



## Lung macrophages are involved in lung injury secondary to repetitive diving<sup>\*#</sup>

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**Abstract:** This study aimed to establish an animal model of decompression-induced lung injury (DILI) secondary to repetitive diving in mice and explore the role of macrophages in DILI and the protective effects of high-concentration hydrogen (HCH) on DILI. Mice were divided into three groups: control group, DILI group, and HCH group. Mice were exposed to hyperbaric air at 600 kPa for 60 min once daily for consecutive 3 d and then experienced decompression. In HCH group, mice were administered with HCH (66.7% hydrogen and 33.3% oxygen) for 60 min after each hyperbaric exposure. Pulmonary function tests were done 6 h after decompression; the blood was harvested for cell counting; the lung tissues were harvested for the detection of inflammatory cytokines, hematoxylin and eosin (HE) staining, and immunohistochemistry; western blotting and polymerase chain reaction (PCR) were done for the detection of markers for M1 and M2 macrophages. Our results showed that bubbles formed after decompression and repeated hyperbaric exposures significantly reduced the total lung volume and functional residual volume. Moreover, repetitive diving dramatically increased proinflammatory factors and increased the markers of both M1 and M2 macrophages. HCH inhalation improved lung function to a certain extent, and significantly reduced the pro-inflammatory factors. These effects were related to the reduction of M1 macrophages as well as the increase in M2 macrophages. This study indicates that repetitive diving damages lung function and activates lung macrophages, resulting in lung inflammation. HCH inhalation after each diving may be a promising strategy for the prevention of DILI.

**Key words:** Repetitive diving; Decompression; Lung injury; Bubble; Macrophage; Inflammation  
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### 1 Introduction

Humans are exploring remote worlds through extensive underwater activities in unstable ambient

pressure, and the reduction of ambient pressure may induce the formation of bubbles in blood vessels and other tissues because of inert gas supersaturation (Vann et al., 2011). It has been confirmed that the bubbles are a major cause of decompression sickness (DCS) (van Liew and Flynn, 2005). However, generally, the amount of bubbles does not correlate directly with the clinical manifestations of DCS, and bubbles can cause damage to the human body without causing severe DCS (Dunford et al., 2002; Marinovic et al., 2010). Bubbles are more likely to form in the veins than in other sites where inert gas supersaturates. These bubbles forming in the vein are finally filtered by the lung through the pulmonary circulation. Thus,

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the lung is a target organ of bubbles formed in the blood and is susceptible to bubble-induced injury. Decompression-induced lung injury (DILI) has been reported in divers after repetitive diving without DCS (Ljubkovic et al., 2010). The pathogenesis of DILI is still poorly understood, and inflammation and oxidative stress induced by circulating bubbles may be the potential mechanisms. Macrophages belonging to the mononuclear phagocyte system (MPS) are an important participant in the innate immunity which is an effective immune response to general signs of infection. As a key cellular component of innate immunity, macrophages are a major player in the first-line defence against the pathogens and the modulation of homeostatic and inflammatory responses (Parisi et al., 2018). Macrophages are not homogenous, and they can be categorized into two subsets as the classically activated (M1) and the alternatively activated (M2). Pro-inflammatory stimuli may result in the shift to M1 cells, which are responsible for the clearance of infected or transformed cells, but simultaneously contribute to tissue injury. Conversely, anti-inflammatory signals may induce the formation of M2 cells, promoting tissue regeneration and wound healing (Liddiard and Taylor, 2015). This phenomenon, termed polarization, results from different microenvironments governing macrophage functionality within the hosting tissues. The polarization of macrophages has been found to be related to some diseases in humans (Aggarwal et al., 2014). Studies have shown that bubbles formed in the blood can induce the inflammatory responses, but the activation of macrophages following decompression is poorly understood (van Liew and Flynn, 2005). Our previous study showed that decompression could induce the activation of macrophages in the blood and lung as shown by the elevated production of factors related to macrophage activation and the increase in the proportion of M1 macrophages in the blood (Han et al., 2017). Hydrogen is the simplest molecule in nature. In recent years, hydrogen has been found to protect against various diseases, mainly by suppressing oxidative stress, inhibiting inflammation, and compromising cell apoptosis (Ohsawa et al., 2007; Sun et al., 2009; Abe et al., 2012). Our previous study has demonstrated that high-concentration hydrogen (HCH) gas (66.7% H<sub>2</sub> and 33.3% O<sub>2</sub>) can affect the polarization of macrophages in a stroke model (Ning et al., 2018). Whether hydrogen gas is

also protective on DILI via inhibiting macrophage polarization is unclear. This study aimed to establish a model of DILI secondary to repetitive diving in mice, investigate the lung macrophage polarization in DILI, and explore the protective effect of HCH on DILI.

## 2 Results

### 2.1 General condition and bubble detection

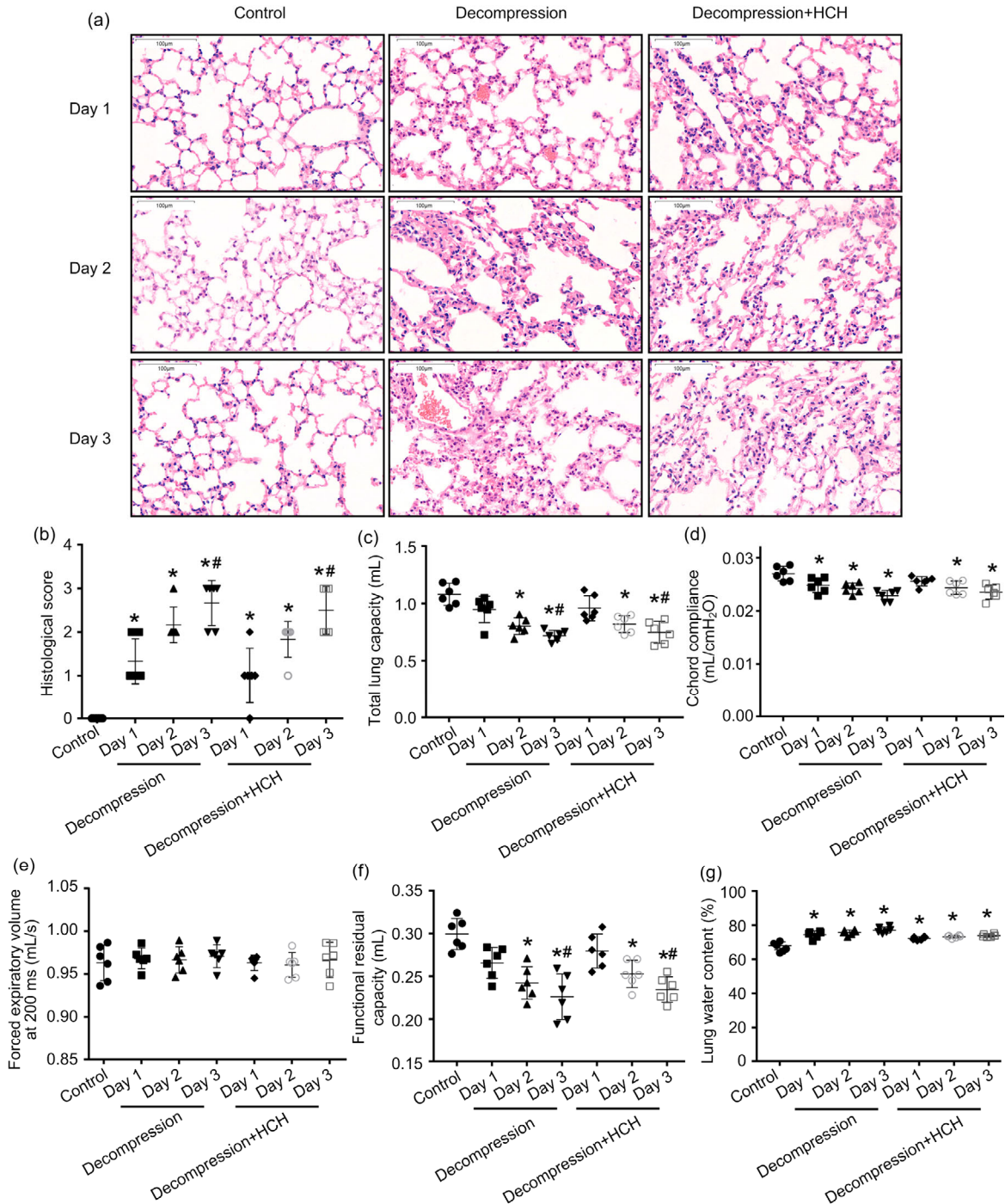
During hyperbaric exposure, no mice developed convulsion and none developed DCS-related symptoms (such as respiratory compromise and paralysis) after decompression. No mice died during the whole study. As shown in the Fig. S1, micro computed tomography (CT) showed that a bubble was found in right atrium after hyperbaric exposure, proving the availability of the decompression protocol.

### 2.2 Repetitive diving causes pathological lung damage

As shown in Figs. 1a and 1b, hematoxylin and eosin (HE) staining revealed no inflammatory exudation in the control group, but repetitive decompression caused damage to the lung tissues. This damage was characterized by structural disruption, edema, hemorrhage, and leukocyte infiltration on HE staining, and the histological scores significantly increased after decompression in three subgroups ( $P < 0.05$ , vs. control group). The histological features of lung injury were improved to a certain extent after HCH treatment, and the histological score in the HCH group was slightly reduced in Day 1, Day 2, and Day 3 three subgroups ( $P > 0.05$ , vs. decompression group). In addition, the histological score increased over time and a significant difference was observed between the Day 1 and Day 3 subgroups in both the decompression and HCH groups ( $P < 0.05$ ).

### 2.3 Repetitive diving compromises lung function

As shown in Figs. 1c–1f, the Cchord and FEV200 (forced expiratory volume at 200 ms) remained stable over time in the three groups, and there were no marked differences in the Cchord or FEV200 among the three subgroups ( $P > 0.05$ ). Total lung capacity (TLC) and functional residual capacity (FRC) remained stable in the control group, but were reduced over time in both the decompression and HCH groups, and significant



**Fig. 1 Organic and histological experiments**

Six hours after decompression or high-concentration hydrogen (HCH) treatment, mice were sacrificed for histological scoring (a, b), evaluation of pulmonary function (c–f), and measurement of lung water content (g). Repetitive decompression caused injuries including disruption of the structure, edema, hemorrhage, and leukocyte infiltration (a). The histological scores significantly increased after decompression (b). HCH treatment partially reversed the lung injury (a, b). The total lung capacity (TLC) and the functional residual capacity (FRC) decreased significantly over time in the decompression and decompression+HCH groups (c, f). However, the Cchord decreased slightly in all groups (d), and there was no change in the forced expiratory volume at 200 ms (FEV<sub>200</sub>) (e). HCH treatment slightly reversed the pulmonary function (c–f). In the presence of HCH treatment, the water content decreased slightly (g). Data are expressed as mean±standard deviation (SD), n=6. \* P<0.05, vs. control group; # P<0.05, vs. Day 1 subgroup. Scale bar=100 μm

differences were noted between the Day 1 and Day 3 subgroups in both the decompression and HCH groups. In addition, the TLC and FRC in the Day 2 and Day 3 subgroups were significantly reduced in the decompression and HCH groups as compared with the control group ( $P<0.05$ ). The TLC and FRC in the HCH group were slightly improved although there were no marked differences between the HCH and decompression groups ( $P>0.05$ ).

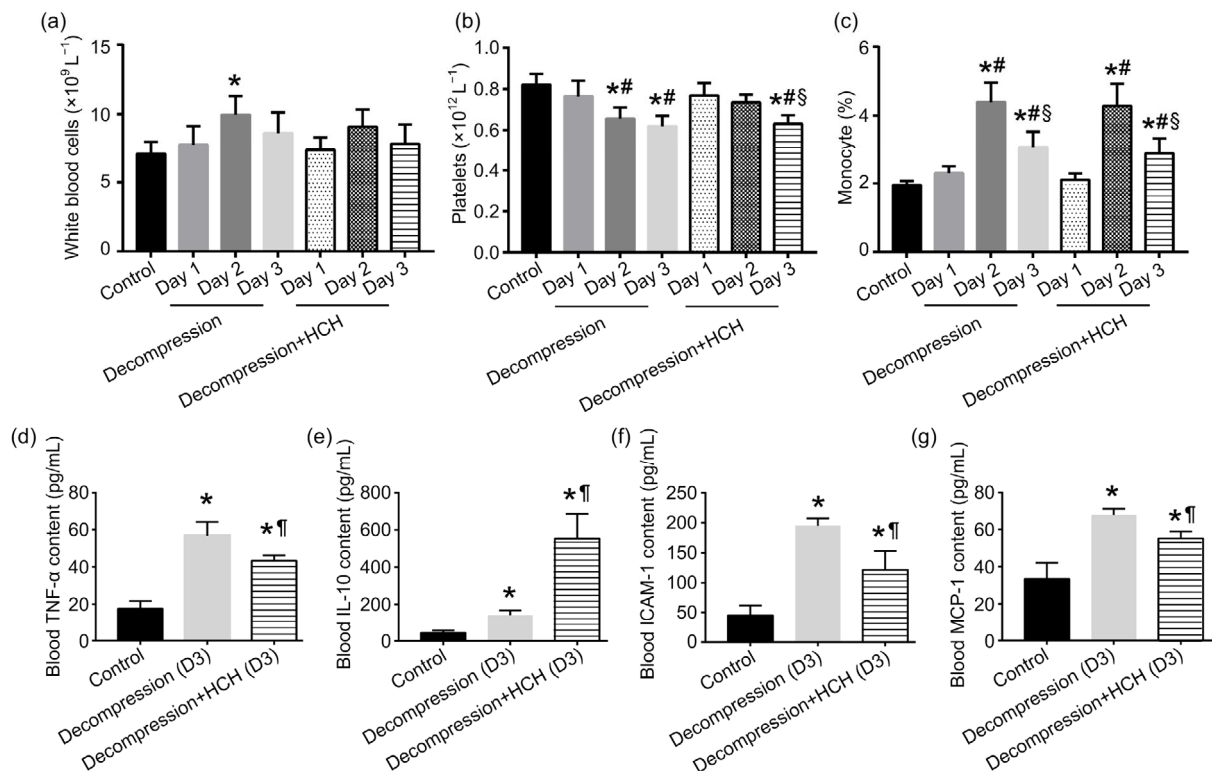
#### 2.4 Repetitive diving increases lung edema

As shown in Fig. 1g, the lung water content increased over time in both the decompression and HCH groups, but there was no significant difference among the three subgroups ( $P>0.05$ ). In addition, the lung water content in both the decompression and HCH groups was significantly higher than that in the control

group at three time points ( $P<0.05$ ). The lung water content was reduced in the HCH group as compared with the decompression group, although no significant difference was observed between them ( $P>0.05$ ).

#### 2.5 Repetitive diving increases blood and lung inflammation

As shown in Fig. 2, the white blood cell (WBC) count, platelet (PLT) count, and monocyte proportion were determined in the blood. Results showed that the WBC count and monocyte proportion increased after one hyperbaric exposure, peaked after two exposures, and thereafter decreased after three exposures. There was no significant difference in the WBC count among the three subgroups of both the decompression and HCH groups ( $P>0.05$ ), but a marked difference was noted in the monocyte proportion among the three

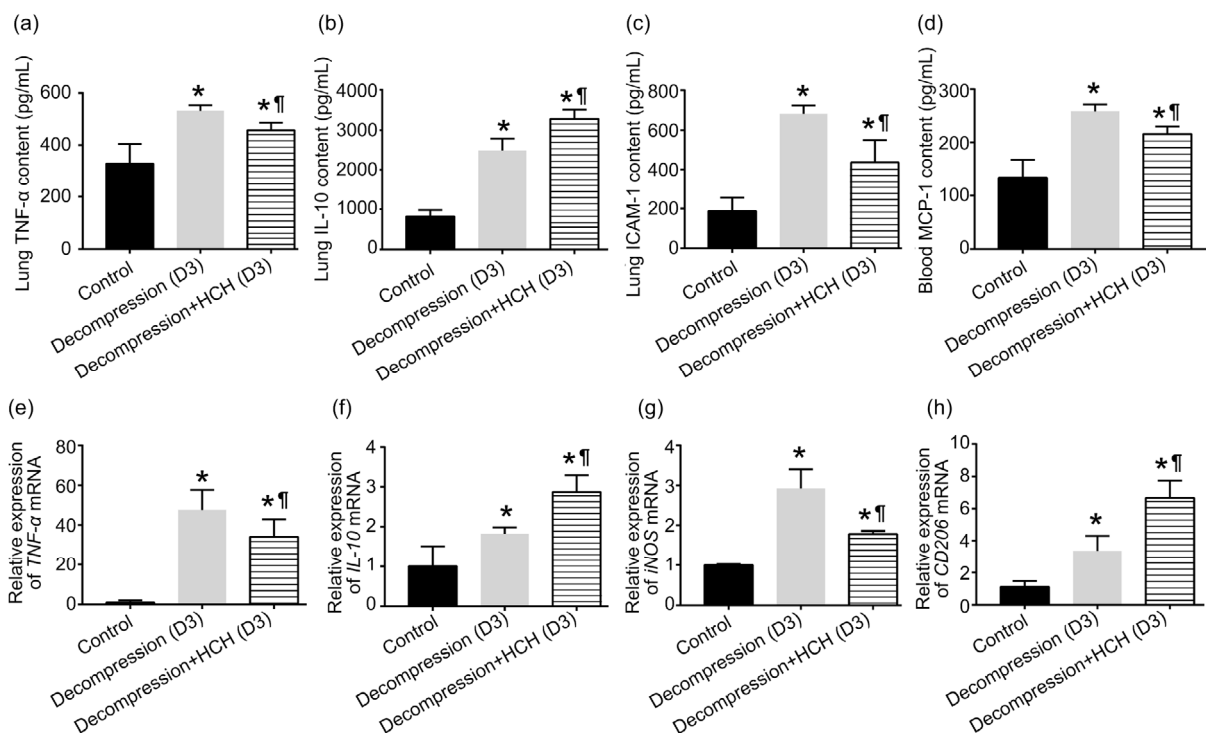


**Fig. 2 Complete blood counting, inflammation, and chemotaxis in the blood**

Six hours after decompression or high-concentration hydrogen (HCH) treatment, mice were sacrificed for blood collection. The amount of white blood cells (WBCs) increased after decompression or HCH treatment (a), and the platelet (PLT) count decreased over time (b). Moreover, the proportion of monocytes had the same trend as WBCs, it raised on Day 1 and Day 2, but decreased on Day 3 (D3) (c). Six hours after final decompression or final HCH treatment, animals were sacrificed for serum cytokine analysis (d-g). Decompression increased the levels of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (d), interleukin-10 (IL-10) (e), intercellular adhesion molecule-1 (ICAM-1) (f), and monocyte chemotactic protein-1 (MCP-1) (g) in the blood. HCH treatment reduced the levels of TNF- $\alpha$  (d), ICAM-1 (f), and MCP-1 (g) as compared with the decompression group, but the IL-10 level increased after HCH treatment (e). Data are expressed as mean $\pm$ standard deviation (SD),  $n=6$ . \*  $P<0.05$ , vs. control group; #  $P<0.05$ , vs. Day 1 subgroup; §  $P<0.05$ , vs. Day 2 subgroup; ¶  $P<0.05$ , vs. decompression group

subgroups in both the decompression and HCH groups. A significant difference in the WBC count was observed between the decompression and control groups only after two exposures. There was marked difference in the monocyte proportion among subgroups of both the decompression and control groups ( $P<0.05$ ). There were slight reductions in both WBC count and monocyte proportion in the HCH group as compared with the decompression group although marked differences were not observed ( $P>0.05$ ). The PLT counts in both the decompression and HCH groups decreased over time. In the decompression group, the PLT counts in Day 2 and Day 3 subgroups were markedly lower than that in the Day 1 subgroup ( $P<0.05$ ); in the HCH group, the PLT count in the Day 3 subgroup was significantly reduced as compared with Day 1 and Day 2 subgroups ( $P<0.05$ ). Of note, the PLT counts in three subgroups of both the decompression and HCH groups were significantly lower than that in the control

group ( $P<0.05$ ), but there was no marked difference between the HCH and decompression groups although it was slightly reduced in the HCH group ( $P>0.05$ ). Since significant difference was mostly observed after three exposures, the inflammation-related factors were detected in the lung and blood by polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA). Results showed the contents of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-10 (IL-10), intercellular adhesion molecule-1 (ICAM-1), and monocyte chemotactic protein-1 (MCP-1) in the blood and lung and the mRNA expression of lung TNF- $\alpha$  and IL-10 increased significantly after three hyperbaric exposures in the decompression group ( $P<0.05$ , vs. control group; Fig. 3). However, HCH treatment significantly reduced the contents of TNF- $\alpha$ , ICAM-1, and MCP-1 as well as the TNF- $\alpha$  mRNA expression, but further elevated the content and mRNA expression of IL-10 ( $P<0.05$ , vs. decompression group; Fig. 3).



**Fig. 3** Evaluating inflammation, chemotaxis, and polarization

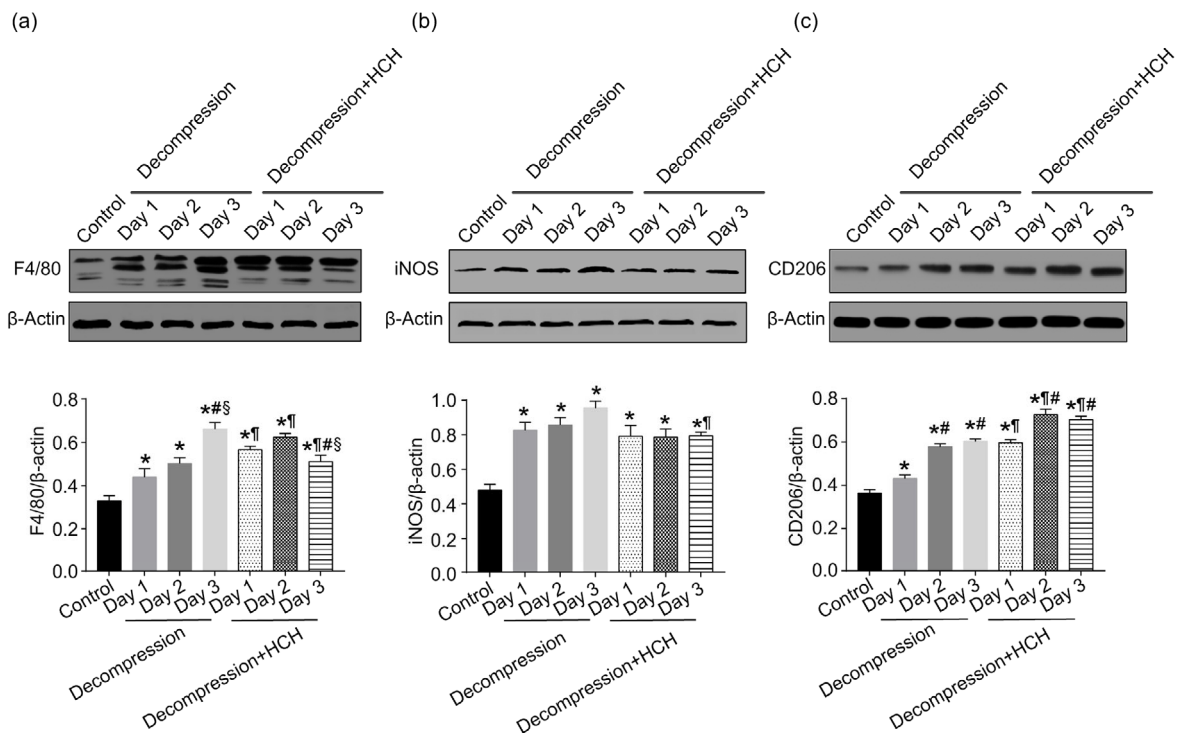
Enzyme-linked immunosorbent assay (ELISA) illustrated that decompression increased the levels of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (a), interleukin-10 (IL-10) (b), intercellular adhesion molecule-1 (ICAM-1) (c), and monocyte chemotactic protein-1 (MCP-1) (d) in the lung. High-concentration hydrogen (HCH) treatment reduced the levels of TNF- $\alpha$  (a), ICAM-1 (c), and MCP-1 (d), compared with the decompression group, but the IL-10 level increased after HCH treatment (b). Quantitative real-time polymerase chain reaction (qRT-PCR) showed that the mRNA expression of TNF- $\alpha$  (e), IL-10 (f), inducible nitric oxide synthase (*iNOS*) (g), and CD206 (h) increased after decompression. Compared with the decompression group, HCH treatment inhibited the mRNA expression of TNF- $\alpha$  (e) and *iNOS* (g), but promoted the mRNA expression of IL-10 (f) and CD206 (h). Data are expressed as mean $\pm$ standard deviation (SD),  $n=6$ . \* $P<0.05$ , vs. control group; \*\* $P<0.05$ , vs. decompression group. D3: Day 3



### 2.6 Repetitive diving regulates macrophage polarization

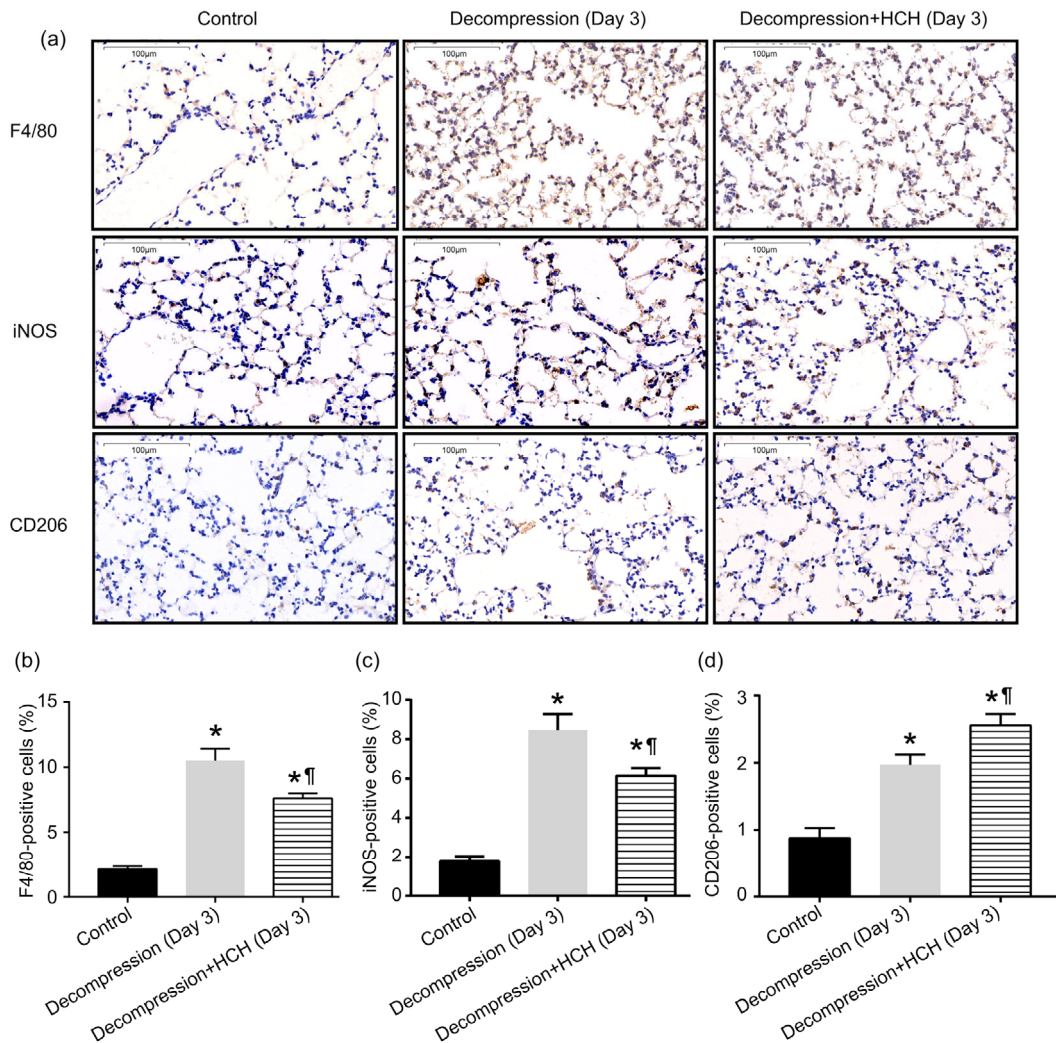
The protein expression levels of F4/80, CD206, and inducible nitric oxide synthase (iNOS) were detected in the lung. The F4/80 expression increased over time in the decompression group and was significantly higher than that in the control group at three subgroups. In the HCH group, the F4/80 expression was markedly higher than that in the control group; it increased after one exposure, peaked after two exposures, but decreased significantly after three exposures ( $P<0.05$ , vs. Day 2 and Day 1 subgroups; Fig. 4a). Of note, the F4/80 expression in the Day 3 subgroup of the HCH group was significantly lower than that in the corresponding subgroup of the decompression group ( $P<0.05$ ). The CD206 protein expression increased significantly after two hyperbaric exposures in both the decompression and HCH groups ( $P<0.05$ , Day 1 subgroup vs. Day 2 subgroup; Fig. 4c), but it slightly increased in the Day 3 subgroup of the decompression group and slightly decreased in the Day 3 subgroup of the HCH group ( $P>0.05$ ). Of note, the CD206

expression was markedly higher in both the decompression and HCH groups than in the control group ( $P<0.05$ ), and was markedly higher in the HCH group than in the decompression regardless of subgroups ( $P<0.05$ ). The iNOS expression in the decompression and HCH groups was significantly higher than that in the control group ( $P<0.05$ ), but it remained stable among subgroups and between the HCH group and the decompression group, although it was slightly lower in the HCH group than in the decompression group ( $P>0.05$ ; Fig. 4b). In addition, immunohistochemistry was done to detect the expression of F4/80, CD206, and iNOS after three hyperbaric exposures. Results showed that hyperbaric exposures significantly increased the expression levels of F4/80, CD206, and iNOS ( $P<0.05$ ), but HCH significantly reduced F4/80 and iNOS expression and dramatically increased CD206 ( $P<0.05$ ; Fig. 5). PCR further confirmed that both *iNOS* and *CD206* mRNA expression increased after hyperbaric exposure, but the *iNOS* mRNA expression decreased and the *CD206* mRNA expression increased after HCH ( $P<0.05$ ; Figs. 3g and 3h).



**Fig. 4 Protein expression of F4/80, iNOS, and CD206**

Six hours after decompression or high-concentration hydrogen (HCH) treatment, animals were sacrificed, and the lungs were collected for western blotting. The expression of F4/80, inducible nitric oxide synthase (iNOS), and CD206 in the lung increased after decompression (a–c) and HCH treatment inhibited the expression of F4/80 and iNOS (a, b) and increased that of CD206 (c) compared with the decompression group. Data are expressed as mean±standard deviation (SD),  $n=6$ . \*  $P<0.05$ , vs. control group; #  $P<0.05$ , vs. Day 1 subgroup; §  $P<0.05$ , vs. Day 2 subgroup; ¶  $P<0.05$ , vs. decompression group



**Fig. 5 Immunohistochemistry for F4/80, iNOS, and CD206**

Six hours after final decompression or final high-concentration hydrogen (HCH) treatment, animals were sacrificed, and the lungs were collected for immunohistochemistry (a). The positive rates of F4/80, inducible nitric oxide synthase (iNOS), and CD206 increased after decompression (b–d), and HCH treatment inhibited the positive rates of F4/80 (b) and iNOS (c), and increased that of CD206 (d) compared with the decompression group. Data are expressed as mean±standard deviation (SD),  $n=6$ . \* $P<0.05$ , vs. control group; † $P<0.05$ , vs. decompression group. Scale bar=100  $\mu\text{m}$

### 3 Discussion

A state of inert gas supersaturation will be achieved when the body is exposed to hyperbaric conditions. Inert gas bubbles may form in the blood vessels and tissues when the inert gas tension (concentration/solubility) exceeds the ambient pressure during the subsequent decompression. The bubbles formed in the body after decompression have been accepted as a major cause of DCS, although the amount of bubbles

does not correlate directly with the clinical manifestations of DCS (Bayne et al., 1985; Dunford et al., 2002). Increasing numbers of studies reveal that not only rapid decompression may cause bubble formation in the body, but a small number of bubbles may also be observed in the blood vessels after diving without protocol violation (Ljubkovic et al., 2011). Although clinical symptoms are not present in these divers, ultrasound examination shows repetitive regular diving can also cause acute interstitial lung edema,

affecting the lung and heart functions (Marinovic et al., 2010). In the present study, we established a mouse model of DILI using repetitive diving. Our results showed that repeated hyperbaric exposures caused significant damage to the lung. This damage was characterized by reduction in lung function, alteration of lung histology (structural disruption and infiltration of inflammatory cells) and lung edema. Of note, the Cchord and FEV200, two indicators reflecting pulmonary resistance, were little affected by repeated hyperbaric exposure, but TLC and FRC, two indicators reflecting lung volume, were reduced significantly after simulated repetitive diving. In addition, mice under this condition did not present with some manifestations of DCS (such as obvious shortness of breath, paralysis, and death). In addition, we investigated the circulating bubbles by micro CT, and bubbles were identified in the heart. These findings suggest the presence of DILI and indicate the successful establishment of a DILI model.

Bubbles formed in the blood may activate blood cells, especially WBCs and PLTs, exhibiting biological effects. The bubbles in the circulation may enter the pulmonary microcirculation, causing mechanical and biochemical effects resulting in inflammation following decompression. Thus, the lung has been regarded as a target organ of bubbles (Butler and Hills, 1979; Kondo et al., 2012). Our results showed that the WBC count and monocyte proportion increased after 1-d exposure and peaked after two hyperbaric exposures, but they thereafter decreased after 3-d exposure, which might be related to accommodation to the environment. However, the PLT count decreased with the increase in the number of hyperbaric exposures. This might be explained by the disruption of PLTs by the intravascular bubbles (Cronin et al., 2016). In addition, pro-inflammatory cytokines in the lung and blood increased following repeated hyperbaric exposure, and this seemed to be related to the number of hyperbaric exposures. In addition, the IL-10 as an anti-inflammatory cytokine also increased markedly after repeated hyperbaric exposure, which may be ascribed to a response to decompression stress. These findings confirm that bubbles induced inflammation in the blood and lung even though DCS is not present.

Recent study also revealed that immune cells in the lung are sensitive to the mechanical change of cyclical force, leading to immunological responses,

especially innate immunity (Solis et al., 2019). Macrophages derived from MPS are an important participant in the inflammatory reaction. It has been confirmed that macrophages can be polarized to classically activated macrophages or M1 cells, which participate in the clearance of either infected or transformed cells and simultaneously contribute to tissue destruction, or to alternatively activated macrophages or M2 cells, which promote tissue regeneration and wound healing (Mills and Ley, 2014). Factors including interferon- $\gamma$  (IFN- $\gamma$ )/lipopolysaccharide (LPS), TNF- $\alpha$ , and others may induce the production of M1 macrophages, which may produce reactive oxygen species (ROS), reactive nitrogen species (RNS), TNF- $\alpha$ , IL-1, IL-12, IL-23, and other chemokines. iNOS is a marker of M1 macrophages. Conversely, anti-inflammatory signals, such as IL-4, IL-13, IL-10, and transforming growth factor- $\beta$  (TGF- $\beta$ ), may induce the production of M2 macrophages, and then these cells can secrete TGF- $\beta$ , vascular endothelial growth factor (VEGF), epithelial growth factor (EGF), and other growth factors. CD206 and arginase-1 (Arg-1) have been proposed as markers of M2 macrophages. Whether decompression could cause lung inflammation and whether macrophages were involved in decompression-induced lung inflammation were poorly understood. Thus, we further investigated the status of lung macrophages following repeated hyperbaric exposures. Results showed that the expression of F4/80, iNOS, and CD206 increased with the increase in the number of hyperbaric exposures. This suggests that decompression can increase both M1 and M2 macrophages, and the increase in total macrophages (F4/80 expression) was mainly attributed to the elevated M1 macrophages (iNOS expression) because the fold change in iNOS expression was significantly larger than that in CD206 expression (M2 macrophages).

Hydrogen is the smallest molecule in nature. In recent years, a variety of studies have confirmed protective effects on organs in some animal models and clinical trials (Ohsawa et al., 2007; Sun et al., 2009; Abe et al., 2012). These are ascribed to its anti-oxidative, anti-inflammatory, anti-apoptotic, and other activities. Since DILI is closely related to inflammation, whether hydrogen gas may exert protective effects on DILI is unclear. In a majority of studies, the concentration of hydrogen gas was lower than 4% because of safety concerns (Ono et al., 2017). In our previous study,



results showed a mixture containing 66.7% hydrogen and 33.3% oxygen produced by water electrolysis via a specifically designed machine could also exert protective effects without safety concerns (Li et al., 2017). In this study, our results further indicated that HCH was also protective against DILI. Although lung function, lung edema, WBC count, or monocyte proportion was not significantly improved after HCH treatment, they tended to improve, which might be ascribed to the small sample size and/or the mild to moderate inflammation in the lung after repetitive diving. Of note, the inflammation-related factors dramatically decreased after HCH treatment. In addition, HCH treatment also reduced the expression of F4/80 and iNOS, but increased CD206 expression. This indicates that HCH can increase M2 macrophages and reduce M1 macrophages to exert anti-inflammatory effects on DILI.

There were several novelties in this study. First, our study for the first time investigated the DILI in a mouse model established by simulated repetitive diving as in a real situation. Generally, larger animals have poorer tolerance to decompression, and mice seem to be resistant to the lung injury secondary to rapid decompression (Fahlman, 2017), which might be ascribed to their rapid metabolism and rapid circulation. In our previous study, mice experienced rapid decompression once to induce lung injury, but there was no marked difference in lung edema (Han et al., 2017). Thus, in this study, repeated hyperbaric exposure was employed to induce lung injury. Second, we evaluated the lung function of mice after simulated repetitive diving, and for the first time revealed that repeated hyperbaric exposure could cause damage to the FRC and TLC. FRC refers to the volume of air in the lungs after a normal relaxed expiration. Physiologically, FRC is very important because it keeps the small airways open and can prevent the complete emptying of the lungs during each respiratory cycle (Selvi et al., 2013). The decreases in FRC are primarily due to decreases in the outward pull of the chest wall and often predispose patients to atelectasis. Our results suggest that repeated hyperbaric exposure may damage the pulmoalveolar surfactant, reducing lung expansion and increasing the likelihood of atelectasis. Third, we further investigated lung macrophages in the mouse model of DILI and explored the protective effects of HCH. Our results showed that repeated hyperbaric exposures increased

the M1 macrophages in the lung. This is consistent with lung inflammation after simulated repetitive diving. Of note, repeated hyperbaric exposure also increased the M2 macrophages, which might be ascribed to a response to bubble-induced inflammation in the lung. Moreover, HCH, as a non-toxic gas, not only inhibited inflammation (reductions in M1 macrophages and pro-inflammatory cytokines), but also increased anti-inflammation (increases in M2 macrophages and anti-inflammatory IL-10). These findings were similar to those reported in our previous study (Ning et al., 2018). It has been reported that hydrogen possesses some advantages in the treatment of diseases (it is sufficiently mild, may not disturb metabolic redox reaction, has favorable distribution characteristics, can diffuse into cellular components, and may metabolize to non-toxic water) (Ohsawa et al., 2007). Moreover, the electrolysis of water to produce hydrogen via a specifically designed machine has no safety concerns, and administration of hydrogen mixture is easy and convenient. Thus, we speculate that inhalation hydrogen gas is promising for use in divers.

This study had several limitations. First, whether repeated hyperbaric exposure causes the long-term adverse effects on lung function was not investigated. Second, bubble-induced inflammation or macrophage activation was not studied in depth. It was reported that cyclical hydrostatic pressure was able to mechanically activate the ion channel, conferring force sensitivity to cells and organisms by allowing the passage of ions across the membrane (Solis et al., 2019), and this will be investigated in our future studies. In addition, the oxygen concentration was at a higher than normal level in the hydrogen mixture produced by the electrolysis of water. Although our previous study showed 66.7% nitrogen and 33.3% oxygen had no significant influence, the effects of elevated oxygen concentration should be confirmed by investigation (Huang et al., 2018).

Overall, this study indicates that repetitive hyperbaric exposure causes an adverse effect on lung function, and that is at least partly related to bubble-induced pulmonary inflammation. Intrapulmonary macrophages are involved in the inflammatory response to simulated repetitive diving, and HCH inhalation after each hyperbaric exposure is helpful for the improvement of pulmonary inflammation and potentially the recovery of lung function.

## Materials and methods

Detailed methods are provided in the electronic supplementary materials of this paper.

## Contributors

Xue-iun SUN and Wen-wu LIU designed the study, wrote and edited the manuscript. Ke NING and Zhen-biao GUAN performed the establishment of animal models and contributed to the study design, data analysis, writing and editing of the manuscript. Hong-tao LU and Ning ZHANG contributed to the study design and data analysis. All authors have read and approved the final manuscript and, therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

## Compliance with ethics guidelines

Ke NING, Zhen-biao GUAN, Hong-tao LU, Ning ZHANG, Xue-jun SUN, and Wen-wu LIU declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed. The study was approved by the Ethics Committee of the Navy Medical University, Shanghai, China (approval No. 20170236).

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## List of electronic supplementary materials

Materials and methods

Fig. S1 Micro computed tomography scan for bubbles

## 中文概要

**题目:** 巨噬细胞参与反复潜水引起的肺损伤

**目的:** 探索反复潜水引起肺损伤的炎症机制以及吸入高浓度氢气 (HCH) 对这种损伤的治疗作用。

**创新点:** 本研究首次在小鼠体内建立并评估减压诱导肺损伤 (DILI) 模型; 首次探索巨噬细胞在 DILI 中的作用; 首次探索呼吸 HCH 对于 DILI 的治疗作用。

**方法:** 将雄性 C57 小鼠随机分为对照组、DILI 组和 HCH 组。DILI 组于 600 kPa 压力下暴露 60 min, 连续 3 d。HCH 组在减压处理后吸入 HCH (66.7% H<sub>2</sub>+33.3% O<sub>2</sub>) 干预。减压操作 6 h 后检测小鼠肺功能和小鼠肺干湿比, 取小鼠肺组织固定进行苏木精-伊红染色, 并取小鼠全血进行血细胞计数实验。取小鼠肺组织提取蛋白并提取血清, 采用酶联免疫吸附测定 (ELISA) 检测炎症因子与趋化因子, 并使用蛋白质免疫印迹 (western blotting) 试验测定小鼠肺内小鼠含生长因子样模体粘液样激素样受体 (F4/80)、巨噬细胞甘露糖受体 (CD206) 和诱导型一氧化氮合酶 (iNOS) 的表达量。使用免疫组化检测小鼠肺组织切片内 F4/80、CD206 和 iNOS 的阳性细胞的比例。提取小鼠肺组织内总信使核糖核酸 (mRNA), 使用荧光实时定量聚合酶链反应测定极化标记蛋白 CD206 和 iNOS 以及炎症因子 *TNF-α* 和 *IL-10* 的基因表达量。

**结论:** 多次减压可导致肺水肿、组织结构破坏和肺功能下降, 病变程度和减压次数有关, 证明模型建立成功。DILI 可以诱导肺内和循环炎症反应的激活, 巨噬细胞可能向肺内迁移趋化并向不同亚型极化, 极化后的巨噬细胞 M1 与 M2 分别参与炎症激活与炎症抑制的过程。吸入 HCH 可以显著改善小鼠肺损伤, 降低肺内炎症反应, 抑制巨噬细胞向 M1 亚型极化并促进其向 M2 亚型极化, 从而证明吸入 HCH 对于 DILI 具有治疗作用。

**关键词:** 反复潜水; 减压; 肺损伤; 气泡; 巨噬细胞; 炎症反应