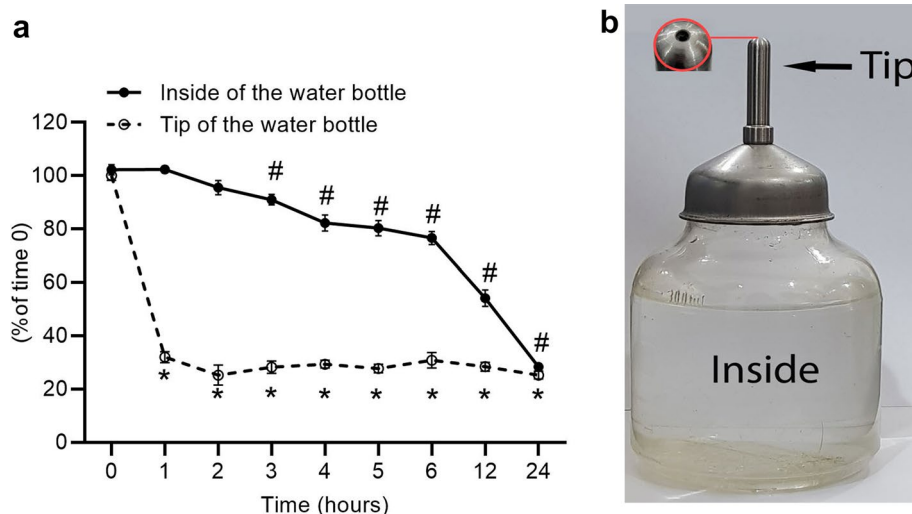


Figure 1. Stability of NaHS solution (30 μM) in drinking water maintained in rat/mice water bottle. After the solution was prepared, samples were obtained from the tips of the water bottles and the inside. Data are mean \pm SE ($n=6/\text{group}$). * and #, $P < 0.05$ compared to time 0. Picture of water bottle indicating the tip (with its aperture) and body. The volume of the tip is about 740 μL .

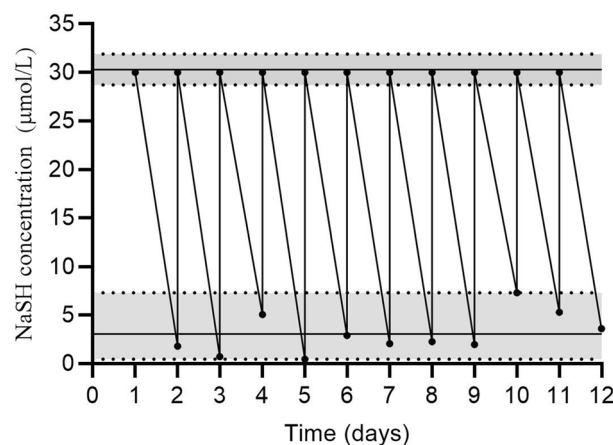


Figure 2. Changes in NaHS concentrations of drinking water to which rats were exposed during the study.

Effect of NaHS administration on body weight, serum total sulfide, and kidney function in rats

Body weight of female rats increased significantly over time (from 205.2 ± 5.2 to 213.8 ± 7.0 g in control and from 204.0 ± 8.6 to 211.8 ± 7.5 g in NaHS-treated rats); however, NaHS administration did not affect body weight (Fig. 3). Body weight of male rats increased significantly over time (from 338.6 ± 8.3 to 352.4 ± 6.0 g in control and from 352.4 ± 5.9 to 363.2 ± 4.3 g in NaHS-treated rats); however, NaHS administration did not affect body weight (Fig. 3).

Serum urea and Cr concentrations were comparable in control and NaHS-treated rats throughout the study. In addition, NaHS treatment did not affect serum urea and Cr concentrations (Table 1).

Baseline serum total sulfide concentrations were comparable in control and NaHS-treated male (8.1 ± 0.5 vs. 9.3 ± 0.2 μM) and female (9.1 ± 1.0 vs. 6.1 ± 1.1 μM) rats. NaHS administration over 14 days did not affect serum total sulfide levels in male and female rats (Fig. 4).

Discussion

The main result of this study is that NaHS-containing drinking water is not stable, and only about one-fourth of the baseline total sulfide was recovered after 24 h following sampling from the tip and inside of rat/mice water bottles. In addition, due to H_2S loss from the NaHS solution, rats were not exposed to a stable concentration of NaHS, and NaHS administration in drinking water did not affect body weight, serum urea and Cr, and serum total sulfide levels.

In this study, the rate of H_2S loss was about 3% per hour of 30 μM NaHS solutions prepared in drinking water. Time-dependent decrease in sulfide concentration has been reported to be 7% per hour in buffer solutions (sodium sulfide, 100 μM in 10 mM PBS, pH = 7.4) over 8 h¹¹. We previously, in defense of intraperitoneal injection of NaHS, reported that the sulfide loss rate from NaHS-containing drinking water in a 54 μM solution was

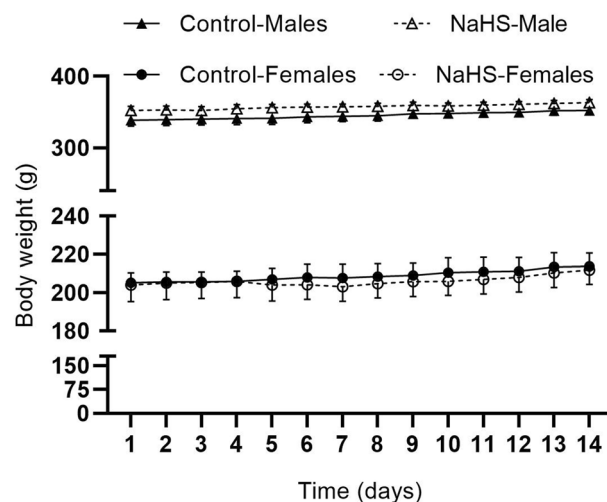


Figure 3. Changes in body weight following administration of NaHS (30 μM) in female and male rats. Data are mean \pm SEM and were compared using two-way mixed (between-within) ANOVA with the Bonferroni posthoc test. $n = 5/\text{group}$ in each sex.

	Time points (days)									
	0		2		4		6		14	
	Control	NasH	Control	NasH	Control	NasH	Control	NasH	Control	NasH
Male										
Urea	52.0 ± 1.7	51.2 ± 0.9	52.0 ± 2.4	50.6 ± 1	51.2 ± 0.6	49.6 ± 0.9	49.2 ± 1.2	51.0 ± 2.2	51.6 ± 0.8	51.2 ± 1.2
Cr	0.88 ± 0.07	0.90 ± 0.03	0.86 ± 0.04	0.94 ± 0.05	0.96 ± 0.07	0.84 ± 0.05	0.90 ± 0.07	0.80 ± 0.01	0.88 ± 0.06	0.92 ± 0.06
Female										
Urea	53.0 ± 0.3	52.4 ± 0.7	50.0 ± 1.1	50.6 ± 1.6	49.6 ± 1.3	50.8 ± 0.9	51.5 ± 0.6	50.6 ± 2.3	49.3 ± 1.9	50.2 ± 0.7
Cr	0.84 ± 0.02	0.84 ± 0.02	0.86 ± 0.02	0.84 ± 0.05	0.96 ± 0.04	0.90 ± 0.08	0.80 ± 0.04	0.82 ± 0.02	0.88 ± 0.09	0.86 ± 0.02

Table 1. Changes in serum urea and creatinine (Cr) (mg/dL) of male and female rats in control and NaSH-treated (30 μ M) (NaSH) groups at different time points. Data are mean \pm SEM and were compared using two-way mixed (between-within) ANOVA with the Bonferroni posthoc test. $n = 5$ /group in each sex.

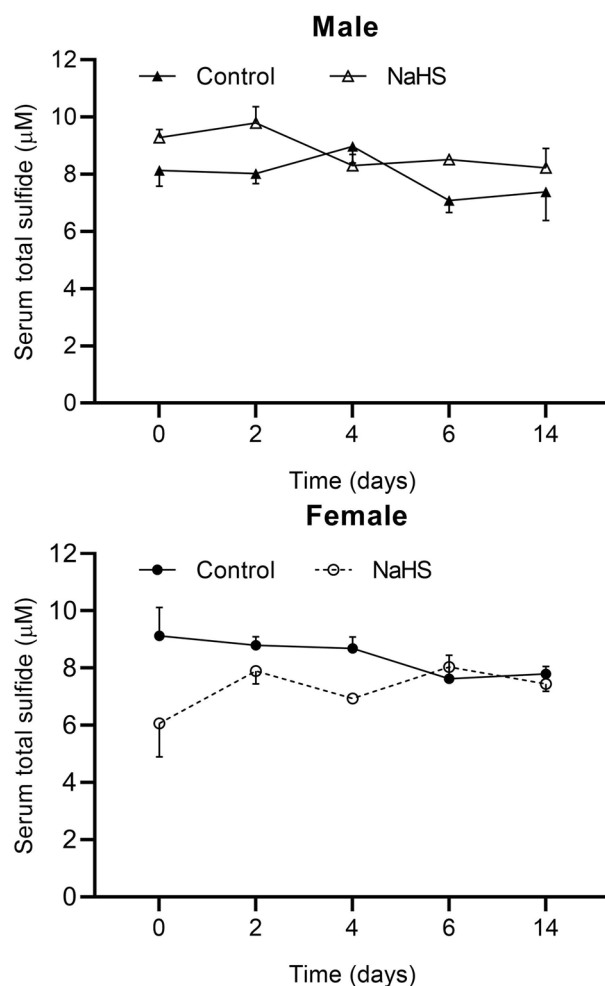


Figure 4. Changes in serum total sulfide concentrations following administration of NaHS (30 μ M) in female and male rats. Data are mean \pm SEM and were compared using two-way mixed (between-within) ANOVA with the Bonferroni posthoc test. $n = 5$ /group in each sex.

about 2.3% per hour (4%/hour for the first 12 h and about 1.4%/hour for the second 12 h after its preparation)⁸. The steady loss of H_2S from NaHS solution has been emphasized in earlier works⁴³ and is mainly done via volatilization and oxidation. Even without bubbling, sulfide loss from stock solutions via H_2S volatilization is fast¹¹. It has been shown that during dilution of stock solution, which took about 30–60 s, about 5–10% of H_2S is lost by evaporation⁶. To avoid H_2S evaporation from the solutions, researchers have adopted some measures, including gentle mixing of solutions¹², covering the stock solutions with parafilm⁶, and minimizing exposure of the solutions to air, as the rate of H_2S evaporation depends on the air–liquid interface¹³. Spontaneous oxidation

of H_2S is primarily due to transition metal ions, particularly ferric iron, which exists as an impurity in water¹³. Oxidation of H_2S causes the formation of polysulfides (sulfur atoms connected with covalent bonds)¹¹. To avoid its oxidation, H_2S -containing solutions are prepared in deoxygenated solvents^{44,45} following purging solutions with argon or nitrogen for 20–30 min to ensure deoxygenation^{11,12,37,44–46}. Diethylenetriamine-pentaacetic-acid (DTPA), a metal chelator (10^{-4} M), prevents auto-oxidation of HS^- in aerobic solutions, which in the absence of DTPA is about 50% in about 3 h at 25°C ^{37,47}. In addition, solutions should be kept in the dark on ice as 1e^- sulfide oxidation is catalyzed by UV light¹¹.

As shown in Fig. 5, when NaHS is dissolved in water, it ionizes to Na^+ and HS^- ; this dissociation depends on pK_1 of the reaction, which is temperature-dependent: $\text{pK}_1 = 3.122 + 1132/T$, where T is within 5 to 30°C and used in degrees of Kelvin (K) and $K = ^\circ\text{C} + 273.15$ ⁴⁸. pK_2 of HS^- is high ($\text{pK}_2 = 19$), thus, it does not yield S^{2-} or minimal amounts of S^{2-} produced at $\text{pH} < 9$ ^{6,49}. Instead, HS^- acts as a base and accepts H^+ from the H_2O molecule, which acts as an acid and is converted to H_2S and OH^- .

Despite evidence that NaHS solutions are not stable, some animal studies used NaHS solutions in drinking water as an H_2S -donating compound^{15–26}, with the duration of intervention ranging from 1 to 21 weeks (Table 2). These studies refreshed NaHS solutions every 12^{15,17,18,24,25} or 24^{19–23} h. Our results indicate that due to H_2S loss from the NaHS solution, rats are not exposed to a stable concentration of the drug, and a large fluctuation in the content of NaHS in the drinking water of rats occurs over 12 or 24 h (see Fig. 2). Two of these studies reported that H_2S content in water was stable for over 24 h²² or only 2–3% H_2S loss was observed over 12 h¹⁵, but they did not provide supporting data or details of the measurement. Two studies stated that the small aperture of water bottles minimizes H_2S evaporation^{15,19}. However, our results showed that this only retards H_2S loss from the inside of the water bottle for 2 h, not 12–24 h. In two studies, it has been stated that we presumed that NaHS content in the drinking water was not changed as we did not see changes in water coloring; thus, oxidation of

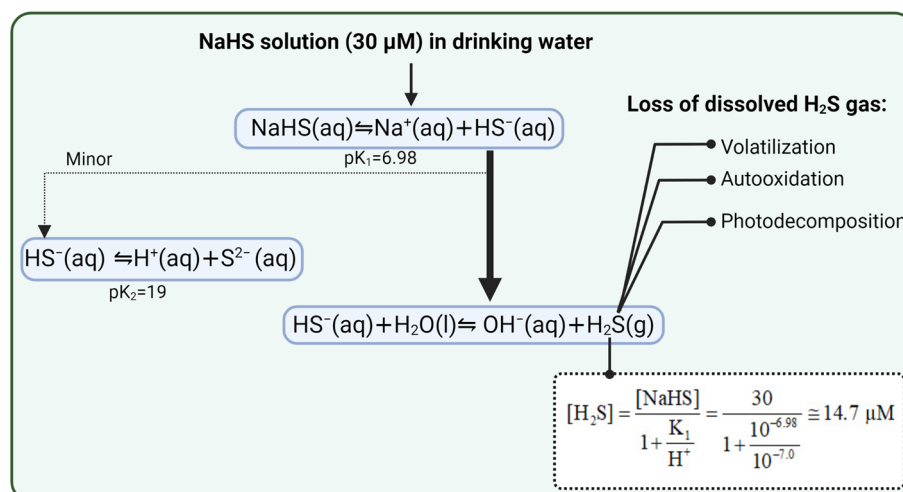


Figure 5. Formation of dissolved H_2S gas in NaHS solution (30 μM). aq, aqueous; g, gas; l, liquid. All calculations were done assuming water $\text{pH} = 7.0$ and water temperature = 20°C . Created with BioRender.com.

Study	Species	NaHS Concentration (μM)	Refreshment of NaHS solution (hours)	Study duration (weeks)	Temperature ($^\circ\text{C}$)	Reference
Sen et al. 2009	Mouse	30	NR	8	NR	16
Sen et al. 2010	Mouse	30	12	8	NR	15
Mishra et al. 2010	Mouse	30	24	8–10	22–24	23
Givvimani et al. 2011	Mouse	30	24	6	NR	19
Qipshidze et al. 2012	Mouse	30	24	4	NR	20
Pozsgai et al. 2015	Mouse	~ 400	24	1	24–25	21
Askari et al. 2018	Rat	30	12	8	20–22	17
Askari et al. 2018	Rat	30	12	8	20–22	24
Shirazi et al. 2018	Rat	30	12	8	20–22	26
Lee et al. 2018	Mouse	30	24	21	NR	22
Lorian et al. 2020	Rat	30	12	8	18–26	25
Lorian et al. 2020	Rat	30	12	8	18–26	18

Table 2. Summary of studies that administrated NaHS in drinking water in rodents. NR not reported.

H₂S due to air is not significant^{19,20}. It is a surprise that such a subjective method is used for assessing the stability of NaHS in water instead of measuring its concentration over time.

H₂S loss from NaHS solutions is pH- and temperature-dependent. Dissolving NaHS in water produces an alkaline solution⁵⁰, as observed in our study. The production of dissolved H₂S gas, following the dissolution of NaHS in water, is pH-dependent⁶. The lower the pH solution, the larger the fraction of NaHS in the form of the H₂S gas molecules, and the more sulfide is lost from aqueous solutions¹¹. None of these studies reported the pH of the drinking water used as the solvent for NaHS. According to WHO guidelines that most countries have adopted, the pH value of drinking water should be in the range of 6.5–8.5⁵¹. The rate of spontaneous H₂S oxidation increased by about tenfold over this pH range¹³. Dissolving NaHS in the water with this pH range yields the concentration of dissolved H₂S gas to be between 1 to 22.5 μM, highlighting the importance of controlling water pH before dissolving the NaHS. In addition, the temperature range reported in the studies mentioned above (18–26 °C) causes about 10% changes in concentration of dissolved H₂S gas in the solution, as a change in the temperature changes pK₁ and a slight change in pK₁ has a substantial effect on the fraction of dissolved H₂S gas⁴⁸. Adding to this issue is the long duration of some studies (5 months)²², during which more variability in temperature is expected to occur.

All studies except one²¹ used 30 μM of NaHS solutions in drinking water. To explain the dose used (i.e., 30 μM), some authors stated that NaHS in the aqueous phase produces exactly equal concentrations of H₂S gas, and since the physiological range of H₂S is between 10 and 100 μM, this dose is in the physiological range^{15,16}. It has also been explained that 30 μM NaHS keeps plasma H₂S levels in the physiological range, i.e., 5–300 μM^{19,20}. Let us consider a concentration of 30 μM of NaHS in water (pH = 7.0, and T = 20 °C), which had been used in some studies to investigate the effects of H₂S. We can calculate the concentration of dissolved H₂S gas to be 14.7 μM, which is about 50% of the initial concentration of NaHS. This value is similar to previous calculations by others under the same conditions^{13,48}.

In our study, NaHS administration did not change body weight; this finding is in line with other studies in male mice^{22,23} and male rats¹⁸; however, two studies reported that NaSH restored reduced body weight in nephrectomized rats^{24,26} and other studies did not report an effect of NaSH administration on body weight^{15–17,19–21,25}. In addition, in our study, NaSH administration did not affect serum urea and Cr, a finding that aligns with another report²⁵.

This study found that NaHS administration in drinking water for 2 weeks did not affect serum total sulfide concentrations in male and female rats. This finding is in line with results reported by Sen et al.¹⁶ that 8-week treatment with 30 μM NaHS in drinking water did not affect plasma sulfide levels in control rats; however, they reported that this intervention restored decreased plasma H₂S levels in nephrectomized mice. Lee et al. also reported that 5 months of treatment with 30 μM NaHS in drinking water increased plasma free sulfide levels by about 26% in aged mice²². Other studies have not reported circulating sulfide changes following NaHS intervention in drinking water.

Seven studies have reported that they used NaHS from Sigma^{15,16,19–23} but did not provide more details about water of hydration, and five studies did not mention the source of NaHS used for their preparations^{17,18,24–26}. NaHS is a hydrated molecule and may have variable waters of hydration, affecting the amount of NaHS needed for preparing a given molar concentration of the solution. For example, in our study, it was as NaHS•1.3 H₂O. Therefore, the actual concentrations of NaHS in these studies may be lower than those reported.

“How could such a short-lived compound exert such sustained effects?” this question has been asked by Pozsgai et al.²¹, who assessed the effects of NaHS on colitis in mice. These authors hoped that future studies would answer this question and speculated that the NaHS solution might contain more stable polysulfides besides H₂S and bisulfides that mediated the NaHS effects²¹. Another possibility is that a very low concentration of NaHS remaining in the solution can provide beneficial effects. Indeed, Olson has provided evidence that the micromolar range of H₂S in the blood is unphysiological, and it should be in the nanomolar range, or it does not exist in blood at all¹³. There is a possibility that H₂S acts through protein sulfhydration, which is a reversible post-translational modification and affects the function, stability, and localization of the many proteins^{52–54}. Indeed, about 10–25% of many liver proteins are sulfhydrated under physiological conditions⁵³. Two studies acknowledged the fast decay of NaHS^{19,23} but surprisingly, they stated that “we controlled the concentration of NaHS in the drinking water by changing the drinking water every day”²³. One study surprisingly claims that “NaHS is a standard H₂S donor which is usually used in clinic instead of H₂S itself”¹⁸.

According to the discussion above indicating that NaHS is lost from solution by volatilization, oxidation, and photodecomposition, some suggestions have been provided for minimizing H₂S loss from solution. First, H₂S evaporation depends on the air–liquid interface¹³ and pH of the solution¹¹; thus, for minimizing loss from volatilization, the size of the aperture of water bottles can be minimized as much as possible, as suggested previously^{15,19} and pH of water can be adjusted at the upper acceptable range (i.e., 6.5–8.5⁵¹) to minimize loss from volatilization¹¹. Second, spontaneous oxidation of H₂S is due to exposure to oxygen and the presence of transition metal ions in drinking water¹³, therefore deoxygenating drinking water using argon or nitrogen^{44,45} and using a metal chelator^{37,47} can decrease sulfide oxidation. Third, to prevent the photodecomposition of H₂S, water bottles can be wrapped in aluminum foils; this practice is also applied to light-sensitive materials, such as streptozotocin⁵⁵. Finally, inorganic sulfide salts (NaHS, Na₂S, and CaS) can be gavaged instead of dissolving in drinking water as has been reported previously^{56–58}; it has been shown that following gavage of radioactive sodium sulfide to rats, it is well absorbed and distributed in almost all tissues⁵⁹. Currently, most studies administer inorganic sulfide salts via the intraperitoneal route; however, this route is minimally used in the clinic⁶⁰. On the other hand, the oral route is the most common and preferred route of drug administration in humans⁶¹. Thus, we suggest oral gavage for assessing the effects of H₂S donors in rodents.

As a limitation, we measured sulfide levels in aqueous solutions and serum using the MB method. Methods for measurement of sulfide include iodometric, spectrophotometric, electrochemical (potentiometric,

galvanic, coulometric, and amperometric), and chromatographic (gas chromatography and HPLC), of which spectrophotometric MB method is the most common approach⁶². A limitation of the MB method for measuring H₂S levels in biological samples is that it measures all sulfur species rather than free H₂S⁶³ because it is done under acidic conditions, which extracts sulfur from biological sources⁶⁴. However, according to the American Public Health Association, MB is the standard method for measuring aqueous sulfide⁶⁵. Therefore, this limitation does not affect our main result on the instability of the NaHS-containing solution. In addition, our study's sulfide measurement recovery was 91% and 93% for NaHS-containing water and serum samples, respectively. These values are in line with the previously reported range (77–92)⁶⁶ and indicate reasonable accuracy of the assay⁴². As a strength, we used both sexes of rats according to the recommendation of the National Institutes of Health (NIH) to avoid over-reliance on male-only animal research in preclinical studies⁶⁷ and include both sexes when possible⁶⁸. Others have emphasized this issue^{69–71}.

In conclusion, the findings of this study indicate that NaHS solution prepared in drinking water cannot be used for H₂S donation as the solution is not stable. This route of administration exposes animals to variable and lower-than-expected amounts of NaHS; data derived, therefore, may not be translated to humans.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Author contributions

A.G., S.J., N.Y., K.K., and R.N.: Conceptualization; A.G., and R.N.: Formal analysis; A.G.: Funding acquisition; All authors wrote the main manuscript text and reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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