Drug Design, Development and Therapy

Dovepress
Taylor & Francis Group

Open Access Full Text Article

ORIGINAL RESEARCH

Study on the Protective Effect of Methyl Rosmarinate on Hypoxic Mice and Their Erythrocytes

Qianwen Guo[1,2,*], Ziyue Yin[1,2,*], Rong Wang[1], Yuemei Sun[1], Anpeng Zhao[1], Wanteng Yao[1], Wenbin Li[1]

[1]Pharmacy of the 940th Hospital of PLA Joint Logistics Surpport Force, Lanzhou, People's Republic of China; [2]School of Pharmacy, Gansu University of Traditional Chinese Medicine, Lanzhou, People's Republic of China

*These authors contributed equally to this work

Correspondence: Wenbin Li, Pharmacy of the 940th Hospital of PLA Joint Logistics Surpport Force, Lanzhou, People's Republic of China, Email yfcs2002@163.com

Objective: We have previously identified methyl rosmarinic (MR) acid as a 2.3-bisphosphoglycerate mutase (BPGM) activator. The present study aimed to verify the protective effect of MR on plateau field hypoxia mice and the mechanism of increased oxygen release capacity of erythrocytes in vivo.

Methods: The anti-hypoxic effect of MR was investigated in a plateau field environment in Specific Pathogen Free -grade healthy BALB/c mice, male and female, and the effect of different doses of MR on the survival time of mice in confined space was investigated in an atmospheric pressure confinement hypoxia experiment, plasma inflammatory markers, oxidative stress indices of myocardial, brain, lung and liver tissues, as well as the histopathological damage and hypoxia in each experimental group were measured by HE staining and hypoxia probe method. Finally, the effects of MR administration to mice on the energy metabolic pathways and metabolites of erythrocytes in vivo were investigated.

Results: After acute plateau entry in mice, the energy metabolic pathway of erythrocytes shifted to the glycolytic pathway as the duration of hypoxia increased. The administration of MR to hypoxic mice further activated Bisphosphoglycerate mutase (BPGM) and increased the levels of glyceraldehyde-3-phosphate and 2.3-bisphosphoglycerate (2,3-BPG), as well as further shifted glucose to the glycolytic pathway and further enhanced the activity of rate-limiting enzymes in the glycolytic pathway.

Conclusions: MR activates BPGM in erythrocytes to produce more 2, 3-BPG from the glycolytic branch, thus exerting a protective effect against injury in hypoxic mice in the highland field.

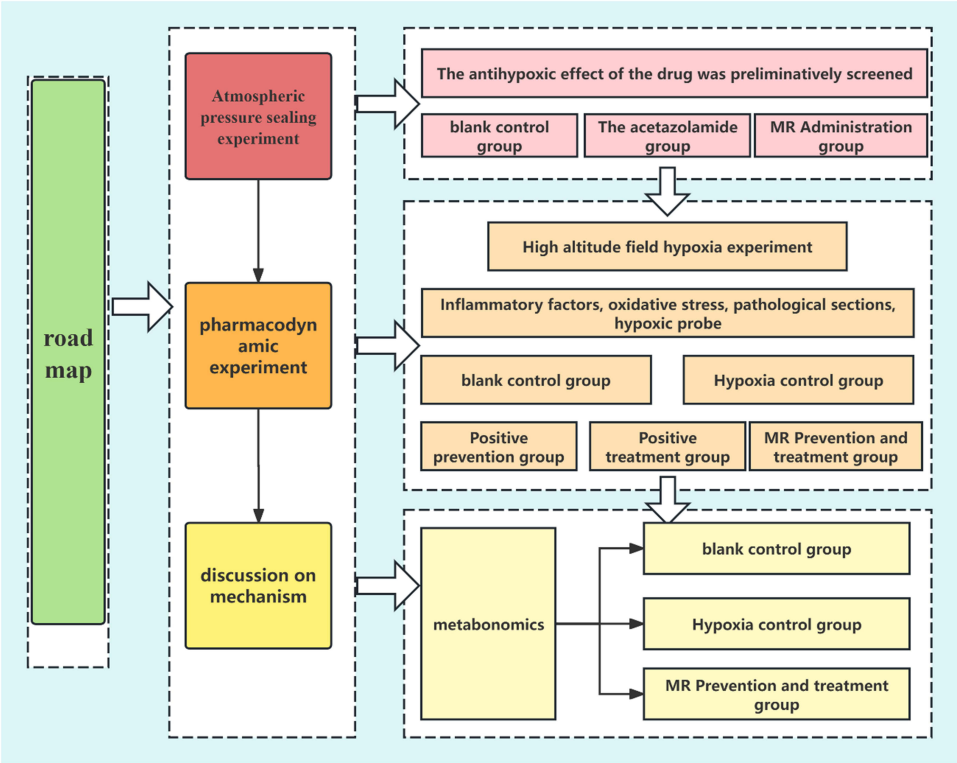
Keywords: erythrocytes, methyl rosmarinate, 2,3-biphosphoglyceric acid, hypoxia, glycolysis

Introduction

The plateau environment is characterized by low pressure and low oxygen, which leads to the decrease of oxygen entering the alveoli and the decrease of available oxygen in the tissues, which has a wide impact on the function and metabolism of the body, causing changes in the cardiovascular system, the blood circulation system and the respiratory system, and the damage of body tissues and organs. The main performance is that the plain people quickly enter the plateau environment will produce a series of uncomfortable reactions such as nausea, headache and insomnia. Hypoxia is the main problem that human beings face when entering the plateau area. Currently, the commonly used anti-hypoxia drugs mainly include acetazolamide, dexamethasone, acetazolamide and Rhodiola rosea.[1,2] However, these drugs are faced with problems such as large side effects or inappropriate access, and the development of new anti-altitude hypoxia drugs has become the focus of research. The main function of red blood cells in the blood system is to carry oxygen and transport and release oxygen to tissue cells, and red blood cells do not consume oxygen themselves. The metabolic

Received: 10 September 2024
Accepted: 3 April 2025
Published: 24 April 2025

Graphical Abstract



pathway of mature red blood cells is mainly glucose glycolysis and pentose phosphate. When stimulated by low oxygen at high altitude, red blood cells activate the glycolysis pathway and Rapoport-Luebering branch, thus increasing the content of 2.3-BPG to increase the oxygen releasing capacity of red blood cell.[3]

In a previous study, MR, a BPGM herbal compound agonist in erythrocytes, was identified through virtual computer screening of more than 300 commonly used herbal components, and it was shown that MR significantly increased 2.3-BPG levels in erythrocytes during in vitro hypoxia experiments. The present study further investigated the protective effect of MR on hypoxic mice and its pathways affecting 2.3-BPG levels in erythrocytes through in vivo experiments in mice.

Rosmarinic acid is widely found in many plants of the Labiatae family, including rosemary, and has a variety of pharmacological activities such as antioxidant, antitumor, anti-inflammatory and antibacterial, but its wide clinical application is hampered by its large polarity that affects its absorption in vivo. MR is a derivative of rosmarinic acid, which is widely found in plants such as Rabdosia rubescens, Rabdosia serra, Radix Salviae Bowleyanae and Perilla frutescens, etc. Compared with rosmarinic acid, MR has reduced polarity of its side chain carboxyl esterification and has better bioavailability, and has pharmacological activities such as anti-cardiovascular smooth muscle cell proliferation,[4] antioxidant,[5] anti-inflammatory[6] and antibacterial,[7] and the activity of MR is stronger than that of rosmarinic acid. The LD_{50} of MR and its toxicological information are not reported at present. By giving MR 50 and 100 mg/kg to the Parkinson's mouse model, Wang S.H. and others found that MR has neuroprotective effects, so high, medium and low doses were designed according to the literature for mouse dosing.[8] Could the increase of 2.3-BPG content in erythrocytes increase the oxygen supply to erythrocytes in tissues and have a protective effect against hypoxia-induced injury? In this study, we further investigated the protective effect of methyl rosmarinic acid on hypoxic mice through atmospheric pressure confinement experiments in mice and hypoxia experiments in the field at plateau.

Materials and Methods

Ethical Statement

This study was approved by the Scientific Research Management Ethics Committee of the 940th Hospital of the Joint Logistic Support Force of the People's Liberation Army (Approval No. 2022KYLL155). All animal work was approved by the Animal Care and Treatment Committee of the 940th Hospital of the Joint Logistic Support Force of the People's Liberation Army (Lanzhou, China). In the use and research of experimental animals, we were adhered to the 3R principle (replacement, reduction, refinement), optimized the design of experiments, standardized the feeding environment and methods, and ensured the reliability, accuracy and scientificity of experimental data.

Chemical and Reagents

Methyl Rosmarinate (Purity>98.0%, Baoji Chenguang Biotechnology Co., Ltd. CAS#99353-00-1); Acetazolamide (Purity>98.0%, Shanghai Yuanye Biotechnology Co., Ltd. CAS#59-66-5).

BCA kit, Mouse TNF-α Elisa kit, Mouse IL-6 Elisa kit, MDA kit, SOD kit, GSH Kit (Nanjing Jiancheng Institute of Biological Engineering); HypoxiaprobeTM-1 Plus Kit (Hypoxiaprobe, USA), 4% Paraformaldehyde (500 mL, Wuhan Xaver Biotechnology Co., Ltd).; Normal Saline (500 mL: 4.5 g, Shijiazhuang Siyao Pharmaceutical Co., Ltd. Batch No. 2007122002); Anticoagulant - heparin sodium injection (Shanghai First Biochemical Pharmaceutical Co., Ltd).

Animals and Experiment Design

30 male and female BALB/c mice, weighing 18–22 g each, were supplied for the study by the 940th hospital (SYXK (Military) 2020–0032). 30 mice were subsequently split into 5 groups (n=6) at random: Blank group, acetazolamide-Positive drug group (200 mg/kg), MR low-dose group (25 mg/kg), MR mid-dose group (50 mg/kg), MR high-dose group (75 mg/kg).

We acquired 60 healthy male and female BALB/c mice, weighing 18–22 g each, from the 940th hospital (SYXK (Military) 2020–0032). The mice were randomly assigned to one of 6 groups: Blank group, hypoxia group, acetazolamide-positive prophylaxis group (200 mg/kg), acetazolamide-positive treatment group (200 mg/kg), MR prophylaxis group (50 mg/kg), MR treatment group (50 mg/kg).

42 healthy BALB/c mice of SPF grade (weighing 18–22 g), male and female, were purchased from Spelford (Beijing) Biotechnology Co., Ltd (Certificate of Conformity No. SCXK (Beijing) 2019–0010) and randomly divided into CON group, HYP and MR groups on the 1st, 3rd and 7th days, with 6 mice in each group.

Atmospheric Pressure Closed Hypoxic Experiment

After the mice were acclimatized for 3 days, each experimental group was administered the drug intraperitoneally for 3 consecutive days, during which the mice were free to drink and eat. After 30 min of drug administration on the third day, the mice were put into 250 mL wide-mouth bottles (filter paper and 5 g sodium lime were prepared in advance in the bottles), and the mouths of the bottles were tightened with rubber stoppers, and the time of starting hypoxia and the time of death were recorded, and the survival time and prolongation rate of mice in each group were counted.

Establishment of an Altitude Field Hypoxia Mouse Model

After the mice were acclimatized and reared for 3 days, the positive prophylactic treatment group and MR prophylactic treatment group were administered intraperitoneally for 3 consecutive days, and the remaining groups were given blank solvent. After drug administration on day 3, the mice were rushed in by a constant temperature box truck to the plateau field SPF level laboratory in Yushu Tibetan Autonomous Prefecture, Qinghai Province (4010 m above sea level), and were hydrated freely with jelly during transportation. The time of arrival at the laboratory was the time of the onset of hypoxia.

Investigation of the Effect of Methyl Rosmarinic Acid on Plasma Inflammatory Factors in Hypoxic Mice in the Field by ELISA

Mice were subjected to orbital blood collection on days 1, 3 and 7 after arrival at the plateau field laboratory, respectively, and the supernatant plasma was centrifuged to determine the plasma levels of TNF-α and IL-6 by ELISA, which was operated strictly according to the kit instructions.

Oxidative Stress to Investigate the Effects of Methyl Rosmarinic Acid on Oxidative Stress Indicators in Field Hypoxic Mouse Tissues

Heart, liver, brain, lung and kidney tissues of mice were excised on day 7 for the detection of oxidative stress indicators. The tissues were washed in pre-chilled saline, blotted on filter paper, weighed and cut into pieces, added to saline at m: v=1:9, ground with a rapid grinder, centrifuged, and frozen in a refrigerator at −80 °C. The total protein concentration, SOD activity, GSH content and MDA content in the tissue homogenate were detected strictly according to the kit instructions.

HE Staining Assay to Investigate the Effect of Methyl Rosmarinic Acid on Histopathological Damage in Mice

The heart, liver, brain, lung and kidney tissues were removed from each group of mice 30 min after drug administration, washed in pre-cooled saline and blotted with filter paper, placed in 4% paraformaldehyde for fixation, and then dehydrated, paraffin-embedded and sectioned after the tissues were fully fixed, and finally stained with HE to observe the pathological changes.

Hypoxia Probe Method to Investigate the Effect of Methyl Rosmarinic Acid on the Degree of Tissue Hypoxia in Mice

After 30 min of administration, mice were injected intraperitoneally with Pimonidazole HCl (60 mg/kg), and tissues of mice were fixed in 4% paraformaldehyde after 30 min. The fixed tissues were dehydrated, trimmed, paraffin embedded, sectioned, dewaxed, quenched endogenous peroxidase in the tissues, and protein blockers were added to give background staining after antigen repair. After incubation of primary antibody for 60 min at room temperature and secondary antibody for 30 min at room temperature, DAB terminated the color development, re-staining, alcohol dehydration, permeabilization and sealing. Finally, a scanner was used for panoramic scanning of fluorescent images.

Mouse Erythrocyte Energy Metabolism Experiment

Blood was collected from blank control mice before entering the plateau, and a flat capillary blood collection tube was used to collect blood through the orbits of the mice. After blood collection, saline was injected into the mice intraperitoneally for rehydration to reduce the damage to the mice. Two to three drops of blood were collected in centrifuge tubes moistened with heparin, and red blood cells were collected after centrifugation and washing with isotonic saline, and 5 μL of red blood cells were diluted into 1000 μL of saline. After entering the plateau, blood was collected from mice in the CON, HYP and MR groups on the 1st, 3rd and 7th days of hypoxia with the same treatment, and the collected samples were promptly frozen in a −80 °C refrigerator.

The samples were thawed, vortexed for 10s and mixed well. Take 50 μL of the sample into a centrifuge tube and add 250 μL of 20% acetonitrile methanol extract. Vortex for 3 min and then centrifuge at 12000 r/min for 10 min at 4 °C. Aspirate 250 μL of supernatant into a centrifuge tube and let it stand for 30 min at −20 °C for 10 min at 12000 r/min at 4 °C. 180 μL of supernatant was passed through the protein precipitation plate and then used for analysis on the machine.

Chromatography Mass Spectrometry Acquisition Conditions

The liquid phase conditions mainly include:

1) Chromatographic column: ACQUITY UPLC BEH Amide column (1.7 μm, 100 mm×2.1 mm i.d).; 2) mobile phase: phase A, ultrapure water (10 mm ammonium acetate, 0.3% ammonia); phase B, 90% acetonitrile/water (V/V); 3)

Flow rate 0.40 mL/min; column temperature 40 °C; injection volume 2 μL; 4) Mobile phase gradient: 5:95 (V/V) for 0–1.2 min A/B, 30:70 (V/V) for 8 min A/B, 50:50 (V/V) for 9.0–11 min A/B, and 5:95 (V/V) for 11.1–15 min A/B.

The mass spectrometry conditions consisted mainly: Electrospray Ionization (ESI) at 550 °C, a mass spectrometry voltage of 5500 V in positive ion mode and −4500 V in negative ion mode, and a gas curtain gas (CUR) of 35 psi. In the Q-Trap 6500+, each ion pair was analyzed according to an optimized declustering voltage (Declustering Potential (DP) and Collision Energy (CE) for scanning detection.

A database of standards was constructed to qualitatively analyze the data detected by mass spectrometry. The analysis was completed using triple quadrupole mass spectrometry in multiple reaction monitoring mode. After obtaining the mass spectrometry analysis data of different samples, the chromatographic peaks of all targets were integrated and quantitatively analyzed by standard curves.

Results

Effect of MR on Survival Time of Mice in Atmospheric Pressure Confinement Hypoxia

The atmospheric pressure confinement experiment is a common experiment to study the anti-hypoxic effect of drugs, and the survival time of mice directly reflects the anti-hypoxic ability of drugs to systemic hypoxia in mice. As shown in Table 1, the survival rate of mice was significantly prolonged by 8.59%, 20.15%, 24.35% and 24.19% in the acetazolamide, MR low, medium and high dose groups compared to the blank control group, with the highest prolongation rate in the MR medium dose group.

Effects of MR on Plasma Inflammatory Factors in Mice with Different Times of Hypoxia in the Field at Plateau

The effects of MR administration on plasma inflammatory factor levels in mice after different hypoxia times for 1, 3 and 7 days are shown in Figure 1. Compared with the blank group, the plasma levels of IL-6 increased by 4.72% and 12.63% on days 1 and 3 in the hypoxia group, and the plasma levels of TNF-α increased by 255.84%, 317.09% and 296.97% on days 1, 3 and 7 in the hypoxia group. The plasma inflammatory factor level of mice in the hypoxia group was significantly higher than that of the blank group, and the highest inflammatory factor level was found on day 3, indicating that the damage was most severe on day 3 of the acute plateau environment. Compared with the hypoxia group, the positive prevention treatment group on days 1 and 7 could significantly reduce inflammatory factor levels, and the positive treatment group had a better effect on day 3. Compared with the hypoxia group, MR at days 3 and 7 of hypoxia, the MR prophylaxis group and MR treatment group could significantly reduce plasma levels of inflammatory factors, and the reduction was significantly stronger than that of positive drugs, and the MR prophylaxis group was more pronounced.

Table 1 The Effect of Different Doses of MR on the Survival Time of Mice in Normobaric Hypoxia (n=6, $\bar{x} \pm s$)

Group	Dose (mg/kg)	Survival time (min)	Increase rate (%)
Blank group	–	26.54±2.08	–
Acetazolamide group	200	28.82±2.54	8.59
MR low-dose group	25	31.89±1.83*	20.15
MR mid-dose group	50	33.00±4.96*	24.35
MR high-dose group	75	32.96±2.10*	24.19

Notes: All data were analyzed by using Least-significant difference and are shown as the means±SD. Compared with the blank group, *P<0.01.

https://doi.org/10.2147/DDDT.S493866

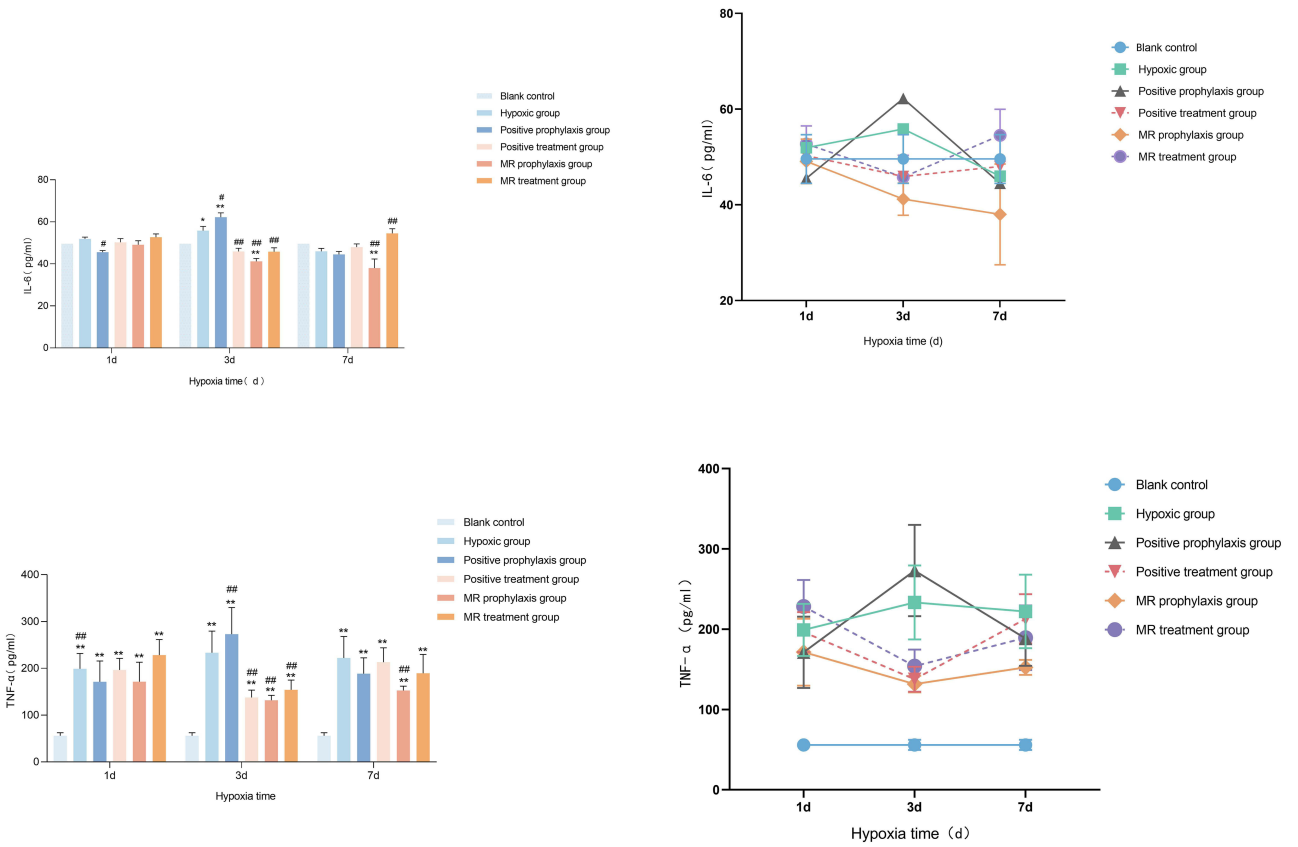


Figure 1 Effect of MR on inflammatory factors in plasma of mice with different times of hypoxia in the field at plateau (n=6). Changes in the levels of IL-6 and TNF-α. All data were analyzed by using Least-significant difference and are shown as the means±SD. Compared with the blank group, $^{\#}$P<0.05, $^{\#\#}$P<0.01; Compared with the hypoxia group, *P<0.05, **P<0.01.

Effects of MR on Tissue Oxidative Stress in Mice with Different Times of Hypoxia in the Field at Plateau

The SOD activity, GSH content and MDA content of MR on oxidative stress indexes in liver, heart, brain, lung and kidney tissues of hypoxic mice are shown in Figure 2. Compared with the blank group, the SOD activity and GSH content of liver, heart, brain, kidney and lung tissues of mice in the hypoxic group were significantly reduced and the MDA content was significantly increased, indicating that hypoxia caused damage to mouse tissues. Compared with the hypoxic group, MR could improve the oxidative stress damage caused by hypoxia better than the positive drug, and the protective effect was stronger in the MR preventive treatment group. By comparing the protective effects of MR on the oxidative stress indexes of the tissues of hypoxic mice, it was found that the MR preventive treatment group had the strongest protective effect on the oxidative stress injury in the heart tissues of hypoxic mice, and the SOD activity significantly increased by 60.87%, the GSH content significantly increased by 112.55%, and the MDA content significantly decreased by 65.85% in the MR preventive treatment group compared with the hypoxic group.

Effect of MR on Tissue Microstructure in Hypoxic Mice in the Field at Plateau

The protective effect of MR on histopathological damage in hypoxic mice in the field at plateau is shown in Figure 3. The histopathological damage of liver is shown in Figure 3. The hypoxic group showed edema and disorganized arrangement of hepatocytes compared with the blank group, as well as a small amount of hepatocyte steatosis with clear round lipid droplets inside. Compared with the hypoxic group, the degree of hepatocyte edema and cytoplasmic sparing in the positive prophylaxis group, the arrangement of hepatocytes became neat in the positive treatment group, and the

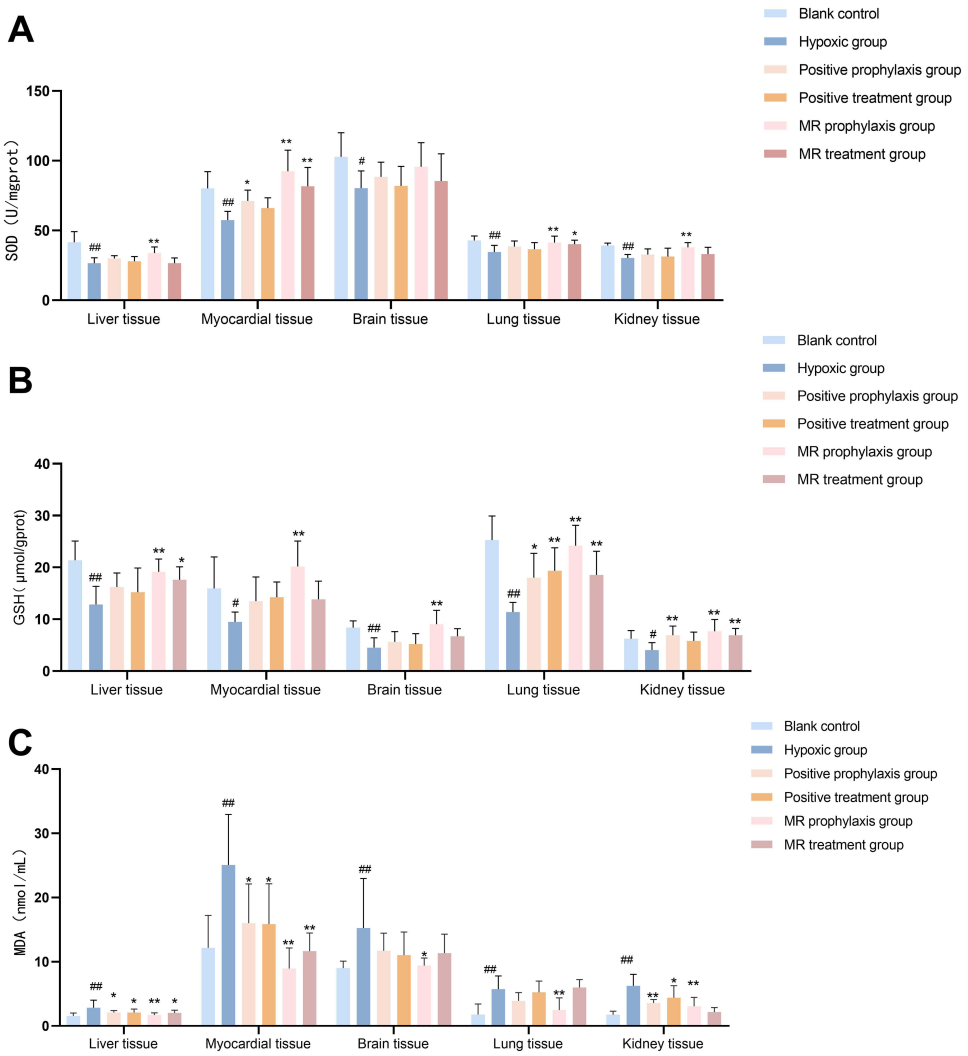


Figure 2 Effect of MR on oxidative stress in tissues of hypoxic mice in the field at plateau. (**A–C**) Change in SOD activity, GSH content and MDA content (*n*=6). All data were analyzed by using Least-significant difference and are shown as the means±SD. Compared with the blank group, $^{\#}P<0.05$, $^{\#\#}P<0.01$; Compared with the hypoxia group, $^{*}P<0.05$, $^{**}P<0.01$.

hepatocytes in the MR prophylaxis and MR treatment groups showed reduced edema and radiolucent arrangement around the central vein, with no other obvious pathological damage.

The cardiac histopathological damage was shown in Figure 3. Compared with the blank group, the cardiac tissue myocardial fibers in the hypoxic group showed wave-like appearance with breakage and irregular arrangement. Compared with the hypoxic group, the myocardial fibers were more neatly arranged and the myocardial cells were basically normal when given positive drugs and MR.

The histopathological damage of the brain was shown in Figure 3. Compared with the blank group, a small number of cone cells in the hippocampal region of the hypoxic group showed necrosis of cell nuclei consolidation, disorganized arrangement, slightly enlarged cell gaps, and slight edema. Compared with the hypoxic group, the positive drug reduced the nucleus fixation phenomenon in the hippocampal area, and MR could make the cone cells in the hippocampal area arranged regularly.

The degree of renal histopathological damage was shown in Figure 3. Compared with the blank group, the hypoxic group had thickened tubular walls and edema, narrowed official lumen, and flattened epithelial cells. Compared with the hypoxic group, the positive drug group and MR administration group showed normal tubular morphology and clear intact glomerular structure, and no obvious pathological damage was observed.

https://doi.org/10.2147/DDDT.S493866

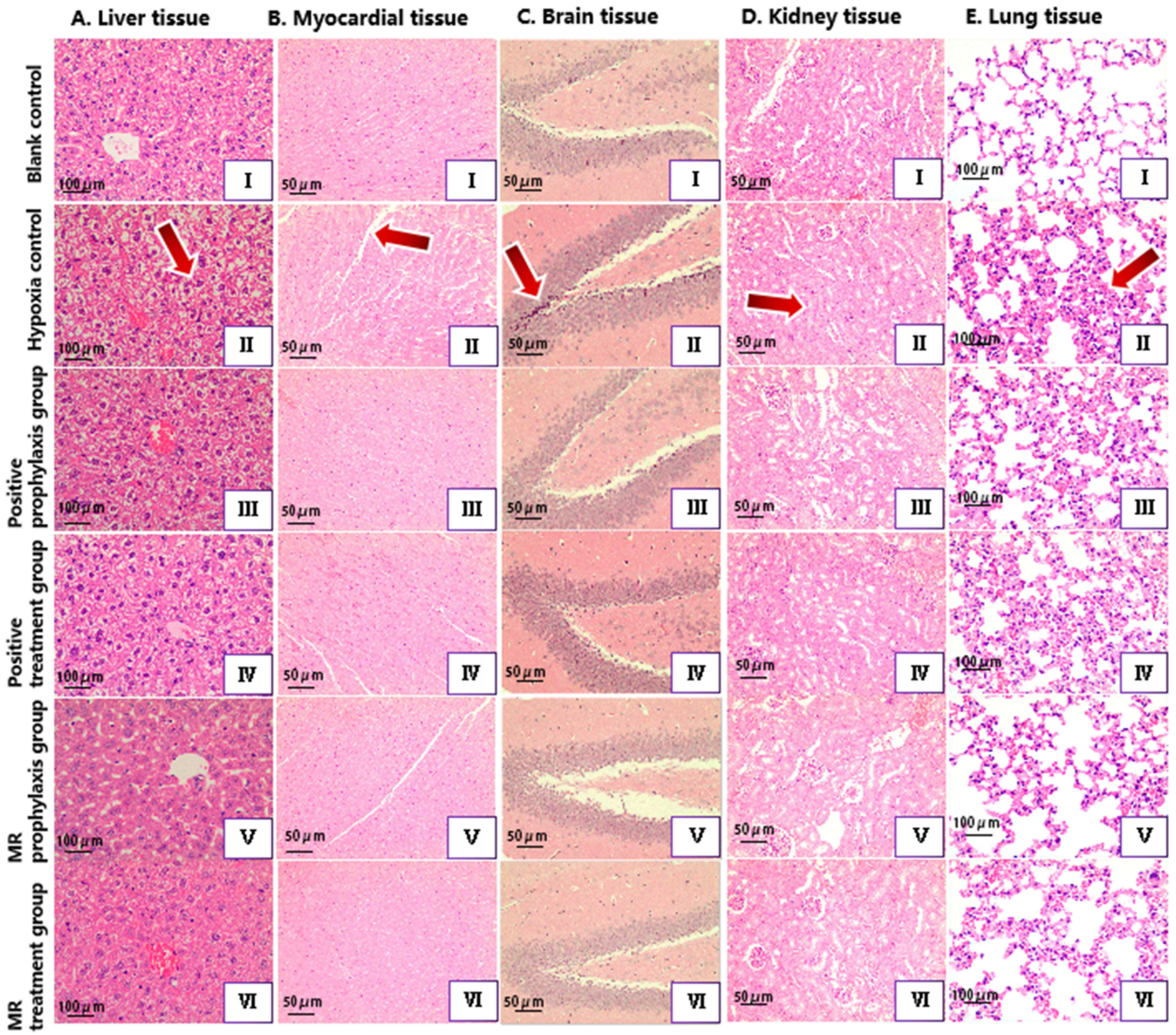


Figure 3 Effect of MR on tissue microstructure in hypoxic mice in the field at plateau. (**A–E**) Changes in the degree of pathological damage to liver (200×), heart (100×), brain (100×), kidney (100×) and lung (200×) tissues in hypoxic mice in the highland field. The red arrow points to the locations within each organization that have significant pathological damage. (I: Blank group; II: Hypoxia group; III: Positive prophylaxis group; IV: Positive treatment group; V: MR prophylaxis group; VI: MR treatment group).

Compared with the blank group, the alveolar wall of the hypoxic group was thickened, the alveolar blood vessels were dilated with congestion, and the epithelial cells were enlarged. Compared with the hypoxic group, the edema was reduced in the positive drug group, and the alveolar epithelial cells were arranged neatly and more normally in the MR administration group, and the protective effect was more obvious in the MR prophylaxis group.

Effect of MR on the Degree of Tissue Hypoxia in Hypoxic Mice in the Field at Plateau

The degree of protection of MR on liver, heart, brain, kidney and lung tissues in hypoxic mice in the field at plateau is indicated by the fluorescence intensity of the hypoxia marker signal pimonidazole, with stronger green fluorescence intensity indicating more severe hypoxia. As shown in Figure 4 and Table 2. Compared with the blank group, mice in the hypoxic group showed significant hypoxic damage to liver, heart, brain, lung and kidney tissues. Both positive prevention treatment group and positive treatment group were able to reduce the fluorescence intensity of hypoxic markers and alleviate the hypoxic injury. MR could significantly reduce the fluorescence intensity of hypoxic markers so that the tissues were not damaged by hypoxia, and the MR prevention treatment group had the best effect, and MR had the

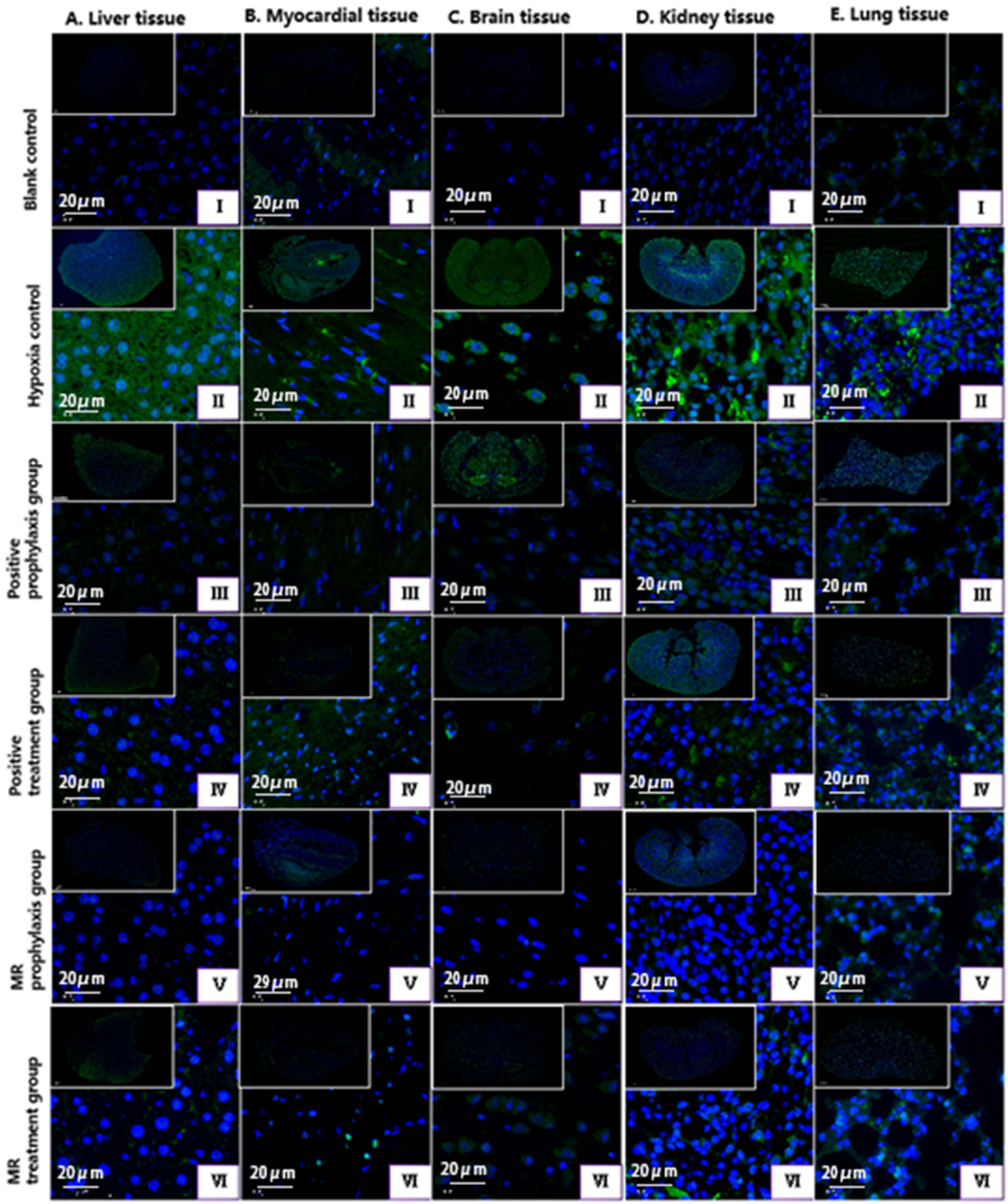


Figure 4 Effect of MR on the degree of tissue hypoxia in hypoxia mice in the field at plateau. (**A–E**) The degree of protection of liver, myocardial, brain, kidney and lung tissues in hypoxia mice in the highland field is indicated by the green fluorescence intensity of the hypoxia marker signal pimonidazole. Blue fluorescence is the cell nucleus. (Panoramic scan image 1×, local scan image 40×I: Blank group; II: Hypoxia group; III: Positive prophylaxis group; IV: Positive treatment group; V: MR prophylaxis group; VI: MR treatment group).

https://doi.org/10.2147/DDDT.S493866

Table 2 The Effect of MR on the Hypoxic Signaling Fluorescence Intensity in Tissues of Mice Subjected to Hypoxia in a High-altitude Environment (n=6, $\bar{x} \pm s$)

Group	Liver	Myocardial	Brain	Kidney	Lung
Blank group	31.81±6.23$^{\#}$	30.73±1.23$^{\#\#}$	30.63±9.78$^{\#\#}$	20.24±4.49$^{\#\#}$	23.15±5.94$^{\#\#}$
Hypoxia group	46.26±1.66	58.33±1.53	53.95±14.29	45.20±5.06	50.93±7.20
Positive prophylaxis group	31.93±5.52*	34.98±1.40**	39.19±1.38	40.72±7.63	40.10±11.45
Positive treatment group	33.82±5.35	29.52±6.98**	44.26±2.90*	37.30±9.81	50.85±1.92
MR prophylaxis group	26.06±2.67**	28.35±3.74**	37.03±0.73	23.98±4.59*	29.81±16.90*
MR treatment group	27.16±14.42**	35.05±1.03**	42.52±4.09	44.16±18.75	38.83±10.67

Notes: All data were analyzed by using Least-significant difference and are shown as the means±SD. Compared with the blank group, $^{\#}$P<0.05, $^{\#\#}$P<0.01; Compared with the hypoxia group, *P<0.05, **P<0.01.

strongest protective effect on cardiac tissues with 51.40% reduction in fluorescence intensity compared with the hypoxic group.

Effect of MR on the Energy Metabolism of Erythrocytes in Hypoxia Mice

Glucose in erythrocytes is metabolized primarily through the pentose phosphate pathway and the glycolytic pathway. As shown in Figure 5A, glucose is catalyzed by rate-limiting enzymes to produce glucose-6-phosphate, which is produced into fractyl-6-phosphate through the glycolysis pathway, and 6-phosphogluconic acid is generated through the pentose phosphate pathway. The effects of plateau hypoxic environment on the energy metabolism pathway of red blood cells in mice were roughly judged by the content of fructose-6-phosphate and 6-phosphogluconic acid in erythrocytes. Figure 5B shows the effect of MR on the proportion of glucose involved in glycolysis and pentose phosphate pathway in mouse erythrocytes at different hypoxic times. The results showed that glucose was metabolized mainly through the pentose phosphate pathway in the normoxic state, and with the increase of hypoxia time, glucose was transferred to the glycolytic pathway. Administration of the drug MR can further increase the transfer of glucose to the glycolytic pathway.

The effect of MR on erythrocyte energy metabolites in plateau field hypoxic mice is shown in Figure 5C and D. Plateau hypoxia enhanced the utilization of glucose and glucose-6-phosphate by erythrocytes, and with the increase of hypoxia time, the content of sedum heptulose 7-phosphate in the pentosiolysis pathway increased, and the content of fructose 1.6-bisphosphate, dihydroxyacetone phosphate, glyceraldehyde-3-phosphate, and 2-phospho-D-glyceric acid in the glycolysis pathway increased. Compared with the corresponding HYP group, MR could further increase the utilization of glucose and glucose-6-phosphate by red blood cells, increase the content of D-ribulose 5-phosphate and sedum heptulose 7-phosphate in the pentosilicolysis pathway, and increase the content of fructose 1.6-bisphosphate, dihydroxyacetone phosphate, glyceraldehyde-3-phosphate, 2-phospho-D-glyceric acid and phosphoenol pyruvate in the glycolysis pathway. Compared with the CON1 group, the levels of 2.3-BPG in red blood cells after 1, 3 and 7 days of hypoxia increased significantly by 114.15%, 212.32% and 227.07%. Compared with the HYP3 group, the level of 2.3-BPG in the MR3 group increased by 17.82%.

HK is the rate-limiting enzyme that catalyzes the first step of glucose, PFK and PK are the rate-limiting enzymes of the glycolytic pathway, and G6PD is the rate-limiting enzyme of the pentose phosphate pathway. Compared with the CON1 group, the HK activity of the HYP3 and HYP7 groups increased by 101.72% and 106.09%, the activity of PFK increased by 18.88% and 46.44%, and the PK activity of the HYP1 and HYP3 groups increased by 82.14% and 65.03%, indicating that hypoxia could significantly increase the activities of the three rate-limiting enzymes HK, PEK and PK in the glycolytic pathway. Compared with the CON1 group, the G6PD activity of the HYP3 and HYP7 groups was enhanced by 256.12% and 138.57%, which also indicated the enhancement of rate-limiting enzyme activity of the pentose phosphate pathway. The results showed that the activity of key enzymes in mouse erythrocytes was greatly enhanced to compensate for hypoxia in the hypoxic environment of plateau field. Rate-limiting enzyme activity in mouse

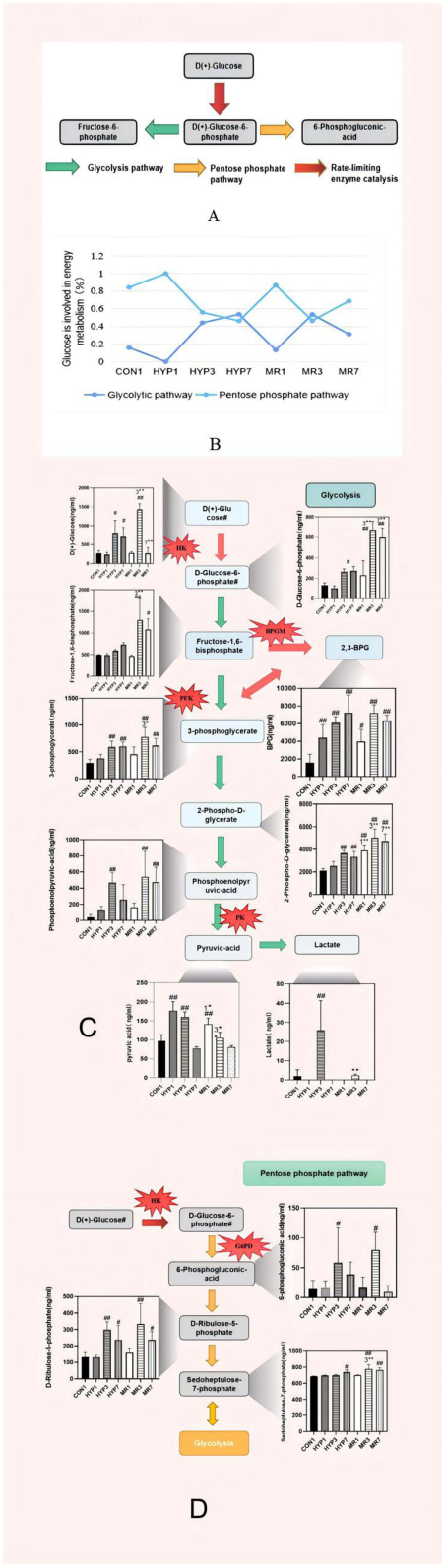


Figure 5 Effects of MR on BPGM activity and energy metabolism in erythrocytes of hypoxia mice (n=6). (**A**) Glucose in erythrocytes is involved in energy metabolism pathways. (**B**) Effect of MR on the proportion of glucose involved in glycolysis and pentose phosphate pathway in erythrocytes of mice at different hypoxic times. (**C**) The erythrocyte glycolysis pathway is involved in the change of glucose ratio in mice at different hypoxic stages (n=6). (HK: Hexokinase; PFK: Phosphofructokinase; PK: Pyruvate Kinase; BPGM: Bisphosphoglycerate Mutase; Red arrow: rate-limiting enzyme pathway; Green arrow: glycolytic pathway). (**D**) The pentose phosphate pathway in erythrocytes in mice at different hypoxic periods was involved in the change of glucose ratio (n=6). (HK: Hexokinase; G6PD: Glucose 6-phosphate dehydrogenase; red arrow: rate-limiting enzyme pathway; Orange arrow: pentose phosphate pathway). All data were analyzed by using Least-significant difference and are shown as the means ±SD. Compared with the blank group, $^{\#}P<0.05$, $^{\#\#}P<0.01$; Compared with the hypoxia group, $^{*}P<0.05$, $^{**}P<0.01$.

https://doi.org/10.2147/DDDT.S493866

erythrocytes was also altered after methyl rosmarinate was administered. Compared with the corresponding hypoxia days, the HK activity of MR1, MR3 and MR7 groups increased by 104.58%, 152.42% and 119.71%, the PFK activity of MR3 and MR7 groups increased by 119.46% and 48.40%, the PK activity of MR7 group increased by 3.66%, and the G6PD activity of MR7 group increased by 6.46%, indicating that MR administration can also enhance the activity of rate-limiting enzyme in the process of erythrocyte energy metabolism.

The results showed that with the deepening of hypoxia, the glucose in red blood cells was greatly transferred from the pentose phosphate pathway to the glycolytic pathway, and the level of 2.3-BPG increased significantly, and the administration of methyl rosmarinate could further increase the entry of glucose into the glycolytic pathway, and could further activate BPGM to increase the level of 2.3-BPG after 3 days of hypoxia.

Discussion

The main function of red blood cells in the blood system is to carry oxygen and to transport and release oxygen to tissue cells, and red blood cells do not consume oxygen themselves. In addition, due to the role of the red blood cell cytoskeleton, red blood cells have a strong deformation ability through blood vessels of different thicknesses (Betz, Lenz, Joanny, and Sykes, 2009).[9] The main gas exchange between erythrocytes and surrounding tissues occurs in the microcirculation, which is strongly influenced by the shape and deformability of erythrocytes, which can be subjected to oxidative stress induced by different drugs and diseases leading to anemia. Under oxidative stress erythrocytes may lose control of their volume and shape, leading to a loss of ability to move in microchannels (Besedina et al, 2021).[10] Erythrocyte aging decreases hexokinase and aldolase activity, indicating severe glycolytic restriction in aged erythrocytes, which may impair erythrocyte viability and is a determinant of normal erythrocyte lifespan (Chapman & Schaumburg, 1967).[11] Storage of erythrocytes in blood banks results in "storage damage", where 2.3-BPG is gradually depleted and Hb oxygen affinity increases. As storage time increases, sulfur dioxide increases and reactive oxygen species accumulate, eventually leading to a gradual loss of the ability of red blood cells to compensate for oxidative stress, resulting in oxidative damage.

Erythrocyte stimulation by plateau hypoxia activates the Rapoport-Luebering branch of the glycolytic pathway, which regulates the levels of 2.3-BPG catalyzed by BPGM. 2.3-BPG is a metabotropic regulator that regulates Hb conformation and reduces Hb oxygen affinity, thereby allowing erythrocytes to release oxygen. Band 3 (also known as anion exchanger) is an important membrane protein in the erythrocyte membrane that is a docking site for deoxyhemoglobin and glycolytic enzymes. Band 3-deoxyhemoglobin-glycolytic enzymes are also called the erythrocyte oxygen tension-based regulatory switch (Issaian et al, 2021).[12] When erythrocytes are normoxic, the end of band 3 binds to glycolytic enzymes and inactivates these enzymes, thereby inhibiting the glycolytic pathway and activating the pentose phosphate pathway; when erythrocytes are hypoxic, the end of band 3 binds to deoxyhemoglobin, inhibiting the pentose phosphate pathway and activating the glycolytic pathway and the Rapoport-Luebering branch, thereby increasing the amount of 2, 3-BPG and increasing the oxygen release capacity of erythrocytes (D'Alessandro & Xia, 2020).[13] This is in line with the results of our study.

The candidate compound MR significantly increased the 2.3-BPG content in normal and hypoxic erythrocytes by virtual screening of small molecules with BPGM in the previous phase. The results of this study showed that MR could significantly increase the survival time of normoxic confined mice, with the highest survival time extension rate at medium dose. MR could significantly reduce the plasma inflammatory factor level in plateau field hypoxic mice, significantly improve the oxidative stress damage in tissues caused by hypoxia, and could reduce the pathological damage of hypoxia on liver, heart, brain, lung and kidney tissues and the degree of hypoxia in mice with better preventive treatment effect. MR could enhance BPGM activity, increase 2.3-BPG content, conversion of glucose to glycolytic pathway and activity of glycolytic pathway rate-limiting enzymes in erythrocytes of hypoxic mice in the highland field. In summary, MR can activate BPGM in erythrocytes to produce more 2.3-BPG from the glycolytic branch, thus exerting a protective effect against injury in hypoxic mice in the field at plateau.

As a preliminary drug experiment, it was confirmed that MR Activated the glycolytic pathway and the Rapoport-Luebering branch, thereby increasing the content of 2.3-BPG to increase the oxygen releasing capacity of red blood cells, which is consistent with the above studies. Subsequently, on this basis, we can continue to explore the anti-hypoxia effect

of methyl rosemary, and detect glycolytic rate-limiting enzyme and BPGM through detection. The expression of isorelated proteins and the mechanism of MR Regulating Hb oxygen supply were discussed.

Data Sharing Statement

About Data Availability Statements All data generated or analysed during this study are included in this published article.

Contacts to Participate

No human subjects were involved in this study.

Contacts to Publish

That the work described has not been published before. That its publication has been approved by all co-authors.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

This work was supported by support from the National Natural Science Foundation of China (Grant No. 82173738), the 940th hospital of PLA Joint Logistics Surpport Force Troop Upbringing Program (Grant No. 2021yxky005), and the other is the All Army Youth Upbringing Program (Grant No. 20QNPY070).

Disclosure

The authors have no relevant financial or non-financial interests to disclose.

References

1. West JB. High-altitude medicine. *Lancet Resp Med*. 2015;3(1):12–13. doi:10.1016/S2213-2600(14)70238-3
2. Bartsch P, Swenson ER. Acute high-altitude illnesses. *N Engl J Med*. 2013;368(24):2294–2302. doi:10.1056/NEJMcp1214870
3. Sun K, Zhang Y, D'Alessandro A, et al. Sphingosine-1-phosphate promotes erythrocyte glycolysis and oxygen release for adaptation to high-altitude hypoxia. *Nat Commun*. 2016;7:12086. doi:10.1038/ncomms12086
4. Liu R, Heiss EH, Waltenberger B, et al. Constituents of Mediterranean spices counteracting vascular smooth muscle cell proliferation: identification and characterization of rosmarinic acid methyl ester as a novel inhibitor. *mol Nutr Food Res*. 2018;62(7):e1700860. doi:10.1002/mnfr.201700860
5. Laguerre M, López Giraldo LJ, Lecomte J, et al. Relationship between hydrophobicity and antioxidant ability of "phenolipids" in emulsion: a parabolic effect of the chain length of rosmarinate esters. *J Agric Food Chem*. 2010;58(5):2869–2876. doi:10.1021/jf904119v
6. So Y, Lee SY, Han AR, Kim JB, Jeong HG, Jin CH. Rosmarinic acid methyl ester inhibits lps-induced no production via suppression of MyD88-dependent and -independent pathways and induction of HO-1 in RAW 264.7 cells. *Molecules*. 2016;21(8):1083. doi:10.3390/molecules21081083
7. Abedini A, Roumy V, Mahieux S, et al. Rosmarinic acid and its methyl ester as antimicrobial components of the hydromethanolic extract of hyptis atrorubens poit. (Lamiaceae). *Evid Based Complement Alternat Med*. 2013;2013:604536. doi:10.1155/2013/604536
8. Wang SH, Ding DY, Sun CY, et al. Effect of methyl rosmarinic acid on 6-OHDA-induced oxidative damage in the brain. *J Yangzhou Univ*. 2017;38 (04):30–34.
9. Betz T, Lenz M, Joanny JF, Sykes C. ATP-dependent mechanics of red blood cells. *Proc Natl Acad Sci U S A*. 2009;106(36):15320–15325. doi:10.1073/pnas.0904614106
10. Besedina NA, Skverchinskaya EA, Ivanov AS, et al. Microfluidic characterization of red blood cells microcirculation under oxidative stress. *Cells*. 2021;10(12):3552. doi:10.3390/cells10123552
11. Chapman RG, Schaumburg L. Glycolysis and glycolytic enzyme activity of aging red cells in man. Changes in hexokinase, aldolase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase and glutamic-oxalacetic transaminase. *Br J Haematol*. 1967;13(5):665–678. doi:10.1111/j.1365-2141.1967.tb08832.x
12. Issaian A, Hay A, Dzieciatkowska M, et al. The interactome of the N-terminus of band 3 regulates red blood cell metabolism and storage quality. *Haematologica*. 2021;106(11):2971–2985. doi:10.3324/haematol.2020.278252
13. D'Alessandro A, Xia Y. Erythrocyte adaptive metabolic reprogramming under physiological and pathological hypoxia. *Curr Opin Hematol*. 2020;27(3):155–162. doi:10.1097/MOH.0000000000000574