1,25-Dihydroxyvitamin D_3 Ameliorates High-Altitude Hypoxic Injury via Modulation of Short-Chain Fatty Acids to Exert a Coordinated Anti-Inflammatory Effect

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Summary

High-altitude hypoxic injury remains a major health threat with limited prophylactic options. This study evaluated the protective effects of 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) against acute high-altitude sickness using parallel human and animal investigations. In the clinical trial, 48 volunteers ascending to 4,200 m received either weight-stratified 1,25-$(OH)_2D_3$ (0.102-0.135 μg/kg/day, n=23) or placebo (n=25) for 5 days. In the animal study, 40 SD rats were exposed to 6,000 m (hypobaric hypoxia) for 48 h with or without 1,25-$(OH)_2D_3$ pretreatment. Results showed that 1,25-$(OH)_2D_3$ significantly reduced Lake Louise scores, alleviated headache, improved oxygen saturation and haemodynamics, suppressed serum pro-inflammatory cytokines (IL-6, IL-1β) and inflammatory cell counts, and attenuated multi-organ (lung, liver, kidney) hypoxic injury. In rats, 1,25-$(OH)_2D_3$ also restored Th1/Th2 immune balance, reduced oxidative stress, and down-regulated four short-chain fatty acids ((R)-3-hydroxyisobutyric acid, (S)-2-hydroxybutyric acid, 2-hydroxybutyric acid, β-hydroxy-isobutyric acid) involved in butyrate metabolism. No toxic effects were observed in normoxic controls. Collectively, 1,25-$(OH)_2D_3$ effectively prevents high-altitude hypoxic injury by suppressing inflammation, restoring immune homeostasis, and modulating short-chain fatty acid metabolism. Our findings support 1,25-$(OH)_2D_3$ as a safe and promising prophylactic agent against high-altitude sickness.

Key words

Hypoxic injury • 1,25-$(OH)_2D_3$ • Immune response • Short-chain fatty acids

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Introduction

High-altitude environments, characterized by hypobaric hypoxia, pose a significant health risk to the rapidly growing number of individuals who reside, work, or travel above 2,500 m – a population exceeding 140 million worldwide [1]. These conditions can trigger a spectrum of acute mountain sicknesses, including Acute Mountain Sickness (AMS), High-Altitude Pulmonary Edema (HAPE), and High-Altitude Cerebral Edema (HACE) [2,3], which collectively represent a serious threat to human health.

Currently, the clinical arsenal for preventing and treating high-altitude sickness is limited and unsatisfactory. The mainstays, hyperbaric oxygen therapy and a handful of chemical drugs, are hampered by

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suboptimal efficacy and significant safety concerns. For instance, acetazolamide can impair exercise tolerance and induce neurological side effects like fatigue and paresthesia [4,5]. Dexamethasone carries the risk of suppressing the hypothalamic-pituitary-adrenal axis and causing hyperglycemia, precluding its long-term or pediatric use [4]. Other agents, such as nifedipine and trimetazidine, offer only limited or symptomatic relief [6,7], while ibuprofen may exacerbate gastrointestinal bleeding under hypoxia [4]. Furthermore, the use of sildenafil for hypoxic pulmonary hypertension remains contentious [8], and bosentan may disrupt acclimatization by reducing diuresis, potentially aggravating AMS [4,9]. Consequently, there is a pressing, unmet need for the development of efficient and reliable prophylactic and therapeutic agents against high-altitude sickness.

Against this backdrop, 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), the most biologically active metabolite of vitamin D_3, emerges as a promising candidate. Beyond its classical role in calcium homeostasis, 1,25-$(OH)_2D_3$ exerts pleiotropic effects, including potent immunomodulatory and anti-inflammatory activities [10-12]. Our previous studies have substantiated its potential in high-altitude settings, demonstrating that it alleviates hypoxic pulmonary edema by inhibiting the complement/ coagulation cascade and modulating autophagy [13], prevents HAPE in rats *via* the B cell receptor/PI3K/AKT/NF-κB pathway [14,15], and protects against hypoxic lung injury [16].

However, critical gaps persist between these promising preclinical findings and clinical application. The clinical efficacy of 1,25-$(OH)_2D_3$ for high-altitude sickness in humans remains unvalidated. Moreover, a comprehensive understanding of its multi-organ protective mechanisms is lacking, and crucially, its role in orchestrating the crosstalk between immune regulation and metabolic homeostasis under hypoxic stress is entirely unexplored.

Based on this rationale, we propose a central hypothesis: 1,25-$(OH)_2D_3$ ameliorates high-altitude sickness by suppressing inflammatory responses, synchronously restoring immune balance, and rectifying metabolic homeostasis.

To test this hypothesis, we will employ an integrated approach combining synchronous human high-altitude field studies and controlled animal experiments. We aim to: 1) elucidate the clinical efficacy of 1,25-$(OH)_2D_3$ by monitoring relevant clinical indicators; 2) decipher its multi-organ protective mechanisms through molecular biological assays in hypoxia models; and 3) identify novel, immune-related metabolic regulators *via* serum metabolomics profiling. This study seeks to unravel the effective mechanisms of 1,25-$(OH)_2D_3$ and provide a robust scientific foundation for developing novel strategies to prevent and treat high-altitude sickness.

Materials and Methods

Research materials

Volunteers were recruited from Qinghai Provincial People's Hospital and Qinghai University between April and June 2022. The recruitment process included: (1) Posting research announcements; (2) Doctors screened qualified candidates according to exclusion/inclusion criteria; (3) Signing informed consent forms. Set specific exclusion and inclusion criteria and recruit 48 volunteers to conduct high-altitude field experiments. Inclusion criteria: (1) first-time travel or work in high-altitude areas; (2) From different plain areas or low altitude provinces (altitude 0-1,500 m); (3) The serum 25-(OH) D level was within the normal range (30-100 ng/ml); (4) Age under 45 years old, gender not limited. Exclusion criteria [17]: (1) History of organic cardiovascular disease, significant arrhythmia, or resting heart rate >100 beats/min; stage 2 hypertension or higher; (2) Chronic respiratory diseases or moderate-to-severe obstructive pulmonary disease; (3) Diabetes mellitus with poor glycemic control; (4) Active upper respiratory infection, acute tracheobronchitis, or pneumonia meeting either criterion: a) Body temperature ≥38 ℃, b) Temperature <38 ℃ with significant symptoms (cough, sputum production, or chest tightness); (5) Prior occurrence at moderate altitude (1,500-3,000 m) of altitude-related symptoms (headache, dizziness, chest tightness); (6) Pregnancy beyond first trimester (>12 gestational weeks); (7) Suffering from blood diseases such as anemia and polycythemia; (8) Being in menopause, with obvious symptoms such as chest tightness and shortness of breath, and excluding organic diseases; (9) Patients with hysteria, epilepsy, and mental illness; (10) Not cooperating with this researcher.

The 48 healthy volunteers who met the inclusion criteria were randomly divided into two groups, G1 group (n=23) and G2 group (n=25). Both groups of volunteers went to the plateau (Maduo County, 4,200 m) together. Before departure, the G1 group (n=23) received calcitriol capsules for drug intervention. The optimal drug dosage

was selected through animal experiments, which was 0.252 μg/kg [18]. The volunteers were divided into three groups according to their body weight: 40 kg, 50 kg, and 60 kg, and the corresponding dosages were calculated using the body surface area direct conversion method [19]. The calculated dosages were 0.102 μg/kg, 0.119 μg/kg, and 0.135 μg/kg, respectively, which were within the normal range of drug use. The G2 group was given an equal number of empty capsules for intervention. The drugs were administered once a day for five days. The clinical symptoms of the two groups of volunteers were observed before and after going to the high-altitude area for five days, and the Lake Louise Score was recorded. Blood gas indexes, blood routine indexes, blood biochemistry, urine routine, lung CT, inflammatory factors, and body metabolism-related hormone indexes were recorded separately through routine examination items in the local hospital. The data collected from G1 group and G2 group five days after entering the high-altitude area were recorded as the VDH5D group and H5D group, respectively.

All individuals had obtained written informed consent before participating in the study and had the right to withdraw at any time. All studies involving human volunteers were conducted in accordance with the World Medical Association‘s Declaration of Helsinki (2000) and followed the rules of the Declaration of Helsinki (1975, revised in 2013) (https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki/).

In order to eliminate the interference factors to the largest extent, after the start of the experiment, we made unified arrangements for all volunteers' daily activities, diet, accommodation and transportation. In particular, we strictly controlled the sunshine time and vitamin D intake (daily diet).

Experimental animals and grouping

Forty Sprague-Dawley (SD) rats, weighing 180-220 g, were purchased from the Laboratory Animal Center of Beijing Weitong Lihua Experimental Animal Technology Co., Ltd. License No. SCXK (Beijing) 2021-0011. They were randomly divided into four groups, with 10 rats in each group. The blank control group (CV, n=10, 2,200 m), the blank control + 1,25-$(OH)_2D_3$ group (NV, n=10, 2,200 m), the high-altitude low-oxygen group (HV, n=10, 6,000 m), and the high-altitude low-oxygen + 1,25-$(OH)_2D_3$ group (MV, n=10, 6,000 m). The blank control group was housed in the animal room of Qinghai University (2,200 m). Calcitriol capsules [Zhengda Pharmaceutical (Qingdao) Co., Ltd (HaiDeWei)] were dissolved in tea oil to form a mixture (1:10). The blank control + 1,25-$(OH)_2D_3$ group (n=10) and the high-altitude low-oxygen +1,25-$(OH)_2D_3$ group (n=10) of SD rats were intraperitoneally injected with the calcitriol mixture, while the remaining two groups were injected with the same dosage of tea oil, once a day for five consecutive days. The high-altitude low-oxygen group and the high-altitude low-oxygen +1,25-$(OH)_2D_3$ group were placed in a low-pressure oxygen chamber at Qinghai University Medical College to simulate a low-oxygen environment at 6,000 m for 48 h. After completing hypoxia stress, rats were anesthetized by intraperitoneal injection of 30 mg/kg pentobarbital sodium. Collect blood samples from the abdominal aorta of rats and use a blood gas analyzer to detect arterial blood gas indicators. Take the venous blood centrifugation supernatant, lung, liver, kidney tissue, and store all samples in liquid nitrogen. All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (1985), National Institutes of Health (NIH), Bethesda, USA.

Collection of Lake Louise Score (LLS) from volunteers

After entering the high-altitude low-oxygen area, Lake Louise Score self-assessment forms were distributed to the two groups of volunteers. In the Lake Louise scoring system, self-reported scores are categorized as mild (3-5 points), moderate (6-9 points), or severe (10-12 points). An LLS score of 3 or higher, accompanied by headache symptoms, clinically indicates AMS, while an LLS score below 3 indicates no AMS [20].

Blood routine, blood biochemistry, and urine routine tests of volunteers

Before and after entering the high-altitude area, fasting venous blood samples were collected from the two groups of volunteers in the early morning for blood routine and blood biochemistry tests, and urine samples were collected for urine routine tests. The above three tests were conducted at Qinghai Provincial People's Hospital before entering the high-altitude area, and at Maduo County People's Hospital in Qinghai Province 5 days after entering the high-altitude area (the normal range of test indicators in the two locations is consistent).

Hematoxylin and eosin (H&E) staining

After anesthesia, lung, liver, and kidney tissues

were collected from rats(Organizational size: 5 cm × 5 cm × 5 cm). The tissues were fixed in 4 % paraformaldehyde and dehydrated using a dehydrator (KD-TS3A). The tissues were then embedded in paraffin using a paraffin embedding system (KD-BM) and a freezing table (KD-BM). The tissue blocks were sliced using a Leica microtome (Leica RM2265), floated on a water bath (HH-3), and picked up on glass slides. The slides were dried using a dryer (KD-H) and then stained. Deparaffinization was performed using xylene, graded ethanol, and absolute ethanol. Hematoxylin staining was performed, followed by water rinsing and bluing with tap water. Eosin staining was performed, and dehydration was performed using graded ethanol and absolute ethanol. Xylene was used for transparency, and neutral resin was used for mounting. After drying, the slides were observed under a microscope. The changes in the structure of alveoli and alveolar septa in lung tissue, hepatocytes and lobules in liver tissue, glomeruli and renal tubules in kidney tissue, as well as the degree of infiltration of red blood cells and inflammatory cells in the three tissues were mainly observed. Histopathological evaluation was performed by two independent pathologists using comparative assessment under light microscopy (RX50 Biological microscope). Key pathological features including inflammatory infiltration, vascular congestion, edema, and cellular degeneration were identified by direct comparison between experimental groups and blank controls. Consensus was required for final determination of alterations.

ELISA detection of Superoxide Dismutase (SOD), Glutathione Peroxidase (GSH-Px) and Malondialdehyde (MDA) levels in rat lung tissue

The lung tissue of rats was weighed and tissue samples were lysed using ice-cold RIPA lysis buffer (Beyotime Biotechnology, Cat# P0013B) containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1 % Triton X-100, 1 % sodium deoxycholate, 0.1 % SDS, and protease/phosphatase inhibitors including sodium orthovanadate, sodium fluoride, EDTA, and leupeptin. Following complete lysis, homogenization was performed at 4 ℃ using a Precellys 24 biological homogenizer (Bertin Instruments, France) with parameters set to 6,000 rpm for 3 cycles (30 s per cycle with 60-second intervals). The homogenate was centrifuged at 12,000× g for 15 min at 4 ℃, and supernatants were collected for ELISA assays. Using ELISA kits (Shanghai Enzyme-linked Biotechnology Co., Ltd) to determine the SOD activity, GSH-Px activity, and MDA content in the lung tissue. Standard curve wells and detection wells were established. The optical density was measured at 450 nm using an ELISA reader, and the SOD activity, GSH-Px activity, and MDA concentration were calculated using the standard curve.

Blood routine, blood biochemistry, and urine routine tests in SD rats

Blood samples were collected from each group of SD rats. The blood samples were tested using a veterinary automatic blood cell analyzer (BC-2800 Veterinary, Shenzhen Mindray Animal Medical Technology Co., Ltd.) to measure relevant blood cell parameters, including white blood cells (WBC), neutrophils (Neu), lymphocytes (Lym), monocytes (Mon), eosinophils (Eos), and basophils (Bas). SD rat serum samples were tested using an automatic biochemical analyzer (Xisen Meikang Sysmex fully automatic biochemical analyzer BX-3010) to measure the levels of albumin (ALB), total bilirubin (TBIL), blood urea nitrogen (BUN), creatinine (CRE), uric acid (UA). Urine samples from SD rats were tested using a URIT-1500 automatic urine analyzer (Shanghai Jumu Medical Equipment Co., Ltd) to measure the urinary protein levels.

Flow cytometry

Heparin sodium tube was used to collect 200-500 μl of blood from the abdominal aorta of SD rats. The blood was then mixed gently with 3-5 times the volume of red blood cell lysis buffer. After incubating at room temperature for 10-15 min, the cells were collected by centrifugation at 4 °C, 450× g for 5 min. The supernatant was discarded and the cells were resuspended in 1 ml of PBS (0.01 M phosphate, pH 7.4, containing 137 mM NaCl). The cells were then incubated with 1 μl of CD3 and CD4 flow cytometry antibodies in a flow cytometry tube for 20 min in the dark. After centrifugation and discarding the supernatant, the cells were treated with 1 ml of 80 % methanol for 5 min and then with 1 ml of 0.1 % PBS-Tween for 20 min. After centrifugation and discarding the supernatant, IL-4 and IFN-γ flow cytometry antibodies were added to the cells and incubated for 20 min in the dark. Finally, the cells were analyzed using a flow cytometer (Shenzhen Weigong Technology).

Serum metabolomics analysis of SD rats

Sample (100 µl) was placed in an EP tube and resuspended with pre-cooled 80 % methanol by vortexing. The sample was then incubated on ice for 5 min and centrifuged at 15,000× g, 4 °C for 20 min. Partial supernatant was diluted with LC-MS grade water to a final concentration containing 53 % methanol. The sample was then transferred to a fresh Eppendorf tube and centrifuged at 15,000× g, 4 °C for 20 min. Metabolomics analysis was performed using liquid chromatography-mass spectrometry (LC-MS) based on the highly sensitive SCIEX QTRAP® 6500+ mass spectrometry platform for targeted metabolomics. The experimental process mainly included sample collection, metabolite extraction, mass spectrometry detection, and data analysis. The SCIEX OS V1.4 software was used to open the mass spectrometry files (.wiff) and perform peak integration and calibration. The peak area (Area) of each chromatographic peak represents the relative quantification value of the corresponding substance. Finally, all integrated peak area data were exported. Quality control (QC) samples were used to ensure the accuracy and reliability of the data results (this work was completed by Beijing Nuohui Zhiyuan Technology Co., Ltd.). Multivariate statistical analysis, including principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA), was performed on the metabolites to reveal differences in metabolic patterns between different groups. Hierarchical clustering analysis (HCA) and metabolite correlation analysis were used to reveal the relationships between samples and between metabolites. Finally, functional analysis, such as metabolic pathway analysis, was used to interpret the biological significance of metabolite-related results.

Statistical analysis

Baseline data and LLS were analyzed using SPSS 28.0 software and Kolmogorov-Smirnov test and Levene were used to test the normality and homogeneity of variance of the data, respectively. If the data before and after the same group experiment conform to normality, paired *t*-test is used for comparison, independent sample *t*-test is used for comparison between two groups, and rank sum test is used for non normality. The remaining human experiment related data were analyzed using GraphPad Prism 9 (La Jolla, San Diego, CA, USA), and Kolmogorov-Smirnov test and F-test were used to test the normality and homogeneity of variance of the data, respectively. If the data before and after the same group experiment were found to be normal, paired *t*-test was used, independent sample *t*-test was used for comparison between the two groups, and rank sum test was used for non normality. The normality of rat experimental data was tested using Kolmogorov-Smirnov test. If the data conforms to normality, ANOVA one-way square check analysis method was used. Otherwise, Kruskal Wallis test method was used. All experimental results are expressed as mean ± standard deviation (Mean ± SD). $P<0.05$ indicates significant differences, while $P<0.01$ indicates extremely significant differences in the data.

Results

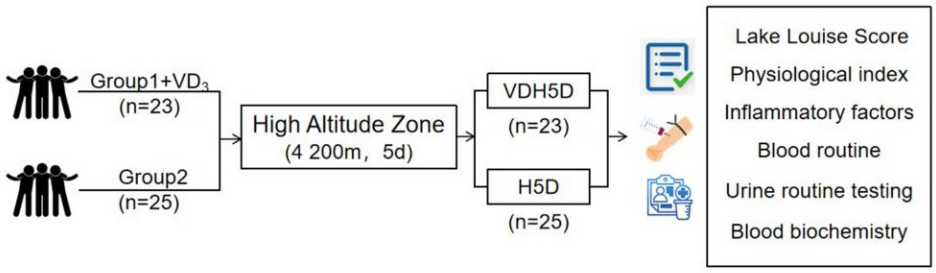
1,25-$(OH)_2D_3$ effectively prevents the occurrence of high-altitude illness in rapid ascent to high altitude individuals

1,25-$(OH)_2D_3$ improves adverse reactions to high altitude in the human body

Figure 1A shows the process of recruiting two groups of volunteers to conduct the experiment in this study. Comparison of baseline data between the two groups of healthy young volunteers showed no significant differences, and both groups had no underlying diseases (Table 1). According to LLS screening for AMS, diagnostic criteria include rapidly ascending to high-altitude areas (>2,500 m), Lake Louise AMS score total of 3 or higher indicates AMS, including at least one point from headache in the setting of a recent ascentor gain in altitude. As shown in Figure 1B, on the first day in the high-altitude area, the Lake Louise Score for the H5D group volunteers was 2.72±0.94, significantly higher than the VDH5D group's score of 1.74±0.72 ($P<0.05$), but neither group's score exceeded 3 points, so it could not be diagnosed as AMS. On the fifth day in the high-altitude area, the H5D group volunteers had a score of 3.40±1.08, exceeding 3, and 21 people had headache reactions, which could be diagnosed as AMS. This score was significantly higher than the VDH5D group's score of 2.09±0.1 ($P<0.05$), with 5 people having headache reactions (Fig. 1B).

Compared with groups G1 and G2, both the VDH5D and H5D groups showed a significant decrease in blood oxygen saturation ($P<0.05$), and an increase in heart rate, systolic blood pressure, and diastolic blood pressure ($P<0.05$). However, the changes in these four indicators were more pronounced in the H5D group (Fig 1C).

A

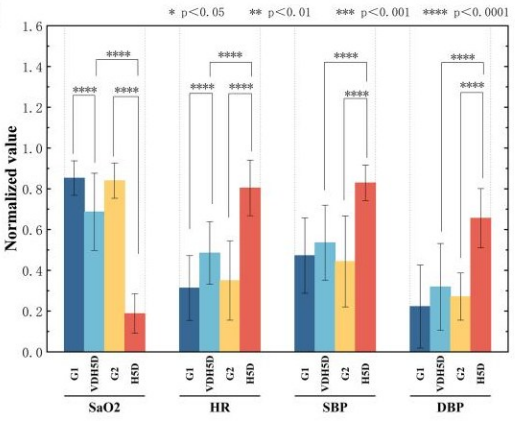


B

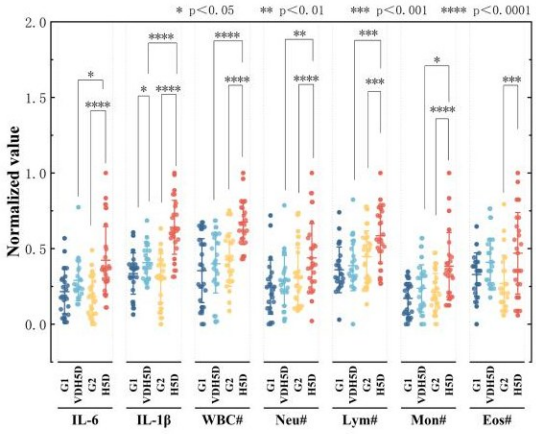
Lake Louise Score

	Day1		Day5	
	VDH5D (n=23)	H5D (n=25)	VDH5D(n=23)	H5D (n=25)
Headache/example	8 (34.8%)	18 (72%)	5 (21.7%)	21 (84%)
Lake Louise Score≥3 /example	4 (17.4%)	14 (56%)	8 (34.8%)	19 (76%)
Louis Lake Score (Mean±SD)	1.74±0.75	2.72±0.94**	2.09±0.10	3.40±1.08##

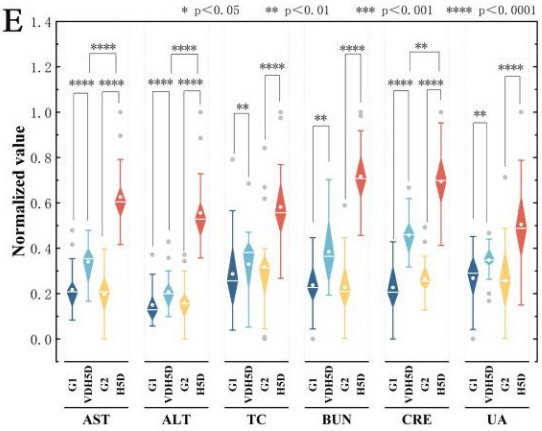
C



D



E

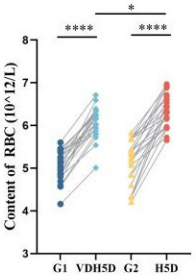


F

Urinary Routine Protein Indicators

	G1	VDH5D	G2	H5D
Negative (-)	22 (100.0%)	19 (82.6%)	24 (100.0%)	10 (40.0%)
Weakly postive (±)	0	3 (13.0%)	0	9 (36.0%)
Postive (+)	0	1 (4.4%)	0	6 (24.0%)
total	23	23	25	25

G



H

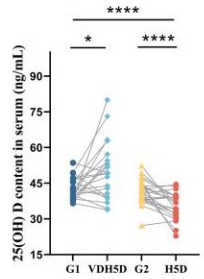


Fig. 1. Vitamin D_3 reduces hypoxic damage in volunteers entering high-altitude hypoxic areas. (**A**) Study flowchart. (**B**) Lake Louise Score of two groups of volunteers on the 1st and 5th days of entering high-altitude areas. * indicates comparison with the VDH5D group on the 1st day, * P<0.05, ** P<0.01; # indicates comparison with the VDH5D group on the 5th day, # P<0.05, ## P<0.01. (**C**) Changes in physiological indicators of the two groups of volunteers before and after 5 days of entering high-altitude areas. (**D**) Changes in inflammatory factors and inflammatory cells of the two groups of volunteers before and after 5 days of entering high-altitude areas. (**E**) Changes in blood biochemical indicators of the two groups of volunteers before and after 5 days of entering high-altitude areas. (**F**) Changes in routine urine protein indicators of the two groups of volunteers on the 1st and 5th days of entering high-altitude areas (-) represents negative (normal), (±) represents weak positive, (+) represents positive. (**G**) Red blood cell count. (**H**) Changes in serum 25-(OH)D levels of the two groups of volunteers before and after 5 days of entering high-altitude areas. G1: data of the group administered with 1,25-$(OH)_2D_3$ before entering high-altitude areas; VDH5D: data of the group administered with 1,25-$(OH)_2D_3$ after 5 days in high-altitude areas; G2: data of the non-medicated group before entering high-altitude areas; H5D: data of the non-medicated group after 5 days in high-altitude areas, * P<0.05, ** P<0.01, **** P<0.001.

Table 1. Baseline characteristics comparison.

Variables	Group 1 (n=23)	Group 2 (n=25)	P
Gender (male/female, n)	14/9	13/12	-
Age (years)	30.00 ± 8.60	30.28 ± 5.21	0.3526
Body mass index (kg/m^2)	22.28 ±1.81	23.26 ± 2.59	0.5912
Heart rate	71.26 ± 3.18	72.00 ± 3.89	0.4488
Systolic blood pressure (mmHg)	120.40 ± 4.07	119.80 ± 4.91	0.9491
Diastolic blood pressure (mmHg)	74.00 ± 3.67	74.88 ± 2.09	0.1952
Pulse blood oxygen Saturation (%)	94.52 ± 0.85	94.40 ± 0.87	0.6286
Underlying disease	No	No	-

1,25-$(OH)_2D_3$ inhibits the increase of red blood cells, inflammatory factors and inflammatory cells induced by hypoxia

Compared with groups G1 and G2, the VDH5D and H5D groups showed an increase in the levels of IL-6 and IL-1β in the serum, but the increase was more pronounced in the H5D group. As follows, and there is almost no difference in the number of red blood cells between the G1 group and the G2 group. Compared with G1 and G2 groups, the red blood cell count in VDH5D and H5D groups significantly increased, but the red blood cell count in H5D group was significantly higher than that in VDH5D group (Fig. 1G). The changes in the numbers of inflammatory cells in the blood routine were similar. Compared with groups G1 and G2, both the VDH5D and H5D groups showed an increase in the numbers of inflammatory cells, but the extent of increase was lower in the VDH5D group (Fig. 1D).

1,25-$(OH)_2D_3$ improves hypoxic liver injury in humans

As shown in Figure 1E, after 5 days of exposure to high-altitude, low-oxygen environment, both the VDH5D and H5D groups showed an increase in serum ALT and AST levels. However, the extent of increase in the VDH5D group was significantly lower compared to the H5D group. As shown in Figure 1H, there was no significant difference in serum 25-(OH)D levels between groups G1 and G2, and both were within the normal range (30-100 ng/ml). In contrast to groups G1 and G2, the VDH5D group showed a slight increase in serum 25-(OH)D levels, while the H5D group exhibited a decreasing trend in serum 25-(OH)D levels.

1,25-$(OH)_2D_3$ ameliorates hypoxic renal injury

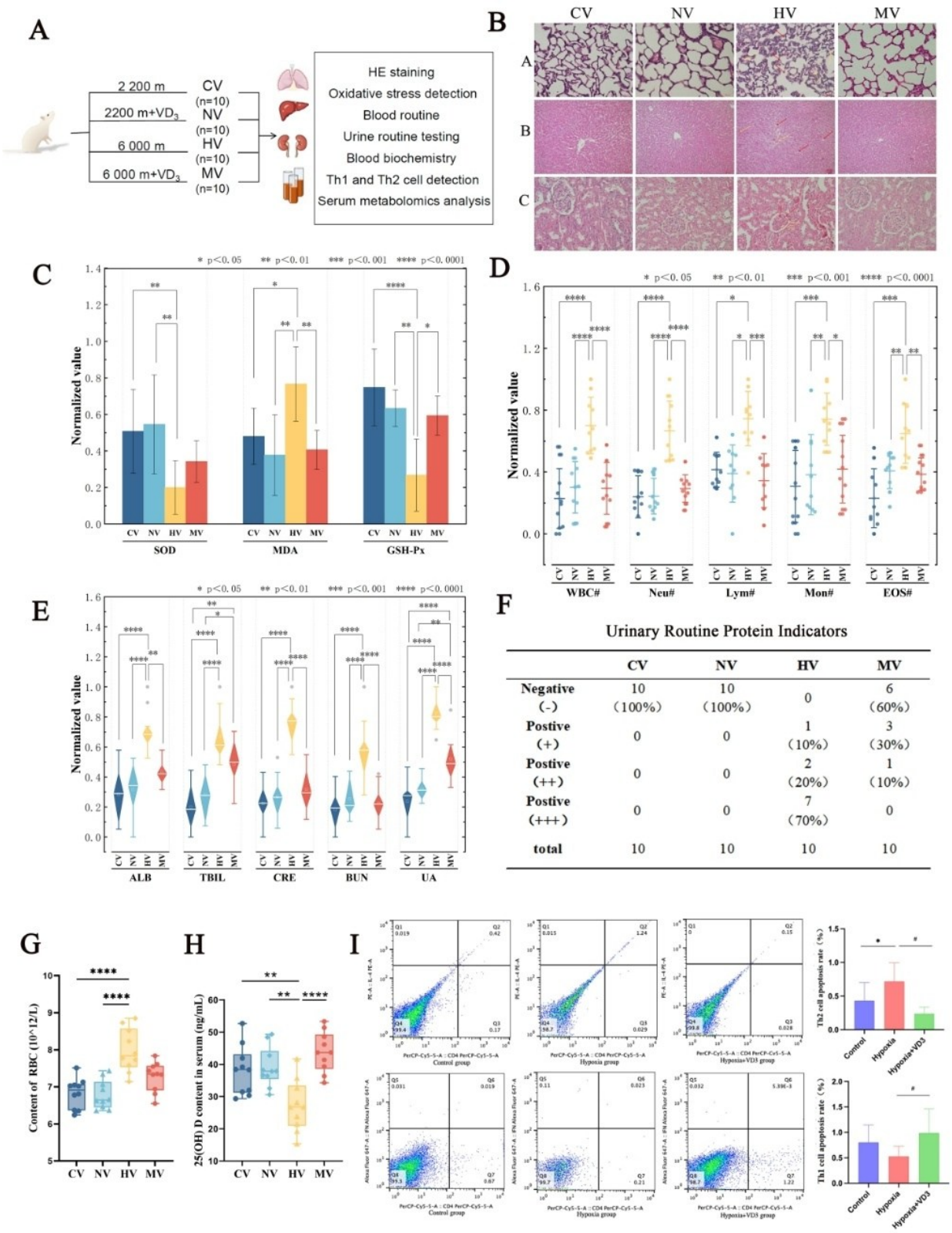
As shown in Figure 1E, compared to Group G1 and G2, the levels of CRE, BUN, and UA in the serum of volunteers in the VDH5D and H5D groups were significantly elevated ($P<0.05$). However, the elevation in the VDH5D group was significantly less than that in the H5D group, with the CRE levels in the VDH5D group being significantly lower than in the H5D group ($P<0.05$). As depicted in Figure 1F, both Group G1 and G2 exhibited negative urine protein results. Following exposure to high-altitude hypoxia, 6 cases (24.0 %) in the H5D group were positive for urine protein, 9 cases (36.0 %) were weakly positive, and 10 cases (40.0 %) were negative. In the VDH5D group, 1 case (4.4 %) was positive, 3 cases (13.0 %) were weakly positive, and 19 cases (82.6 %) were negative. The positive rate of urine protein in the H5D group after exposure to high-altitude hypoxia was significantly higher than in the VDH5D group.

In summary, rapid ascent to high-altitude hypoxic regions can lead to numerous adverse reactions in humans. Hypoxia results in a reduction of oxygen inhaled into the lungs, decreased saturation of blood oxygen, reduced oxygen utilization efficiency in blood circulation, and elevated heart rate and blood pressure, which further cause hypoxic injuries to multiple organs such as the lungs, liver, and kidneys. Observation of the aforementioned phenotypes indicates that 1,25-$(OH)_2D_3$ effectively ameliorates human adverse reactions to high-altitude hypoxia and hypoxic injuries; however, its mechanism of action still requires further exploration. Constructing acute hypoxic injury rat models to conduct animal experiments combined with metabolomic analysis is particularly important for elucidating the mechanism of action of 1,25-$(OH)_2D_3$ in preventing and treating high-altitude diseases.

1,25-$(OH)_2D_3$ ameliorates hypoxic injury in SD rats

1,25-$(OH)_2D_3$ ameliorates hypoxia-induced injury in the lung, liver, and kidney tissues of SD rats

Figure 2A is the flowchart of the animal experiment in this study. As shown in Figure 2B/



	CV	NV	HV	MV
Negative (-)	10 (100%)	10 (100%)	0	6 (60%)
Postive (+)	0	0	1 (10%)	3 (30%)
Postive (++)	0	0	2 (20%)	1 (10%)
Postive (+++)	0	0	7 (70%)	0
total	10	10	10	10

Fig. 2. Vitamin D_3 reduces hypoxic damage in SD rats. (**A**) Study flowchart. (**B**) H&E staining of lung, liver, and kidney tissues in four groups of rats. (**C**) Oxidative stress indicators in four groups of rats. (**D**) Changes in routine blood inflammatory cells in four groups of rats. (**E**) Changes in blood biochemical indicators in four groups of rats. (**F**) Changes in routine urine protein indicators in four groups of rats. (**G**) Changes in Th1 and Th2 cell apoptosis in rats. (**H**) Changes in serum 25-(OH)D levels in four groups of rats. (**I**) Detection of changes in Th1/Th2 cells by flow cytometry. CV: blank control group; NV: blank control group administered with 1,25-$(OH)_2D_3$; HV: high-altitude hypoxia group; MV: high-altitude hypoxia group administered with 1,25-$(OH)_2D_3$. For high-resolution histopathological sections, see Supplementary Figure 4. This image should be used as a reference for interpreting tissue microstructure. Red arrows indicate areas of structural damage in the respective tissues. Yellow arrows indicate areas of red blood cell or inflammatory cell infiltration. * P<0.05, ** P<0.01, **** P<0.0001.

Supplementary Figure 4, the histological structures of the pulmonary alveoli, liver lobules, and renal glomeruli in the CV and NV groups were normal; in the HV group, the pulmonary alveolar septa widened, the pulmonary alveoli enlarged, the liver cellular structure was disrupted, with cells arranged in a disorganized manner, showing signs of rupture, necrosis, and vacuolation. The renal tissue exhibited abnormalities in the renal tubular structure, with tubular epithelial cell shedding, unclear nuclear outlines, and substantial infiltration of red blood cells and inflammatory cells in the lung, liver, and kidney tissues. The MV group rats showed reduced tissue damage, with structures tending to be more intact, approaching those of the CV and NV groups.

1,25-$(OH)_2D_3$ attenuates oxidative stress in SD rats

As shown in Figure 2C, there were no significant differences in oxidative stress indicators in the lung tissues of rats in the CV, NV, and MV groups, and these indicators were comparable. In comparison with the CV group, the activities of SOD and GSH-Px in the lung tissues of the HV group were significantly decreased ($P<0.05$), while MDA levels were significantly increased ($P<0.05$). In contrast to the HV group, the MV group exhibited an increasing trend in the activities of SOD and GSH-Px, and a significant decreasing trend in MDA levels ($P<0.05$).

1,25-$(OH)_2D_3$ inhibits the elevation of red blood cells and inflammatory cell numbers in SD rats subjected to hypoxic injury

The number of red blood cells in the CV and NV groups is similar and there is no significant difference. The number of red blood cells in the HV group is significantly higher than that in the CV and NV groups. Compared with the HV group, the MV group showed a certain degree of decrease in red blood cell count, but it was not significant (Fig. 2G). As shown in Figure 2D, there were no significant differences in the numbers of inflammatory cells in the serum of rats in the CV, NV, and MV groups, which approached normal levels. In comparison with the CV and NV groups, the HV group exhibited a significant increase in the numbers of white blood cells, lymphocytes, neutrophils, monocytes, and eosinophils ($P<0.05$). Conversely, when compared to the HV group, the MV group showed a significant decrease in the numbers of inflammatory cells ($P<0.05$).

1,25-$(OH)_2D_3$ ameliorates hypoxic liver injury in SD rats

As shown in Figure 2E, compared to the CV group, the HV group exhibited a significant elevation in both ALB and TBIL levels ($P<0.05$). In contrast to the HV group, the MV group demonstrated a decreasing trend in TBIL levels and a significant decrease in ALB levels ($P<0.05$). As depicted in Figure 2H, no significant differences were observed in the serum levels of 25-(OH)D among the CV, NV, and MV groups, which were found to be comparable. In comparison to the CV group, the HV group showed a significant reduction in serum 25-(OH)D levels ($P<0.05$). Conversely, the MV group demonstrated a significant elevation in serum 25-(OH)D levels compared to the HV group.

1,25-$(OH)_2D_3$ improves hypoxic kidney injury in SD rats

As shown in Figure 2E, compared to the CV group, the serum levels of CRE, BUN, and UA in the HV group significantly increased ($P<0.05$). Compared to the HV group, the serum levels of CRE, BUN, and UA in the MV group significantly decreased ($P<0.05$).As shown in Figure 2F, compared to the CV group, the HV group had 10 cases (100 %) of positive urine protein, indicating a significant increase in the urine protein positivity rate. Compared to the HV group, the MV group had 6 negative cases (60 %) and 4 positive cases (40 %), showing a decrease in the urine protein positivity rate.

1,25-$(OH)_2D_3$ induces increased Th2 cell count in hypoxic SD rats

Flow cytometry was used to observe changes in Th1/Th2 cells in rats. As shown in Figure 2I, the numbers of Th1 and Th2 cells in the Control group and the Hypoxia+VD_3 group were similar without significant differences. Compared to the Control group, the number of Th2 cells in the Hypoxia group significantly increased ($P<0.05$), and the number of Th1 cells decreased. Compared to the Hypoxia group, the number of Th2 cells in the Hypoxia+VD_3 group significantly decreased ($P<0.05$), and the number of Th1 cells significantly increased ($P<0.05$).

1,25-$(OH)_2D_3$ downregulates serum short-chain fatty acids in SD rats to exert synergistic anti-inflammatory effects

A metabolomic analysis of rat serum was conducted to further explore the protective mechanism of 1,25-$(OH)_2D_3$ against hypoxic injury. The TIC overlay analysis showed good technical reproducibility of metabolites (Fig. S1). The Pearson correlation coefficient

of QC samples ranged from 0.989 to 1.000 (Fig. S1), demonstrating good detection stability and high data quality, laying a foundation for subsequent studies.

Pairwise comparisons between the CV, HV, and MV groups were performed with PLS-DA statistical analysis (Fig. 3A-B), showing clear separation between different groups, indicating good grouping. The model evaluation parameters R^2 and Q^2 obtained through orthogonal partial least squares discriminant analysis were close to 1 (Fig. 3C-D), indicating significant differences between the models and strong predictive power, allowing for further analysis. After refining the targeted metabolomic data analysis with VIP>1.0 and P<0.05 and fold change ≥2 as criteria, we found significant differences in metabolites between groups, detecting a total of 664 metabolites (Fig. S2). There were 217 differential metabolites between HV and CV, with 155 metabolites showing an upward trend and 62 a downward trend (Fig. 3E). There were 88 differential metabolites between MV and HV, with 14 showing an upward trend and 74 a downward trend (Fig. 3F). The intergroup comparison further elucidated the differences in metabolites between groups.

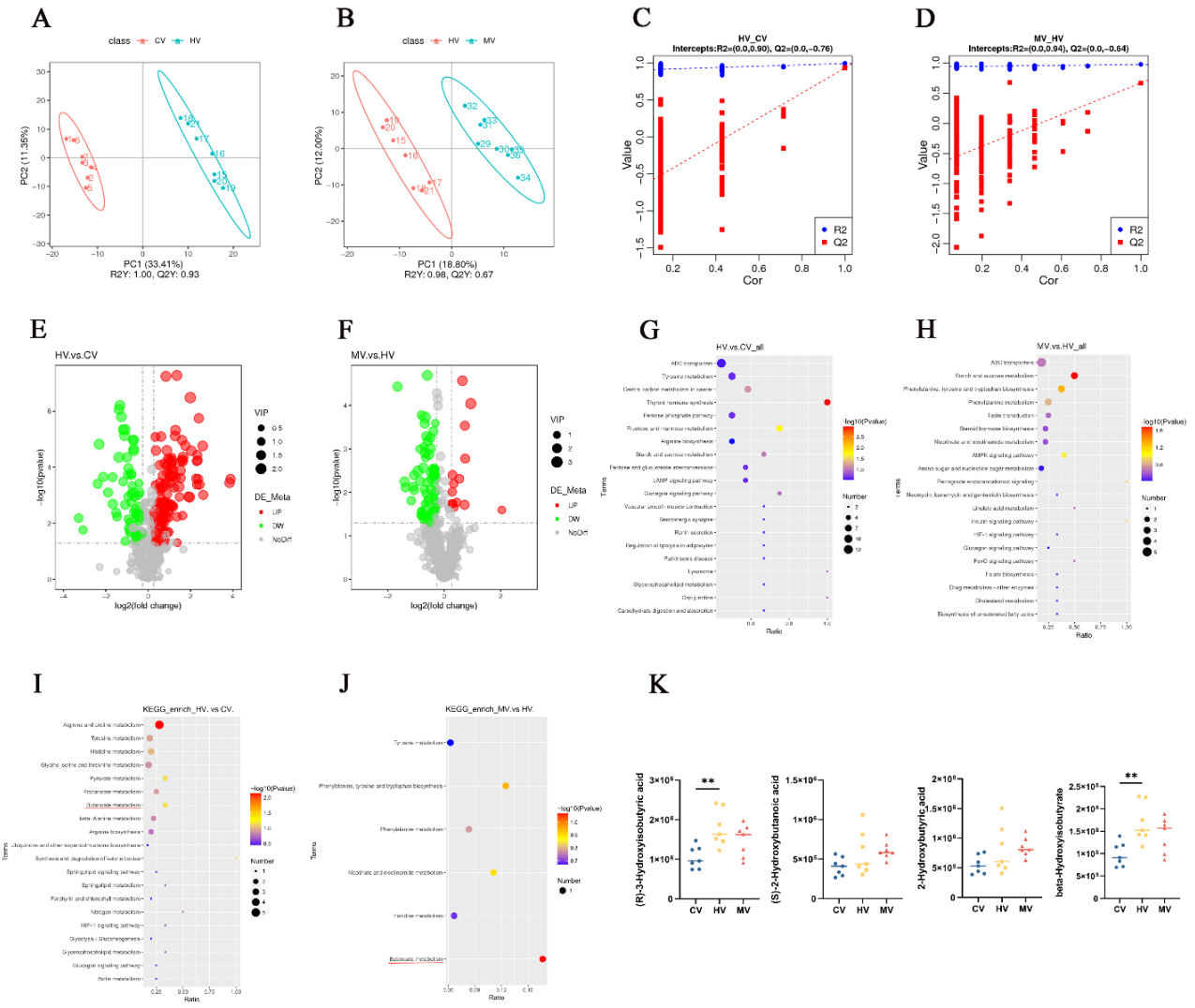


Fig. 3. Effects of Vitamin D_3 on serum metabolites in rats. (**A**) PLS-DA analysis of HV and CV groups. (**B**) PLS-DA analysis of MV and HV groups. (**C**) OPLS-DA analysis of HV and CV groups. (**D**) OPLS-DA analysis of MV and HV groups. (**E**) Volcano plot of differential metabolites between HV and CV groups. (**F**) Volcano plot of differential metabolites between MV and HV groups. (**G**) KEGG enrichment bubble chart of differential metabolites between HV and CV groups. (**H**) KEGG enrichment bubble chart of differential metabolites between MV and HV groups. (**I**) KEGG enrichment bubble chart of organic acid metabolites between HV and CV groups. (**J**) KEGG enrichment bubble chart of organic acid metabolites between MV and HV groups. (**K**) Screening of four key differential organic acid metabolites. CV: blank control group; NV: blank control group administered with 1,25-$(OH)_2D_3$; HV: high-altitude hypoxia group; MV: high-altitude hypoxia group administered with 1,25-$(OH)_2D_3$.

After performing KEGG enrichment analysis on the differential metabolites (Fig. 3G-H), the differential metabolites in the HV vs. CV comparison group were mainly enriched in thyroid hormone synthesis, fructose, and mannose metabolism ($P<0.05$). The differential metabolites in the MV vs. HV comparison group were mainly enriched in starch and sucrose metabolism ($P<0.05$). Based on the KEGG enrichment analysis, we screened key differential metabolic pathways of organic acids and selected key metabolites in these pathways for further analysis (Fig. S3). The results showed that both analyses annotated the butyrate metabolism pathway (Fig. 3I-J). Using the criteria of VIP>1.0 and $P<0.05$ and fold change ≥2, we further analyzed key metabolites in the butyrate metabolism pathway. Among them, we identified four differential metabolites regulated by 1,25-$(OH)_2D_3$: (R)-3-hydroxyisobutyric acid, (S)-2-hydroxybutyric acid, 2-hydroxybutyric acid, and β-hydroxyisobutyric acid (Fig. 3K).

Compared to the CV group, the HV rats experienced oxidative stress and inflammatory responses due to hypoxia. To cope with acute hypoxia, the body produced a large amount of short-chain fatty acids, resulting in increased levels of the four butyrate salts (R)-3-hydroxyisobutyric acid, (S)-2-hydroxybutyric acid, 2-hydroxybutyric acid, and β-hydroxyisobutyric acid to adapt to the hypoxic environment and mitigate hypoxic damage. Conversely, compared to the HV group, the levels of these four butyrate salts in the MV rats showed a decreasing trend after treatment with 1,25-$(OH)_2D_3$, approaching the levels seen in the normal group.

Safety of 1,25-$(OH)_2D_3$ administration

To investigate the safety of 1,25-$(OH)_2D_3$ administration, normal rats were given 1,25-$(OH)_2D_3$ intervention and designated as the NV group. Comparisons were made with the CV group to observe whether 1,25-$(OH)_2D_3$ had any toxic side effects on the rats. H&E staining of lung, liver, and kidney tissue sections showed no differences between the NV and CV groups, with both displaying normal tissue structures and no pathological damage (Fig. 2B/Fig. S4). Comparing the NV group with the CV group, the levels of ALB and TBIL were similar and showed no significant differences, both remaining within the normal reference range [25] (Fig. 2E). The serum levels of 25-(OH)D in both groups were also similar, showing no significant differences and remaining within the normal range (36-53 ng/ml) (Fig. 2H). 1,25-$(OH)_2D_3$ did not cause a significant increase in ALB and TBIL levels in normal rats, indicating no toxic effects on the liver. Comparing the NV group with the CV group, the serum levels of CRE, BUN, and UA were similar and showed no significant differences, all remaining within the normal reference range [21] (Fig. 2E). No individuals in either group tested positive for urine protein (Fig. 2F), indicating that 1,25-$(OH)_2D_3$ did not exert toxic side effects on the kidneys.

Discussion

Our integrated study, combining human field interventions and controlled animal experiments, provides compelling evidence that 1,25-$(OH)_2D_3$ pretreatment effectively mitigates the multifaceted adverse effects of acute high-altitude hypoxia. The collective data demonstrate that 1,25-$(OH)_2D_3$ not only alleviates clinical symptoms of AMS but also confers significant protection against multi-organ damage through a coordinated mechanism involving the suppression of systemic inflammation, restoration of immune balance, and modulation of metabolic homeostasis.

The clinical efficacy of 1,25-$(OH)_2D_3$ was first evidenced in our human trial. Volunteers receiving 1,25-$(OH)_2D_3$ exhibited significantly lower Lake Louise Scores (LLS) and a marked reduction in the incidence of headache compared to the control group. This clinical improvement was associated with better physiological adaptation, as reflected in the amelioration of hypoxia-induced reductions in arterial oxygen saturation and elevations in heart rate and blood pressure. These findings confirm that 1,25-$(OH)_2D_3$ enhances the body's resilience to hypoxic stress, aligning with its known role in regulating various physiological processes [10].

At the tissue level, H&E staining in our rat model confirmed that acute hypoxic stress induces significant pathological injury, characterized by erythrocyte congestion and inflammatory cell infiltration in the lung, liver, and kidneys. 1,25-$(OH)_2D_3$ pretreatment substantially attenuated these pathological changes, indicating robust multi-organ protection. This morphological preservation was consistent with the improvement in functional biomarkers. The hypoxic environment triggered a pronounced systemic inflammatory response, as indicated by significant increases in pro-inflammatory cytokines (IL-1β, IL-6) and circulating immune cells (leukocytes, lymphocytes,

and neutrophils) in both humans and rats. 1,25-$(OH)_2D_3$ effectively suppressed this inflammatory cascade, underscoring its potent anti-inflammatory properties, which are critical in conditions like HAPE where inflammation is a key driver of pathology [22,23].

Beyond broad anti-inflammatory effects, we delved deeper into the immunoregulatory actions of 1,25-$(OH)_2D_3$ by investigating T-helper cell responses. Flow cytometry analysis revealed that hypoxia disrupts the Th1/Th2 balance, decreasing the number of pro-inflammatory Th1 cells while increasing that of anti-inflammatory Th2 cells. This shift towards a Th1-dominant state promotes the release of cytokines like IFN-γ, thereby exacerbating inflammation [24,25]. 1,25-$(OH)_2D_3$ treatment reversed this imbalance by significantly increasing Th1 cell and decreasing Th2 cell. This fine-tuning of the adaptive immune response aligns with previous reports that 1,25-$(OH)_2D_3$ can ameliorate experimental colitis by inhibiting Th1/Th17 cells [26,27], and now extends its relevance to hypoxic immune dysregulation.

Concurrent with immune activation, hypoxic stress also induced significant oxidative damage, as demonstrated by decreased activities of the antioxidant enzymes SOD and GSH-Px and increased levels of MDA in rat tissues. 1,25-$(OH)_2D_3$ pretreatment effectively counteracted this oxidative stress, thereby mitigating subsequent organ dysfunction. This was particularly evident in the liver and kidneys. The increases in serum ALT, AST, ALB, and TBIL following hypoxia indicated hepatocellular injury, likely compounded by increased blood viscosity from polycythemia [28]. Notably, acute hypoxic liver damage impaired the synthesis of 25-(OH)D, the precursor to 1,25-$(OH)_2D_3$, creating a vicious cycle. Exogenous 1,25-$(OH)_2D_3$ not only broke this cycle by increasing serum 25-(OH)D levels but also directly protected against hepatocellular injury. Similarly, renal dysfunction, marked by elevated CRE, BUN, UA, and proteinuria, hallmarks of hypoxia-induced renal vasoconstriction and microthrombosis [29-31] was significantly ameliorated by 1,25-$(OH)_2D_3$.

Perhaps the most intriguing finding of this study lies in the metabolic dimension of the action to 1,25-$(OH)_2D_3$. Our metabolomic analysis revealed that hypoxic stress triggers a substantial increase in several branched short-chain fatty acids (SCFAs), including (R)-3-hydroxyisobutyric acid and β-hydroxyisobutyric acid. This may represent a compensatory mechanism to mitigate hypoxic damage. Crucially, 1,25-$(OH)_2D_3$ pretreatment normalized the levels of these SCFAs. Given that SCFAs are key microbial metabolites known to recruit and modulate immune cells in peripheral tissues [32], we postulate that the modulation of the SCFA profile is a novel pathway through which 1,25-$(OH)_2D_3$ coordinates its anti-inflammatory and immunoregulatory functions. This suggests a potential crosstalk between 1,25-$(OH)_2D_3$ and gut microbiota-derived metabolites in orchestrating the host's defense against hypoxic insult.

Conclusions

This study shows that 1,25-$(OH)_2D_3$ can effectively prevent hypoxic damage in rats by reflecting changes in indicators such as H&E staining of tissue, blood biochemistry, routine urine tests, and serum 25-(OH)D levels. It is safe and reliable, showing no toxic side effects. This study demonstrates that 1,25-$(OH)_2D_3$ may effectively prevent high-altitude sickness by reducing the body's inflammatory response, promoting the differentiation of Th1 and Th2, increasing the number of Th2 cells, balancing immune homeostasis in a hypoxic environment, and downregulating serum short-chain fatty acid levels to exert anti-inflammatory effects in synergy with SCFAs. It is inferred that 1,25-$(OH)_2D_3$ may be a potential therapeutic drug for preventing and treating high-altitude sickness in high-altitude areas.

Ethics Declarations

Human Ethics and Consent to Participate declarations: This research has passed the ethical review of medical science research by the Medical College of Qinghai University, and has been approved by the Ministry of Science and Technology of the People's Republic of China to collect Chinese human genetic resources. The batch number is: [2024] CJ0016, allow the collection of human samples. All volunteers were aware of the experimental content and voluntarily participated. In case of emergencies, all volunteers have the right to choose to withdraw from the experiment. All studies involving human volunteers were conducted in accordance with the World Medical Association's Declaration of Helsinki (2000) and followed the rules of the Declaration of Helsinki (1975, revised in 2013) (https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki/). **Animal Ethics:** The animal handling measures involved in the implementation of this project were carried out in

accordance with the national "Guidelines for Ethical Review of Experimental Animal Welfare" (GB/T35892-2018) and have been approved by the Animal Ethics Committee of Qinghai University. All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (1985), National Institutes of Health (NIH), Bethesda, USA.

Limitations of the Work

The following are the main limitations and confounding factors that may affect the results of our study: ① Sample size limitation: there is a problem of insufficient sample size in this study. This may affect the statistical significance and generalizability of the results. ② Lack of dietary factors: During the experiment, we uniformly arranged the activities and diet of volunteers, but did not specifically detect and evaluate the intake of vitamin D in their daily diet. This factor may affect the research results, as vitamin D plays an important role in immune regulation. ③ Consideration of compensation mechanisms: There may be other compensation mechanisms in the study that may obscure or alter the results we observe to some extent. ④ Due to the focus of this study on preliminary screening of multiple organs, a qualitative comparison method was used instead of semi quantitative scoring. In the future, in-depth mechanism research will adopt blind evaluation and standardized scoring systems (such as Ishak score). More research is needed to delve deeper into these potential confounding factors. We will consider these limitations in future research to enhance the comprehensiveness and reliability of the study.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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