Original Article High-fat diet promotes epithelial-mesenchymal transition through enlarged growth of opportunistic pathogens and the intervention of saturated hydrogen

Xiangjie Qiu¹, Ousman Bajinka¹, Lili Wang¹, Guojun Wu¹, Yurong Tan^{1,2}

¹Department of Medical Microbiology, Xiangya School of Medicine, Central South University, Changsha, China; ²China-Africa Research Center of Infectious Diseases, School of Basic Medical Sciences, Central South University, Changsha 410078, Hunan, China

Received September 10, 2020; Accepted February 25, 2021; Epub June 15, 2021; Published June 30, 2021

Abstract: Objectives: This study investigated the effects and mechanism of high-fat diet on the epithelial-mesenchymal transition (EMT) of respiratory tract and the intervention of saturated hydrogen on it. Methods: 80 five-week-old C57BL6/J male mice were randomly divided into normal control group, H₂ group, high-fat (HF) group and HF+H₂ group, making 20 mice in each group. The weights of the mice were measured on weekly basis. Six mice from each group were executed at every second week. Blood samples were collected for lipid testing. Lung tissues were collected for 16S rRNA gene sequencing, HE staining, immunofluorescence and quantitative real-time PCR (qPCR). Results: Compared with the control group, the mice in the HF group showed increased inflammatory cell infiltration, decreased expression of e-cadherin (E-cad) and increased expression of Twist. There were significant differences in the composition of bacteria in the lung, and the expression of isocitrate lyase (ICL) genes in *Pseudomonas aeruginosa, Staphylococcus aureus* and *Acinetobacter baumannii*, which were significantly associated with asthma were seen with a significant increasing trend. After the treatment of saturated hydrogen, the changes in lung microbial population, lung tissue infiltration of inflammatory cells and the transformation of epithelial stroma caused by high-fat diet were moderately alleviated. Conclusion: High-fat diet can promote inflammation and EMT in the lung by enlarging the growth of glyoxylic acid cycle-dependent bacteria, and the pathological process are partly alleviated by saturated hydrogen.

Keywords: Saturated hydrogen, high-fat diet, pulmonary microecology, glyoxylic acid cycle, epithelial-mesenchymal transition

Introduction

High-fat diet is not only associated with type 2 diabetes (T2DM), hypertension and other metabolic diseases, but is also studied to be correlating significantly with asthma and other respiratory diseases. Based on the previous studies, the incidence of asthma and asthmatic diseases in children with high-fat diet is significantly higher than children without high-fat diet [1, 2], however, the pathogenicity underpinning highfat diet with asthma is still in the mystery. Asthma is a chronic airway inflammatory disease, which often occurs with airway remodeling [3, 4]. Airway remodeling is based on chronic airway inflammation, which leads to the irreversible changes of airway structure. Subepithelial fibrosis is one of the core characteristics of airway remodeling, which is associated with the severity of asthma and one of the main causes of death in patients with severe asthma [5, 6]. Airway epithelial mesenchymal transition (EMT) is a cell biological process in which epithelial cells are transformed into stromal phenotypes through a specific process, which plays an important role in airway remodeling of asthma [7]. Early prevention and treatment of airway remodeling and reduction of fibrosis in asthmatic patients is an intervention that do not only control the level of asthma but also enhance the prognosis.

Modern science is blessed with 16S rRNA gene sequencing technology that enhanced microbial characterization and ascertained more than 30 bacterial species planted in the lungs [8].

Going by the published studies, the diversity of microbes in the lung is biological imperative in maintaining the normal airway function, and in addition to bacterial, fungi and viruses constitute the intrapulmonary microecology. Studies based on common respiratory diseases, alteration or changes in the diversity of microbes are associated with asthma and pulmonary fibrosis [9-11]. A number of studies meet a consensus that in respiratory diseases such as asthma and chronic obstructive pulmonary diseases (COPD), abnormal increase in abundance of species such as Pseudomonas aeruginosa, Acinetobacter baumannii, Staphylococcus aureus, Klebsiella pneumoniae, and Haemophilus influenzae was found [12, 13].

The glyoxylic acid cycle is a metabolic pathway in prokaryotes, lower eukaryotes, and plants, and it is essential for the energy metabolism. Studies have shown that the glyoxylate cycle mostly exists in many opportunistic pathogens. These opportunistic pathogens use the host's fatty acid degradation products to synthesize substances required for their normal growth and reproduction, split macrophages from the inside, and then expand in abundance in the host [14]. Fatty acids are decomposed into acetyl-CoA by beta-oxidation, which form citric acid with oxaloacetate produced from two acetates through the glyoxylic cycle. Under the action of aconitase, citric acid produces isocitrate, which is broken by isocitrate lyase (ICL) to produce glyoxylic acid and succinic acid. Next, glyoxylic acid and acetyl-CoA produce malic acid under the catalysis of malic acid synthetase and malic acid is dehydrogenated to oxaloacetic acid under the catalysis of malic acid dehydrogenase. The succinic acid produced in this cycle can be used to synthesize sugars and other cellular components, which defined ICL as the most critical enzyme in the glyoxylic acid cycle [15].

Number of studies have shown that viral infection and high-fat diet can lead to the accumulation of fatty acids in cells. Therefore, we speculate that the high-fat airway microenvironment caused by high-fat diet leads to an imbalance of airway microecology through the promotion of the glyoxylate cycles of opportunistic pathogens. And the abnormal increase of opportunistic pathogens mediates chronic airway inflammation, thus promoting the occurrence of asthma and airway remodeling.

Hydrogen (H₂) has anti-oxidant, anti-inflammatory, and anti-apoptotic properties and is the most abundant chemical element in the world [16]. In 2007, Ohsawa et al [17] confirmed that inhaling 2% hydrogen could lead to selective scavenging of hydroxyl radicals (OH) and peroxynitrite anions (ONOO⁻), which significantly enhance cerebral ischemia-reperfusion injury in rats. After long-term drinking of hydrogenrich water by obese mice with T2DM, liver malondialdehyde level, fat content, blood sugar, and blood total glyceride (TG) levels all decreased [18, 19], suggesting that H₂ could improve metabolic disorders of lipids and glucose, as well as the metabolic syndrome. Therefore, we use saturated hydrogen as a treatment factor to explore whether it can improve the EMT induced by high-fat diet through regulating intrapulmonary microecology.

Materials and methods

Animal grouping

A litter of five-week-old C57BL6/J male mice weighing 16-20 g (purchased from Hunan Tiangin Biological Technology Co., Ltd.) were acclimatized for a week during 12 hrs of a light/ dark cycle at a constant temperature of 25°C and supplied with sterile water (once a day). The mice were randomized into the control, H₂, HF and HF+H₂ groups (n = 20 each). The mice in the Control and H₂ groups were fed with sterile water and low-fat control feed, while HF and H₂+HF group animals were given sterile water and 60% high-fat model feed (purchased from Nantong Trophy Feed Technology Co., Ltd.). Additionally, mice in the control and HF groups were given 0.5 ml of saline intragastrically once a day, and those in the H₂ and HF+H₂ groups were given 0.5 ml of saturated hydrogen saline once a day. Saturated hydrogen saline was prepared at the Center of Modern Analysis and Detection of Central South University by dissolving molecular hydrogen into normal saline at high pressure (13.5 Mpa). The formulated saturated hydrogen solution was freshly prepared once every week and stored in aluminum packaging to ensure the hydrogen concentration remained > 0.6 mmol/L. The weights of the mice were taken once in a week. Six mice from each group were sacrificed in the 2nd, 4th, and 6th weeks from the onset of the experiment. Blood, and lung tissues were collected for subsequent analyses. All mice were fed and the samples were collected in sterile condition. This study was conducted according to the Declaration of Helsinki and approved by the Medical Ethical Committee of the Xiangya School of Medicine. The same experiments were repeated 3 times.

Body weights and blood lipid concentrations in peripheral blood

Mice were weighed on day 0, 7, 14, 21, 28, 35, and 42, and the figures in kilogram were recorded appropriately. Blood samples were collected from veins using the eyeball extraction method; $35 \ \mu$ l of blood was collected from each mouse for blood lipid tests according to the specific operation steps of blood lipid tester and blood lipid test card (Aikang Biotechnology Co., Ltd., Hangzhou, China).

Hematoxylin-eosin (HE) staining

After anesthetizing mice with ether, lung tissues were excised and rinsed with a 0.9% sodium chloride solution. The resected lung tissues were fixed in a 10% formaldehyde solution for 48 hrs, embedded in paraffin, and cut into 4 µm-thick sections. The tissue sections were then heated to 60°C for 2 hrs, dewaxed with xylene (twice, 15 min each), and dehydrated using an increasing alcohol gradient (75%, 95%, and 100%) for 5 min each. HE staining was performed, and the specimens were observed for pathological morphology under an optical microscope.

Real-time quantitative PCR (qPCR)

Primers were designed using the Primer Premier 5.0 program (Supplementary Table 1) and synthesized by Dingguochangsheng Biotechnology Co., Ltd., Beijing, China. Lung tissues were collected from each mouse of 4 groups and total RNAs were extracted with trizol. RNA purities were analyzed using a nucleic acid analyzer. cDNA was synthesized by reverse transcription and amplified using a qPCR kit (TansGen Biotech Technology Co., Ltd., Beijing, China) according to the manufacturer's instructions. The reaction mixture consisted of 1.5 µl cDNA, 12.5 µl SYBR Green gPCR Master Mix, 1.5 µl each of the forward and reverse primers, and diethylpyrocarbonate (DEPC) water, making a total volume of 25 µl. qPCR cycling conditions were: 94°C for 3 min, and 35 cycles of [94°C for 45 s, 51°C for 45 s, and 72°C for 45 s], and the relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method [20].

Immunofluorescence

Excised lung tissue sections were processed as described above and boiled with 0.01 M citrate buffer for antigen retrieval. The tissues were then washed twice with PBS (3 min each), fixed with 4% paraformaldehyde for 5 min. washed with phosphate buffered solution (PBS) again (same condition as the first wash), blocked with normal goat serum for 20 min, and incubated overnight with 5 µl of E-cad (bs-10009R, Beijing Bioss Biological Technology Co., Ltd.) or Twist (bs-2441R, Beijing Bioss Biological Technology Co., Ltd.) rabbit antimouse primary antibody in a wet box at 4°C. On the following day, the tissue sections were washed thrice with PBS, incubated with a CY3conjugated goat anti-rabbit secondary antibody (GB21303, Wuhan Seville Biotechnology Co., Ltd.) at room temperature for 1 h, washed three times with PBS, and counterstained with 4'6-diamidino-2-phenylindole (DAPI) (100 ng/ ml) for 10 min. After removing moisture from the tissues, the slides were sealed with an antifluorescence quenching agent.

16S rRNA gene sequencing technology

DNA was extracted from lung tissues of the mice. The extraction qualities of DNA were detected by 0.8% agarose gel electrophoresis. and the DNA was quantified by ultraviolet spectrophotometer. Primers were designed with microbial ribosomal RNA as the target and according to the conserved region in the sequence. Sample specific Barcode sequences were added to further PCR amplification of rRNA gene variable regions (single or consecutive multiple) or specific gene fragments. PCR amplification was performed by NEB's Q5 highfidelity DNA polymerase, and the number of cycles was strictly controlled to keep the number of cycles as low as possible while ensuring the same amplification conditions for the same batch of samples. PCR amplification products were detected by 2% agarose gel electrophoresis, and the target fragments were gelled and recovered using AXYGEN gel recovery kit. Based on the preliminary quantitative results of electrophoresis, fluorescence quantification was performed for the PCR amplification of the recovered products. The fluorescent reagent was the Quant-IT[™] PicoGreen dsDNA Assay Kit and the quantitative instrument was the Microplate reader (BioTek, FLx800). According to the fluorescence quantitative results and the sequencing quantity demand of each sample, the samples were mixed according to the corresponding proportion. Illumina MiSeq sequencing was used as an example to prepare a sequencing Library using Illumina's TruSeq Nano DNA LT Library Prep Kit, and then highthroughput sequencing was performed on the machine (Detected in Shanghai paisenno Biotechnology Co., Ltd.).

Statistical analysis

The SPSS21.0 software was used for statistical analysis, and the data were expressed as mean \pm standard deviation. The *t*-test was used for comparisons between two groups, variance analysis was used for comparisons among multiple groups, and the least significant difference (LSD) was used for comparisons between intra-group differences. *P*-value < 0.05 was considered statistically significant.

Results

HF diet induced increased body weights and dyslipidemia, which were alleviated by saturated hydrogen

As shown in **Figure 1A**, the average weights of the mice in the HF+H₂ group on the 42^{nd} day was 34.05 ± 2.15 g, 20% higher than that of mice in the Control group (27.45 ± 1.15 g), meaning that the average weight of mice in the HF+H₂ group met the weight standard of obese mice. The average weights of mice in the H₂ and HF+H₂ groups decreased when compared with those in the corresponding Control and HF groups, as shown in **Figure 1B**, indicating that saturated hydrogen inhibited the increases in body weights of mice.

On day 28, total cholesterol (TC) and low-density lipoprotein (LDL) in the HF group increased significantly compared with those in the Control group, and LDL in the $HF+H_2$ group decreased considerably compared with the HF group (**Figure 1C, 1E**). On day 42, TG, TC, and LDL in the HF group increased markedly compared with those in the Control group, which decreased substantially after saturated hydrogen treatment (**Figure 1C-E**). At the same time, high-density lipoprotein (HDL) in the HF group decreased remarkably compared with the Control group, and it increased dramatically after saturated hydrogen treatment (**Figure 1F**), suggesting that saturated hydrogen reduces the levels of TC, TG, and LDL and increases the level of HDL in the peripheral blood of obese mice.

HF diet promotes inflammation and EMT, which were partly alleviated by saturated hydrogen

It can be seen from the HE staining results of the lung histopathological sections (Figure 2A) that, on day 42, the infiltration degree of inflammatory cells around the bronchus in HF group increased compared with that of the Control group, with a small amount of inflammatory cells infiltrating in the lumens and a little shedding of bronchial cilia. Compared with the HF group, the pathological changes were decreased in the HF+H₂ group. The results of qPCR showed that the expression of E-cadherin was significantly decreased, and twist was significantly increased in the HF group compared with those in the Control group. Compared with the HF group, the expression of E-cadherin increased, and twist decreased in the HF+H₂ group, but there was no significant statistical difference (Figure 2B). The results of immunofluorescence were in consistent with the results of qPCR (Figure 2C, 2D). These results have shown that HF diet can lead to inflammation of lung tissue and accelerate the process of EMT of respiratory tract. Saturated hydrogen can alleviate the inflammation and slow down the process of EMT in lung tissue induced by highfat diet.

The diversity and difference of microflora in the lungs

The length distribution of 16S rRNA sequencing was shown in <u>Supplementary Figure 1</u>. The rarefaction sparse curve (Chao1), Specaccum species accumulation curve and rank abundance curve in alpha diversity analysis tend to be gentle, indicating that the sequencing depth is enough to reflect the diversity and richness of the microbial community and the total number of OTUs in the community will no longer increase significantly with the addition of new samples



Figure 1. HF diet induced increased body weights and dyslipidemia, which were alleviated by saturated hydrogen (n = 6). A. Changes of weight with time. B. Weight of mice on day 42. C-F. Represented the changes of TC, TG, LDL and HDL in peripheral blood of mice with time. (*P < 0.05, **P < 0.01 and ***P < 0.001 vs. Control; +P < 0.05, ++P < 0.01 and +++P < 0.001 vs. Control; #P < 0.05 and ##P < 0.01 vs. HF).

(Figure 3A). According to the alpha diversity index (Chao1, ACE, Shannon, Simpson) (Figure 3B), we found the Shannon and Simpson indexes in the HF group were significantly higher than those of the Control group. But there were no significant differences of these indexes between HF+H₂ and HF group.

Discriminant analysis by PLS-DA partial least squares and beta diversity based on weighted

UniFrac NMDS UPGMA cluster analysis chart of UniFrac distance matrix (**Figure 3C**) showed that there were obvious differences in the structure of microbial community between the HF group and the Control group, and also slight differences between the hydrogen treatment groups and the non-hydrogen treatment groups, however, the differences within the same group were small. The results showed that the animal classification model was effective.



Figure 2. HF diet promotes inflammation and EMT, which were partly alleviated by saturated hydrogen (n = 6). A. HE staining of lung tissues (400×). B. Showed the expression of E-cad, N-cad, twist, snail and slug mRNA in lung tissues on day 42 using qPCR. C. Immunofluorescence of E-cad protein in lung tissues (400×). D. Immunofluorescence of twist protein in lung tissues (400×) (*P < 0.05 vs. Control).

Identification of key microbes with significant difference among 4 groups

The number of OTU at different taxonomic level was shown in Supplementary Figure 2. Using R software, cluster analysis and heat map (Figure 4A) were made for the first 50 genera of abundance, which showed that there were significant differences among different groups. Compared with the Control group, the abundance of the common opportunistic pathogens Acinetobacter. Pseudomonas. Corvnebacterium, Strepcoccus, Clostridium, Haemophilus and Porphyromonas increased significantly in the HF group, among which, Acinetobacter, Clostridium and Porphyromonas decreased significantly after H₂ treatment. There was also a significant decrease in Bifidobacterium in the HF group compared with that of the Control group (Figure 4B, 4C). These results showed that HF diet significantly alters the diversity of microbes in the lungs, inducing opportunistic pathogens increased while probiotic microbes decreased. Saturated hydrogen can inhibit the increases of *Acinetobacter*, *Clostridium* and *Porphyromonas* induced by high-fat diet.

Functional prediction of bacterial metabolism

Next, the bacterial metabolism was predicted in KEGG, COG and rfam3 functional spectrum databases by PICRUSt functional prediction analysis. The predicted functional spectrum data were clustered according to the abundance distribution of functional groups or the similarity between samples, and the functional groups and samples were sorted according to the clustering results. Using R software, the functional groups in the top 50 of the abundance were clustered and analyzed, and a heat map (**Figure 5A**) was drawn, showing significant differences between the HF group and



Figure 3. Sequencing quality and microecological diversity analysis of lung in each group. A. Rarefaction sparse curve, Specaccum species accumulation curve and abundance rank curve for Alpha diversity analysis are presented. B. There are Alpha diversity index (microbial diversity index) Chao1, ACE, Shannon and Simpson statistical charts in order. C. It is the principal component analysis graph (PCA), multidimensional scale analysis graph (MDS) and sample clustering analysis graph (UPGMA) of Beta diversity analysis. (**P < 0.01 and ****P < 0.0001 vs. Control; ++P < 0.01 and ++++P < 0.0001 vs. Control).

the Control group. R software was used to calculate the number of common functional groups of each sample, and the proportion of common and unique functional groups of each sample were visualized through Venn diagram. The results showed that the number of functional groups in the HF group was significantly increased compared with the Control group, which was decreased after saturated hydrogen treatment (**Figure 5B**).

Violin diagram was drawn to show the abundance distribution of the predicted functional groups in each sample (Figure 5C). The results showed that the abundance of bacteria involved in cell motility, cell growth and death in HF group was increased significantly compared with the Control group, while there was no significant difference between HF and HF+H, group; The abundance of bacteria involved in the transcription of genetic information processing in HF group was decreased significantly compared with the Control group, while there was no significant difference between HF and HF+H₂ group; The abundance of bacteria involved in neurodegenerative diseases in HF group was increased significantly compared with the Control group, while it was significantly decreased after H₂ treatment; The abundance of bacteria involved in lipid metabolism, energy metabolism, amino acid metabolism, metabolism of terpenoids and polyketides in HF group was significantly increased compared with the Control group, while the glycan biosynthesis and metabolism, glucose metabolism in HF group were decreased compared with the Control group. There was no significant difference between HF and HF+H, group in these changes. The abundance of bacteria involved in the body's immune system in HF group was decreased significantly and the endocrine system and circulatory system was increased significantly compared with the Control group. There was no significant difference between HF and HF+H₂ group in these changes. These results suggest that HF leads to significant changes in the metabolism of bacterial flora in the lungs and saturated hydrogen may play a role especially in the decrease of the abundance of bacteria involved in neurodegenerative diseases.

HF promoted the increases of glyoxylic acid cycle-dependent bacteria

To investigate whether changes in microbes in the lungs of mice on a high-fat diet were due to the increases of glyoxylic acid cycle-dependent bacteria, we designed primers using the ICL sequences, a key enzyme in the glyoxylic acid cycle, to detect the expression of ICL genes in representative pathogens significantly associated with the development of asthma. The results of qPCR (Figure 6) showed that the ICL gene expression in P. aeruginosa, S. aureus and A. baumandii in the HF group was significantly increased compared with those of the Control group, and P. aeruginosa in the HF group was significantly decreased after hydrogen treatment. These results suggest that HF may promote the growth of glyoxylic acid cycledependent bacteria in the lung, while H₂ had moderate protective effects. However, K. pneumoniae, C. albicans and X. maltophilia showed no significant changes.

Discussion

Hyperlipidemia is a significant risk factor associated with airway hyperresponsive diseases (AHD), but the mechanism remains unclear. In the present study, the results obtained from qPCR and immunofluorescence showed that HF diet induced significant airway inflammation and EMT with a significant reduction in the expression of E-cad and increase in the expression of twist, indicating that HF diet may affect the occurrence and development of AHD through inflammation and EMT. Molecular identification techniques have shown the presence of bacteria in the lower respiratory tract of healthy subjects, which is imperative to maintaining the normal airway function. And changes in airway microflora may trigger many various respiratory diseases [21]. Therefore, we



Figure 4. The difference analysis of microflora in the lung of each group and the identification of key microbes. A. At the genus level, cluster analysis of the top 50 bacteria in each sample. Red represents the genus with higher abundance in the corresponding sample, while green represents the genus with lower abundance. B. Cluster analysis diagram of taxa with significant differences among groups. From the inner circle to the outer circle are phylum, class, order, family, and genus. The fan area represents the abundance of bacteria. C. The abundance of the first 20 taxa with the most significant differences in each group. In the figure, the abscissa is the first 20 taxon with the most significant difference, and the ordinate is the sequence quantity of each taxon in various groups. The "fat and thin" of "violin" reflects the density of sample data distribution, and the wider the width, the more samples corresponding to this sequence quantity. The border of the boxplot represents the Interquartile range (IQR), the horizontal line represents the median value, the upper and lower tentacles represent the IQR range of 1.5 times beyond the upper and lower quartile, and the symbol "•" represents the extreme value beyond the range.

further identified whether the inflammation and EMT were induced by the changes of pulmonary flora and its metabolism. Our results showed that HF diet can change the number and microbial species in the lung, leading to significant increases of some opportunistic pathogens such as Acinetobacter. Pseudomonas, Corynebacterium, Strepcoccus, Clostridium, Haemophilus and Porphyromonas. These increased opportunistic pathogens are believed to mediate the occurrence of chronic airway inflammation, airway hyperresponsiveness and other pathological changes [22]. After treatment with saturated hydrogen, Acinetobacter, Clostridium and Porphyromonas was significantly reduced. Moreover, the abundance of bacteria involved in cell motility, cell growth and death, neurodegenerative diseases, lipid metabolism, energy metabolism, amino acid metabolism, metabolism of terpenoids and polyketides in HF group was increased significantly compared with the Control group, while the transcription of genetic information processing, glycan biosynthesis and metabolism, glucose metabolism and the body's immune system in HF group was decreased significantly compared with the Control group. The increased lipid metabolism and decreased body's immune system may be closely related to the occurrence of AHR [23, 24].

Many opportunistic pathogens, which exist glyoxylates, can synthesize their own pathogenic substances through glyoxylate cycle by using the fatty acids in the body, and then amplify in the host [25-30]. Number of studies have shown that high-fat diet can lead to the accumulation of fatty acids in cells. Therefore, in order to investigate whether the imbalance of airway microecology during HF diet is caused by promoting the glyoxylic acid cycle of opportunistic pathogens, we detected the expression of ICL gene of several opportunistic pathogens intensively related to respiratory diseases. The results showed that the ICL genes of *P. aeruginosa*, *S. aureus* and *A. baumannii* in the lungs of mice fed with HF diet had abnormal high expression, indicating the HF diet activated the glyoxylic acid cycle of these opportunistic pathogens. However, we did not find any significant changes in ICL genes of fungi, and we speculated that this may be associated with the antagonistic effect of bacteria on fungi.

In addition, our results showed that saturated hydrogen treatment could alleviate the inflammation and slow down the process of EMT in lung tissue induced by HF diet. Moreover, saturated hydrogen could inhibit the growth and expansion of Acinetobacter, Clostridium, Porphyromonas and decrease the abundance of bacteria involved in neurodegenerative diseases, indicating that saturated hydrogen can influence the bacteria and its metabolism in the lung. Especially, hydrogen saturation can inhibit the increase of ICL gene expression of P. aeruginosa in the lung induced by HF diet. But how does hydrogen regulate the Microbiota in the lungs? Studies have shown that H₂ can regulate the level of oxidative stress markers, reduce the level of malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), 8-hydroxy-2 deoxyguanosine (8-OHdG), myeloperoxidase (MPO) and other lipid peroxidation products, and increase the levels of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH PX) and other antioxidant enzymes. Thus, it can selectively neutralize the hydroxyl radicals (OH) and peroxynitrite anions (ONOO⁻) in the body to play an antioxidant role [31-34]. In addition, hydrogen can act as a gas signal molecule and exert anti-inflammatory effect by inhibiting MAPK, NF-kB and other signaling pathways [35, 36]. Is it indirect act on Microbiota through reducing the pulmonary oxidative response or as a messenger molecule to regu-





Figure 5. Prediction of microbial metabolism. A. The heat map of clustering analysis of functional groups in the first 50 abundances. Red represents the functional group with higher abundance in the corresponding sample, while green represents the functional group with lower abundance. B. Venn diagram. Each ellipse represents a set of samples. The overlapping regions between the ellipses indicate the common functional groups among each group, and the number of each block indicates the number of common or unique functional groups contained in the block. C. Analysis diagram of KEGG database for prediction of microbial metabolism function. In the figure, the abscissa is the second functional group of KEGG, and the ordinate is the relative abundance of each functional group in each group. The "fat and thin" of the "violin" reflects the density of the distribution of sample data. The wider the width, the more samples there are under this abundance. The border of the boxplot represents the Interquartile range (IQR), the horizontal line represents the median value, the upper and lower tentacles represent the IQR range of 1.5 times beyond the upper and lower quartile, and the symbol "•" represents the extreme value beyond the range.



Figure 6. HF promoted the increases of glyoxylic acid cycle-dependent bacteria (n = 6). A-F was the ICL expression level of *P. aeruginosa*, *S. aureus*, *A. baumannii*, *K. pneumoniae*, *C. albicans and X. maltophilia* in the lung tissues of each group detected by qPCR. (**P* < 0.05 and **P < 0.01 vs. Control; #*P* < 0.05 vs. HF).

late the body's immune system or direct act? We recommend further *in vitro* and *in vivo* studies to reveal the exact mechanism.

In conclusion, this study found that the HF diet can promote the growth of opportunistic pathogens via glyoxylic acid cycle, thus inducing inflammation and EMT of airway. We report here for the first time that saturated hydrogen has a significant effect on the growth and metabolism of bacteria in the lung, which may provide new ideas and research basis as a new therapeutic approach.

Acknowledgements

This work was supported by Grant 31670121 and 31771277 from National Natural Science Foundation of China and Central South University Postgraduate Innovation Project (2021).

Disclosure of conflict of interest

None.

Address correspondence to: Yurong Tan, Department of Medical Microbiology, Xiangya Medical College, Central South University, No. 88, Xiangya Road, Kaifu District, Changsha City 810008, Hunan Province, China. Tel: +86-18711187944; E-mail: yurongtan@csu.edu.cn

References

- [1] Lin YP, Kao YC, Pan WH, Yang YH, Chen YC and Lee YL. Associations between respiratory diseases and dietary patterns derived by factor analysis and reduced rank regression. Ann Nutr Metab 2016; 68: 306-314.
- [2] Patel S, Custovic A, Smith JA, Simpson A, Kerry G and Murray CS. Cross-sectional association of dietary patterns with asthma and atopic sensitization in childhood in a cohort study. Pediatr Allergy Immunol 2014; 25: 565-571.
- [3] Silva RA, Almeida FM, Olivo CR, Saraiva-Romanholo BM, Martins MA and Carvalho CR. Airway remodeling is reversed by aerobic training in a murine model of chronic asthma. Scand J Med Sci Sports 2015; 25: e258-266.
- [4] Han L, Wang L, Tang S, Yuan L, Wu S, Du X, Xiang Y, Qu X, Liu H, Luo H, Qin X and Liu C. ITGB4 deficiency in bronchial epithelial cells directs airway inflammation and bipolar disorder-related behavior. J Neuroinflammation 2018; 15: 246.
- [5] Sachdeva K, Do DC, Zhang Y, Hu X, Chen J and Gao P. Environmental exposures and asthma development: autophagy, mitophagy, and cellular senescence. Front Immunol 2019; 10: 2787.
- [6] Qin Q, Chen X, Feng J, Qin L and Hu C. Low-intensity aerobic exercise training attenuates airway inflammation and remodeling in a rat model of steroid-resistant asthma. Chin Med J (Engl) 2014; 127: 3058-3064.

- [7] Tan M, Liu C, Huang W, Deng L, Qin X and Xiang Y. CTNNAL1 inhibits ozone-induced epithelialmesenchymal transition in human bronchial epithelial cells. Exp Physiol 2018; 103: 1157-1169.
- [8] Giannella M, Valerio M, Franco JA, Marin M, Bouza E and Muñoz P. Pacemaker infection due to Mycobacterium fortuitum: the role of universal 16S rRNA gene PCR and sequencing. Diagn Microbiol Infect Dis 2007; 57: 337-339.
- [9] Erb-Downward JR, Thompson DL, Han MK, Freeman CM, McCloskey L, Schmidt LA, Young VB, Toews GB, Curtis JL, Sundaram B, Martinez FJ and Huffnagle GB. Analysis of the lung microbiome in the "healthy" smoker and in COPD. PLoS One 2011; 6: e16384.
- [10] O'Dwyer DN, Ashley SL, Gurczynski SJ, Xia M, Wilke C, Falkowski NR, Norman KC, Arnold KB, Huffnagle GB, Salisbury ML, Han MK, Flaherty KR, White ES, Martinez FJ, Erb-Downward JR, Murray S, Moore BB and Dickson RP. Lung microbiota contribute to pulmonary inflammation and disease progression in pulmonary fibrosis. Am J Respir Crit Care Med 2019; 199: 1127-1138.
- [11] Huang YJ, Erb-Downward JR, Dickson RP, Curtis JL, Huffnagle GB and Han MK. Understanding the role of the microbiome in chronic obstructive pulmonary disease: principles, challenges, and future directions. Transl Res 2017; 179: 71-83.
- [12] Garcia-Nuñez M, Millares L, Pomares X, Ferrari R, Pérez-Brocal V, Gallego M, Espasa M, Moya A and Monsó E. Severity-related changes of bronchial microbiome in chronic obstructive pulmonary disease. J Clin Microbiol 2014; 52: 4217-4223.
- [13] Huang YJ, Sethi S, Murphy T, Nariya S, Boushey HA and Lynch SV. Airway microbiome dynamics in exacerbations of chronic obstructive pulmonary disease. J Clin Microbiol 2014; 52: 2813-2823.
- [14] Timm J, Post FA, Bekker LG, Walther GB, Wainwright HC, Manganelli R, Chan WT, Tsenova L, Gold B, Smith I, Kaplan G and McKinney JD. Differential expression of iron-, carbon-, and oxygen-responsive mycobacterial genes in the lungs of chronically infected mice and tuberculosis patients. Proc Natl Acad Sci U S A 2003; 100: 14321-14326.
- [15] Qiu X, Ye Q, Sun M, Wang L, Tan Y and Wu G. Saturated hydrogen improves lipid metabolism disorders and dysbacteriosis induced by a high-fat diet. Exp Biol Med (Maywood) 2020; 245: 512-521.
- [16] Ohno K, Ito M and Ichihara M. Molecular hydrogen as an emerging therapeutic medical gas for neurodegenerative and other diseases.

Oxid Med Cell Longev 2012; 2012: 3531-3552.

- [17] Ohsawa I, Ishikawa M, Takahashi K, Watanabe M, Nishimaki K, Yamagata K, Katsura K, Katayama Y, Asoh S and Ohta S. Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. Nat Med 2007; 13: 688-694.
- [18] Kamimura N, Nishimaki K, Ohsawa I and Ohta S. Molecular hydrogen improves obesity and diabetes by inducing hepatic FGF21 and stimulating energy metabolism in db/db mice. Obesity (Silver Spring) 2011; 19: 1396-1403.
- [19] Song G, Lin Q, Zhao H, Liu M, Ye F, Sun Y, Yu Y, Guo S, Jiao P, Wu Y, Ding G, Xiao Q and Qin S. Hydrogen activates ATP-binding cassette transporter A1-dependent efflux ex vivo and improves high-density lipoprotein function in patients with hypercholesterolemia: a doubleblinded, randomized, and placebo-controlled ttrial. J Clin Endocrinol Metab 2015; 100: 2724-2733.
- [20] Li L, Liu M, Kang L, Li Y, Dai Z, Wang B, Liu S, Chen L, Tan Y and Wu G. HHEX: a crosstalker between HCMV infection and proliferation of VSMCs. Front Cell Infect Microbiol 2016; 6: 169.
- [21] Martin C, Burgel PR, Lepage P, Andréjak C, de Blic J, Bourdin A, Brouard J, Chanez P, Dalphin JC, Deslée G, Deschildre A, Gosset P, Touqui L and Dusser D. Host-microbe interactions in distal airways: relevance to chronic airway diseases. Eur Respir Rev 2015; 24: 78-91.
- [22] Anthony CT, Stuart ET, Marco PA, Nicolas R, Burkhard T and Dominik H. Current concepts: host-pathogen interactions in cystic fibrosis airways disease. Eur Respir Rev 2014; 23: 320-332.
- [23] Rebecca KV, Jakob S, Bo LKC and Hans B. Blood lipid levels associate with childhood asthma, airway obstruction, bronchial hyperresponsiveness, and aeroallergen sensitization. J Allergy Clin Immunol 2016; 137: 68-74.
- [24] Deborah LC, Shyamala D, Anna-Karin L, Jane EW and Akira Y. Lipid metabolites as regulators of airway smooth muscle function. Pulm Pharmacol Ther 2009; 22: 426-435.
- [25] Michael CL and Gerald RF. Life and death in a macrophage: role of the glyoxylate cycle in virulence. Eukaryot Cell 2002; 1: 657-62.
- [26] Ha S, Shin B and Park W. Lack of glyoxylate shunt dysregulates iron homeostasis in Pseudomonas aeruginosa. Microbiology (Reading) 2018; 164: 587-599.
- [27] Lorenz MC, Bender JA and Fink GR. Transcriptional response of Candida albicans upon internalization by macrophages. Eukaryot Cell 2004; 3: 1076-1087.

- [28] McKinney JD, Honer zu Bentrup K, Munoz-Elias EJ, Miczak A, Chen B, Chan WT, Swenson D, Sacchettini JC, Jacobs WR Jr and Russell DG. Persistence of mycobacterium tuberculosis in macrophages and mice requires the glyxylate shunt enzyme isocitrate lyase. Nature 2000; 406: 735-738.
- [29] Höner Zu Bentrup K, Miczak A, Swenson DL and Russell DG. Characterization of activity and expression of isocitrate lyase in Mycobacterium avium and Mycobacterium tuberculosis. J Bacteriol 1999; 181: 7161-7.
- [30] Hamilton AJ, Jeavons L, Youngchim S, Vanittanakom N and Hay RJ. Sialic acid-dependent recognition of laminin by Penicillium marneffei codidia. Infect Immun 1998; 66: 6024-6026.
- [31] Xie K, Yu Y, Pei Y, Hou L, Chen S, Xiong L and Wang G. Protective effects of hydrogen gas on murine polymicrobial sepsis via reducing oxidative stress and HMGB1 release. Shock 2010; 34: 90-7.
- [32] Qiang S, Tomohiro K, Kosuke M, Ximei P, Qing S, Donna BS, John PP, Timothy RB, Xuejun S, Christian AB, Yoshiya T and Atsunori N. Oral intake of hydrogen-rich water inhibits intimal hyperplasia in arterialized vein grafts in rats. Cardiovasc Res 2012; 94: 144-53.

- [33] Hideaki O, Tsutomu I, Takashi Y, Hiroaki F, Hisaharu S, Mitsuru M, Hiroshi T, Shigeo O and Ikuroh O. Protection of the retina by rapid diffusion of hydrogen: administration of hydrogenloaded eye drops in retinal ischemia-reperfusion injury. Invest Ophthalmol Vis Sci 2010; 51: 487-92.
- [34] Cui W, Gao C, Fang P, Lin G and Shen W. Alleviation of cadmium toxicity in Medicago sativa by hydrogen-rich water. J Hazard Mater 2013; 260: 715-24.
- [35] Shi Q, Chen C, Deng WH, Wang P, Zuo T, Zhao L, Yu J, Zhao KL, Mei FC, Li C, Wang GR and Wang WX. Hydrogen-rich saline attenuates acute hepatic injury in acute necrotizing pancreatitis by inhibiting inflammation and apoptosis, involving JNK and p38 mitogen-activated protein kinase-dependent reactive oxygen species. Pancreas 2016; 45: 1424-1431.
- [36] Li Q, Yu P, Zeng Q, Luo B, Cai S, Hui K, Yu G, Zhu C, Chen X, Duan M and Sun X. Neuroprotective effect of hydrogen-rich saline in global cerebral ischemia/reperfusion rats: up-regulated tregs and down-regulated miR-21, miR-210 and NFκB expression. Neurochem Res 2016; 41: 2655-2665.

	1 1 2		
Gene	Forward primers (5'-3')	Reverse primers (5'-3')	Product (bp)
nodal	ACCATGCCTACATCCAGAGCC	CCTGCCATTGTCCACATAAAGC	116
slug	AGAAGCCCAACTACAGCGAACT	AGCTGCCGACGATGTCCATA	149
snail	TTGTAACAAGGAGTACCTCAGCC	AGCCAGACTCTTGGTGCTTGT	302
e-cad	CGACCGGAAGTGACTCGAAAT	TCAGAACCACTGCCCTCGTAAT	188
twist1	CCCACCCCACTTTTTGACGA	CAGTGGCTGATTGGCAAGAC	82
n-cad	CAATGGAGACTGCACGGACG	TCCCGCCGTTTCATCCATAC	135
Candida albicans	CATCATCTCAACAAGCCGACAA	TGAGCAACGTGGATTGGGTCTA	106
Pseudomonas aeruginosa	TCCTGGACTGCATCACCTCG	GCTTGGCGTTCGGAATCAC	126
klebsiella pneumonia	TGGGCGGTAAAGTGCTGGTT	TCCGGTTTCGACGTTTCACA	289
Acinetobacter baumannii	AGACGGCGTTATCGTTGCTC	CAGAAGCTAAACGAGTTGGACGA	203
Xanthomonas maltophlia	GAAGGCATCCAGCGCAACT	GGTGTTGTTGATGCGCTTGAC	312
Staphylococcus aureus	AAGGGGAATCAGCGGGAGA	TTGTCCGCCTTTACCACTGTG	248
β-actin	GTGACGTTGACATCCGTAAAGA	GTAACAGTCCGCCTAGAAGCAC	287

Supplementary Table 1. Primers for quantitative polymerase chain reaction



Supplementary Figure 1. Distribution of sequence length. The abscissa is the length distribution of all sample sequences, and the ordinate is the total number of sequences corresponding to each length value.



Supplementary Figure 2. Statistical results of OTU in each group. A-F was the number of OTU of each group at each taxonomic level. (*P < 0.05 and **P < 0.01 vs. Control).