INFLAMMATORY RESPONSE AND IMMUNOTOXICITY OF LUNG TISSUE IN RATS INDUCED BY LOW DOSE HYDROGEN SULFIDE EXPOSURE

Kexian Li, Jingjing Fang*, Lu Jiang, Xinhong Xu, Ren Xiomeng

Navy Medical Centre, 880 Xiangyin Road, Shanghai 200433, China, e-mail 552417745@qq.com

1. Introduction

Hydrogen sulfur (H_2S) exposure exists in many scenes, including livestock, agriculture, petroleum processing, geothermal power generation, coke ovens, food processing and waste & sewage treatment, causing mainly Respiratory and Central nervous system symptoms [1-2]. It is now widely believed that H_2S acts as a signaling molecule, showing some potentially beneficial therapeutic effects at physiologically active concentrations [3-5]. However, these are proper for very low concentrations, toxicity of H_2S is still very high in environment exposure level, and gaseous H_2S is still one of the most common substances for acute poisoning deaths in working environments. The effects of long-term low-level H_2S exposure are difficult to accurately estimate due to the unknown mechanism of chronic toxicity. Symptoms mainly include visual complications, olfactory disorders, fatigue, nausea, respiratory stimulation, asthma and headache [6-7]. Lung tissue, as an organ that comes into direct contact with pollutant air, has a wide range of sensitive epithelial cells, and is one of the main target organs for H_2S exposure. Studies of rats with gene knockout confirmed that the lungs were more susceptible to toxic effects from elevated endogenous H_2S concentrations than the liver or kidneys [8]. There is a close relationship between respiratory system and immune system. Low-dose exposure effect of H_2S was researched, which provide a basis for the evaluation of health damage of odor substances and treatment of new targets.

2. Materials and methods

2.1 Animals

Healthy male and female Sprague-Dawley (SD) rats of eight months old weighing 200±20g at the beginning of the experiment were used (SIPPR-BK laboratory animal Co. Ltd. Shanghai China). Sixty SD rats were randomly divided into 2 groups: the control group (C group, n=30), which were exposed to clean air and H_2S exposure group (E group, n=30), which were exposed to 0.5±0.1 ppm odor gas.

2.2 H_2S inhalation exposure

After the acclimation housing, the rats were dealt with H_2S in a 0.6 m^3 inhalation exposure chamber (Model 8050D, Hope-med Co. Ltd., Tianjin China). The concentration of the gas was monitored by a portable instrument (Konor Model JA903, Konor Electronics, Shenzhen China). A H_2S gas cylinder was used (Haizhou Gas Co. Ltd., Shanghai China) and the gas was lead to the chamber after decompressing. The exposure concentration of H_2S was 0.5±0.1 ppm. The inhalation time was 4 hours per day, continuing for 90 days. The rats were sacrificed every 30 days to collect the lung tissue and blood sample.

2.3 Immune molecule and cytokine measurements

Blood samples were collected and centrifuged at 2000 rpm for 10 min at 4°C, and the serum obtained was stored at -20°C until analyzed for biochemical parameters. Immune molecule and cytokines in the serum and lung tissue were determined by a murine specific ELISA system (ENLEY DRAGON Wellscan MK 3, Thermo, USA). Homogenates of pulmonary tissues were prepared to get the amount of total protein, and the data of tissue were expressed as per mg protein. Quantification of cytokine levels were assessed using a commercially available porcine cytokine multiplex immunoassay kit (Aushon BioSystems, Inc, Billerica, Mass) according to the manufacturer's protocol. Cytokine concentrations are expressed in picograms per milliliter.

2.4 Histopathological examination

Tissues were prepared for histopathological analysis using routine techniques. After immersion fixation for 24 h, the sample tissues were cryo protected using sucrose solutions at increasing concentrations (from 15% to 30%) and then frozen in nitrogen. Next, using a cryostat (Leitz, Digital 1702, Oberkochen, Germany) at −20°C, each piece was cut at 4 μm thickness intervals and the slice were placed on glass slides and stained using hematoxylin and eosin (HE) to assess tissue morphology. Histopathological parameters were obtained using an Olympus®Bx50 optical microscope (Olympus, Tokyo, Japan).

2.5 TUNEL assay

Fluorescence microscopic assays were run using the in situ apoptosis detection kit, Fluorescein (Roche, Rotkreuz, Switzerland), following the manufacturer's protocol. Pictures were taken with a Leica DFC 350 FX, cooled fluorescence black and white camera, in a uniform random sampling mode, generating at least nine different pictures per chamber.

2.6 Real-time PCR

Real-time PCR test was performance at seven gene levels related to inflammatory factors and repair in rat lung tissue after 90 days of exposure to H_2S, which are representative genes associated with inflammatory reactions, tissue damage, and post-injury repair (Table 1). Lung tissue weighing approximately 50 mg was collected with aseptic technique. Single step method was applied for extracting total RNA from pneumonocytes with acid guanidinium thiocyanate-phenol-chloroform extraction. The specimens of rat lung tissue were subjected to semi-quantitative reverse transcription-polymerase chain reaction amplification with the primers. After electrophoresis, the gel is placed on a UV light box and a picture of the fluorescent ethidium bromide-stained DNA separation pattern is taken with a Polaroid camera, and then integral absorbance was calculated. Ratio of target gene to inter-reference was regarded as relative amount of gene expression.

2.7 Statistical analysis

Body weight, hematological and blood biochemical parameters were analyzed following the algorithm. Before the comparison, the data was firstly tested the normally distribution and homogeneity of variance. The data significance differences between treatment group and control group was determined using a two-tailed t-test if variance was homogeneous or distribution was normally, or else nonparametric test was used. The software used in the analysis was SPSS version 21 (IBM Inc., Armonk, New York, USA). The values are presented as mean ± SEM and differences were considered statistically significant if the P value was <0.05.

3. Results and discussion

3.1 Low concentration of H_2S exposure affects immune function and alters the level of expression of pulmonary tissue and peripheral blood

Cytokines such as Tumor necrosis factor (TNF), Interferon (IFN) and Interleukin (IL) are the material bases for immune damage. Tumor necrosis factor (TNF) are mainly produced by activated macrophages, NK cells and T lymphocytes. As an important cytokine, it can induce a variety of signaling pathways such as apoptosis, cell survival, inflammation and immunity. Among workers exposed to H2S for a long time, the expression level of inflammatory leukocyte interleukin-8 (IL-8) increased significantly[9], suggesting that H_2S was immune to organism. Long-term exposure to volcanic-induced H2S-specific air pollutants can affect lung macrophages, promoting the production of inflammatory cytokine TNF-α, which promotes deterioration of inflammatory processes in lungs. Excessive inflammatory responses lead to lung damage and fibrosis. The inflammatory factor TNF-α activates signaling pathways such as NF-B and plays a key role in the onset of chronic inflammation. Continuous stimulation of TNF-α induces transcriptional memory of some inflammatory response genes and produces faster, stronger and more sensitive responses to subsequent TNF-α stimuli. The group used SD rats and expose them to H_2S for 30, 60 and 90 days. Tests on serum and lung tissue showed an upward trend in both TNF-α and TNF-β in the exposure group and the control group. The differences between the two groups in the pre- and mid-exposure periods were significant. However during later stages the differences were reduced.

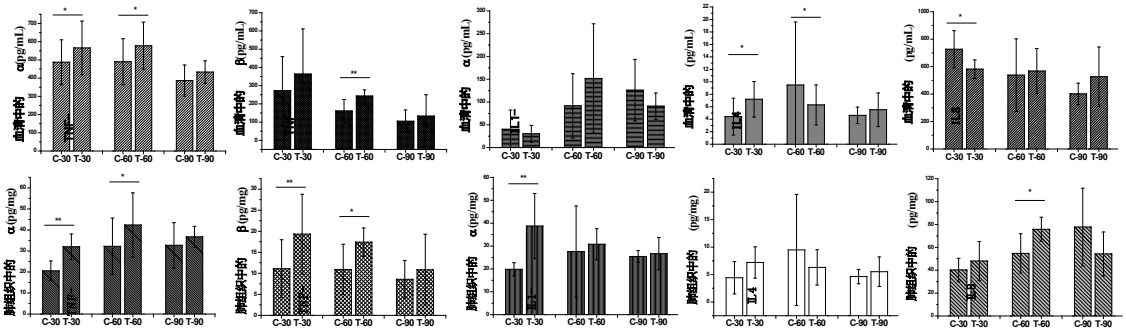

Figure 1. Changes in Serum and lung tissue TNF-α, TNF-β, IL1a, IL4, and IL8 levels in SD rats at 0.5 ppm H_2S exposure for 30, 60 and 90 days. C represents control group, T exposure group, * P<0.05, and ** P<0.01, n = 10.

3.2 Low concentrations of H_2S exposure causes inflammatory effects in tissues, increases levels of apoptosis or procedural necrosis

After 90 days of 0.5 ppm concentration of H_2S sub-chronic exposure, rat lung tissue showed damages of mainly inflammatory apoptosis, permeable changes, fibrin and mononucleosis, alveoli wall thickening. H2S exposure is similar to methanol exposure. When the exposure period was extended from 30 days to 90 days, there was no significant increase in symptoms, and female rats tolerated better than male rats (Figure 2). HE staining showed normal alveoli sacs, alveoli, bronchial tubes in the control group, and no signs of congestion or collapse in the alveoli sacs. There were significant changes in the exposure group. Thin bronchial tubes showed significant goblet cell. After exposure, there are significant protruding goblet cell in the thin bronchial tube; The end of the fine bronchial contracts; Some of the alveoli sac, alveoli and alveoli space is not visible; Alveoli of exposed animal

congests; Red blood cells, fibrin, and monocytes effuse; Thickening of the alveoli wall is observed, especially in the female exposure group. TUNEL fluorescent staining of the exposure group showed more positive apoptosis cells (green).

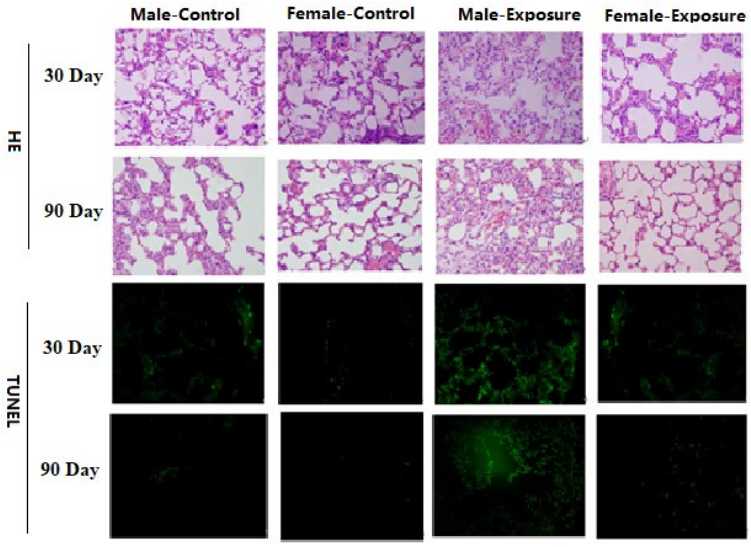

Figure 2. HE staining and cell TUNE fluorescence staining of SD rat lung tissue after exposure to 0.5ppm H_2S for 30 days and 90 days.

3.3 Low concentration of H_2S exposure affects immune function and alters the level of inflammatory gene expression in pulmonary tissue

Compared to control group, there was a significant increase in IL1α, TNF-α, and Bcl-3 expression in rat lung tissue in exposed group. The ratio of value of exposure group to that of control group was 1.3, 1.7 and 1.8 respectively. IL8 and Cflar have an increasing trend with no statistical differences. Scgb1a1 expression is significantly reduced. The ratio of value of exposure group to that of control group was 0.7. Marco has a downward trend with no statistical differences (Table 2). Low concentrations of H_2S exposure induced expression levels of inflammatory factors TNF-α, IL1α, IL8, and mRNA of apoptosis factor Casp8 gene Cflar, inhibited the expression of clara cell secretion proteins and macrophage receptor gene Marco. It suggests that low concentration of H_2S sub-chronic exposure induces excessive inflammatory reaction, reduces the ability of cell auto repair, and causes damage effect of cell tissue. Macrophages are the first defensive barrier to respiratory system. Lower expression levels of macrophage receptors suggest that the body's ability to remove H_2S is reduced and the risk of body damage is further increased. Scgblal is a clara cell secretion protein, a representative repair marker for inflammatory damage to pulmonary tissue, suggesting a decrease in tissue self-healing ability. Inflammatory factor is the result of the effect of tissue organ damage. It also increases the degree of damage to tissue organs by promoting inflammatory responses. It is a closed-loop physiological process of mutual cause and effect. Therefore, we consider TNF-α to be a key immune indicator of inflammatory injury to H_2S exposure.

Table 1. Primer sequence of real-time fluorescent quantitative PCR.

Gene	Primer	length
IL1α	F:5′AGCCTGTGTTGCTGAAGGAGATTC3′ R:5′GGGAAAGCTGCGGATGTGAAGTAG3′	121 bp
IL8	F:5′CACCTTCCCATACATTGTCCTCTCTG3′ R:5′TCTGAGCGGCGGCATCTACC3′	143 bp
Marco	F:5′GGAACACTAGCTGCTGAGGACAAG3′ R:5′CAGTTGCTCCTGGCTGGTGTG3′	138 bp
TNF-α	F:5′ACCACCAAGCGGAGGAGCAG3′ R:5′GCCTTGTCCCTTGAAGAGAACCTG3′	149 bp
Scgb1a1	F:5′ATCACTGTGCTCATGCTGTCCATC3′ R:5′GTCTGAGGCAGGGTTGAAAGGC3′	144 bp
Bcl3	F:5′GCTGCTGAACCTGCCTACTCAC3′ R:5′GGCAATGTGGAGAGGCGTGTC3′	121 bp
Cflar	F:5′GGGTGCTGCTGATGGAGATTGG3′ R:5′AACTCTTGTCCTTGGCTACCTTGC3′	111 bp
ACTB	F:5′TGTCACCAACTGGGACGATA3′ R:5′GGGGTGTTGAAGGTCTCAAA3′	165 bp

Table 2. Real-time fluorescence quantitative PCR test results for inflammatory factors in rat lung tissue.

Gene	ID Number	Absolute expression		Target gene /ACTB，$\times 10^{-3}$		Exposure / Control
		Control	Exposure	Control	Exposure	
IL1α	24493	418	603	221.0	285.0*	1.3
IL8		139	194	73.5	91.7	1.2
Marco	367391	7.5	6.7	4.0	3.2	0.8
TNF-α	24835	5.9	11.0	3.1	5.2**	1.7
Scgb1a1	25575	19099	14862	10100	7024*	0.7
Bcl3	680611	1.5	3.0	0.8	1.4**	1.8
Cflar	117279	12.9	17.8	6.8	8.4	1.2
ACTB		1891	2116			

Note: ACTB, actin; IL1 alpha, leukocyte interleukin 1α; IL8, leukocyte interleukin-8; Marco, macrophage collagen structure receptor; Cflar: CASP8 apoptosis regulatory factor; Bcl-3, B lymphocyte tumor factor-3; TNF-α, tumor necrosis factor-α; Scgb1a1, clara cell secretion protein;* $P<0.01$, ** $P<0.05$.n=10

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