Regular Article

Extract of *Salvia przewalskii* Maxim Repair Tissue Damage in Chronic Hypoxia maybe through the RhoA/ROCK Signalling Pathway

Yafeng Wang,*,a Delong Duo,a Yingjun Yan,a Rongyue He,a Shengbiao Wang,a Aixia Wang,a and Xinan Wu,*,b

*a Department of pharmacy, Qinghai Provincial People's Hospital, Xining 810000, China; and b Department of pharmacy, The First Hospital of Lanzhou University, Lanzhou 730000, China.

*Correspondence e-mail: gyyxgzz@outlook.com; xinanwu6511@163.com
Summary

*Salvia przewalskii* Maxim is a traditional Chinese herbal medicine and is known to have antibacterial, antiviral, anti-oxidant, anti-thrombotic and anti-depressant properties. However, the major active components of *S. przewalskii* and its anti-hypoxic effects are still unclear. This study probed the major active component and anti-hypoxic activity of *S. przewalskii*. The major active components of *S. przewalskii* were detected by HPLC. The anti-hypoxic effects of *S. przewalskii* were detected in mice and a rat model of hypoxic preconditioning. The results showed that there are eight active components, including sodium danshensu, rosmarinic acid, lithospermic acid, salvianolic acid B, dihydrotanshinone I, cryptotanshinone, tanshinone I and tanshinone IIA, and each component showed a certain anti-hypoxic effect. Moreover, *S. przewalskii* enhanced anti-hypoxia in mice, which was manifested as prolonged survival time in acute hypoxic preconditioning and the amelioration of acute hypoxia-induced changes in the activity of superoxide dismutase (SOD) and lactate dehydrogenase (LDH). In addition, *S. przewalskii* also repaired tissue damage in chronic hypoxia by downregulating HIF-1α, PCNA, Bcl-2, CDK4, CyclinD1 and P27Kip1 and inhibiting pro-inflammatory cytokines and the RhoA/ROCK signalling pathway. Our findings provide new insight into the anti-hypoxic effect of *S. przewalskii* as a promising agent for high-altitude pulmonary hypertension treatment.

Keywords

*Salvia przewalskii* Maxim; extract; repair tissue damage; RhoA/ROCK signalling pathway
INTRODUCTION

Global hypobaric hypoxia occurs at high altitudes. Approximately 140 million people live in the plateau area at altitudes of 2500 m.\cite{1} In addition, many people temporarily reach plateau altitudes due to tourism, skiing, mountaineering and other reasons. Acute exposure to high altitude may cause acute mountain sickness.\cite{2} High-altitude pulmonary hypertension (HAPH) is a severe health consequence of chronic exposure to hypoxia and is also a frequently occurring disease in the high-altitude regions (with a prevalence of up to 15\%).\cite{3} HAPH is characterized by increased pulmonary vascular resistance, pulmonary vasoconstriction and vascular remodelling of pulmonary arterioles,\cite{4} and HAPH is the leading cause of death from altitude sickness.\cite{5}

*Salvia przewalskii* Maxim (SP) is also known as “big Danshen” and is mainly produced in the western regions of China (Gansu, Qinghai, and Tibet). The main pharmacological activities of SP are similar to those of *Salvia miltiorrhiza* Bunge (known as ‘Danshen’ in Chinese) (SM), which is a well-known traditional Chinese medicine that has pharmacological functions in treating angina pectoris, stroke, atherosclerosis, myocardial infarction, liver fibrosis and hepatitis in clinical practice.\cite{6-8} Moreover, it has been demonstrated that SM and SP have marked anti-oxidant, anti-inflammatory, anti-thrombotic, antibacterial and antiviral activities.\cite{9,10}

In China, there are numerous commercial pharmaceutical dosage forms of SM, including granules, oral liquids, and dripping pills. Dripping pills have been approved for phase III clinical trials by the Food and Drug Administration (FDA),\cite{11,12} while SP is not included in the Chinese Pharmacopoeia and is only included in local standards such as Gansu Province Traditional Chinese Medicine Quality Standard and Yunnan Provincial Drug Standard. The active components of SP are still unclear, and it is urgent to determine its pharmacological
activity. In this study, the major active component and anti-hypoxic effects of SP, which will be beneficial for the development and clinical application of SP, were detected.

**MATERIALS AND METHODS**

**Chemicals and Reagents**  HPLC grade acetonitrile and methanol were provided by Thermo Fisher Scientific (MA, USA). Tanshinone IIA (ST8020, purity: 99.5%), cryptotanshinone (SC8640, purity: 99.0%), tanshinone I (ST8010, purity: 98.0%), salvianolic acid B (SS8100, purity: 96.2%), rosmarinic acid (SR8190, purity: 96.2%), dihydrotanshinone I (SD8290, purity: 98.0%) and sodium danshensu (SS8600, purity: 96.2%) were all purchased from Solarbio (Beijing, China). Acetazolamide (ACTZ) was purchased from Sine Pharm (Shanghai, China). Sildenafil was purchased from Pfizer (New York, USA). *S. przewalskii* samples were collected from Minhe County of Qinghai Province and identified by Professor Quanming Ma at the Department of Pharmacy, People's Hospital of Qinghai Province, according to the identification standards of the Yunnan Provincial Drug Standard (2013 Edition). The medicine was stored in a 4°C refrigerator at People's Hospital of Qinghai Province.

**Preparation of *S. przewalskii* Suspension**  *S. przewalskii* collected from Minhe (Qinghai Province, China) was cleaned, dried and pulverized, and extracted with 70% ethanol under ultrasound conditions (power 180 W, frequency 40 kHz) for 50 min. The extract solution was centrifuged, dried and ground to obtain extract powder. The desired concentration of the *S. przewalskii* suspension was prepared by distilling the extract powder with deionized water.

**Preparation of Test Samples and a Mixed Standard Sample**  The extract powder of *S. przewalskii* dissolved in 500 µL of 80% methanol and then filtered through 0.22 µm nylon mesh into sample vials. The mixed standard sample was prepared by combining 21.827 mg of sodium danshensu, 13.71 mg of rosmarinic acid, 26.45 mg of shikonic acid, 16.42 mg of salvianolic acid
B, 13.85 mg of dihydrotanshinone I, 15.74 mg of cryptotanshinone, 29.60 mg of tanshinone I, and 15.05 mg of tanshinone IIA in a 100 mL volumetric flask and making up the volume using 80% methanol as the solvent.

**Detection of the Major Active Components of S. przewalskii by HPLC**  
Analysis of the test samples and mixed standard sample analysis was performed with an Agilent 1290 Infinity LC system coupled to an ultraviolet-visible (UV-vis) detector (Agilent, USA). Chromatographic separation of the test samples and mixed standard sample was performed on a SunFire C-18 threaded column (4.6 × 250 mm, 5.0 μm, Waters, USA) maintained at 26°C. The mobile phase consisted of solvent A (0.1% formic acid in acetonitrile, v/v) and B (0.1% formic acid in water, v/v). The post time was set to 3 min for equilibration. The detection method of *S. przewalskii* was further validated by inspecting its linear range, precision, repeatability, and recovery rate according to FDA guidance for validation of bioanalytical methods.

**Activity of S. przewalskii Against Acute Hypoxia**  
All experiments using animals followed protocols approved by the Institutional Animal Care and Use Committee of People’s Hospital of Qinghai Province (PHQP-181102-01). To detect the activity of *S. przewalskii* against acute hypoxia, an acute hypoxia model was established in Kunming mice. In brief, all mice were assigned to five groups (*n*=6): (I) the normal group (the mice were administered deionized water by gavage); (II and III) the ACTZ groups (the mice were administered ACTZ at doses of 0.5 g/kg and 0.2 g/kg body weight every day for 7 days); and (IV, V and VI) the *S. przewalskii* groups (the mice were administered different doses of SP (0.5 g/kg, 1.0 g/kg and 2.0 g/kg body weight) every day for 7 days. All mice were housed by group in facilities in a closed hypoxia environment. The survival of the mice in the different treatment groups was recorded, and the heart and brain tissues were carefully isolated immediately. These tissues were frozen in liquid nitrogen and homogenized with a tissue homogenizer at 4°C, and then the homogenates were
Biological and Pharmaceutical Bulletin Advance Publication

The supernatants were transferred to a new tube and stored at -80°C until analysis. The concentrations of malondialdehyde (MDA) and superoxide dismutase (SOD) in the heart and brain were detected. Moreover, lactate dehydrogenase (LDH) activity was detected in the heart and brain using commercial kits according to the manufacturer's protocols (the three kits were all purchased from Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Activity of *S. przewalskii* Against Chronic Hypoxia

Seventy Sprague-Dawley (SD) rats (7 weeks old) weighing 160–180 g were housed in groups in a facility at a controlled relative humidity (45–65%) and temperature (22 ± 2°C). Feed and drinking water were supplied to the rats *ad libitum*. All rats were randomly assigned to five groups (*n*=14): (a) the normal group (the rats were raised in Xining; altitude approximately 2260 m); (b) the hypoxia group (the rats were raised in Maduo; altitude approximately 4260 m); and (c, d and e) the SP treated groups (the rats were administered different doses of SP (0.5 g/kg, 1.0 g/kg and 2.0 g/kg body weight every day) by gavage and raised in Maduo; altitude approximately 4260 m). The experiment lasted for four weeks. Adaptive feeding for one week before starting the experiment. All animals were anaesthetized with phenobarbital (intraperitoneal injection, 30 mg/kg), and the mean pulmonary arterial pressure (mPAP) of all animals was detected by a pulmonary artery pressure recorder at the end of the experiment. All animals were sacrificed under anaesthesia, and the lung, heart, liver, spleen, kidney and brain were carefully isolated immediately. The heart was excised rapidly, placed into ice-cold normal saline to remove the blood and weighed. Then, the atria were removed, and the hearts were separated into the right ventricle (RV) and left ventricle plus septum (LV+SEP) and weighed separately. Finally, the right ventricular hypertrophy index (RVHI) was calculated according to the relative weight ratio of the RV to the LV+SEP. Some tissues were fixed with 10% formalin, dehydrated with gradient alcohol, embedded in paraffin, sliced with a microtome and stained with haematoxylin eosin (H&E) for pathological...
observations by light microscopy. Some lung tissues were frozen in liquid nitrogen and then transferred and stored at -80°C until analysis.

Total mRNA from the lung was isolated using Trizol reagent (Shanghai Pufei Biotechnology Co., Ltd, Shanghai, China) following the manufacturer’s protocol. Complementary DNA synthesis was performed using the HiScript 1st Strand cDNA Synthesis Kit (TIANGEN, Beijing, China). Real-time quantitative PCR (RT-qPCR) was carried out using the SYBR Master Mixture system (TaKaRa, Dalian, China) on the ABI-7900HT system (Applied Biosystems, USA). The relative expression of genes was calculated using the $2^{-\Delta\Delta Ct}$ method. The details of the primers used for RT-qPCR were as the follows:

- GAPDH (forward primer: 5’-AATGGTGAAGGTCGGTGTGAAC-3’, reverse primer: 5’-AGGTCAAT GAAGGGTTCGGTGAAC-3’);
- HIF-1α (forward primer: 5’-CCAGATTCAAGATCAGCCAGC-3’, reverse primer: 5’-GCTGTACCATCAAGATCAGCCAGC-3’);
- PCNA (forward primer: 5’-GAGCTTGGGAATGGGAACA-3’, reverse primer: 5’-AGCTGAACTGGCTCATTCGGGAACA-3’);
- MCP-1 (forward primer: 5’-CTATGCAGGTCTCTGTCACGCTTC-3’, reverse primer: 5’-CAGCCGA CTCATTGGGATCA-3’);
- NF-κB (forward primer: 5’-CGACGTATTGCTGTGCCTTC-3’, reverse primer: 5’-TTGAGATCTGCCAGGTGGTA-3’);
- Bcl-2 (forward primer: 5’-GACTGAGTACCTGAACCGGCATC-3’, reverse primer: 5’-CTGAGCGTCTTCAGAGACA-3’);
- CDK4 (forward primer: 5’-CAACGCCTGTGGATATGTGGAG-3’, reverse primer: 5’-CTTCTGGAGG CAATCCAATGAG-3’);
- CyclinD1 (forward primer: 5’-TACCGCACAACCGCCTTC-3’, reverse primer: 5’-AAGGGCTTCAA TCTGTTTCTG-3’);
P27kip1 (forward primer: 5’-CGAATGCTGGCACTGTGGA-3’, reverse primer:
5’-CATTCAATGGA GTCAGCGATATGTA-3’);

RhoA (forward primer: 5’-CAGCAAGGACCAGTTCCCAGA-3’, reverse primer:
5’-AGCTGTGTCCC ATAAAGCCAACTC-3’);

ROCK1 (forward primer: 5’-TGCCAATAGTCCTTGGGTTCT-3’, reverse primer:
5’-CAAGGTCTC CACCAGGCATGTA-3’);

ROCK2 (forward primer: 5’-CACACAGTGCTTGTCAAAGTGC-3’, reverse primer:
5’-TGGATTGCA GGTTGAAGTAAGA-3’)

Activity of the Major Active Components of *S. przewalskii* Against Pulmonary Arterial Hypertension Induced by Low Pressure and Hypoxia Eighty-eight SD rats (7 weeks old, weighing 160–180 g) were randomly assigned to eleven groups (n=8): (a) the normal group; (b) the hypoxia group; (c-j) the major active components of *S. przewalskii* groups; and (k) the sidenafil group. The rats were raised in hypobaric oxygen chamber except normal group, and were administered drug every day by gavage according to the following doses based on each content of active components: rosmarinic acid (10.1 mg/kg), salvianolic acid B (6.2 mg/kg), dihydrotanshinone I (1.9 mg/kg), cryptotanshinone (3.9 mg/kg), tanshinone I (2.8 mg/kg), tanshinone IIA (6.7 mg/kg), sodium danshensu (1.1 mg/kg), lithospermic acid (1.0 mg/kg), and sidenafil group (5.4 mg/kg). Meanwhile, the hypoxia group was administered distilled water every day by gavage according to body weight. Feed and drinking water were supplied to the rats *ad libitum*. The experiment lasted for four weeks. At the end of the experiment, the mPAP and RVHI were detected according to the described method of chronic hypoxia.

**Statistical Methods** All results were presented as mean ± standard deviation (SD). One-way analysis of variance was applied to analyze the difference among groups. The results
were considered significant at \( P<0.05 \). All statistical analyses were conducted with the GraphPad Prism (version 5.0) software.

RESULTS

**Major Components of *S. przewalskii***  To detect the major active components of SP, HPLC analysis was used to compare SP extract with standard components. The HPLC analysis showed that the major components and contents of SP extract were sodium danshensu (0.398%), rosmarinic acid (4.334%), lithospermic acid (0.373%), salvianolic acid B (2.526%), dihydrotanshinone I (0.668%), cryptotanshinone (0.716%), tanshinone I (0.946%) and tanshinone IIA (1.311%) (Fig. 1A, B and C).

**Activity of *S. przewalskii* Against Acute Hypoxia**  The activity of SP extract against acute hypoxia was evaluated by an anoxia tolerance assay. As indicated in Fig. 2, 0.5 g/kg ACTZ and 1.0 g/kg SP extract markedly prolonged the anoxia survival time of mice \(( P<0.01 \), suggesting that the both medicines have certain effects against acute hypoxia.

**Effects of *S. przewalskii* Extract on the Levels of SOD, MDA and LDH in the Brain and Heart**  The effect of SP extract on the levels of SOD, MDA and LDH in the brain and hearts of the experimental and control mice is presented in Fig. 3. The results indicated that the levels of SOD and LDH were significantly increased in the brain and hearts of the experimental mice compared with the controls \(( P<0.05 \text{ or } P<0.01 \) (Fig. 3A and C). The level of MDA decreased in the SP extract-treated mice compared to the control mice \(( P<0.05 \text{ or } P<0.01 \) (Fig. 3B).

**S. przewalskii Extract Can Prevent and Treat Highland Hypoxic Pulmonary Hypertension**  A test of chronic hypoxia was performed at an altitude of 4260 m. The results showed that the mPAP and RVHI were significantly increased in the chronic hypoxia groups.
compared with the controls (P<0.01) (Fig. 4A and B). The chronic oral administration of SP extract (daily for 4 weeks) significantly decreased the levels of mPAP and RVHI compared with that in the chronic hypoxia groups and showed a dose-dependent effect (P<0.01) (Fig. 4A and B).

**Tissue Analysis**  The control group showed normal structure and morphology of the lung, heart, liver, spleen and kidney. However, the hypoxia group rats showed a reduced number of alveoli, the disordered structure, red blood cell leakage, thickening of the alveolar septum, seriously congested capillaries and thickening of the bronchial tube wall (Fig. 5A). The myocardial fibres were indistinct, the myocardial gap was enlarged and disordered (Fig. 5B). The hepatocytes were swollen and disorganized, and the hepatic sinuses and central veins were severely congested (Fig. 5C). The capsule of the spleen was markedly thickened and the cells were disorderly arranged, and the splenic sinus was severely congested (Fig. 5D). Glomerular compensatory hypertrophy with a smaller glomerular cystic lumen, swelling of renal tubular epithelial cells, loose cytoplasm, and a smaller lumen were observed (Fig. 5E). However, the pathology of the lung, heart, liver, spleen and kidney was gradually improved in the hypoxia group upon treatment with 0.5 g/kg SP (Fig. 5A-E), and the hypoxia+2.0 g/kg SP group showed basically normal organization form and structure (Fig. 5A-E).

**Mechanism of the Effects of *S. przewalskii* Extract Against Chronic Hypoxia**  To better understand the molecular mechanism of the effect of SP against chronic hypoxia, we performed RT-qPCR to detect the gene expression of HIF-1α, PCNA, MCP-1, NF-κB, Bcl-2, CDK4 and CyclinD1 in the lung. Our results showed that mRNA expression levels of HIF-1α, PCNA, MCP-1, NF-κB, Bcl-2, CDK4 and CyclinD1 were significantly increased in hypoxia group rats compared with the control groups (P<0.01) (Fig. 6A-G). SP extract inhibited the expression of HIF-1α, PCNA, MCP-1, NF-κB, Bcl-2, CDK4 and CyclinD1 mRNA in a
dose-dependent manner (Fig. 6A-G). To investigate the effect of SP extract on the RhoA/ROCK signalling pathway in the lung, we performed RT-qPCR for RhoA, ROCK1, ROCK2 and P27kip1. The results showed that RhoA, ROCK1, ROCK2 and P27kip1 mRNA expression in lung tissues was upregulated by hypoxia treatment at a high altitude compared with the control (P<0.01) and that the mRNA levels of RhoA, ROCK1, ROCK2 and P27kip1 decreased in a dose-dependent manner in SP extract-treated rats compared to control rats for 4 weeks (P<0.05 or P<0.01) (Fig. 6H-K).

**Effect of the Eight Components of *S. przewalskii* Extract on Pulmonary Hypertension Induced by Low Pressure and Hypoxia**

The hypoxia was simulated in hypobaric cabin. The mPAP and RVHI were significantly increased in hypobaric cabin rats compared with controls at the end of the experiment (P<0.01) (Fig. 7A and B). The mPAP was significantly decreased in all hypoxia+drug groups except for the component of rosmarinic acid compared with hypoxia group (P>0.05); and among them, salvianolic acid B and tanshinone IIA were the most effective (Fig. 7A). Meanwhile, the RVHI was significantly decreased in all hypoxia+drug groups except sildenafil compared with hypoxia group (P>0.05) (Fig. 7B). The results indicated that the eight components of *S. przewalskii* extract showed preventive and therapeutic effect on pulmonary hypertension induced by low pressure and hypoxia.

**DISCUSSION**

Folklore and herbal medicines play important role in primary health care in many low- and middle-income countries. Medicinal plants commonly contain many bioactive constituents that have multiple biological activities.13) Salvianolic acid B is one of abundant molecule isolated from the aqueous fraction of *S. miltiorrhiza* Bunge and has been shown to exert various anti-oxidative and anti-inflammatory effects.14,15) Salvianolic acid B is
associated with protective and therapeutic properties in various pathological conditions, including neuroinflammation, ischaemic-reperfusion injury, renal failure and neurological disorders. A recent study revealed that salvianolic acid B shows anti-tumour properties through p38 activation-mediated reactive oxygen species generation exerts anti-emphysema effects via the JAK2/STAT3/VEGF-dependent stimulation of lung cell proliferation and migration and inhibits the induction of lung cell death. Danshensu is an active component with wide cardiovascular and neuroprotective effects, as well as effects on mitochondrial function and cell survival. Tanshinone I and Tanshinone IIA also exhibit anti-oxidant and anti-inflammatory effects. Cryptotanshinone (CT) is a type of tanshinone that has been reported to have cytotoxic anti-tumour effects. In the present study, we demonstrated that extracts from SP contain eight components, namely, sodium danshensu, rosmarinic acid, lithospermic acid, salvianolic acid B, dihydrotanshinone I, cryptotanshinone, tanshinone I and tanshinone IIA. The bioactivity of SP extract may be associated with these components.

HAPH, defined as mPAP>25 mmHg, is a severe health consequence of chronic exposure to hypoxia with a prevalence of up to 15%. High altitude exposure enhanced formation of reactive oxygen species (ROS) by mitochondria electron transport chains, xanthine oxidase/reductase, NADPH oxidase, nitric oxide synthase enzymes, and inflammatory process and decrease the activity and cellular defence systems of antioxidant enzyme. ACTZ, is a carbonic anhydrase inhibitor, has long been standard prophylaxis for acute mountain sickness. In our study, anoxia tolerance assay showed that 0.5 g/kg ACTZ and 1.0 g/kg SP extract markedly prolonged the anoxia survival time of mice. And SP extract could effectively inhibit the oxidative damage by enhancing antioxidant (SOD) levels and reducing oxidative stress (MDA) levels. Meanwhile, increasing LDH can promote capacity for lactate efflux and elevate cardiac glucose uptake.
In chronic hypoxia experiment, SP extract significantly decreased the levels of mPAP and RVHI, exhibited significant anti-hypoxic effects and reversed the destruction/loss of tissue structure due to hypoxia. HIF-1α is a central regulator of the global response to hypoxia.\(^{32}\) PCNA plays critical roles in eukaryotic DNA replication and replication-associated processes.\(^{33}\) Bcl-2 is the first inhibitor of apoptosis through the regulation of mitochondrial outer membrane permeabilization leading to the irreversible release of intermembrane space proteins and subsequent caspase activation and apoptosis.\(^{34}\) CDK4 belongs to the cyclin-dependent kinase family and plays an important role in regulating the cell cycle. Phosphorylation by the CyclinD1/CDK4 kinase complex promotes cell cycle entry and proliferation.\(^{35}\) P27Kip1 is a CDK inhibitor that regulates CDK activity and plays a vital role in controlling progression through the G1 phase of the cell cycle.\(^{36}\) MCP-1 and NF-κB are one of inflammatory mediators and pro-inflammatory transcription factors. SP extract reverses alveolar structural destruction, alveolar septum thickening and capillary congestion of lung induced by hypoxia via the downregulation HIF-1α, PCNA, Bcl-2, CDK4, CyclinD1 and P27Kip1 to promote tissue damage repair; meanwhile, the expression of pro-inflammatory cytokines (MCP-1 and NF-κB) is inhibited. RhoA regulates cellular functions such as proliferation, apoptosis, contraction and motility, and ROCKs are the best characterised downstream targets of RhoA.\(^{37,38}\) The inhibition of RhoA and its downstream target ROCKs further inhibits the RhoA/ROCK signalling pathway and plays a significant role in the anti-hypoxic effects of SP extract.

In order to evaluate the activity of the eight active components of *S. przewalskii* against pulmonary arterial hypertension, hypobaric cabin was used to simulate hypoxia. Sildenafil can reduce pulmonary artery pressure, and received approval for the treatment of adult pulmonary arterial hypertension in the US and the EU,\(^{39}\) so sildenafil was chosen as the reference. The results showed that the mPAP was significantly decreased in all active
components of *S. przewalskii* groups (P<0.01) except rosmarinic acid; and the all active components significantly decreased RVHI (P<0.05 or P<0.01) and the effect was better than sildenafil. Further research is needed to test the relationship between structure and function of the main components of *S. przewalskii*.

**CONCLUSIONS**

SP extract contains eight components (sodium danshensu, rosmarinic acid, lithospermic acid, salvianolic acid B, dihydrotanshinone I, cryptotanshinone, tanshinone I and tanshinone IIA), and each component showed a certain anti-hypoxic effect. SP extract markedly prolonged the anoxia survival time and inhibited the oxidative damage in acute hypoxia. SP extract repairs tissue damage in chronic hypoxia by downregulating HIF-1α, PCNA, Bcl-2, CDK4, CyclinD1 and P27Kip1, inhibiting pro-inflammatory cytokines and inhibiting the RhoA/ROCK signalling pathway.

**Acknowledgements** Authors wish to thank Plateau Medical Research Center of Medical College of Qinghai University for the great support and help. This research was funded by Science and Technology Plan Project of Qinghai Province (2018-ZJ-786).

**Conflict of Interest** The authors declare no conflict of interest.
REFERENCES

1) Pratali L, Cavana M, Sicari R, Picano E. Frequent subclinical high-altitude pulmonary edema detected by chest sonography as ultrasound lung comets in recreational climbers. *Crit. Care. Med.*, **38**, 1818–1823 (2010).

2) Barry PW, Pollard AJ. Altitude illness. *BMJ*, **326**, 915–919 (2003).

3) Leon-Velarde F, Maggiorini M, Reeves JT, Aldashev A, Asmus I, Bernardi L, Ge RL, Hackett P, Kobayashi T, Moore LG, Penaloza D, Richelet JP, Roach R, Wu T, Vargas E, Zubieta-Castillo G, Zubieta-Calleja G. Consensus statement on chronic and subacute high altitude diseases. *High Alt. Med. Biol.*, **6**, 147–157 (2005).

4) Cardiology (ESC), European Respiratory Society (ERS), International Society of Heart and Lung Transplantation (ISHLT), Galíé N, Hoeper MM, Humbert M, Torbicki A, Vachiery JL, Barbera JA, Beghetti M, Corris P, Gaine S, Gibbs JS, Gomez-Sanchez MA, Jondeau G, Klepetko W, Opitz C, Peacock A, Rubin L, Zellweger M, Simonneau G. Guidelines for the diagnosis and treatment of pulmonary hypertension. *Eur. Respir. J.*, **34**, 1219–1263 (2009).

5) Hackett PH, Roach RC. High-altitude illness. *N. Engl. J. Med.*, **345**, 107–114 (2001).

6) Wang H, Gao XM, Zhang BL. Tanshinone: an inhibitor of proliferation of vascular smooth muscle cells. *J. Ethnopharmacol.*, **99**, 93–98 (2005).

7) Fei YX, Wang SQ, Yang LJ, Qiu YY, Li YZ, Liu WY, Xi T, Fang WR, Li YM. *Salvia miltiorrhiza* Bunge (Danshen) extract attenuates permanent cerebral ischemia through inhibiting platelet activation in rats. *J. Ethnopharmacol.*, **207**, 57–66 (2017).

8) Yu J, Gao H, Wu C, Xu QM, Lu JJ, Chen X. Diethyl Blechnic, a Novel Natural Product Isolated from *Salvia miltiorrhiza* Bunge, Inhibits Doxorubicin-Induced Apoptosis by Inhibiting ROS and Activating JNK1/2. *Int. J. Mol. Sci.*, **19**, 1809 (2018).

9) Lin R, Wang WR, Liu JT, Yang GD, Han CJ. Protective effect of tanshinone IIA on human umbilical vein endothelial cell injured by hydrogen peroxide and its mechanism. *J. Ethnopharmacol.*, **108**, 217–222 (2006).

10) Matkowski A, Zielińska S, Oszmiański J, Lamer-Zarawska E. Antioxidant activity of extracts from leaves and roots of *Salvia miltiorrhiza* Bunge, *S. przewalskii* Maxim., and *S. verticillata* L. *Bioresour. Technol.*, **99**, 7892–7896 (2008).

11) Hao PP, Jiang F, Chen YG, Yang J, Zhang K, Zhang MX, Zhang C, Zhao YX, Zhang Y. Evidence for traditional Chinese medication to treat cardiovascular disease. *Nat. Rev. Cardiol.*, **12**, 374 (2015).

12) Jia C, Han S, Wei L, Dang X, Niu Q, Chen M, Cao B, Liu Y, Jiao H. Protective effect of compound Danshen (*Salvia miltiorrhiza* Bunge) dripping pills alone and in combination with carbamazepine on kainic acid-induced temporal lobe epilepsy and cognitive impairment in rats. *Pharm. Biol.*, **56**, 217–224 (2018).

13) Li X, Luo Y, Wang L, Li Y, Shi Y, Cui Y, Xue M. Acute and subacute toxicity of ethanol extracts from *Salvia przewalskii* Maxim in rodents. *J. Ethnopharmacol.*, **131**, 110–115 (2010).

14) Chen T, Liu W, Chao X, Zhang L, Qu Y, Huo J, Fei Z. Salvianolic acid B attenuates brain damage and inflammation after traumatic brain injury in mice. *Brain Res. Bull.*, **84**, 163–168 (2011).

15) Durairajan SSK, Yuan Q, Xie L, Chan WS, Kum WF, Koo I, Liu C, Song Y, Huang JD, Klein WL, Li M. Salvianolic acid B inhibits A beta fibril formation and disaggregates preformed fibrils and protects against A beta-induced cytotoxicity. *Neurochem. Int.*, **52**, 741–750 (2008).

16) Hao YB, Xie TP, Korotcov A, Zhou YF, Pang XW, Shan L, Ji H, Sridhar R, Wang P, Califano J, Gu X. Salvianolic acid B inhibits growth of head and neck squamous cell carcinoma in vitro and in vivo via cyclooxygenase-2 and apoptotic pathways. *Int. J. Cancer*, **124**, 2200–2209 (2009).

17) Wang ZS, Luo P, Dai SH, Liu ZB, Zheng XR, Chen T. Salvianolic acid B induces apoptosis in human glioma U87 cells through p38-mediated ROS generation. *Cell Mol. Neurobiol.*, **33**, 921–928 (2013).

18) Sha W, Zhou Y, Ling ZQ, Xie G, Pang X, Wang P, Gu X. Antitumor properties of Salvianolic acid B against triple-negative and hormone receptor-positive breast cancer cells via ceramide-mediated apoptosis. *Oncotarget*, **9**, 36331–36343 (2018).
19) Dhapare S, Sakagami M. Salvianolic acid B as an anti-emphysema agent I: In vitro stimulation of lung cell proliferation and migration, and protection against lung cell death, and in vivo lung STAT3 activation and VEGF elevation. *Palm. Pharmacol. Ther.*, 53, 107–115 (2018).

20) Yu PF, Wang WY, Eerdun G, Wang T, Zhang LM, Li C, Fu FH. The role of P-glycoprotein in transport of danshensu across the blood–brain barrier. *Evid. Based Complement Altern. Med.*, 2011, 713523 (2011).

21) Chong CM, Su H, Lu JJ, Wang Y. The effects of bioactive components from the rhizome of *Salvia miltiorrhiza* (Danshen) on the characteristics of Alzheimer's disease. *Chin. Med.*, 14, 19 (2019).

22) Liu S, Han Z, Trivett AL, Lin H, Hannifin S, Yang, Oppenheim JJ. Cryptotanshinone has curative dual anti-proliferative and immunotherapeutic effects on mouse Lewis lung carcinoma. *Cancer Immunol. Immunother.*, 68, 1059–1071 (2019).

23) Saraf RS, Datta A, Sima C, Hua J, Lopes R, Bittner M. An in-silico study examining the induction of apoptosis by Cryptotanshinone in metastatic melanoma cell lines. *BMC Cancer*, 18, 855 (2018).

24) Simonneau G, Montani D, Celermajer DS, Denton CP, Gatzoulis MA, Krowka M, Williams PG, Souza R. Haemodynamic definitions and updated clinical classification of pulmonary hypertension. *Eur. Respir. J.*, 53, 1801913 (2019).

25) Winterbourn CC. Reconciling the chemistry and biology of reactive oxygen species. *Nat. Chem. Biol.*, 4, 278–286 (2008).

26) Arya A, Sethy NK, Singh SK, Das M, Bhargava K. Cerium oxide nanoparticles protect rodent lungs from hypobaric hypoxia-induced oxidative stress and inflammation. *Int. J. Nanomedicine*, 8, 4507–4520 (2013).

27) Ramanathan L, Gozal D, Siegel JM. Antioxidant response to chronic hypoxia in the rat cerebellum and pons. *J. Neurochem.*, 93, 47–52 (2005).

28) Bärtsch P, Swenson ER. Acute high-altitude illnesses. *N. Engl. J. Med.*, 368, 2294–2302 (2013).

29) Aamand R, Dalsgaard T, Jensen FB, Simonsen U, Roepstorff A, Fago A. Generation of nitric oxide from nitrite by carbonic anhydrase: a possible link between metabolic activity and vasodilation. *Am. J. Physiol. Heart Circ. Physiol.*, 297, H2068–H2074 (2009).

30) Horscroft JA, Kotwica AO, Laner V, West JA, Hennis PJ, Levett DZH, Howard DJ, Fernandez BO, Burgess SL, Ament Z, Gilbert-Kawai ET, Vercekual A, Landis BD, Mitchell K, Mythen MG, Branco C, Johnson RS, Feelsch M, Montgomery HE, Griffin JL, Grocott MPW, Gaiger E, Martin DS, Murray AJ. Metabolic basis to Sherpa altitude adaptation. *Proc. Natl Acad. Sci. U.S.A.*, 114, 6382–6387 (2017).

31) Holden JE, Stone CK, Clark CM, Brown WD, Nickles RJ, Stanley C, Hochachka PW. Enhanced cardiac metabolism of plasma glucose in high-altitude natives: adaptation against chronic hypoxia. *J. Appl. Physiol.*, 79, 222–228 (1995).

32) Han J, He Y, Zhao H, Xu X. Hypoxia inducible factor-1 promotes liver fibrosis in nonalcoholic fatty liver disease by activating PTEN/p65 signaling pathway. *J. Cell Biochem.*, 120, 14735–14744 (2019).

33) Hou Y, Ji N, Zhang H, Shi X, Han H, Lin S. Genome size-dependent pcna gene copy number in dinoflagellates and molecular evidence of retroposition as a major evolutionary mechanism. *J. Phycol.*, 55, 37–46 (2019).

34) Kale J, Osterlund EJ, Andrews DW. BCL-2 family proteins: changing partners in the dance towards death. *Cell Death Differ.*, 25, 65–80 (2018).

35) Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev.*, 13, 1501–1512 (1999).

36) Chu IM, Hengst L, Slingerland JM. The Cdk inhibitor p27 in human cancer: prognostic potential and relevance to anticancer therapy. *Nat. Rev Cancer.*, 8, 253–267 (2008).

37) Somlyo AP, Somlyo AV. Signal transduction by G-proteins, rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II. *J. Physiol.*, 522, 177–185 (2000).

38) Etienne-Manneville S, Hall A. Rho GTPases in cell biology. *Nature*, 420, 629–635 (2002).

39) Chanu P, Gao X, Bruno R, Claret L, Harnisch L. A modeling and simulation-based assessment of the impact of confounding factors on the readout of a sildenafil survival trial in pulmonary arterial hypertension. *J. Pharmacokinet. Pharmacodyn.*, 46, 499–509 (2019).
**Fig. 1.** HPLC profile of SP extract. (A) HPLC chromatogram of mixed standard solution. (B) HPLC chromatogram of SP extract. 1: Sodium danshensu, 2: rosmarinic acid, 3: lithospermic acid, 4: salvianolic acid B, 5: dihydrotanshinone I, 6: cryptotanshinone, 7: tanshinone I, and 8: tanshinone IIA. (C) The contents of the major components. (Color figure can be accessed in the online version.)
Fig. 2. The anoxia survival time of mice. The administration of 0.5 g/kg ACTZ and 1.0 g/kg SP extract markedly prolonged the anoxia survival time. ** P<0.01. (Color figure can be accessed in the online version.)
Fig. 3. Effects of treatment with SP extract (0.5, 1.0 and 2.0 g/kg) or the reference standard, ACTZ (0.2 and 0.5 g/kg), on the levels of SOD (A), MDA (B) and LDH (C) in the brain and hearts of acute hypoxia mice. * P<0.05, ** P<0.01. (Color figure can be accessed in the online version.)
Fig. 4. SP extract prevents and treats highland hypoxic pulmonary hypertension. The chronic oral administration of SP extract significantly decreased the levels of mPAP (A) and RVHI (B) compared with that in the chronic hypoxia groups and showed a dose-dependent effect. ** P<0.01. (Color figure can be accessed in the online version.)
Fig. 5. H&E staining of the main organs in the different groups (200×). (A) Lung, (B) heart, (C) liver, (D) spleen, and (E) kidney, magnification: 4×. (Color figure can be accessed in the online version.)
Fig. 6. The effects of SP extract on the mRNA expression of HIF-1α (A), PCNA (B), MCP-1 (C), NF-κB (D), Bcl-2 (E), CDK4 (F) and CyclinD1 (G), and the RhoA/ROCK signalling pathway (H-K) in the lung, as determined by RT-qPCR. *P<0.05, **P<0.01. (Color figure can be accessed in the online version.)
Fig. 7. The eight components of *S. przewalskii* extract showed preventive and therapeutic effect on pulmonary hypertension induced by low pressure and hypoxia. The component of rosmarinic acid did not show a significant decrease effect on mPAP (A). Sildenafil did not decrease RVHI compared with hypoxia group (B). * P<0.05, ** P<0.01. (Color figure can be accessed in the online version.)