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Systemic effects of H_2S inhalation at human equivalent dose of pathologic halitosis on rats

Defne Yalçın Yeler[a], Murat Aydin[b]* , Mehmet Gül[c], Turgay Hocaoğlu[d], Hakan Özdemir[e] and Melike Koraltan[a]

aDepartment of Oral and Maxillofacial Radiology, Faculty of Dentistry, Cumhuriyet University, Sivas,Türkiye; bPrivate Practice, Adana, Türkiye; cDepartment of Histology and Embryology, Faculty of Medicine, İnönü University, Malatya, Türkiye; dDepartment of Oral and Maxillofacial Surgery, Faculty of Dentistry, Cumhuriyet University, Sivas,Türkiye; eDepartment of Periodontology, Faculty of Dentistry, Osmangazi University, Eskisehir, Türkiye

ABSTRACT

Objectives: Halitosis is composed by hundreds of toxic gases. It is still not clear whether halitosis gases self-inhaled by halitosis patients cause side effects. The aim of the study was to investigate the effect of H_2S inhalation at a low concentration (human equivalent dose of pathologic halitosis) on rats.

Materials and methods: The threshold level of pathologic halitosis perceived by humans at 250 ppb of H_2S was converted to rat equivalent concentration (4.15 ppm). In the experimental group, 8 rats were exposed to H_2S via continuous inhalation but not the control rats. After 50 days, blood parameters were measured and tissue samples were obtained from the brain, kidney and liver and examined histopathologically to determine any systemic effect.

Results: While aspartate transaminase, creatine kinase-MB and lactate dehydrogenase levels were found to be significantly elevated, carbondioxide and alkaline phosphatase were decreased in experimental rats. Other blood parameters were not changed significantly. Experimental rats lost weight and became anxious.

Histopathological examination showed mononuclear inflammatory cell invasion in the portal areas, nuclear glycogen vacuoles in the parenchymal area, single-cell necrosis in a few foci, clear expansion in the central hepatic vein and sinusoids, hyperplasia in Kupffer cells and potential fibrous tissue expansion in the portal areas in the experimental rats. However, no considerable histologic damage was observed in the brain and kidney specimens.

Conclusions: It can be concluded that H_2S inhalation equivalent to pathologic halitosis producing level in humans may lead to systemic effects, particularly heart or liver damage in rats.

Introduction

Halitosis is mainly attributed to volatile sulphur compounds (VSCs) and most notably hydrogen sulphide (H_2S). Other gases of the sulphur family such as methyl mercaptan and dimethyl sulphide and many organic compounds and nitrogen-based volatiles are also present in the oral cavity [1,2].

Acute accidental exposures to high concentrations of H_2S have been quantitatively studied but the dose-response relationship for human toxicity due to H_2S exposure is not well-documented. According to the National Homeland Security Research Center, it is estimated that continuous exposure to H_2S in the air or drinking water for longer than 24 h at a concentration of 1.2 ppm or for 30 or 90 days at a concentration of 0.85 ppm could produce adverse health effects in the general population. Adverse health effects are not expected when concentrations are at or below these limits [3]. However, studies demonstrate that even at low concentrations, VSCs are highly toxic to tissues even in nanogram levels [4–6].

H_2S directly contributes to periodontal pathogenesis [7], increases the permeability of periodontal tissues and allows penetration of bacterial products and prostaglandins [6]. Moreover, H_2S plays an important role in acute inflammatory processes [8], enhances neutrophil migration [9], results in a significant increase in the production of TNF-α, IL-1β, and IL-6 in monocytes [10], and inhibits proliferation of epithelial cells [11] and osteoblasts [12], or leads to apoptosis in osteoblasts [13], and triggers apoptosis and causes genomic DNA damage in human gingival epithelial cells [14]. Deformation of cellular shape, vacuolization, disintegration of intercellular connections (desmosomes) and loss of collagen fibrils have been demonstrated in the rat gingival epithelia [15]. When rat gingival tissue was exposed to low (equivalent to levels of human halitosis) concentrations of H_2S via inhalation for 50 days, an inflammatory cell response and osteoclastic activity were seen [16]. Even a single topical application of NaHS (H_2S donor) caused osteoclast differentiation in the rat gingival sulci [16] and even a physiologic concentration (0.05 ng/ml) of an oral malodorous compound induced osteoclast differentiation within 4 days in a cell culture medium [17].

Other VSCs also increase production of IL-1 and prostaglandins, and activate collagenase [18], and thereby reduce collagen synthesis [15,19]. Long-term exposure (100 ng/ml) to

VSCs may cause cell death and DNA damage in human fibro-blasts [20], by suppressing reconstruction of basal membranes.

Total VSC or H_2S concentration in mouth air even from healthy subjects fluctuates up to 2 ppm. Several times this concentration is found in periodontal patients [21]. Apparently, patients with Type 1 (oral) halitosis continuously inhale a low level of self-produced H_2S for hours every day and perhaps for years. Given these facts, there is no reason to not think that patients with Type 1 halitosis can be systemically affected from self-produced H_2S. It is not clear whether self-inhalation of H_2S at a pathologic halitosis level causes any systemic effect and this has not been truly investigated.

The aim of the present study was to investigate if there were any potential systemic effects of H_2S inhalation (at human equivalent dose of pathologic halitosis) on rat tissues.

Material and methods

A total of 16 male Wistar rats (8-weeks-old, weighing between 245 and 250 g) were divided into two groups. H_2S group ($n = 8$) was exposed to H_2S for 50 days while the control group received fresh air. Both groups were kept in hardened polyfluorovinyl chambers and exposed to 24-h light/dark cycle and controlled temperature ($25 \pm 3\,°C$). All animals were allowed free access to powdered food and drinking water. The experimental protocol was approved by the Ethics Committee for Animal Research of Cumhuriyet University School of Medicine (Protocol ID.2012/0297).

Criteria for setting H_2S concentrations in rats

There is no consensus on the threshold for human halitosis. Although socially intolerable level of halitosis has been considered as an H_2S concentration above 700 ppb in the literature, 75, 100, 110, 125, 150 and 250 ppb have been established by investigators [2,15]. For H_2S, a total concentration of 250 ppb (0.25 ppm) was considered as the threshold level for minimal pathologic halitosis [1].

As a rule, a biologic effect occurs when a living organism is exposed to a toxic gas, the extent of which depends on many factors including the minute volume and the surface area of the extrathoracic region (nose, mouth, nasopharynx, oropharynx, laryngopharynx, and larynx). Since respiratory parameters greatly differ between humans and rats, the human threshold concentration for halitosis (250 ppb) must be converted to rat equivalent concentration by using the following formula [22]:

$$RGDR(ET) = \left(VE/SA(ET)\right)_a \div \left(VE/SA(ET)\right)_h$$

where VE is the minute volume and SA(ET) is the surface area of the extrathoracic region for the rat (a) and human (h). $VE_{(a)} = 0.275\ m^3/day$, $VE_{(h)} = 20\ m^3/day$, $SA(ET)_a = 15\ cm^2$, $SA(ET)_h = 200\ cm^2$.

$$RGDR(ET) = (0.275/15) \div (20/200) = 0.18$$

When calculating the rat equivalent dose, an uncertainty factor (UF) of 3 was applied for interspecies extrapolation because dosimetry adjustment was applied to calculate the human-equivalent lowest observed adverse effect level [LOAEL(HEC)] [22].

$$0.250 \times UF \div RGDR(ET) = 0.250 \times 3 \div 0.18 = 4.15\ ppm$$
$$(or\ 4155\ ng/mL)$$

Contrary to what we supposed, according to this calculation, a higher concentration of H_2S was needed to obtain the same effect in rats as in humans. As a result, it was found that rat threshold concentration for halitosis was 16.64-fold greater than the human threshold concentration for halitosis.

Experimental design

An H_2S cylinder (20000 ppm) was connected to a gas-mixing chamber (8000 cm^3) through gas regulators (Airgas Y14-C445F, Radnor, PA, USA) and a flow meter (GRV-150 GK0010G, Honsberg Instruments, GmbH, Reimscheid, Germany). Fresh air was obtained from the atmosphere with an air compressor through another flow meter to the gas-mixing chamber. (Figure 1). A 1440.3 mL/min (0.3 mL/min of H_2S + 1440 mL/min of fresh air) gas mixture was sent to the main chamber. To avoid causing negative or positive air pressure in the chamber, used air in the main chamber (0.1575 m^3) was passively exhausted with a pipe without aspirating.

Single-aspiration volume of one rat is 0.6–2 mL, and inspiration frequency is 70–90/min [23] thus, each rat consumes 180 mL/min of air. Eight rats would need at least approximately 1440 mL/min of air in the main chamber. Premixing gas ratio, H_2S, and fresh air concentration and volume were calculated using a computer program written by one of the investigators (M. A.). H_2S concentration in the exhaust air was monitored by a gas detector that was capable of measuring a range of 0–500 ppm, with a 0.1 ppm resolution (GasBadge, IndSci, PA) 3 times per day (a total of 150 measurements in 50 days).

Thus, it was possible to confirm the accuracy of actual gas concentration passing through the chamber during the experiment.

Histopathological and biochemical evaluations

Blood samples were obtained from both groups of rats immediately before they were sacrificed by ketamine at the end of the experiment. Biochemical analyses were carried out on blood samples as shown in Table 1. Then, histologic samples were obtained from the liver, brain and kidney. Each sample was placed in 10% neutral buffered formalin solution for 48 h before embedding in paraffin. Tissue specimens were cut into 5 μm thick sections, mounted on slides, stained with haematoxylin-eosin, and then examined under light microscope.

To quantify the degree of histopathologic damage, 10 different microscope fields (×40) from each slide were scored separately for hepatocyte necrosis, inflammatory cell
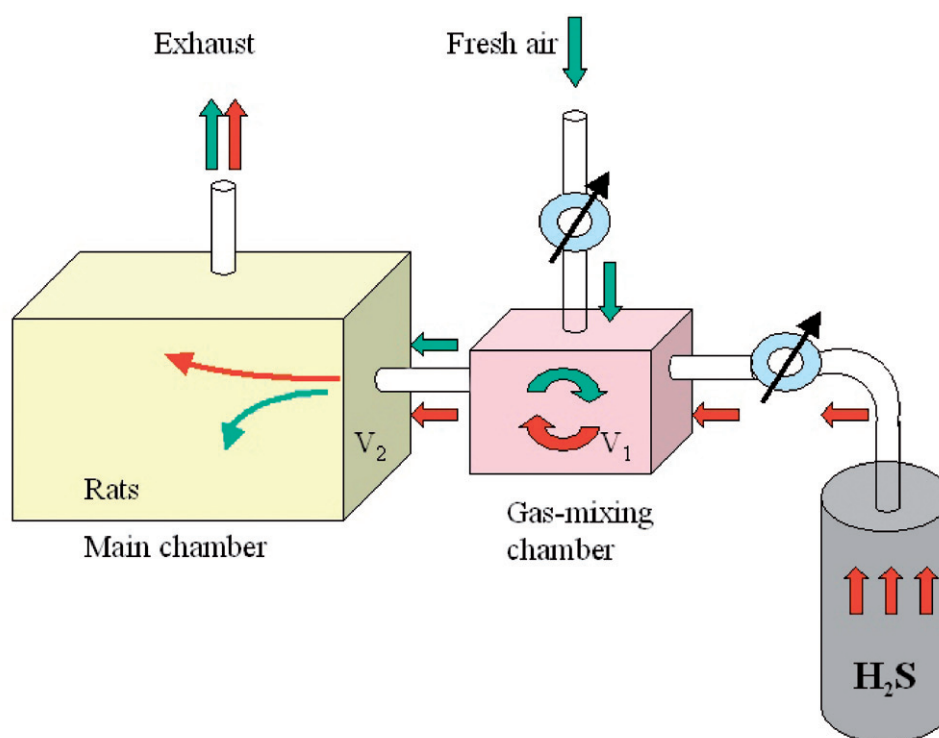
Figure 1. The H2S tube (20,000 ppm 150 bar 10 L) flow rate was adjusted to 0.3 mL/min, while the fresh air flow rate was set to 1440 mL/min. Both gases were pre-mixed in a chamber (V1 = 8000 cm^3). Under these conditions, rats were exposed to 4.15 ppm H$_2$S from the main chamber (V2 = 0,1575 m^3).

Table 1. Results of biochemical analyses of whole blood for rats breathing fresh air (control) or hydrogen sulfide.

	Control group ($n = 8$)	H$_2$S group ($n = 8$)	p-Value
Alanine transaminase (ALT) (U/L)	62.8 (47–89)	55 (44–82)	.334
Albumin (gr/dl)	7.79 (3.43–15.9)	6.18 (4.9–12.2)	.341
Alkaline phosphatase (ALP)[a] (gr/dl)	152 (124–168)	77.6 (9.43–153)	.015
Aspartate transaminase (AST)[a] (U/L)	14.5 (95–145)	149.3 (98–266)	.008
Blood urea nitrogen (BUN) (mg/dl)	24.7 (24–25)	20.8 (17.3–25.8)	.335
Ca (gr/dl)	10.38 (10.1–10.59)	10.68 (10.08–11.06)	.129
Carbondioxide (CO2)[a] (mmol/L)	21.13 (15.9–31.2)	13.78 (10.2–18.9)	.021
Cl (mmol/L)	96.6 (93–98)	103.5 (101–108)	.050
Cholesterol (Chol) (mg/dl)	64.5 (50–71)	86.8 (56–115)	.074
Creatinine (Cre) (mg/dl)	0.38 (0.34–0.42)	0.36 (0.29–0.45)	.466
Creatine kinase-MB (CK-MB)[a] (U/L)	199.4 (88–360.1)	296 (146.2–394.6)	.016
Glucose (Glu) (mg/dl)	135 (124–151)	128.5 (106–143)	.432
High-density lipoprotein HDL (mg/dl)	51.5 (42–59)	49 (40–93)	.103
K (mmol/L)	6.07 (5.9–6.45)	6.18 (5.43–7.58)	.782
Lactate dehydrogenase (LDH)[a] (U/L)	343.6 (204–584)	921 (686–1972)	.004
Low-density lipoprotein LDL (mg/dl)	8 (7–9)	10.5 (5–20)	.334
Na (mmol/L)	137.5 (137–140)	140 (137–145)	.151
Total protein (gr/dl)	7.95 (7.57–8.22)	8.07 (5.7–9.13)	.830
Triglyceride (mg/dl)	83.5 (47–104)	86.83 (38–205)	.910

[a]Shaded rows indicate statistically significant changes.

infiltration and sinus dilation using a scale of 0–3 (0, none; 1, mild; 2, moderate; 3, severe).

Blood sample or biopsy collection or histopathologic assessment was blindly made by different investigator.

Statistical analysis

Assuming $\alpha = 0.05$, $\beta = 0.20$ and $1-\beta = 0.80$, it was estimated that 8 rats in each group would be needed to conduct the study in order to achieve 80% power calculating by G-Power 3.1v software. Comparison of biochemistry parameters between groups were analysed using Mann–Whitney U-test.

The weight-loss between groups was analysed using Wilcoxon test. The frequency of histopathologic damage scores were analysed using a chi-square test A p-value below .05 was considered statistically significant and the data were analysed with SPSS 22.0.

Results

H$_2$S concentration inside the chamber fluctuated between 4.0 and 4.2 ppm throughout the experiment. The average initial weight of the rats in the H$_2$S group ($n = 8$) was 248.3 grams (range, 245–250 g) and showed a significant decrease

Table 2A. Effect of H$_2$S inhalation on liver tissue.

Damage score	Hepatocyte necrosis (%)		Inflammatory cell infiltration (%)		Sinus dilatation in liver (%)	
	H$_2$S	Control	H$_2$S	Control	H$_2$S	Control
0*	0	100	0	100	0	100
1*	50	0	50	0	50	0
2*	50	0	50	0	50	0
3*	0	0	0	0	0	0

*$p = 0.00$, Statistically significant;.
(0, None; 1, mild; 2, moderate; 3, severe).

Table 2B. Effect of H$_2$S inhalation on brain and kidney tissue.

	Brain tissue (%)						Kidney tissue (%)					
	Vascular damage		Necrosis		Inflammatory cell infiltration		Vascular damage		Necrosis		Inflammatory cell infiltration	
	H2S	Control	H2S	Control	H2S	Control	H2S	Control	H2S	Control	H2S	Control
0	100	100	100	100	100	100	100	100	100	100	100	100
1	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0

(0, None; 1, mild; 2, moderate; 3, severe).

to 239 grams (range, 230–242) ($p = .044$) at the end of the experiment. Animals progressively became anxious over 50 days, with greatest anxiety appeared in the last 18 days.

Average values of blood parameters of both groups are listed in Table 1.

Mononuclear inflammatory cell invasion in the portal areas, dilation of the central sinusoidal vein, nuclear glycogen vacuoles in the parenchymal area, fibrotic degenerations, necrosis in a few foci and hyperplasia in Kupffer cells were seen in the H$_2$S group (Table 2A, Figure 2) but no significant changes were observed in brain and kidney biopsies of the H$_2$S group compared to control specimens (Table 2(B))

Discussion

There is not a comprehensive practical laboratory guide on the normal range of rat blood parameters. The values of blood parameters obtained from control rats were assumed that as normal values when comparing them with those of H$_2$S group. This is one of the limitations of the experiment. Another limitation of the study is related to the analysis of a small number of blood parameters due to the low volume of blood that could be collected from one rat at a time. It would have been better to analyse endocrine and immunologic markers or the amounts of sulphur gases in the systemic circulation if more blood could have been collected from each rat.

Cardiac muscle

It is known that mainly heart, brain and lungs are affected from H$_2$S intoxication [24,25]. In the literature, 37 µg/ml of H$_2$S was detected in the cardiac muscle of the experimental rats, while 10, 20, 25 and 30 µg/ml for the kidney, liver, lung and brain, respectively, as early as 20 min after being exposed to H$_2$S (110 µg/m^3) [25]. Creatine kinase (CK) is found in striated muscle, brain, heart and kidneys and CK-MB is the most relevant isoenzyme of CK since it is a good indicator of cardiac muscle injury. It is present in large quantities in myocardium. CK-MB is usually elevated after the onset of a myocardial damage [26]. As expected, creatine kinase MB (CK-MB) was drastically increased by 148% in the H$_2$S group. This may be due to a potential damage in the cardiac muscle.

It was previously demonstrated that breathing H$_2$S at 80 ppm for 6 h markedly reduced heart rate, body temperature, respiratory rate and physical activity, whereas blood pressure remained unchanged in mice. Also others reported changes in blood gas tensions and electrolyte concentrations that could be responsible for the cardiovascular abnormalities in mice breathing H$_2$S [27].

On the other hand, in a similar experiment, lactate dehydrogenase (LDH), a well-known marker of cell destruction in the organism, was monitored to ascertain apoptosis following incubation of a cell culture with 100 ng/ml of H$_2$S [20]. In that study, LDH level increased more than 2.5 fold, indicating potential cardiac muscle injury. LDH is released during tissue damage (e.g., hypoxia, necrosis) and elevated. LDH is seen in a wide variety of cardiovascular diseases including heart failure and myocarditis as well as carcinomas and liver diseases [26].

Aspartate transaminase (AST) is found in high concentrations in cardiac and skeletal muscles and in lesser amounts in kidneys, brain, lungs, pancreas, spleen, white cells and erythrocytes. AST and alanine transaminase (ALT) are elevated in hepatic disorders and many other conditions including earlier stages of chemical injury of the liver and heart failure. When there is an increase in the AST/ALT ratio or AST alone, it is likely to be related to a disease of the hepatocytes [26]. In the H$_2$S group, AST/ALT ratio increased from 0.23 to 2.71; this usually indicates liver damage and also may lead to potential hepatocellular necrosis as indicated in Figure 2(D).
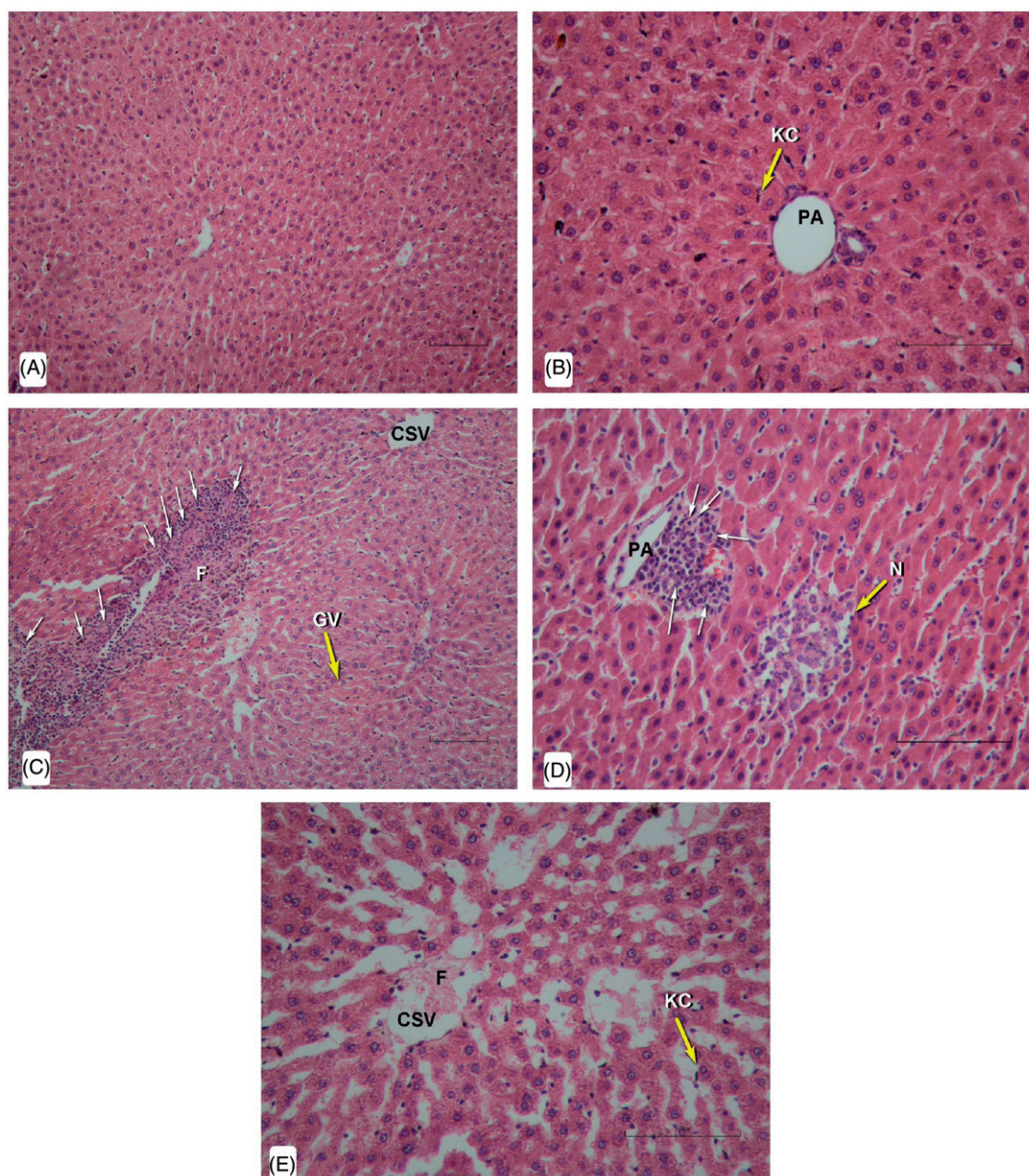
Figure 2. Histopathologic changes in hepatocellular tissue (HE stain, under light microscope ×40). From the control group: A- intact hepatocellular tissue, B- portal area (PA) and Kupffer cells (KC). From the H_2S group: C- mononuclear inflammatory cell invasion (narrow arrows) in portal areas, central sinusoidal vein (CSV), nuclear glycogen vacuoles (GVs) in the parenchymal area and fibrous tissue (F) expansion in the portal areas. D- Mononuclear inflammatory cell invasion (narrow arrows) in the portal areas, necrosis in a few foci (N). E- Clear expansion in the central sinusoidal vein, and hyperplasia in Kupffer cells (KC).

Respiratory alkalosis

Blood CO_2 level significantly decreased (65.2%) by H_2S exposure in the experimental rats. This indicated that an over respiratory alkalosis has developed which can be explained by hyperventilation of the rats due to inadequate oxygenation as a result of inhaling H_2S throughout the experiment. This finding matches closely with other literature findings. It was previously shown that both carbondioxide production and oxygen consumption were reduced by 46% and 30%, respectively, in mice breathing H_2S at 80 ppm for 30 min and both parameters returned to baseline levels within 10 min after cessation of H_2S inhalation [27].

Blood chlor level increased however it was statistically lesser significant ($p = .05$) compared to LDH ($p = .004$) or AST ($p = .008$). Chlor usually elevates to compensate alkalosis in most cases [26]. In this study, slightly increasing chlor can be explained with a result of compensation mechanism for alkalosis.

Bone and liver

Normally, 80% of the serum alkaline phosphatase (ALP) activity originates from liver and bone. Serum ALP provides a useful but nonspecific indication of infiltrative and inflammatory liver or bone disease. ALP may also give information on bone turnover within specific bone compartments. As an unexpected finding, ALP decreased in the H_2S group (by 51%) although there was an obvious hepatic failure. It is

difficult to explain this reduction in ALP but it may be related to anaemia [26]. Neither erythrocytes nor haemoglobin were monitored during the experiment. However, it has already been demonstrated that H_2S inhalation causes bone resorption and apoptosis in osteoblasts [13,15], and even a low concentration (0.05 ng/ml) of sulphurous gases induces osteoclast differentiation within 4 days [28].

Kidneys and brain

No signs of tubular malfunction or significant changes in Na, K, Ca, BUN, creatinine, albumin and total protein were observed in the H_2S group. Biopsy specimens from the kidneys did not show visible microscopic changes in the H_2S group (data not shown). This showed absence of damage in the kidneys.

Although it has been demonstrated that H_2S selectively affects the brain stem [29–31], no visible evidence for brain damage were observed in the H_2S group microscopically.

Potential mechanisms for systemic effects

It is not clear what mechanisms are involved in the systemic effects observed in the present study. However, it is well-known that free H_2S gas is biologically highly active when inhaled, and immediately enters the red cell where it becomes completely oxidized. Haem catalyses sulphide oxidation to elemental sulphur, thiosulfate or sulphate [32]. However, free H_2S is not found in human serum [33,34] or tissues [33]. It was found that exogenously applied sulphide is rapidly removed from the blood circulation by oxidation to sulphate, or methylated to methylsulphide, or converted to sulphide-containing proteins but appears in the acid-labile sulphide (ALS) form and in the dithiothreitol-labile forms. ALS values of 1.3–18.8 μM in blood of non-fatal cases of H_2S poisoning [35] and of 50–117 μM in the blood of fatal cases have been measured [32] while the plasma sulphide concentration (23.8 ± 0.2 μM) of mice did not change by breathing 80 ppm H_2S for 30 min [27]. In this experiment, ALS or any other H_2S metabolites could have resulted in heart or liver damage. ALS concentration was not measured in this experiment, although in the literature, a large variation in ALS values is seen in various reports, with some authors reporting a value of 26 nmol/g for ALS in the liver [36] and others providing a value of 112.2 nmol/g [37].

On the other hand, mechanism of the weight loss is in accordance with literature findings. A previous study showed that subchronic H_2S inhalation at 80 ppm led to reduced food consumption in rats throughout the experiment (90 days) with no alterations in haematological indices, serum chemistries nor gross pathology but an increased incidence of olfactory neuronal loss and rhinitis, bronchiolar epithelial hypertrophy and hyperplasia [38].

In conclusion, it is suggested that low-level H_2S inhalation (equivalent to pathologic halitosis level in humans) may cause systemic effects including weight loss, anxiety, and heart and liver damage in rats.

ORCID

Murat Aydin http://orcid.org/0000-0003-0030-8999

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