Polarization of macrophages in the blood after decompression in mice

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Abstract

The veins are a major site of bubble formation after decompression and the lung is a target organ of bubbles. Bubble-induced inflammation has been implicated in the pathogenesis of decompression sickness (DCS). Macrophages play a central role in the inflammation, and macrophage polarization is closely related to the pathogenesis of some lung diseases. This study aimed to investigate the blood macrophage polarization in mice after decompression. BALB/c mice were exposed to hyperbaric air for 60 minutes, and rapid decompression was performed to induce DCS. Slow decompression and hyperoxia (150 kPa, 60 minutes) served as control groups, and hyperbaric oxygen (HBO; 250 kPa, 60 minutes) was employed for DCS treatment. Macrophage phenotype was determined by flow cytometry, and cytokines related to macrophage polarization were measured by enzyme-linked immunosorbent assay. Our results showed rapid decompression significantly induced the shift to M1 phenotype, which was not observed in slow decompression group, HBO and hyperoxia groups. These changes were consistent with the change in blood tumor necrosis factor α level. Moreover, any treatment could significantly increase the M2 macrophages, but blood interleukin-10 remained unchanged after different treatments. In addition, the blood and lung levels of monocyte chemoattractant protein-1 and intercellular adhesion molecule-1 increased significantly after rapid decompression, but reduced markedly after HBO treatment. Taken together, rapid decompression is able to induce the shift to M1 phenotype in blood macrophages, which may then migrate into the lung involving decompression-induced lung injury.

Key words: hyperbaric air; macrophage polarization; decompression sickness; hyperoxia; blood; inflammation; bubble; mouse

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Introduction

The exposure to hyperbaric conditions may incur an inert gas load, and a state of inert gas supersaturation will be achieved in the blood and tissues when the inert gas tension (concentration/solubility) exceeds the ambient pressure during the subsequent decpmpression, and inert gas bubbles may form under this condition. The bubbles forming in the blood vessels and tissues are a major cause of decompression sickness (DCS), although it does not correlate directly with clinical manifestations.[1,2]

Of all the sites where bubbles form from supersaturated dissolved gas, bubbles most easily form in the veins. Bubbles forming in the veins may exert mechanical and biochemical effects, directly or indirectly activating leukocytes in the blood and subsequently resulting in inflammation.[3,4] Macrophages derived from mononuclear phagocytes (MP) are an important participant in the inflammatory reaction. Under the influence of the different microenvironments, the migrated monocytes give rise to a variety of MP subtypes, including mucosal macrophages, dendritic cells, and tissue-associated Langherans cells of skin, perivascular macrophages, Kupffer cells of liver, and brain microglial cells.[5] Macrophages may be activated along two main functional pathways. Pro-inflammatory stimuli result in classically

activated macrophages or M1-cells, which participate in the clearance of either infected or transformed cells, but simultaneously contribute to tissue destruction. Conversely, anti-inflammatory signals induce alternatively activated or M2-macrophages that will activate cellular programs, promoting tissue regeneration and wound healing. This is also known as the macrophage polarization.[6]

This study aimed to investigate the blood macrophage polarization in mice with DCS.

Materials and Methods

Animals

A total of 72 male BALB/c mice weighing 18.1 ± 3.2 g were purchased from the Experimental Animal Center of the Second Military Medical University, China and house in an environment with a 12/12 light-dark cycle and given *ad libitum* access to water and food. This study protocol was approved by the Ethics Committee of the Second Military Medical University, Shanghai, China (No. 20160161).

Grouping and hyperbaric exposure

Mice were randomly divided into five groups (n = 12 per group): normal control group, rapid decompression (rDec) group, slow decompression (sDec) group, hyperoxia group and treatment group. In the normal control group, mice were placed in a chamber without pressurization; in the rDec group, mice were exposed to hyperbaric air at 700 kPa for 60 minutes and then decompressed to 100 kPa within 1 minute; in the sDec group, sDec was performed according to the animal air decompression table; in the hyperoxia group, mice were exposed to oxygen (98.3%) at 150 kPa for 60 minutes; in the treatment group, mice were treated with pure oxygen at 250 kPa for 60 minutes immediately after rDec.

Blood collection and lymphocyte separation

After decompression, mice were intraperitoneally anesthetized with 10% chloral hydrate at 0.3 mL/100 g. Then, blood samples were collected from the eyes within 30 minutes, anti-coagulated with heparin and processed for lymphocyte separation by density gradient centrifugation with lymphocyte separation medium (Dakewei Biotech Co., Ltd., Shenzhen, China).

Flow cytometry

Lymphocytes were harvested and incubated with CD16/32 (Biolegend Biotech Co., Ltd., San Diego, CA, USA) on ice for 10 min. Then, cells were divided into two parts: the first part was treated with PE-CD11c, APC-CD11b and FITC-F4/80 (Biolegend Biotech Co., Ltd.); the second part was treated with APC-CD11b and FITC-F4/80. Cells in the first part were washed and subjected to flow cytometry. Cells in the second part were fixed in fixation buffer in dark at room temperature for 20 minutes, and then centrifuged at 350 × g for 5 minutes. Cells were harvested and treated in permeability buffer in dark at room temperature for 10 minutes. After washing twice in 2% bovine serum albumin, cells were incubated in dark with PE-CD206 (Biolegend Biotech Co., Ltd.) for 20 minutes at room temperature. After washing in 2% bovine serum albumin, cells were harvested for flow cytometry (B&D Biosciences, San Jose, CA, USA).

Collection of the lung and blood samples

After different treatments, mice were intraperitoneally anesthetized with 10% chloral hydrate at 0.3 mL/100 g. Then, blood samples were collected from the eyes and the lungs was collected (one was used for enzyme-linked immunosorbent assay (ELISA) and the other for lung edema detection).

ELISA

Lungs were collected as above mentioned and stored at –80°C for use. Lungs were homogenized in lysis buffer (Beyoutime Biotech Co., Ltd., Nantong, Jiangsu Province, China) and total protein was extracted. ELISA was performed for the detection of blood tumor necrosis factor (TNF)-α, blood interleukin-10 (IL-10), monocyte chemoattractant protein-1 (MCP-1) and intercellular adhesion molecule-1 (ICAM-1) in the lung and blood.

Measurement of lung water content

Lungs were collected as abovementioned, 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%.

Statistical analysis

Statistical analysis was performed with SigmaPlot 11.0 (Systat Software, Inc., CA, USA). Data are expressed as the mean ± standard deviation (SD). Comparisons were performed with one way analysis of variance (ANOVA) among groups, followed by Student-Newman-Keuls method. A value of $P < 0.05$ was considered statistically significant.

Results

Lung water content

The lung water content was 78.15 ± 0.35% in the normal control group, 80.84 ± 0.65% in the rDec group, 79.12 ± 0.34% in the sDec group, 79.56 ± 0.42% in the treatment group and 78.25 ± 0.47% in the hyperoxia group. The lung

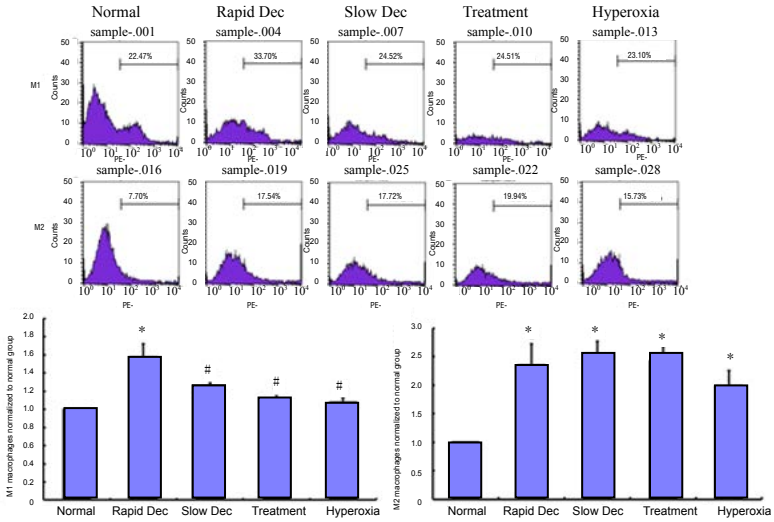

Figure 1: Proportion of M1 macrophages and M2 macrophages in the blood after different treatments.

Note: Normal control group: Without pressurization; rapid Dec group: exposed to hyperbaric air at 700 kPa for 60 minutes and then decompressed to 100 kPa within 1 minute; slow Dec group: slow Dec was performed according to the animal air Dec table; hyperoxia group: exposed to oxygen (98.3%) at 150 kPa for 60 minutes; treatment group: treated with pure oxygen at 250 kPa for 60 minutes immediately after rapid Dec. The data were normalized to the normal control group, and expressed as the mean ± standard deviation (SD), and analysed by one way analysis of variance followed by Student-Newman-Keuls method. *P < 0.05, *vs*. the normal control group; #P < 0.05, *vs*. rapid Dec group. Dec: Decompression.

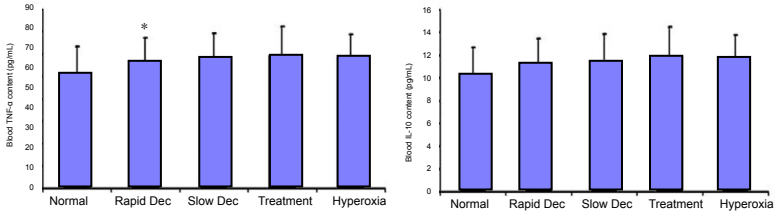

Figure 2: Blood tumor necrosis factor α (TNF-α) and interleukin-10 (IL-10) contents in the blood of rats different groups (enzyme-linked immunosorbent assay).

Note: Normal control group: Without pressurization; rapid Dec group: exposed to hyperbaric air at 700 kPa for 60 minutes and then decompressed to 100 kPa within 1 minute; slow Dec group: slow Dec was performed according to the animal air Dec table; hyperoxia group: exposed to oxygen (98.3%) at 150 kPa for 60 minutes; treatment group: treated with pure oxygen at 250 kPa for 60 minutes immediately after rapid Dec. The data were expressed as the mean ± standard deviation (SD), and analysed by one way analysis of variance followed by Student-Newman-Keuls method.*P < 0.05, *vs*. normal control group. Dec: Decompression.

water content in the rDec group was significantly higher than in the normal control group ($P < 0.05$), and the lung water content in the sDec group and treatment group was only slightly higher than that in the normal control group ($P > 0.05$). The lung water content was comparable between the normal control group and hyperoxia group ($P > 0.05$).

Macrophage phenotype

In the rDec group, the proportion of M1 macrophages increased significantly when compared with the normal control group ($P < 0.05$), but it reduced dramatically in the sDec and treatment groups ($P < 0.05$, *vs*. the rDec group). Moreover, hyperoxia exposure slightly increased the proportion of M1 macrophages ($P > 0.05$, *vs*. the normal control group), and there was no significant difference between the sDec group and treatment group ($P > 0.05$).

The proportion of M2 macrophages increased markedly in the rDec group, sDec group, treatment group and hyperoxia group ($P < 0.05$, *vs*. the normal control group). However, it was comparable among the former four groups ($P > 0.05$; **Figure 1**).

Blood and lung cytokines

The blood TNF-α in the rDec group was significantly higher than in the normal control group ($P < 0.05$), but it in the sDec group and treatment group was lower than that in the rDec group even though significant difference was not observed. There was no significant difference between the sDec group and treatment group ($P > 0.05$). In addition, the blood TNF-α in the hyperoxia group was also slightly higher than that in the normal control group ($P > 0.05$; **Figure 2**).

The blood IL-10 in the rDec group, sDec, treatment group and hyperoxia group was slightly higher than that in the normal control group ($P > 0.05$), and there was no significant difference among the former four groups ($P > 0.05$; **Figure 2**).

The blood and lung MCP-1 contents increased dramatically after rDec as compared to the normal control group ($P < 0.05$), but they in the sDec group and treatment group were significantly lower than those in the rDec group ($P < 0.05$). In addition, the blood and lung MCP-1 in the treatment group were still higher than those in the sDec

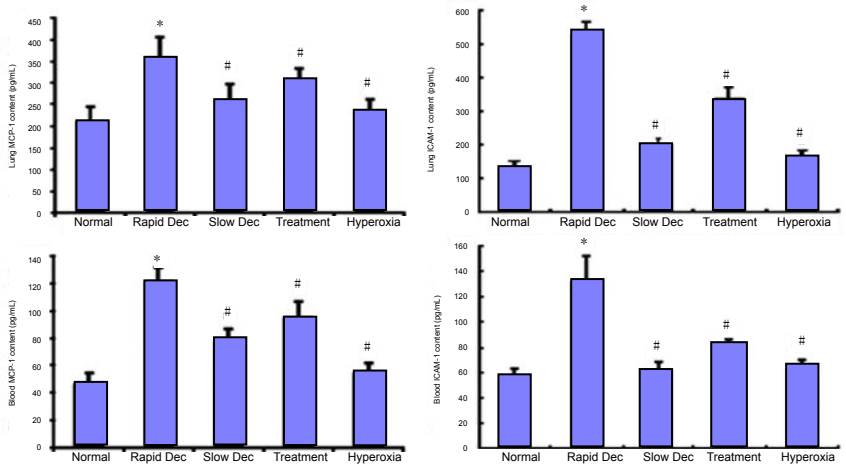

Figure 3: Contents of monocyte chemoattractant protein-1 (MCP-1) and intercellular adhesion molecule-1 (ICAM-1) in the lung and blood of different groups (enzyme-linked immunosorbent assay).

Note: Normal control group: Without pressurization; rapid Dec group: exposed to hyperbaric air at 700 kPa for 60 minutes and then decompressed to 100 kPa within 1 minute; slow Dec group: slow Dec was performed according to the animal air Dec table; hyperoxia group: exposed to oxygen (98.3%) at 150 kPa for 60 minutes; treatment group: treated with pure oxygen at 250 kPa for 60 minutes immediately after rapid Dec. The data were expressed as the mean ± standard deviation (SD), and analysed by one way analysis of variance followed by Student-Newman-Keuls method. $^{*}P < 0.05$, *vs.* the normal control group; $^{\#}P < 0.05$, *vs.* rapid Dec group. Dec: Decompression.

group (**Figure 3**). The blood and lung MCP-1 content in the hyperoxia group was slightly higher than that in the normal control group ($P > 0.05$). The changes in lung and blood ICAM-1 were similar to those in MCP-1 (**Figure 3**).

Discussion

In this study, we investigated the blood macrophage polarization in mice after different treatments. Our results showed the M1 macrophages and M2 macrophages increased significantly after rDec, and treatment with hyperbaric oxygen (HBO) was able to reduce M1 macrophages, but had no influence on M2 macrophages. In addition, hyperoxia exposure failed to markedly increase M1 macrophages, but significantly increased M2 macrophages in the blood of mice.

These results suggest that rDec may increase pro-inflammatory macrophages, which is also confirmed that decompression induced inflammation is involved in the pathogenesis of DCS. Under hyperbaric air, the oxygen partial pressure is 147 kPa. To exclude the influence of hyperoxia on the shift to M1 phenotype, mice was exposed to pure oxygen at 150 kPa for 60 minutes. Results showed this failed to significantly increase M1 macrophages, which implies that the shift to M1 phenotype is related to the rDec or precisely to the bubbles formed in the blood vessels. This is further confirmed by the fact that the proportion of M1 macrophages remains unchanged after sDec. HBO has been accepted as an effective therapy for DCS and may exert protective effects on DCS *via* different mechanisms: it can directly increase the oxygen content of ischemic sites after bubble embolism; it can reduce the bubble volume/number; it can increase anti-inflammatory cytokines; it can induce the expression of anti-oxidases. Herein, we further evaluated the influence of HBO on the macrophage phenotype in DCS mice. Results showed HBO at 250 kPa significantly reduced pro-inflammatory macrophages, indicating the anti-inflammatory effects of HBO.[7,8]

However, either rDec or sDec with hyperoxia exposure could increase M2 macrophages significantly. The hyperbaric air exposure (rDec group and sDec group) and hyperoxia exposure shared elevated oxygen partial pressure, which might increase in M2 macrophages. Of note, HBO therapy following DCS failed to further increase or decrease M2 macrophages. Thus, the therapeutic effects of HBO might have no relationship with the increase in anti-inflammatory macrophages. Of note, as shown in **Figure 1**, the absolute proportion of M1 phenotype macrophages was significantly higher than that of M2 phenotype macrophages. There might be two factors related to this phenomenon: i) there was inflammation in the mice before exposure; ii) the binding activity of antibody used for M2 detection was poorer than that of antibody used for M1 detection. More investigations are needed to elucidate this issue in our future studies. Thus, in this study, we used the relative proportion after normalization to normal control group.

We further detected the blood cytokines related to macrophage polarization. TNF-α is an important cytokine related to the shift to M1 phenotype, and IL-10 is an anti-inflammatory cytokine and related to the shift to M2 phenotype. Our results showed blood TNF-α increased significantly after rDec, but remained unchanged in the sDec group. In addition, HBO therapy reduced blood TNF-α, and hyperoxia had little influence on the blood TNF-α. These findings further confirm above findings that rDec (but not hyperoxia) induces the shift to M1 phenotype and HBO may inhibit the shift to M1 phenotype in case of DCS. However, the blood IL-10 level remained stable after rDec or sDec, and HBO or hyperoxia exposure had no influence on the blood IL-10 level. Maybe other anti-inflammatory cytokines contribute to the induction of M2 phenotype under this condition.

The lung is a target organ of DCS.[9] With the development of technique for ultrasound detection,[10] increasing studies reveal that not only rDec, but also decompression without protocol violation may cause venous gas embolism, resulting in lung injury.[11,12] Our study also confirmed that lung edema was present following DCS. Moreover, HBO therapy significantly improved the lung edema. MCP-1 is one of the

key chemokines that regulate the migration and infiltration of monocytes/macrophages.[13] Our results showed the content of MCP-1 in the blood and lung increased significantly after rDec, which was reduced dramatically after HBO therapy. ICAM-1 is an important adhesion molecule and mediates the adhesion between leukocytes and endothelial cells.[14] Our results showed the changes in the lung and blood ICAM-1 were similar to those in MCP-1. As mentioned above, the lung is a target organ of bubbles formed in the veins, and it is also exposed to hyperoxia during hyperbaric air exposure. Thus, the lung is susceptible to damage during DCS. The elevated MCP-1 and ICAM-1 in the blood and lung may induce the chemotaxis of blood macrophages to the lung, further deteriorating lung inflammation.

Taken together, rDec is able to induce the shift to M1 phenotype, which may be related to the bubbles formed in the blood because M1 macrophages slightly increase after sDec and HBO therapy reduces them significantly, and these macrophages may migrate into the lung due to the elevated MCP-1 and ICAM-1 in the blood and lung, leading to the deterioration of lung injury in case of DCS. In addition, hyperoxic environment seems to induce the shift to M2 phenotype in blood. Of note, whether bubbles directly or indirectly induce the shift of macrophage phenotypes is unclear. There is evidence showing that the production of microparticles is elevated following diving.[3,15,16] Thom et al.[16] found that microparticles could initiate decompression-induced neutrophil activation, and our group also showed bubbles could induce the production of microparticles in endothelial cells *in vitro*.[17] Whether microparticles may further induce the shift of macrophage phenotype will be validated in our future studies.

Author contributions
WWL designed this study and drafted the paper; CHH and PXZ conducted the experiment; WGX, RPL, and JJX provided advice on the experiment. All the authors approved the final version of the manuscript.
Conflicts of interest
There was no conflict of interest in this study.
Research ethics
The study protocol was approved by the Ethics Committee of the Second Military Medical University at Shanghai, China.
Data sharing statement
The datasets analyzed during the current study are available from the corresponding author on reasonable request.
Plagiarism check
Checked twice by iThenticate.
Peer review
Externally peer reviewed.

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