Supplementary materials:

Lung macrophages are involved in lung injury secondary to repetitive diving

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Materials and methods

1. Animals and Experimental protocol

A total of 84 male BALB/c mice weighing (18.0±1.8) g were purchased from the Experimental Animal Center of the Naval Military Medical University, China. This study was approved by the Ethics Committee of the Naval Military Medical University. Animals were housed in a specific pathogen free environment and given *ad libitum* access to food and water with a natural day/night cycle in accordance with the Guideline for the Animal Care and Use of the Naval Medical University. Animals were randomly divided into the following groups: a) Control group: mice were exposed to nomobaric air in the chamber; b) Decompression group: mice were exposed to hyperbaric environment in the chamber once daily for consecutive 3 days; c) HCH group: immediately after each hyperbaric exposure, animals were exposed to a 66.7% hydrogen and 33.3% oxygen mixture for 1 h at normal pressure. In decompression group and HCH group, mice were subdivided into 1-day subgroup, 2-day subgroup ad 3-day subgroup, in which mice were sacrificed after hyperbaric exposure once, twice and thrice, respectively. The experimental protocol is shown in Figs. 1c and 1d.

2. Establishment of decompression-induced lung injury model

Mice were compressed with air (79% Nitrogen; 21% Oxygen) in a transparent hyperbaric rodent chamber (Type RDC150-300-6, NMU, Shanghai, China; Fig. 1a). Mice were pressurized to 600 kPa within 5 min at a rate of 100 kPa/min, which was maintained for 60 min. Thereafter, decompression was carried out linearly to normobaric pressure at a rate of 200 kPa/min (Fig. 1e). The chamber was ventilated continuously with compressed air during the hyperbaric exposure to avoid carbon dioxide (CO_2) accumulation, and soda lime was used to absorb carbon dioxide (CO_2) produced in the chamber. Mice were exposed to hyperbaric environment once daily for consecutive 3 days to simulate repetitive diving.

3. HCH treatment

After each hyperbaric exposure, mice received inhalation of HCH for 60 min. HCH treatment was performed as previously described (Li et al., 2017). In brief, animals were placed in a chamber that was flushed with 66.7% hydrogen and 33.3% oxygen produced with the BYT-JP-H03 hydrogen generator (Asclepius, Kunshan, China), which was designed to electrolyze water to produce mixed gas (Fig. 1b).

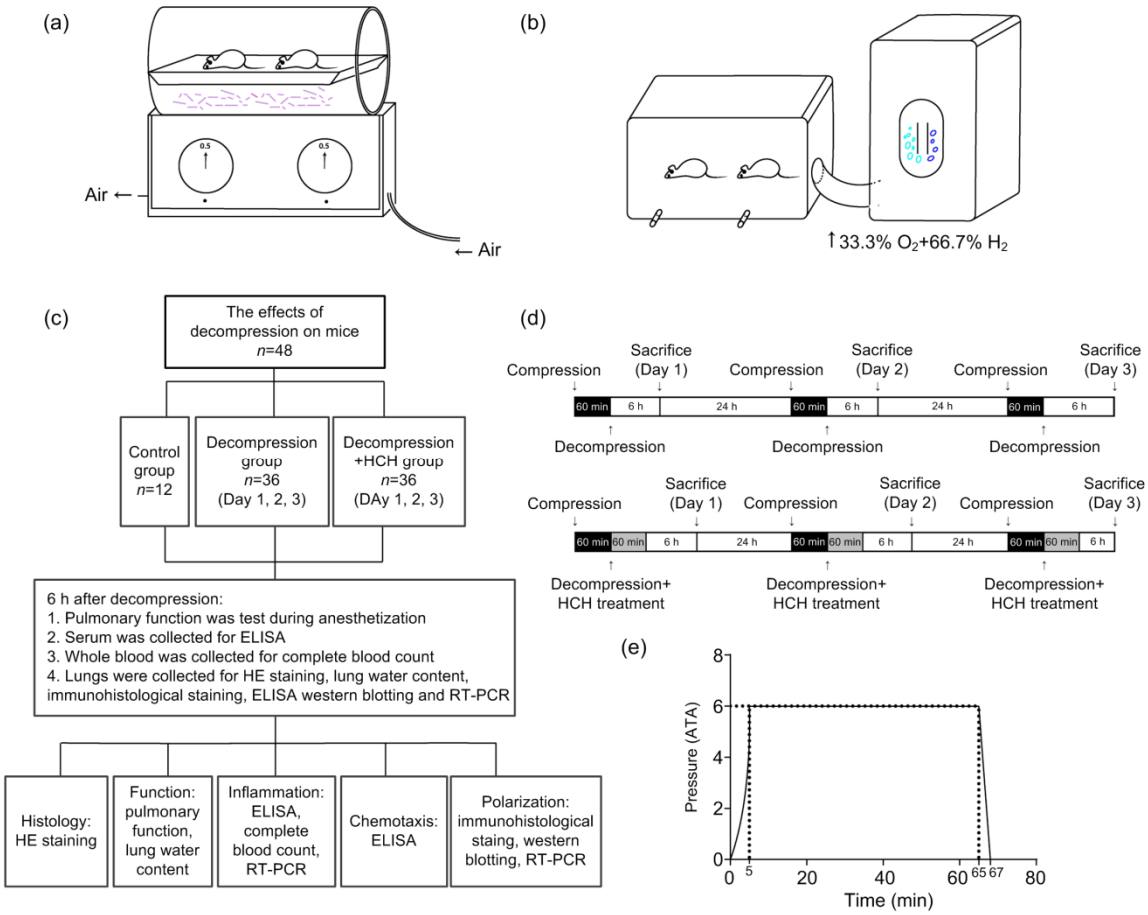

Fig. 1 Animal groups and protocols

(a) For the establishment of model, mice were compressed with air in a transparent hyperbaric rodent chamber. (b) For HCH treatment, mice were exposed to 66.7% hydrogen (H_2) and 33.3% oxygen (O_2). (c) Mice were divided into three groups, and subdivided into 1-d, 2-d, and 3-d subgroups. Histology, pulmonary function, inflammation, chemotaxis, and polarization were evaluated at 6 h after decompression or treatment. (d) Mice in the decompression groups were decompressed once a day for three consecutive days, mice were sacrificed at each time point in subgroups. Mice in the decompression+HCH group were administrated with HCH after decompression daily. (e) For decompression, the pressure was raised to 600 kPa and then maintained for 60 min. Thereafter, decompression was carried out linearly to ambient pressure. ATA: atmosphere absolute; ELISA: enzyme-linked immunosorbent assay; HCH: high concentrations of hydrogen; HE: hematoxylin and eosin; RT-PCR: real-time polymerase chain reaction

4. Sample collection

Six hours after hyperbaric exposure, mice were intraperitoneally anesthetized with 1% (w/v) sodium pentobarbital at 40 mg/kg. The blood samples were collected from the supraorbital vessels within 30 min and divided into two parts: one was anti-coagulated with heparin for complete blood counting (CBC) (BC280 Vet, Mindray, Shenzhen, China) and the other was stored at 4 °C for enzyme linked immunosorbent assay (ELISA). Lung tissues were collected and divided into three parts: one was stored at −80 °C for protein and DNA extraction; one was fixed in formalin for histological examination and immunohistochemistry; one was processed for lung edema detection.

5. Pulmonary function test

Six hours after hyperbaric exposure, the mice underwent the tracheotomy after being intraperitoneally anesthetize. The pulmonary function was test using invasive lung function assessment (Model CRFM100,

EMMS, Britain). The total lung capacity (TLC), chord compliance (Cchord), forced expiratory volume at 200 ms (FEV200) and functional residual capacity (FRC) were measured.

6. Measurement of lung water content

Lungs were collected as above mentioned, and the water on the lungs was removed. The lungs were weighed (wet weight), and then placed into an incubator at 60 °C. The lungs were weighed once daily (dry weight) until the dry weight remained stable. The lung edema was evaluated by calculating the lung water content as follow: lung water content=[(wet weight–dry weight)/wet weight]×100%.

7. ELISA

Lung tissues were collected after three hyperbaric exposure. Tissue proteins were extracted using total protein extraction reagent (PMSF, protease inhibitor and phosphorylase inhibition were added to the protein lysate at 1:100). The protein concentration was determined by the BCA method. The contents of tumor necrosis factor-α (TNF-α) (Abcam, Cambridge, MA, USA), interleukin-10 (IL-10) (MultiSciences Biotech, Co., Hangzhou, China), intercellular cell adhesion molecule-1 (ICAM-1) (Elabscience Biotechnology, Wuhan, China) and monocyte chemoattractant protein-1 (MCP-1) (Jiancheng Bioengineering Institute, Nanjing,) were detected by using the commercial ELISA kits following the manufacturer's instructions. The absorbance at 450 nm was measured using a microplate reader (Synergy 2, BioTek Anaheim, CA, USA) and the level of each protein was calculated based on the standard curve.

8. Histological examination

Histological examination was performed as previously described (Li et al., 2017). Briefly, lung tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 4-μm sections. Lung sections were subjected to hematoxylin and eosin (H&E) staining. The edematous area was measured under a light microscope at a magnification of 100×, and 10 randomly selected fields were selected from each section. Histological scoring was scaled as described previously (Zhang et al., 2016). Briefly, score 0 represents normal histology; score 1 represents slight leukocyte infiltration and capillary congestion; score 2 represents mild leukocyte infiltration, perivascular edema, partial damage of pulmonary structures, and hemorrhage; score 3 represents intense leukocyte infiltration and destruction of pulmonary structures.

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9. Immunohistochemistry

After three hyperbaric exposures, lung tissues were collected and processed for immunohistochemistry with the following antibodies: F4/80 (Abcam, Cambridge, MA, USA ,1:1000), iNOS (Cell Signaling Technology, Danvers, MA, USA, 1:800), CD206 (Abcam, Cambridge, MA, USA, 1:1000). Immunohistochemistry was evaluated as reported previously (Ye et al., 2015). Briefly, the lung sections were de-paraffinized, re-hydrated and washed three times with PBS. Then sections were incubated with primary antibodies overnight at 4 °C and washed with PBS. Sections were incubated with secondary antibodies at 37 °C for 30 min and washed three times with PBS for 5 min. After being incubated with 0.05% (w/v) 3,3-diaminobenzidine tetrahydrochloride dehydrate, and counterstained with hematoxylin, and dehydrated, sections were mounted for microscopic examination (Carl Zeiss Jena, Oberkochen, Germany). Photographs were captured at a magnification of 100×, and positive cells were counted by two investigators in a blind manner. Positive cells were expressed as the percentage of positive cells per field.

10. Quantitative real-time polymerase chain reaction (RT-PCR)

After three hyperbaric exposures, lung tissues were collected, and total RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). The RNA concentration was measured with a microplate reader (Synergy 2, BioTek Anaheim). The cDNA of the sample was obtained using an mRNA reverse transcription reagent and the sample was subjected to real-time fluorescent quantitative PCR using SYBR Green Master mix (Roche, Basel, Switzerland). The primers (Sangon Biotech, Shanghai, China) used were as follows: TNF-α, 5′-CATCTTCTCAAAATTCGAGTGACAA-3′ (forward) and 5′-TGGGAGTAGAC AAGGTACAACCC-3′ (reverse); IL-10, 5′-CTTACTGACTGGCATGAGGATCA-3′ (forward) and 5′-GCAGCTCTAGGAGCATGTGG-3′ (reverse); iNOS, 5′-CCTTGGTGAAGGGACTGAGC-3′ (forward) and 5′-CAACGTTCTCCGTTCTCTTGC-3′ (reverse); and CD206, 5′-ACCTGGCAAGTATCCACAGC ATTG-3′ (forward) and 5′-TGTTGTTCTCATGGCTTGGCTCTC-3′ (reverse). Total RNA was reverse transcribed in a 50-μL mixture containing 2 μg of total RNA and Prime Script RT master mix (TaKaRa, Tokyo, Japan) at 25 °C for 10 min, 42 °C for 42 min, and 85 °C for 5 min. Real-time PCR was performed in triplicate using a real-time PCR system (Stepone Plus TM, Foster City, CA, USA): 94 °C for 3 min; 94 °C for 30 s; 68 °C for 40 s; 72 °C for 1 min; a total of 40 cycles. The mRNA expression of target genes was normalized to that of GAPDH as an internal reference.

11. Western blotting

After each hyperbaric exposure, lung tissues were harvested for the detection of F4/80, CD206 and iNOS protein expressions with antibodies against F4/80 (Abcam, 1:1000), iNOS (Cell Signaling Technology, 1:1000) and CD206 (Abcam, 1:1000). Western blotting was performed as reported previously (Ye et al., 2015). In brief, after extraction of total protein, the protein concentration was determined. Then, the proteins in each group were subjected to SDS-PAGE and subsequently transferred onto nitrocellulose filter membrane (NC) membranes (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The resulting membranes were blocked in 5% non-fat dry milk and incubated with specific primary antibodies over night at 4 °C. Subsequently, the membranes were washed and incubated with LI-COR IRDye 800CW goat anti-rabbit (Li-Cor Biosciences, Lincoln, NE, USA; 1:10000) and 680LT goat anti-mouse secondary antibodies (Li-Cor Biosciences, 1:10000) in TBS-T at room temperature in dark room for 60 min. Bands were visualized using Li-Cor odyssey scanner (Gene Company Limited, Shanghai, China) according to the manufacturer's instructions. The resulting bands were quantified using the Li-Cor odyssey Software (Li-Cor Biosciences). The protein expression was normalized to that of β-actin (Abcam, 1:1000) as a reference.

12. Statistical analysis

Quantitative data were expressed as mean±standard deviation (SD) if normal distribution was present. By means of one-way analysis of variance (ANOVA) followed by Tukey test, we performed statistical analysis. Unpaired Student *t* test was used where appropriate. Statistical analyses were performed using GraphPad Prism version 7.00 (La Jolla, CA, USA). A value of $P<0.05$ was considered statistically significant.

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