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A growth chamber for chronic exposure of mammalian cells to H₂S

David A. Hanna^{a,1}, Victor Vitvitsky^{a,b,1}, Ruma Banerjee^{a,*}

^aDepartment of Biological Chemistry, University of Michigan Medical School, Ann Arbor, MI, 48109-0600, USA

^bCenter for Theoretical Problems of Physico-Chemical Pharmacology, Russian Academy of Sciences, Moscow, 109029, Russia

Abstract

H₂S is a redox-active signaling molecule that exerts an array of cellular and physiological effects. While intracellular H₂S concentrations are estimated to be in the low nanomolar range, intestinal luminal concentrations can be significantly higher due to microbial metabolism. Studies assessing H₂S effects are typically conducted with a bolus treatment with sulfide salts or slow releasing sulfide donors, which are limited by the volatility of H₂S, and by potential off-target effects of the donor molecules. To address these limitations, we describe the design and performance of a mammalian cell culture incubator for sustained exposure to 20–500 ppm H₂S (corresponding to a dissolved sulfide concentrations of ~4–120 μM in the cell culture medium). We report that colorectal adenocarcinoma HT29 cells tolerate prolonged exposure to H₂S with no effect on cell viability after 24 h although 50 ppm H₂S (~10 μM) restricts cell proliferation. Even the lowest concentration of H₂S used in this study (i.e. ~4 μM) significantly enhanced glucose consumption and lactate production, revealing a much lower threshold for impacting cellular energy metabolism and activating aerobic glycolysis than has been previously appreciated from studies with bolus H₂S treatment regimens.

1. Introduction

Since its discovery as a signaling molecule [1], an array of physiological effects has been associated with hydrogen sulfide (H₂S) in the cardiovascular, nervous, and gastrointestinal systems (reviewed in Ref. [2]). Cells maintain low steady-state levels of H₂S [3–5], which is the product of at least three enzymatic reactions in eukaryotic sulfur metabolism [6,7]. Steady-state intracellular concentrations of sulfide are dictated by the relative rates of its synthesis and its clearance by the mitochondrial sulfide oxidation pathway, which oxidizes H₂S to persulfide, thiosulfate, and sulfate [8,9]. The first step in the sulfide oxidation

*Corresponding author. rbanerje@umich.edu (R. Banerjee).

¹Equal contributors.

Declaration of competing interest

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Appendix A. Supplementary data

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pathway is catalyzed by sulfide quinone oxidoreductase (SQOR), a coenzyme Q-dependent flavoprotein, which resides in the inner mitochondrial membrane and feeds electrons from sulfide oxidation to the electron transport chain (ETC) [10]. Low concentrations of sulfide are rapidly cleared by this pathway, stimulating mitochondrial bioenergetics [11,12], but high concentrations of H₂S inhibit complex IV in the ETC [13]. Typical concentrations of intracellular H₂S span 10–80 nM in various tissues [3–5] but can increase in response to hypoxia, ER stress, or sulfur amino acid restriction [14–19]. Due to its inherent volatility and rapid loss from the culture medium ($t^{1/2}$ –4 min from 6 cm plates at 37 °C) [20], maintaining a constant concentration of H₂S is impractical in a conventional CO₂ incubator.

Most studies on the mechanism of H₂S signaling have focused on the roles of protein persulfides, a reactive and non-specific post translational modification resulting from the reaction of sulfide with oxidized cysteines [21,22]. In contrast, the direct interaction of sulfide with the ETC as a substrate (via SQOR) and as an inhibitor (via complex IV), represents a less studied mechanism by which H₂S can impact cellular redox status and rewire metabolism [23,24]. Exogenous H₂S (35 μM) transiently inhibits the oxygen consumption rate in colonocytes, which returns to basal levels following its clearance [11]. Complex IV inhibition by H₂S has pleiotropic metabolic effects that emanate from the mitochondrion and ripple out to the cytoplasm. H₂S induces reductive stress, stimulates aerobic glycolysis, reductive carboxylation of glutamine and lipid biogenesis [20,25]. Complex IV inhibition also promotes rewiring within the ETC so that electrons from H₂S oxidation are recycled via reversal of complex II, using fumarate as a terminal electron acceptor [26].

Colonic epithelial cells are adapted to chronic H₂S exposure at concentrations that can range from 0.2 to 2.4 mM [27,28]. Therefore, to study the effect of sustained H₂S exposure on host cell biology, culture conditions that provide stable delivery of the gas at the desired concentration are needed. Sustained exposure to sulfide would more closely simulate the local environment at the gut microbe interface, and allow assessment of its impact on colon physiology. The two common strategies for supplying exogenous sulfide to cells are by addition of sulfide salts (Na₂S or NaHS) or sulfide donors that release H₂S in the culture medium or following cellular uptake [29] or targeting to a specific compartment, e.g. mitochondrion [30]. The effectiveness of these methods is limited by the volatility of H₂S and incomplete information about release rates, leading to uncertainty about the sulfide concentration as well as concerns about potential off-target effects of the pro-drug scaffolds. To simulate the effects of chronic exposure with sulfide salts, multiple and periodic additions of sulfide have been used, which lead to pulsatile changes in H₂S concentration [25]. On the other hand, the sulfide donor GYY4137 at a concentration of 400 μM, reportedly leads to accumulation of up to ~20 μM sulfide in the culture medium over 3 days [31].

Custom built gastight polypropylene chambers (i.e. Tupperware) large enough to temporarily house mice have been used to deliver a constant concentration of H₂S mixed with air via tubing connected to cylinders [32,33]. Similarly, a custom chamber for culturing *Caenorhabditis elegans* has been described to study the effects of chronic exposure to 50 ppm H₂S on thermotolerance, lifespan, and stress response [34–36]. In this study, we describe the assembly of a chamber that is designed along similar lines for mammalian

cell culture. We also describe the rigorous characterization of chamber performance (i.e., stability of dissolved and atmospheric H₂S concentration over time), variation in dissolved sulfide concentration as H₂S in the atmosphere is dialed between 20 and 500 ppm, and its effects on cell viability and glycolysis. The availability of an H₂S cell culture incubator will enable investigations on the impact of chronic sulfide exposure on host cell metabolism in 2D and 3D cultures.

2. Material and methods

2.1. Materials

The human colorectal adenocarcinoma cell line HT29 was obtained from the American Type Culture Collection. RPMI 1640 with glutamine and with [Cat. # 22400] or without 25 mM HEPES [Cat. # 11875], fetal bovine serum (FBS; Cat. # 10437), penicillin/streptomycin mixture (Cat. # 15140), phosphate buffered saline (PBS; Cat. # 10010), and Dulbecco's PBS (DPBS; Cat. # 14040) were from Gibco. Cylinders of 5000 ppm H₂S in N₂ (500 L, "A33" 5000 ppm H₂S with 1% accuracy; #3130) or breathing air containing 5% CO₂ were from Cryogenic Gases (Detroit, MI, USA). The following materials were purchased from the indicated vendors: Sierra SmartTrak[®] C100 mass flow controllers (Model #C100L-DD-1-OV1-SV1-PV2-V2-V2-S0, Sierra Instruments, Monterey, CA, USA) and sulfide mass flow controller (Model #C100L-DD-1-ON1-SK1-PV2-V2-V2-S0-A1, Sierra Instruments), 1/16" ID 1/8" OD fluorinated ethylene propylene (FEP) tubing (Cole-Parmer #06406-62), T-connectors (BioRad Cat #731-8229) and platinum cured silicone Masterflex pump tubing with 1/16" ID 1/8" OD (VWR # 96410-14).

2.2. Basic design of an H₂S atmosphere-containing mammalian cell culture chamber

The design of the H₂S growth chamber for mammalian cell culture followed the scheme described previously for *C. elegans* culture [34]. The chamber design is described briefly here and in greater detail in Appendix. A two-tiered 18 L Thermo Scientific Heratherm Compact Microbiological Incubator (Fisher Scientific, Cat # 50125590H) was placed in a fume hood and maintained at 37 °C. The incubator was fitted with inlet and outlet tubes for independent control of gas flow and atmospheric composition in each chamber (Fig. 1). Rubbermaid[®] Brilliance Tupperware containers (760–2800 ml volume) served as control and H₂S chambers and the size was varied as needed to accommodate 1–15 plates (6-well, 6 or 10 cm) per chamber. Gases passed through at a flow rate such that the atmosphere in each chamber was replaced within ~3 min (i.e., 0.33 × chamber volume/min). The gas in the "control chamber" was a humidified mixture of 95% breathing air and 5% CO₂, which enabled use of a standard bicarbonate-buffered cell culture medium. The gas in the "H₂S chamber" comprised 20–500 ppm H₂S in a humidified mixture of 95% breathing air and 5% CO₂. The atmosphere in the H₂S chamber was regulated with mass flow controllers for diluting 5000 ppm H₂S to 0.4–10% of the total flow rate (Table S1). For example, to achieve an atmosphere containing 50 ppm H₂S, 5000 ppm H₂S was diluted to 1% with the humidified mixture of breathing air and CO₂.

2.3. Analysis of outlet H₂S from the atmospheric chamber

The actual concentration of H₂S in the chamber was estimated by measuring the concentration of H₂S exiting the chamber. For this, gas samples were collected with a gas-tight syringe through the outlet Tygon tubing exiting the H₂S chamber. The samples were analyzed immediately following collection, using a gas chromatograph connected to Sulfur Chemiluminescent Detector SCD 355 (Agilent), as described previously [37].

2.4. Cell culture conditions and calculation of dissolved sulfide concentration

HT29 cells were cultured in RPMI 1640 ± 25 mM HEPES medium supplemented with 10% FBS, 100 units/mL penicillin and 100 µg/mL streptomycin. The cells were maintained in humidified cell culture incubators at 37 °C containing a 5% CO₂ atmosphere. While the initial experiments were conducted in a regular RPMI medium, RPMI with HEPES was routinely used later to decrease extracellular acidification, which was observed at 50 ppm H₂S. A difference in the magnitude of H₂S-dependent activation of glycolysis in medium ± HEPES was not observed.

The expected sulfide concentration in the culture medium at a given sulfide concentration in the gas phase was estimated as described previously [37]. Briefly, equation (1) describes the distribution of H₂S between the gas and liquid phases where D, which has a value of 1.6, describes the equilibrium ratio between the concentration of H₂S in the gas and liquid phases at 37 °C [3]. Equations (2) and (3) describe the dissociation of H₂S in the liquid phase where S_T is the total sulfide concentration in the liquid phase and pKa = 6.8 [38].

$$[\text{H}_2\text{S}]_{\text{Liq}} = D * [\text{H}_2\text{S}]_{\text{Gas}} \quad [1]$$

$$S_T = [\text{H}_2\text{S}]_{\text{Liq}} + [\text{HS}^-] \quad [2]$$

$$[\text{HS}^-] = [\text{H}_2\text{S}]_{\text{Liq}} * 10^{(\text{pH} - \text{pKa})} \quad [3]$$

Using these equations and the H₂S level that was set (i.e., dialed) in the gas phase (Table 1), the total sulfide concentration in the liquid phase was calculated using equation (4):

$$S_T = D * \left([\text{H}_2\text{S}]_{\text{Gas}} + [\text{H}_2\text{S}]_{\text{Gas}} * 10^{(\text{pH} - \text{pKa})} \right) \quad [4]$$

2.5. Metabolite analysis

Cells were seeded in 6-well plates at a density of 1 × 10⁶ cells per well each containing 2 mL culture medium and allowed to settle overnight in a conventional incubator. The next morning, the medium was replaced, and the plates were moved into the control and H₂S chambers, which were pre-equilibrated with the respective gases for 15–30 min. Cells were cultured at the desired concentration of H₂S for 20–24 h and aliquots of conditioned medium were collected at the desired time intervals for analysis of sulfide, glucose, and lactate

concentrations. For this, the plates were briefly removed from the chamber for sample collection, which took ~2 min, before being returned quickly.

For H₂S analysis, 45 µL of the culture medium was mixed with 2.5 µL of 1 M unneutralized Tris in a microcentrifuge tube and rapidly frozen in dry ice. The samples were derivatized with monobromobimane (MBB) and analyzed by HPLC as described previously [20]. For glucose and lactate analyses, 45 µL of medium was mixed with 90 µL of 5% HClO₄, vortexed, and stored at -20 °C until further use. The glucose and lactate concentrations were measured as described previously [20] using the D-GLUCOSE-HK kit (Megazyme) and L-Lactate assay kit (Cayman Chemical), respectively, according to the manufacturers' protocols. The rate of glucose consumption was estimated by subtracting the final glucose concentration in the medium from the initial value and dividing the difference by the duration of the experiment. The rate of lactate production was estimated by subtracting the initial lactate concentration in the medium from the final value and dividing the difference by the duration of the experiment.

2.6. Cell proliferation

To measure proliferation, cells were split at a density of 2×10^6 cells per 6 cm plate containing 4 mL complete cell culture medium (RPMI, supplemented with 10% FBS, 100 units/mL penicillin and 100 µg/mL streptomycin). Cells were maintained overnight in a regular cell culture incubator in a humidified atmosphere of ambient air containing 5% CO₂ at 37 °C. The next day, the medium was replaced with fresh medium (8 mL per plate) and 3–4 plates each were placed in the control and H₂S chambers. After 20–24 h, the medium was aspirated and the cells were washed with PBS and 0.5 mL of 0.05% trypsin (Gibco) was added per plate. When the cells detached, 1 mL of complete cell culture medium was added per plate and the cell suspension was mixed 1:1 with 0.1% trypan blue solution (Gibco). Cell concentration in the suspension was measured using the Cellometer Auto T4 cell counter (Nexcelom Bioscience).

3. Results and discussion

3.1. Chamber performance

The H₂S chamber exhibited stable H₂S levels in the gas phase over 24 h of monitoring (Fig. 2A). Between 20 and 250 ppm H₂S, the measured levels in the atmosphere were lower compared to the desired level set by mass flow controllers; at 500 ppm H₂S, there was excellent correspondence between the two values within experimental error (Fig. 2B). The deviation at concentrations < 250 ppm H₂S likely reflects the performance of both the chamber setup and the mass flow controllers although the overall correlation between the set and measured H₂S concentrations show a linear relationship (Fig. 2B inset).

The dissolved total H₂S concentration (i.e., HS⁻ plus H₂S) in the cell culture medium increased initially and then remained largely stable for up to 24 h between 25 and 100 ppm, but decreased between ~8 and 24 h at 250 and 500 ppm H₂S (Fig. 2C, Table 1). The dissolved H₂S concentration (averaged between 4 and 24 h), exhibited a linear dependence on the dialed H₂S concentration (Fig. 2D). Thus, the atmospheric chamber allows cell

culture at an (average) dissolved sulfide concentration ranging from ~4 to 120 μM over a period of 24 h (Table 1).

3.2. Exposure of cells to H_2S activates glycolysis

The slow decrease in the dissolved H_2S concentration after an initial accumulation (Fig. 2C) might be explained by extracellular acidification, which was indicated by yellowing of the culture medium in plates exposed to high H_2S . Acidification shifts the gas-liquid equilibrium for sulfide distribution toward the gas phase by protonation of HS^- and volatilization of the resulting H_2S , thereby decreasing the dissolved sulfide concentration. Culture medium acidification is a measure of glycolysis flux, leading to lactate accumulation (Fig. 3A–D) as observed previously after bolus treatment of cells with H_2S [20]. Maximal activation of glycolysis was seen at 100 μM sulfide under these conditions, while almost no activation was seen at 50 μM sulfide due to its rapid clearance by cells as seen previously at low sulfide concentrations [20]. In contrast, in the H_2S chamber, a 1.6-fold activation of glycolysis was observed at ~3–5 μM (at a set H_2S concentration of 25 ppm), and a 3.4-fold activation was observed at ~12–13 μM H_2S (at a set concentration of 50 ppm) (Fig. 3C). Lactate accumulation increased between 1.4-fold (25 ppm) and 4.1-fold (50 ppm) (Fig. 3D). Thus, cultivation of cells in an atmosphere of H_2S , reveals their actual sensitivity to H_2S and the significantly lower steady-state concentration at which energy metabolism shifts from oxidative phosphorylation to glycolysis.

3.3. Impact of prolonged H_2S exposure on cell viability and proliferation

The antiproliferative effect of H_2S on HT29 cells was demonstrated previously in an H_2S treatment regimen that involved repeated bolus treatments with relatively high sulfide concentrations (100–300 μM) [11]. In contrast, the growth restrictive effect of H_2S on HT29 cells grown in the H_2S chamber was observed after 24 h even at 50 ppm H_2S (i.e., dissolved sulfide = $12 \pm 6 \mu\text{M}$) and was even more significant at 100 ppm H_2S (Fig. 4A). However, the fraction of dead cells was not significant at any H_2S concentration (Fig. 4B). These data indicate that the decreased cell count observed at 50 ppm H_2S reflects restricted cell division rather than increased cell death.

3.4. H_2S growth chamber: limitations and opportunities

Herein, we report the assembly, characterization and utility of an H_2S incubator that can simulate chronic exposure to sulfide at dissolved concentrations ranging from ~4 to 120 μM as the concentration in the atmosphere is dialed between 20 and 500 ppm. While cell viability is not impacted in this concentration range, our study reveals a much lower threshold for eliciting cellular responses to H_2S (e.g. increased glycolysis and decreased proliferation) than has been previously recognized. This difference is explained in part by the rapid loss of H_2S from the culture medium in the regular incubator due to its volatilization and its rapid clearance by cells [20], in contrast to sustained exposure to an H_2S atmosphere in the chamber.

A major advantage of an H_2S cell culture chamber as described here is that it permits prolonged exposure to relatively stable concentrations of H_2S , which can be dialed over a 20-fold range. By changing the H_2S concentration in the cylinder one can potentially further

extend the available concentration range. At the high end of H₂S concentration, the problem with medium acidification can be solved by either decreasing the starting cell density and/or by increasing the volume of culture medium per well.

On the other hand, a limitation of the setup is the possible dependence of the dissolved sulfide concentration on experimental parameters such as the gas flow rate, chamber volume, number of plates and the volume of culture medium in the chamber, necessitating standardization each time the setup is changed. Furthermore, since multiple sample collections result in exposure to the ambient atmosphere and disturbs the gas-liquid equilibrium established in the chamber, the setup is better suited for long term experiments with endpoint data collection. Some of these issues could potentially be addressed by modifying the setup to permit continuous monitoring of dissolved H₂S, glucose or lactate using the appropriate electrodes.

The mammalian H₂S cell culture chamber opens the doors to studies on the impact of H₂S on mitochondrial bioenergetics, cellular metabolism and signaling. The incubator design will also allow modulation of gases entering the chamber, e.g., low O₂ and sulfide, to better simulate the gut environment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

No data was used for the research described in the article.

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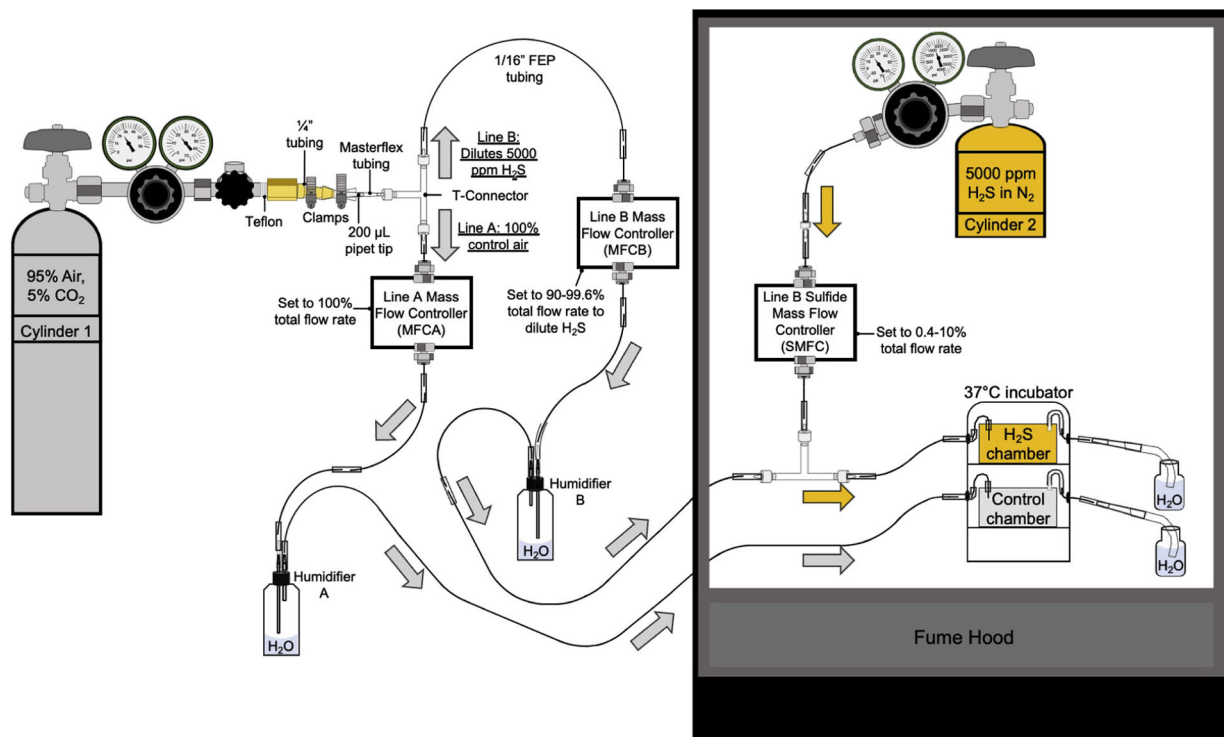


Fig. 1.

Scheme showing design of the H₂S atmosphere chamber. Gas from cylinder 1 (95% air, 5% CO₂) is split into two lines at a T-connector. Line A delivers gas to the “control chamber” (gray box) whereas Line B connects with cylinder 2 (5000 ppm H₂S in N₂) at a second T-connector before entering the “H₂S chamber” (yellow box). Gas flow is independently controlled in each line (A and B) by separate mass flow controllers (MFCA and MFCB), and the gas is humidified in each line by passage through plastic wash bottles (humidifiers A and B). Gas flow from cylinder 2 is controlled by the line B sulfide mass flow controller (SMFC). The direction of breathing air/CO₂ mixture flow in lines A and B is indicated by the gray arrows. The yellow arrows indicate flow of H₂S-containing gas. Other components used in chamber construction are as labelled and include clamps for securing tubing on the brass outlet of cylinder 1, pipet tips that serve as adaptors for transitioning from thinner to wider tubing, and tubing for air flow to and from the Tupperware chambers that are housed within a Thermo Scientific Heratherm Compact Microbiological Incubator. The gas exiting the chambers bubbles into 150 mL bottles filled with water to a height of 10 cm to provide a positive pressure inside the chambers and prevent contamination of the chamber atmosphere with ambient air. The bubbling of gases exiting the chambers also serves as a useful indicator of gas flow. The thinnest tubing (1/16” inner diameter fluorinated ethylene propylene (FEP)) is joined at different connection sites (i.e., between FEP tubing, pipet tips, and T-connectors) using Masterflex tubing, which is more malleable and permits tight connections at the various joints in lines A and B. The air flow at MFCA was set to 100% of the combined rate of air flow from MFCB and SMFC, which was used to achieve the desired H₂S atmosphere between 20 and 500 ppm H₂S.

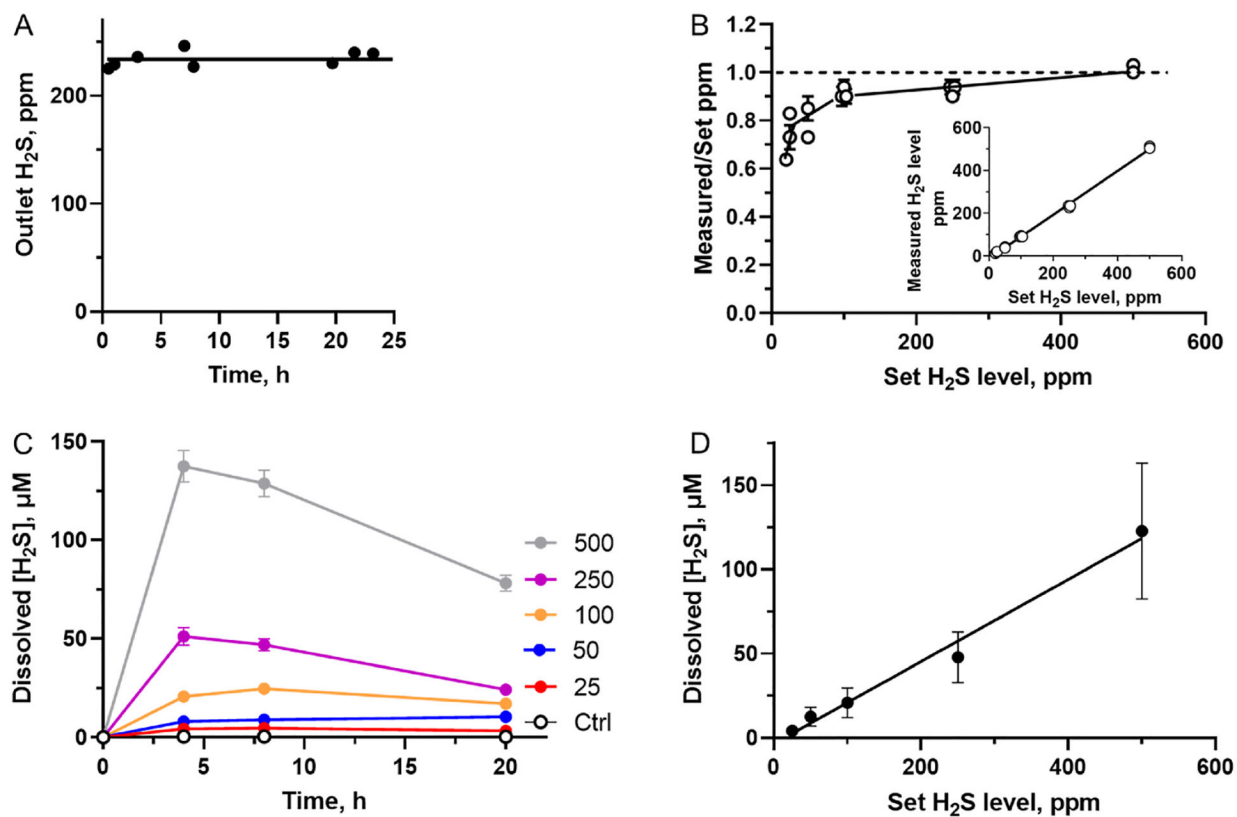


Fig. 2. H₂S chamber assessment. A – H₂S concentration in the gas exiting the atmospheric chamber (760 mL) with an inlet flow rate of 250 mL/min and H₂S set at 250 ppm; B – The ratio between the measured and set H₂S levels increases with increasing set H₂S levels. The dashed line indicates a ratio of 1, i. e. a complete correspondence between the set and obtained values. *Inset* – Relationship between H₂S levels in the gas exiting the chamber and the set H₂S levels. Data represent the mean \pm SD of 13 experiments; C – Time-dependent changes in the dissolved H₂S concentration in HT29 cell culture medium (RPMI 1640 + HEPES) at the indicated set concentrations of H₂S in ppm. The data represent the mean \pm SD, n = 3. D – Dependence of the average measured dissolved sulfide concentration between 20 and 24 h of culture of HT29 cells on H₂S levels set in the inlet gas. Data represent the mean \pm SD, n = at least 9.

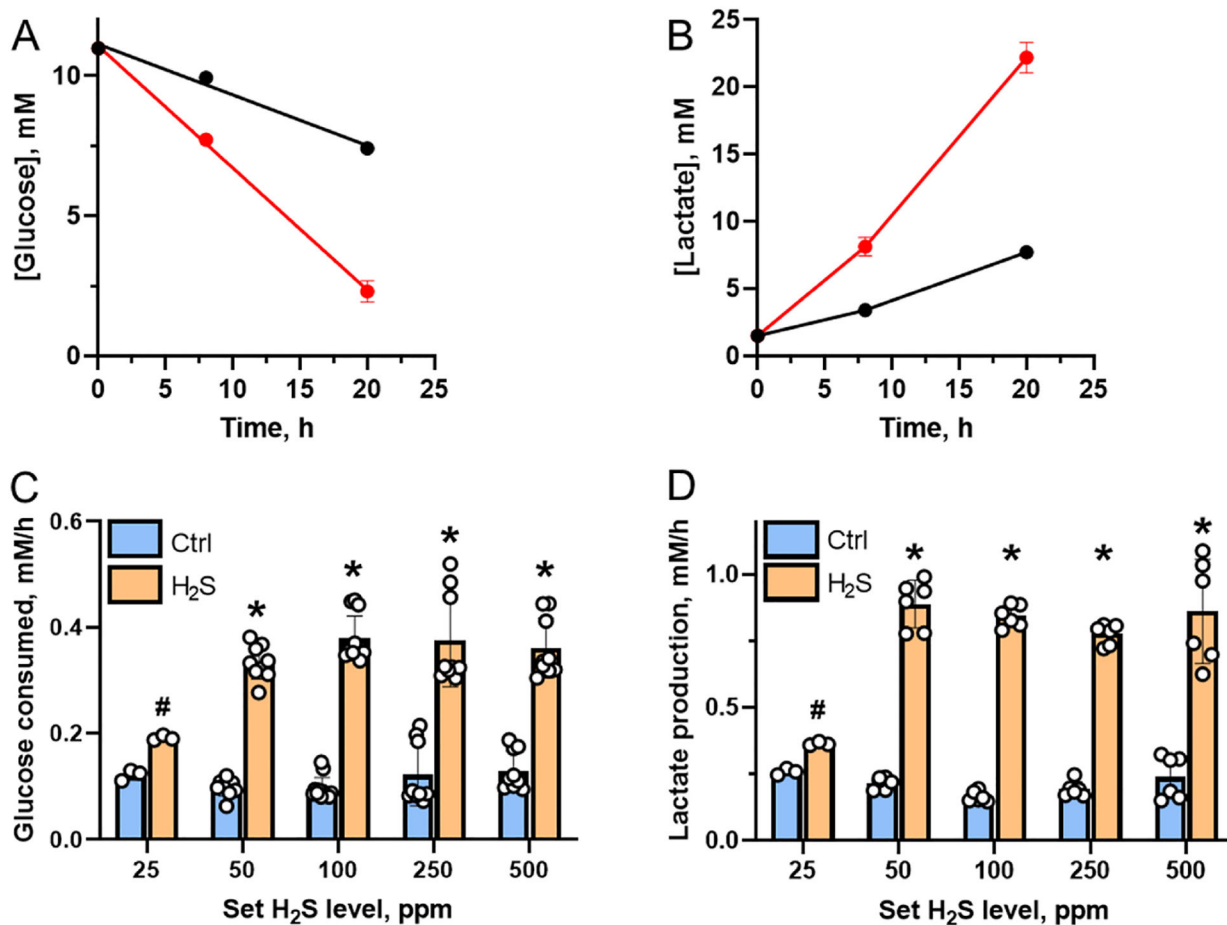


Fig. 3.

Activation of aerobic glycolysis in HT29 cells cultured in the H₂S chamber. A,B – Representative kinetics of glucose consumption (A) and extracellular lactate accumulation (B) in the absence (*black*) or presence of 500 ppm H₂S (*red*). Data are mean \pm SD, n = 3. C, D – Summary of glucose consumption (C) and lactate production (D) at 20–24 h at different set concentrations of H₂S. # and * indicate statistically significant differences from control values with p < 0.001 and p < 0.0001, respectively.

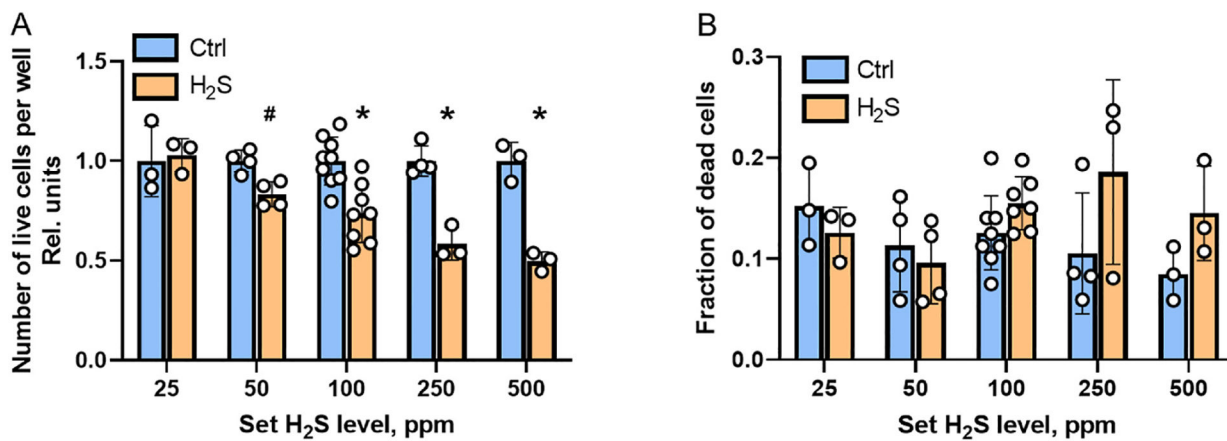


Fig. 4. The effect of sulfide on cell proliferation. A – Cell proliferation (monitored by the number of live cells after 24 h) is unaffected at \pm 25 ppm H₂S but decreases at 50 ppm H₂S. # $p < 0.01$ * $p < 0.001$. B – A significant change in the fraction of dead cells is not seen after 24 h between HT29 cultures grown in the control versus H₂S chamber. Each data point represents an independent experiment.

Table 1Concentrations of dissolved sulfide in the culture medium at different set levels of H₂S.^a

Set H ₂ S level in gas phase		Measured H ₂ S level in gas phase		Expected (calculated) [Sulfide] in cell culture medium	Actual (measured) [Sulfide] in cell culture medium, (μM)		
ppm	μM	ppm	μM		All time points	4–8 h	Final conc. 20–24 h
25	1.1	20	0.9	7.1	4.1 ± 0.9	4.5 ± 0.8	3.3 ± 0.4
50	2.2	39	1.7	13.9	12 ± 6	12 ± 6	13 ± 5
100	4.5	91	4.1	32.4	21 ± 9	22 ± 9	20 ± 8
250	11.1	231	10.3	82.2	48 ± 15	51 ± 18	43 ± 10
500	22	507	23	180.4	123 ± 40	147 ± 32	81 ± 6

^aData were obtained with 1×10^6 HT29 cells/well with 2 mL medium/well in 6-well plates. The plates were placed in a 760 mL chamber with a gas flow of 250 mL/min. Data represent mean ± SD from at least three replicates.

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