High-flow hydrogen inhalation might suppresses the immune function of middle-aged participants: a self-controlled study

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Abstract

Hydrogen inhalation therapy has been proven to be safe and effective in disease treatment in multiple clinical reports, but the gas flow rates used in different studies vary greatly. Since there is no upper limit for the safe concentration of hydrogen, this study tested the effects of high-flow (not high concentration) hydrogen inhalation on immune function. From October 2019 to January 2020, 20 adult participants (31–60 years old) were enrolled in a self-controlled study to check the immune function in peripheral blood lymphocyte subsets before and after a 2-week hydrogen inhalation protocol. The participants inhaled hydrogen for 2 or 4 hours each day. After 2 weeks of hydrogen inhalation, statistically significant changes were observed in follicular helper T cells, helper and cytotoxic T cells, natural killer and natural killer T cells, and gamma delta T cells, generally suggesting a decrease in their proportions. These results show that high-flow hydrogen inhalation has an inhibitory effect on the immune function of healthy participants. The study protocol received ethical approval from the Ethics Committee of Fuda Cancer Hospital, Jinan University on December 7, 2018 (approval No. Fuda20181207).

Key words: B cell; high-flow hydrogen; immune function; inhalation; middle-aged people; natural killer T cell; natural killer cell; T cell

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Introduction

Hydrogen gas is a promising gas stage medicine, with a potential role in preventing cell injury from various sources.[1,2] Hydrogen gas is generated in small amounts by the hydrogenase enzymes of certain microbiota in the human gastrointestinal tract from unabsorbed carbohydrates in the intestine.[3] These carbohydrates are degraded and metabolized, and the resulting hydrogen is partially diffused into blood flow where it is released and can be detected in exhaled breath.[4] As the lightest molecule in nature, hydrogen gas exhibits appealing penetration properties, as it can rapidly diffuse through cell membranes.[5]

Previously, case reports[6-9] and a real-world survey[10] have demonstrated that high-flow hydrogen inhalation (flow rate: 3 L/min; hydrogen/oxygen: 66.7%/33.3%; 4 hours each day) was safe and effective for patients with nasopharyngeal carcinoma, non-small cell lung cancer (NSCLC), and other tumors. Although high-flow (not high concentration) hydrogen inhalation can improve the immune function of patients with advanced cancers, it is not clear how it affects the immune function of people without cancer. The purpose of this study is to evaluate the impact of high-flow hydrogen on immune cells function in middle-aged participants, using accurate and comprehensive immunoassay methods.[11]

Participants and Methods

Participants

Twenty middle-aged (31–60 years old) participants were recruited in the study from October 2019 through January 2020 at Xu Kecheng Care Health Studio of Guangdong Province, China. This study was a self-control study, comparing the changes of immune indexes before and after hydrogen inhalation for 2 weeks, so there was no control group. The enrollment criteria were that subjects must be 31–60 years of age, without chronic diseases or with mild chronic diseases that were controlled with medication. The exclusion criteria were that subjects must not have malignant tumors, organ transplants, cardiac pacemakers, or refractory chronic diseases that are difficult to control with medication. The study protocol received ethical approval from the Ethics Committee of Fuda Cancer Hospital, Jinan University on December 7, 2018 (approval No. Fuda20181207). Written informed consent was obtained from each participant in accordance with the *Declaration of Helsinki*.

Hydrogen inhalation method

Hydrogen was produced by a hydrogen-oxygen nebulizer (AMS-H-03, Shanghai Asclepius Meditec Co., Shanghai, China). The patients remained seated or recumbent and inhaled a mixture of hydrogen (66.7%) and oxygen (33.3%) through a nasal tube with a gas flow rate of 3 L/min. Participants were randomly divided into two groups by random number table (**Figure 1**). Hydrogen inhalation sessions were for 2 (n = 10) or 4 (n = 10) hours per day for 2 weeks in Xu Kecheng Care Health Studio of Guangdong Province, China.

Immunophenotype evaluation

Up to 5 mL of peripheral blood was extracted from elbow vein in all enrolled patients before and after 2 weeks of hydrogen inhalation. Peripheral blood mononuclear cells were isolated

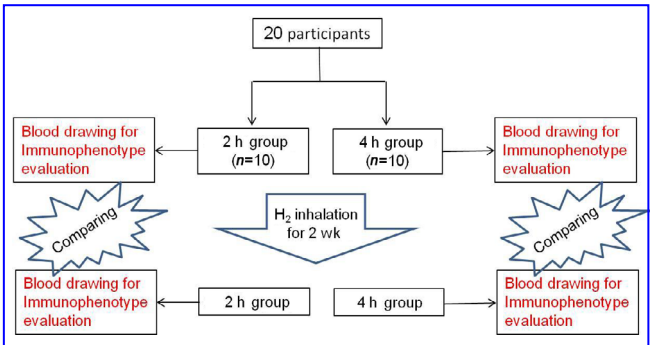

Figure 1: A trial flow chart.

using Ficoll solution and labeled with fluorescent antibodies (BD Biosciences, San Jose, CA, USA). T lymphocytes, natural killer (NK) cells, natural killer T (NKT) cells and gamma delta T (γδ T) cells were analyzed using flow cytometry (FACSanto II; BD Biosciences) by a professional third-party inspection center (Shuangzhi Purui Medical Laboratory Co., Ltd., Wuhan, Hubei Province, China).

Statistical analysis

The calculation method of sample size was based on MedSci Sample Size tools (MSST) software (MedSci, Shanghai, China). The general clinical data of participants were all compared using a two-way analysis of variance before hydrogen inhalation. Cell subsets between and within groups were compared by one-way analysis of variance analysis and Bonferroni's multiple comparison test. Statistical differences were indicated by $P < 0.05$. All analyses and figures were produced using Prism 5.0 software (GraphPad, San Diego, CA, USA).

Results

Clinical data of adult participants with high-flow hydrogen inhalation

A total of 20 participants were recruited consecutively to participate in the study. The general clinical data of the cohort are shown in **Table 1**.

Table 1: General clinical data of adult participants with high-flow hydrogen inhalation

Parameters	2 h group (*n*=10)	4 h group (*n*=10)	*P*-value
Sex			> 0.05
Female	5	6	
Male	5	4	
Age (yr)			> 0.05
31–40	5	4	
41–50	4	4	
51–60	1	2	
Chronic diseases that drugs can control			> 0.05
Hypertension	3	2	
Hyperlipidemia	2	3	
Type 2 diabetes	1	2	

Note: Data are expressed as number, and were analyzed by two-way analysis of variance.

Changes of T cell subsets of adult participants with high-flow hydrogen inhalation

We analyzed four types of immune cells from the peripheral blood of subjects: total T cells, $CD4^+$ T cells, $CD8^+$ T cells, and regulatory T cells. No significant difference in the numbers of ratios of any cell type was found within or between the two hydrogen inhalation groups (**Additional Figure 1**). The changes in the proportions of a variety of different functional subsets of $CD4^+$ and $CD8^+$ T cells were further analyzed. These subsets included: terminally differentiated cells, memory cells, anti-virus cells, functional cells, and exhausted cells. No significant differences in the proportions of $CD4^+$ T cell subtypes (**Additional Figure 2**) and $CD8^+$ T cell subtypes (**Additional Figure 3**) were found within or between the two hydrogen inhalation groups.

We also analyzed the changes in the proportions of T helper (Th) cells and cytotoxic T (Tc) cell subsets. Compared with the baseline, the proportion of Th1 cells decreased in the 2 hours group, and the proportion of Th17 cells decreased in the 4 hours group (both $P < 0.05$, **Figure 2A** and **C**). No significant differences in the proportions of Th2 cell in the two groups (**Figure 2B**). The proportion of Tc1 and Tc2 cells decreased (both $P < 0.05$) in the 2 hours group, and the proportion of Tc17 cells decreased ($P < 0.05$) in the 4 hours group (**Figure 2D–F**).

Comparing with the baseline, follicular helper T (Tfh) cell subtypes Tfh1 and Tfh2 both showed a decrease in their proportions (both $P < 0.05$), while Tfh17 cells showed a significant increase in their proportions ($P < 0.001$) in the 2 hours group, but these changes were not observed in the 4 hours group (**Figure 3**).

Changes of NKT and NK cells of adult participants with high-flow hydrogen inhalation

We analyzed the changes in the proportions of NK and NKT cells, and observed that the proportion of NKT cells decreased in the 4 hours group ($P < 0.05$, *vs*. baseline; **Figure 4A**). The ratio of NK cells and activated NK cells did not change significantly before and after hydrogen inhalation (**Figure 4B** and **C**). The proportion of killer NK cells also decreased in the 4 hours group ($P < 0.05$; **Figure 4D**), and the proportion of anti-virus NK cells decreased significantly in both groups (both $P < 0.01$; **Figure 4E**).

Changes of γδ T cells of adult participants with high-flow hydrogen inhalation

The changes in the proportions of γδ T cells and their subsets were also analyzed. Comparing with the baseline, no statistical changes were found in the proportions of total γδ T cells (**Figure 5A1**). The proportion of Vdelta (Vδ) 1 cells decreased in the 2 hours group ($P < 0.05$; **Figure 5A2**), and the proportion of the anti-virus subset decreased significantly in both groups (both $P < 0.01$; **Figure 5B2**). The proportion of the Vδ2 cells) killer subset decreased significantly in the 2 hours group ($P < 0.01$; **Figure 5C1**), and the proportion of anti-virus Vδ2 cells decreased in the 2 hours group and decreased significantly in 4 hours group ($P < 0.05$ and $P < 0.01$; **Figure 5C2**).

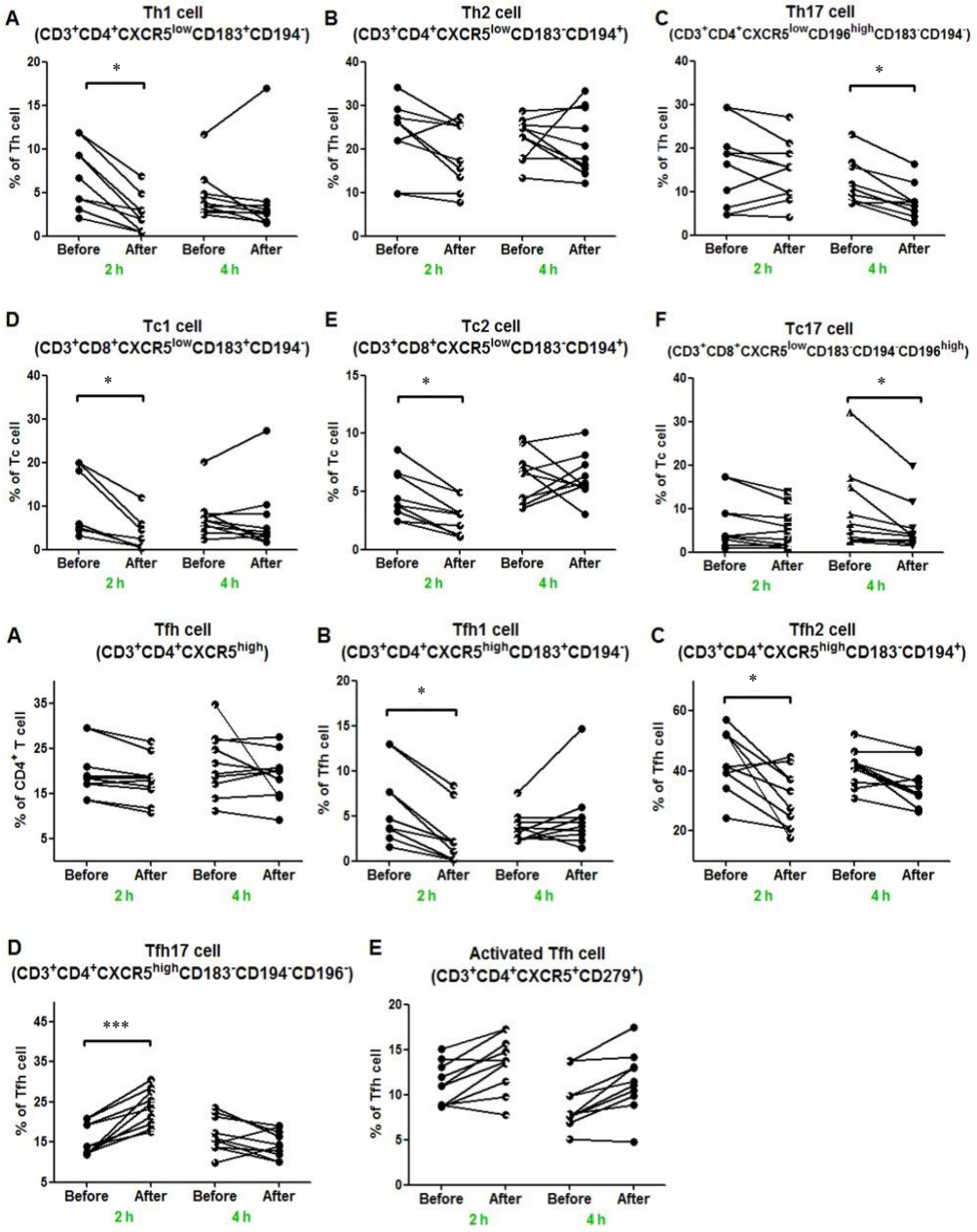

Figure 2: Immunoassays of helper T (Th) cell and cytotoxic T (Tc) cell subsets before and 2 weeks after 2 or 4 hours high-flow hydrogen inhalation in adult participants.
Note: (A–C) Quantitative results of Th1 (A), Th2 (B) and Th17 (C) subsets, respectively. (D–F) Quantitative results of Tc1 (D), Tc2 (E) and Tc17 (F) subsets, respectively. *P < 0.05 (one-way analysis of variance analysis followed by Bonferroni's multiple comparison test).

Figure 3: Immunoassays of follicular helper T cell (Tfh) and its subsets before and 2 weeks after 2 or 4 hours high-flow hydrogen inhalation in adult participants.
Note: (A) Quantitative results of Tfh cells. (B–E) Quantitative results of Tfh1 (B), Tfh2 (C), Tfh17 (D) and activated subsets (E), respectively. *P < 0.05, ***P < 0.001 (one-way analysis of variance analysis followed by Bonferroni's multiple comparison test).

Discussion

Immune cell senescence refers to the phenotypic characteristics of individual lymphocytes, regardless of their immune function which does not necessarily decrease during aging.[12-14] Immune cell exhaustion refers to the decrease in immune function due to excessive free radicals in the mitochondria, causing the cell to lose its function and enter programmed apoptosis regardless of age.[15] Senescence and exhaustion do not indicate the inevitable and progressive decline of all immune functions, but rather constitute a highly dynamic process of remodeling and adaptation.[16] Thus, we tested whether hydrogen gas inhalation can rescue the function of exhausted and senescent lymphocytes, as hydrogen gas was reported to protect the mitochondrial function of human lymphocytes.[17-19] By comparing the immune functions of advanced non-small cell lung cancer patients before and after hydrogen inhalation, we found that 2 weeks of hydrogen inhalation was enough to reverse multiple senescent or exhausted cell subsets into functional subsets,[20] so we used the same hydrogen inhalation duration during this study. There were no obvious changes in any T cell subsets due to hydrogen inhalation in either cell number, memory phenotype, differentiation phenotype or senescent or exhausted phenotype. This result indicates that 2 weeks of hydrogen inhalation will not significantly change the number or proportion of major T lymphocyte subsets in health persons. The number and function of immune cells of patients with advanced NSCLC are significantly lower than those of middle-aged participants, which becomes the basis for the high-flow hydrogen therapy to play a therapeutic role.[20] Therefore, it can be speculated that the high-flow hydrogen therapy is a regulatory therapy that can restore the damaged immune system to function, but has little effect on the normal immune system.

Cytokines are the central mediators of immune responses, and Th cells are dedicated cytokine-producing cells. By producing effector cytokines, Th cells play critical roles during

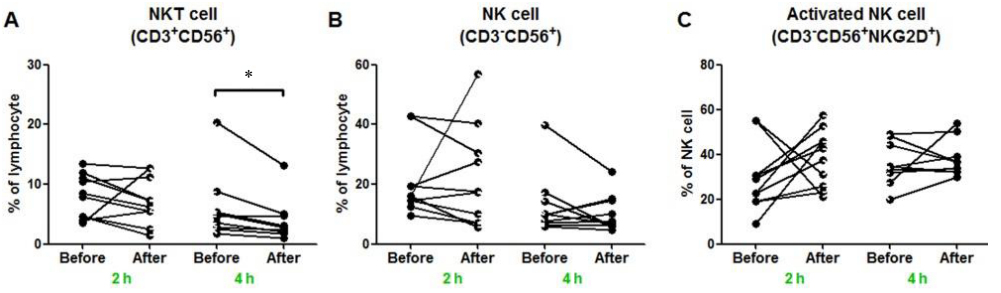

Figure 4: Immunoassays of NKT and NK cell before and 2 weeks after 2 or 4 hours high-flow hydrogen inhalation in adult participants.
Note: (A) Quantitative results of NKT cell. (B) Quantitative results of NK cells. (C–E) Quantitative results of activated (C), killer (D) and anti-virus subsets (E) of NK cells. *$P < 0.05$, **$P < 0.01$ (one-way analysis of variance analysis followed by Bonferroni's multiple comparison test). NK: Natural killer; NKT: natural killer T.

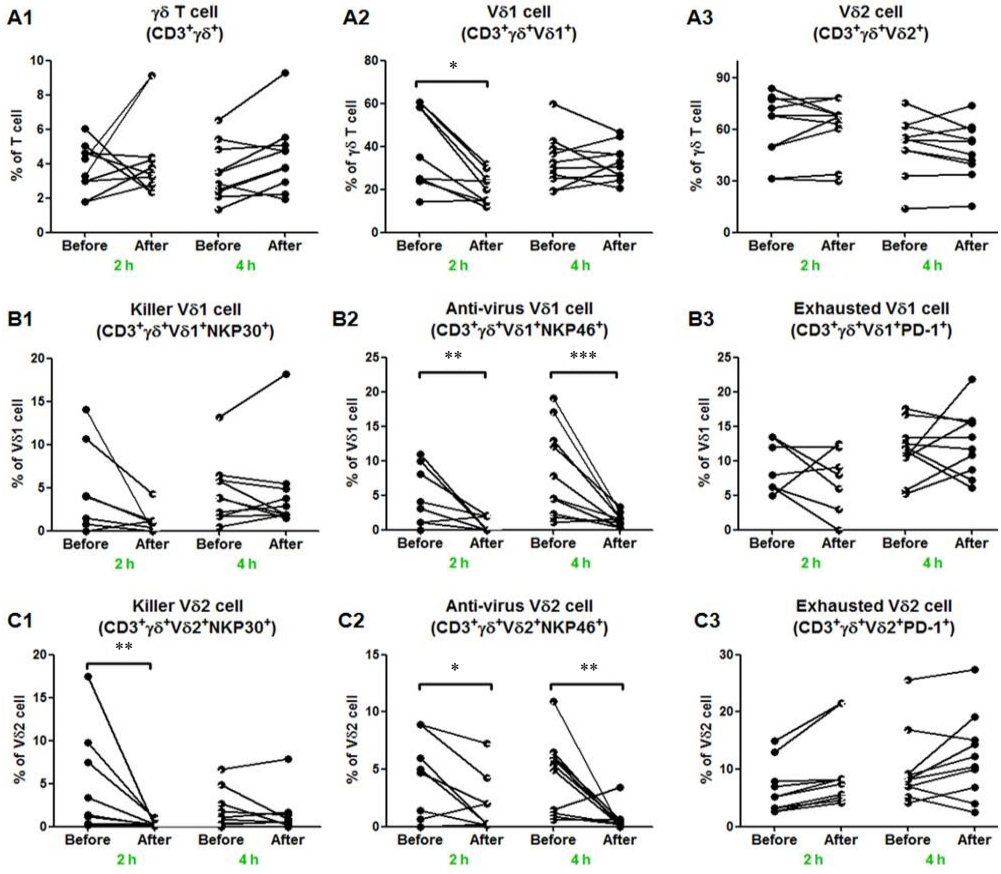

Figure 5: Immunoassays of γδ T cell and subsets before and 2 weeks after 2 or 4 hours high-flow hydrogen inhalation in adult participants.
Note: (A1–3) Quantitative results of γδ T cell and its subsets. (B1–3) Quantitative results of γδ 1 subsets. (C1–3) Quantitative results of γδ 2 subsets. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (one-way analysis of variance analysis followed by Bonferroni's multiple comparison test). γδ: Gamma delta.

adaptive immune responses to infections, and distinct Th subsets are involved in conferring protective immunity to different pathogens.[21] Th1 and Th2 cells produce interferon-γ and interleukin-4, respectively, while Th17 cells produce IL-17. While Th1 cells are mainly important for host defense against intracellular bacteria and viruses, they are also responsible for the development of tumor immunity and organ-specific autoimmunity.[22] Th2 cells are responsible for the pathogenesis of inflammatory asthmatic and allergic diseases.[23] Th17 cells are essential for orchestrating immune responses to extracellular bacteria and fungi, and they are also responsible for some autoimmune diseases and severe asthma.[24] After hydrogen inhalation, every functional subset of Th cells was inhibited, representing an overall decline in the immune function of Th cells after hydrogen inhalation. Tc cells are divided into three classes. Tc1 cells can kill intracellular pathogens and tumors by releasing granzymes and perforin into the immunological synapse,[25] while Tc2 cells can mediate the propagation of allergy and arthritis,[26] and Tc17 cells take part in the propagation of autoimmunity, immunity to viral infections and

tumor responses.[27] All three kinds of Tc cells were simultaneously decreased after hydrogen inhalation, which represents a reduction in the ability of the immune system to eliminate abnormal cells. Generally, the inhibitory effect of hydrogen gas absoption was observed in both Th and Tc in the blood across most cellular subtypes, indicating that overall immune function was disturbed.

Tfh cells are resident in lymph nodes and the spleen because their purpose is to help B cells, while non-Tfh effector cells such as Th and Tc cells are destined to predominantly leave lymphoid tissues and traffic to sites of infection or inflammation.[28] Tfh1, Tfh2 and Tfh17 cells can also co-express Tfh and Th cell associated genes, and effectively act as Th1, Th2 and Th17 cells in the germinal center of lymph nodes or the spleen.[29] The changes in Tfh cells were observed only in the 2 hours group, but in these subjects the proportion of Tfh1 and Tfh2 cells decreased, and the proportion of Tfh17 cells increased significantly. This indicates that the ability to clear malignant cells or virus infections in lymphoid tissues is weakened, but that the ability to fight bacteria or fungi is strengthened.[28] Why there is an effect after 2 hours of hydrogen inhalation, but no effect in the 4 hours group may be explained from the body's self-regulation and the microenvironment of lymph nodes in further study.[28,29]

The immune repertoire of NK,[30] NKT[31] and γδ T[32] cells play important regulatory roles in infection and tumor immunity. Here we found that the total number of NKT cells decreased after hydrogen inhalation, while neither the total number of NK cells nor γδ T cells changed significantly. Multiple functional subsets of NK cells were significantly reduced, including killer NK cells, anti-virus NK cells, anti-virus Vδ1 cells, killing Vδ2 cells, and anti-virus Vδ2 cells), while no subsets were increased. The reduction in the proportion of so many functional subsets, which were more pronounced in the 4 hours group than in the 2 hours group, is likely to reflect a weakening of the subject's positive anti-infection and anti-tumor immune capabilities. Based on the detection of Th, Tc and Tfh cells, it can be considered that the effect on lymphocytes of 2 weeks of hydrogen inhalation is a broad reduction in lymphocyte function, which will reduce the body's anti-infection and anti-tumor abilities, and is therefore not a method for preventing tumorigenesis.

Prior to this study, we recruited 20 patients with advanced NSCLC, who underwent high-flow (not high concentration) hydrogen therapy for 2 weeks (4 hours per day, also 3 L/min, 66.7% hydrogen, 33.3% oxygen) before receiving standard treatments.[20] Before hydrogen inhalation, abnormally high indexes of the patients included exhausted Tc cells, senescent Tc cells, and killer Vδ1 cells. After 2 weeks of hydrogen therapy, the number of exhausted and senescent Tc cells decreased to within the normal range, and there was an increase in the number of killer Vδ 1 cells. Abnormally lower indexes included functional Th and Tc cells, Th1, NKT cell, NK cell, and Vδ2 cells. After 2 weeks of hydrogen therapy, all six cell subsets increased to within the normal range. For advanced NSCLC patients, the immune senescence involved nearly all lymphocyte subsets, and 2 weeks of hydrogen treatment significantly improved most of these indexes.[20] The effects of hydrogen therapy on advanced NSCLC patients and middle-aged non-tumor participants in this study appear to be contradictory. The reason for this may be that the lymphocytes of patients with progressive tumors and those of non-tumor patients are quite different. The subgroups indicating senescence and failure in lymphocytes of cancer patients are significantly increased compared to those of non-tumor patients, so harmful free radicals in mitochondria may be neutralized by a large flow of hydrogen, improving the health of the cell.[18]

In general, most of the immunological indicators of middle-aged participants did not change significantly after 2 weeks of high-flow hydrogen inhalation, and a small number of anti-infection and anti-tumor cell subtypes showed a decrease, reflecting certain adverse reactions. There are still some limitations on this study. The sample size was small, and the age group involved in the study was not broad enough. Further research and improvement are needed.

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Author contributions
Design of the study: JBC and FM; data collection: XFK; data analysis: JBC and XFK; manuscript writing: JBC and FM. All authors approved the final version of the manuscript.

Conflicts of interest
The authors have no conflicts of interest to declare.

Financial support
None.

Institutional review board statement
This study protocol received ethical approval from the Ethics Committee of Fuda Cancer Hospital of Jinan University on December 7, 2018 (approval No. Fuda20181207).

Declaration of participant consent
The authors certify that they have obtained all appropriate consent forms from the participants. In the forms, the participants have given their consent for the images and other clinical information to be reported in the journal. The participants understand that the names and initials will not be published.

Biostatistics statement
The statistical methods of this study were reviewed by the biostatistician of Fuda Cancer Hospital of Jinan University, China.

Data sharing statement
Individual participant data that underlie the results reported in this article, after deidentification (text, tables, figures, and appendices). Study protocol and informed consent form will be available immediately following publication, without end date. Results will be disseminated through presentations at scientific meetings and/or by publication in a peer-reviewed journal.

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Additional files
Additional Figure 1: Immunoassays of T cell subsets before and 2 weeks after 2 or 4 hours high-flow hydrogen inhalation in adult participants.
Additional Figure 2: Immunoassays of $CD4^+$ T cell subsets before and 2 weeks after 2 or 4 hours high-flow hydrogen inhalation in adult participants.

Additional Figure 3: Immunoassays of $CD8^+$ T cell subsets before and 2 weeks after 2 or 4 hours high-flow hydrogen inhalation in adult participants.

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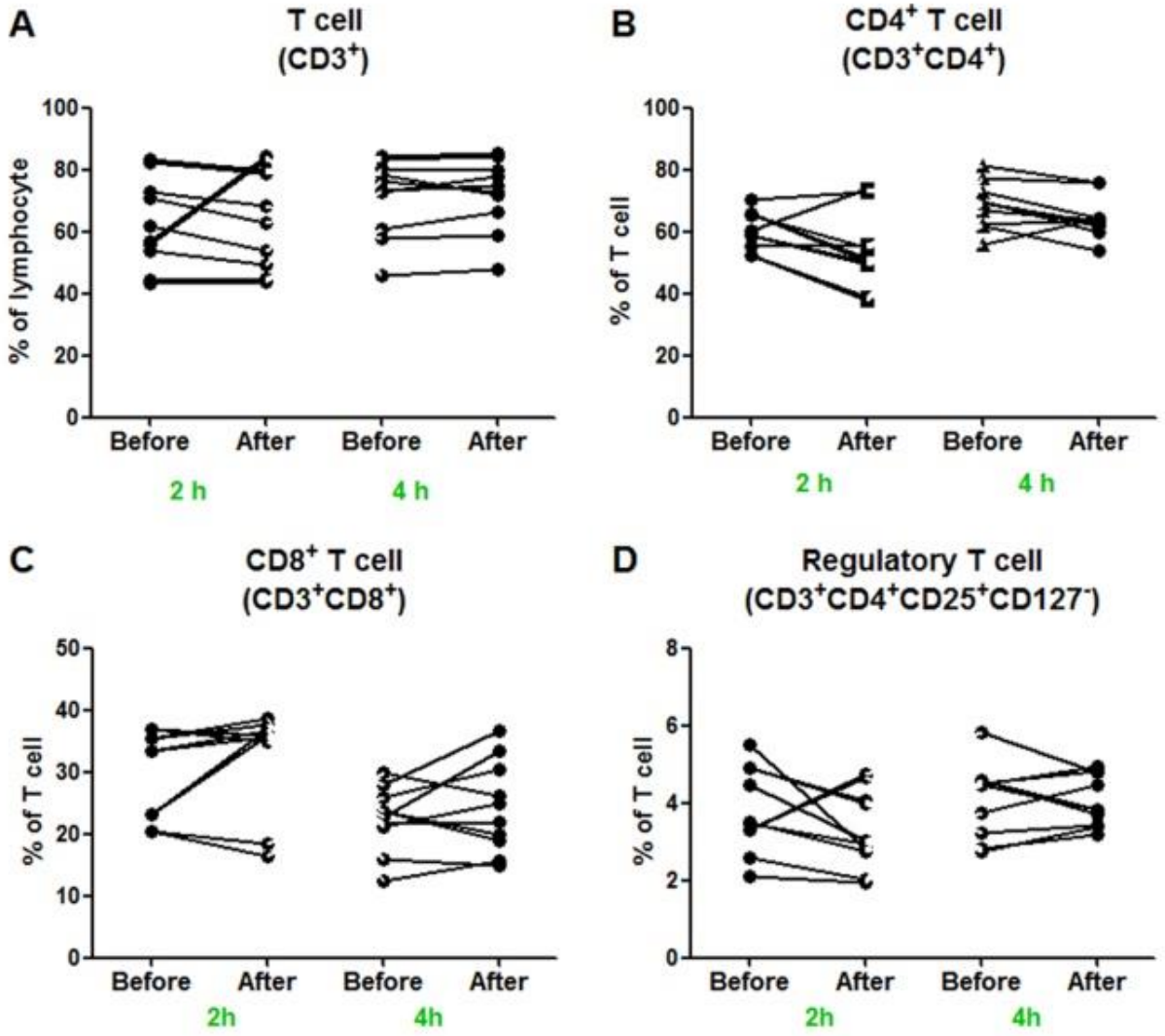
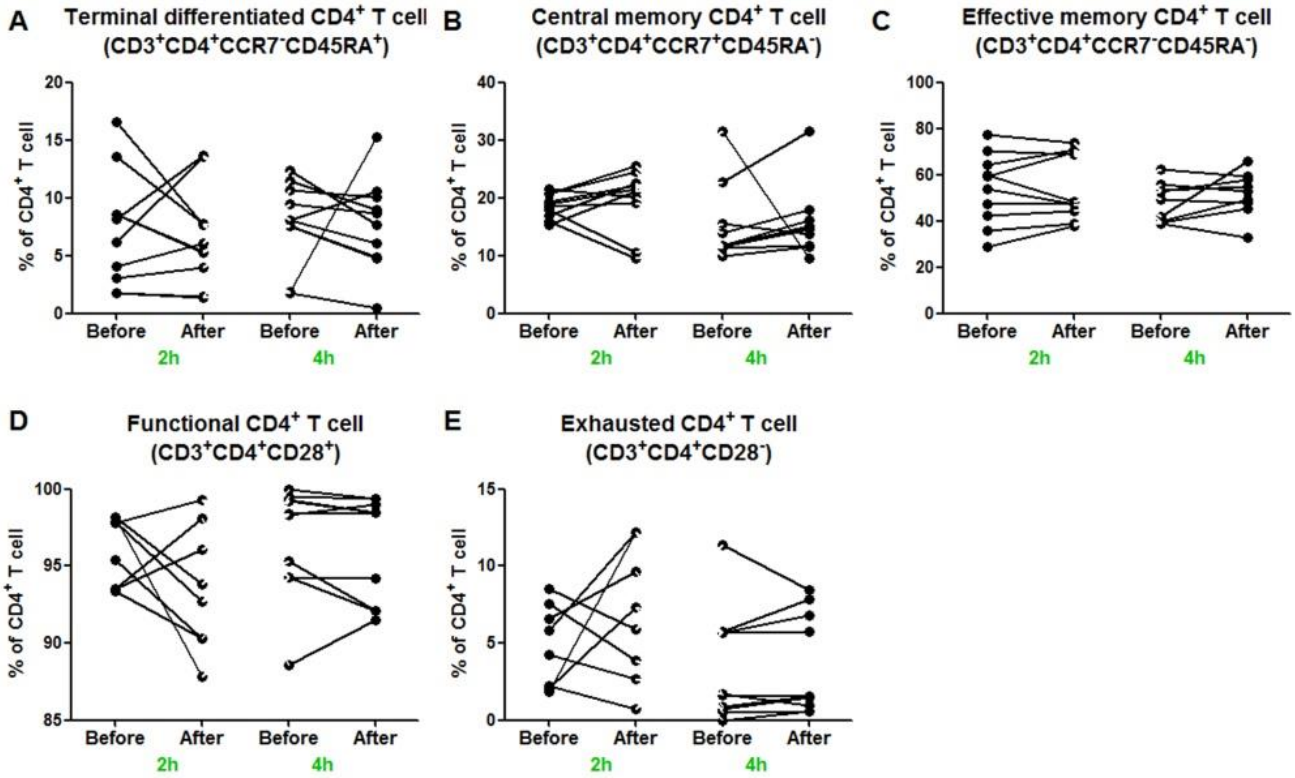


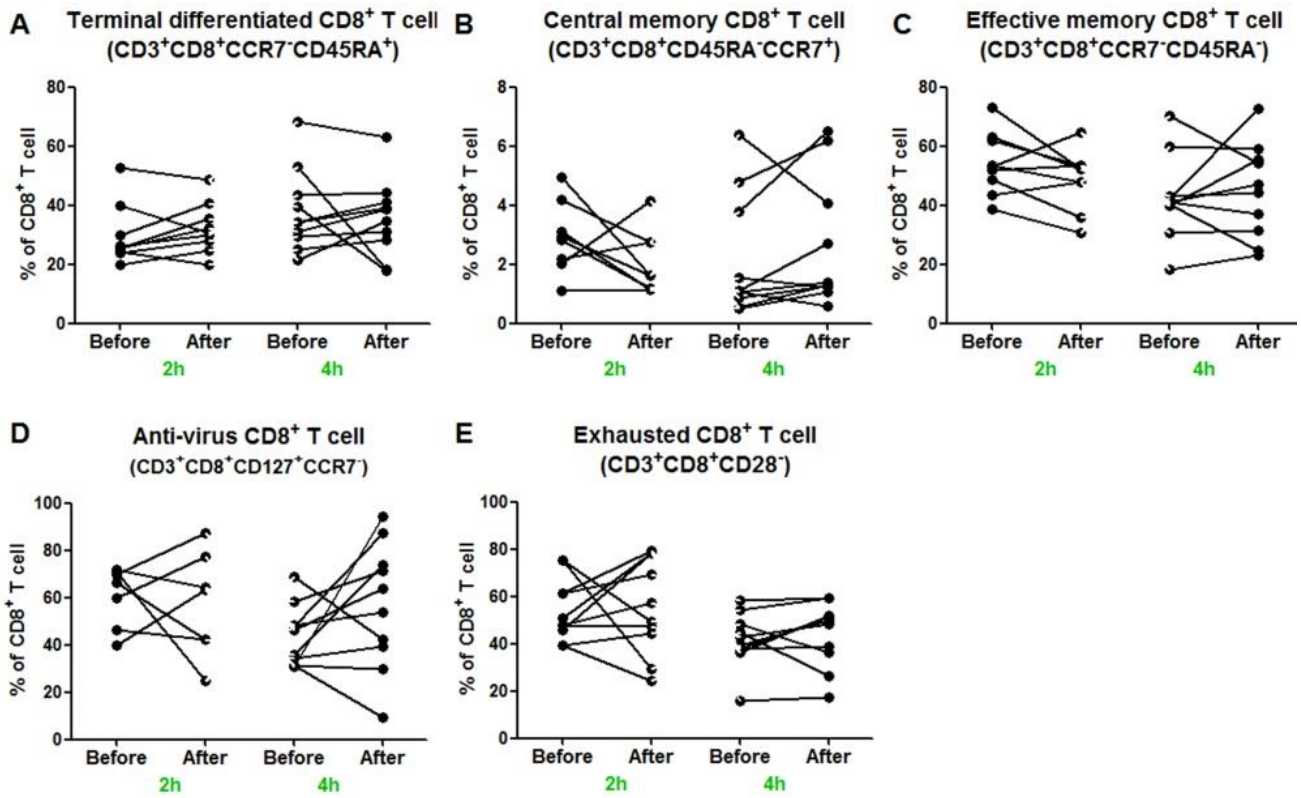
Additional Figure 1: Immunoassays of T cell subsets before and two weeks after 2 or 4 hours high-flow hydrogen inhalation in adult participants.

(A-D) Quantitative results of total T cells (A), $CD4^+$ T cells (B), $CD8^+$ T cells (C), and regulatory T cells (D). Data were analyzed by one-way analysis of variance analysis followed by Bonferroni's multiple comparison test.



Additional Figure 2: Immunoassays of $CD4^+$ T cell subsets before and two weeks after 2 or 4 hours high-flow hydrogen inhalation in adult participants.

(A-E) Quantitative results of terminally differentiated subsets (A), central memory subsets (B), effective memory subsets (C), functional subsets (D) and exhausted subsets (E), respectively. Data were analyzed by one-way analysis of variance analysis followed by Bonferroni's multiple comparison test.



Additional Figure 3: Immunoassays of CD8$^+$ T cell subsets before and two weeks 2 or 4 hours high-flow hydrogen inhalation in adult participants.

(A-E) Quantitative results of terminal differentiated subsets (A), central memory subsets (B), effective memory subsets (C), functional subsets (D) and exhausted subsets (E), respectively. Data were analyzed by one-way analysis of variance analysis followed by Bonferroni's multiple comparison test.