Salvia miltiorrhiza alleviates hypoxia-induced nerve injury associated with Ngf/Akt intracellular signaling pathway

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Research

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Abstract

Background: *Salvia miltiorrhiza* (Danshen), a traditional Chinese herbal medicine, can effectively improve the high-altitude adverse reactions of high-altitude patients. While, the mechanism of how they exert neuroprotective effect to intervene the hypoxic at high altitudes is still not well understood.

Methods: The study established high altitude hypoxia mouse model and CoCl₂ -induced PC12 cell hypoxia model, the protective effects of *S. miltiorrhiza* radix extract (SE), Tanshinone IIA (Tan IIA) and salvianic acid A sodium (SAS) on hypoxia model were studied in vitro and in vivo.

Results: The results showed that SE, Tan IIA, and SAS are able to improve biochemical level in high altitude hypoxia mouse model, increase in Ca²⁺ concentration, and decrease in MMP, inhibit apoptosis in CoCl₂ -induced PC12 cell hypoxia model by activating Akt signaling pathway, protecting neurons, thus improving the oxygen carrying capacity of brain tissue.

Conclusion: This study confirms the efficacy of SE, Tan IIA, and SAS with respect to therapeutic treatment of hypoxia, shown that *S. miltiorrhiza* and its active monomers can protect neurons by activating Ngb/Akt intracellular signaling pathway, and attenuate cerebral anoxia and neuronal damage, subsequently nerve injury caused by hypoxia at high altitude. Providing important information for the clinical treatment of nerve injury caused by hypoxia at high altitude.

Introduction

The normal oxygen partial pressure of human tissue is 2–9% (14–65 mm Hg), whereas the oxygen partial pressure of intake air is 21% (160 mm Hg). Tissue hypoxia occurs when the outside air pressure is low or oxygen transport and use are blocked. Hypoxia is common stress reported in humans and animals[1]. Studies have shown that hypoxia can cause diseases of cardiovascular system, immune system, nervous system and many other physical disorders-even life-threatening pulmonary edema or cerebral edema[2]. These common diseases are closely related to tissue or cell hypoxia. Therefore, the pathogenesis of and interventions used in hypoxia-related diseases are currently hot research topics.

In hypoxic research, the study of anoxia injury of the nervous system is the most popular topic. Neuroglobin (Ngb) is mainly distributed in the neurons and retina cells, and it has been of high concern since found in 2000[3]. As the third kind of globins, Ngb has the ability of carrying oxygen similar to hemoglobin and myoglobin[4]. A large body of research has shown that the oxygen binding and neuron-specific expression properties make neuroglobin a new target molecule for the study of neuronal hypoxia and ischemia[5]. Sun et al. showed that antisense-mediated knockdown of neuroglobin rendered cortical neurons more vulnerable to hypoxia, whereas overexpression of neuroglobin conferred protection of cultured neurons against hypoxia[6]. A similar effect was observed in neuroblastoma cell line SH-SY5Y in which neuroglobin over-expression enhanced cell survival under conditions of anoxia or oxygen and glucose deprivation[7]. Other studies demonstrated that Ngb expression increased significantly in

neurons under hypoxic conditions, and Ngb has the ability of scavenging superoxide[8–10]. Therefore, Ngb can be an important target in the study of hypoxia in the nervous system.

It is of great significance to explore safe and effective drugs for the prevention of hypoxia at high altitudes. Recently, protective additives of hypoxia injury are mainly focused on natural extracts based on the free radical scavenging effects. The traditional Chinese Medicine Department of Tibet Military Region General Hospital has treated 181 patients with high-altitude sleep disorder syndrome since 2006. The clinical practice has proven that the traditional Chinese medicine which can promote blood circulation and remove stasis, could effectively improve the high-altitude adverse reactions of patients. According to the theory of traditional Chinese medicines, *S. miltiorrhiza* (*Salvia miltiorrhiza*) radix, also known as DanShen in Chinese, is a traditional Chinese medicine, which is used to promote blood circulation and relieve vessel stasis; it has been widely used in clinics in China for the treatment of cardiovascular diseases[11–13]. Tanshinone IIA (Tan IIA) and salvianic acid A sodium (SAS) are the main natural active ingredients purified from *S. miltiorrhiza* radix, SAS is a major water-soluble component extracted and Tan IIA is a diterpene quinone[14]. *S. miltiorrhiza* can inhibit the aggregation function of blood platelets, anticoagulation, improve the activity of fibrinolytic enzyme, reduce blood lipid and regulate blood. It can promote blood circulation and remove blood stasis, expand coronary artery, improve myocardial ischemia and improve hypoxia response at high altitudes[15].

The basic and clinical research has confirmed that *S. miltiorrhiza* and its active monomers have protect effect on hypoxic brain damage [16]. However, the mechanism of how *S. miltiorrhiza* and its active monomers exert the neuroprotective effect to directly intervene the hypoxic at high altitudes is still not well understood. Previous studies have purified Ngb *in vitro* and preliminarily explored the hypoxic neuroprotective effect of Ngb[17]. Therefore, this study takes Ngb as the key point, combining *in vivo* and *in vitro* research, to observe the intervention effect of *S. miltiorrhiza* radix and its active monomers on high-altitude hypoxia and to explain the pharmacological mechanism of *S. miltiorrhiza* radix and its active monomers on high-altitude hypoxia injury.

Materials And Methods

Drugs and reagents

S. miltiorrhiza radix (lot 1805031) was obtained from Beijing Jin Chongguang Pharmaceutical Co., Ltd. (Beijing, China), Tanshinone IIA (Tan IIA) (≥98% purity; lot 20170908), salvianic acid A sodium (SAS) (≥98% purity; lot 171001) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetazolamide (ACZ) (≥98% purity; lot B27044) was purchased from Beijing BioDee Biotechnology Co., Ltd. (Beijing, China). LY294002 (≥99.5% purity, CAS No.154447-36-6) was purchased from MedChemExpress LLC(USA, New Jersey). Kits of glutathione peroxidase (GSH-Px, Cat. No. A005), total superoxide dismutase (T-SOD, Cat. No. A001-1), Catalase (CAT), and malondialdehyde (MDA, Cat. No. A003-1) were purchased from Nianjing Jiancheng Bioengineering Institute (Nanjing, China); Hypoxia Inducible Factor 1 α (HIF-1α) was purchased from Quanzhou Konodi

Biotechnology Co., Ltd. RPMI-1640 and fetal bovine serum (FBS) are Hyclone products obtained from Thermo Fisher Scientific (Waltham, MA, USA). Dichlorofluorescein diacetate and a Cell Counting Kit-8 (CCK-8) were obtained from Sigma Millipore (Darmstadt, Germany). Apoptosis detection kit and fluo-3-acetoxymethyl ester were purchased from Biosea Biotechnology (Beijing, China). The reactive oxygen species (ROS) fluorescent probe, dihydroergotamine, was purchased from Vigorous Biotechnology (Beijing, China). TransScript™ First-Step quantitative reverse transcriptase polymerase chain reaction SuperMix and SuperMix were purchased from Promega Corporation (Fitchburg, WI, USA). Applied Biosystems Fast SYBR® Green Master Mix was purchased from Thermo Fisher Scientific. The tissue protein extraction kit was obtained from Cowin Biotech Company Ltd. (Beijing, China). bicinchoninic acid (BCA) was purchased from Applygen Technologies Inc (Beijing, China). Polyvinylidene difluoride membrane and the ECL® kit were purchased from Amersham (now GE Healthcare, Arlington Heights, IL, USA). Protein markers were purchased from Genstar (Beijing, China). Ngb Polyclonal Antibody (PA5-76950) were purchased from Invitrogen Corporation (Carlsbad, USA). Akt Polyclonal Antibody (YT0175), Akt (phospho Ser473) Polyclonal Antibody (YP0006) were purchased from ImmunoWay Biotechnology Company (Texas, USA). Other analytical reagents were purchased from Promega (Beijing) Biotech Co., Ltd. (Beijing, China), low pressure and low oxygen animal test cabin borrowed from the Academy of military medicine (model: flydwc50-1b, Guizhou Fenglei Aviation Ordnance Co., Ltd., China).

Preparation of Decoctions.

Fifty grams of *S. miltiorrhiza* were extracted using deionized water and ethanol (1:1, 500 mL) for 1 h during microboiling under reflux. The extracts were filtered through three layers of gauze, and the drug extraction was then repeated. The filtrates were combined and were then concentrated to 250 mL at 60–70°C under reduced pressure.

For the in vivo experiments, samples were shaken, calibrated, and stored at 4°C. Based on a preliminary test, ACZ was prepared with normal saline according to 60 mg/mL. Tan ⅡA was prepared with soybean oil for injection at a concentration of 6 mg/mL. SAS was prepared with soybean oil for injection at 12 mg/mL.

For the in vitro experiments, SE was centrifuged at 1200 rpm for 3 min and filtered with 0.2 µm filter membrane to prepare 1 g/mL in Phosphate buffer solution (PBS), SAS, and Tan IIA dissolved in Dimethyl sulfoxide (DMSO). Through the CCK-8 test, we chose the dose of SE (1 mg/mL), Tan ⅡA (40 µM), and SAS (100 µM), which has no toxic effect on cell viability according to the follow-up test.

Animals and treatments

Seven-week-old male BALB/c mice weighing 20–25 g were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. and fed at the specific pathogen free (SPF) level animal center of the Academy of Military Medical Sciences (Beijing, China). The mice were kept in an SPF animal facility at a constant temperature of 23°C and relative humidity of 40%–60%. The protocol was approved by the

Committee on the Ethics of Animal Experiments of the animal center of the Academy of Military Medical Sciences. The animal experiments were conducted in accordance with the Guiding Principles in the Use of Animals in Toxicology.

Group assignment and drug administration

Mice were randomly divided into six groups (n=8 per group): vehicle, ACZ, SE, SAS, and Tan IIA groups. Animals were treated with ACZ, SE, SAS, and Tan IIA for 10 days by intraperitoneal injection or gastric lavage as shown in Table 1. Continuous administration lasted for 10 days. On the 4th day, 30 min after daily administration, the rats were exposed to hypobaric hypoxia, which increased at a rate of 10 m/s and was maintained at 3000 m for 10 min; and then increased to 4500 m and stayed for 10 min; final increased to 6000 m, after 16 h of hypoxia exposure, it decreased to sea level at a rate of 15 m/s. Mice in the control group were fed in normoxic environment. After the last hypoxia exposure for 15 h, the mice were dissected. Group assignment and drug administration as shown in Table 1.

Histopathologic examination

Daily changes in body weight and clinical signs. For macroscopic damage, brain were fixed in 10% buffered formalin. A single pathologist, who was blinded to the treatment, duration, and genotype of the sample, examined and scored brain representative from three mice per group. Hematoxylin and eosin (H&E)- Nissl-stained sections were assessed according to a standard staining protocol.

Determination of the levels of MDA / HIF-1 α and activities of T-SOD, GSH-Px, and CAT

Mice were sacrificed by cervical dislocation. The blood of each mouse was obtained from the aortaventralis. The brain were immediately removed and washed with ice-cold normal saline. The left brain were weighed and stored at -80°C, and then homogenized with a phosphate buffer (50 mmol/L, pH 7.0) containing 0.1 mmol/L EDTA before use. The brain homogenate was divided into two parts. One part was centrifuged at 2054 g for 10 min at 4°C, and the supernatant was used for MDA, GSH-Px, T-SOD, and CAT tests. Another part was used for direct determination of HIF-1 α activity. The blood and brain homogenate were centrifuged at 2054 g for 10 min at 4°C to obtain serum and brain supernatant for MDA, GSH-Px, T-SOD, CAT, and HIF-1 α tests. All parameters were determined using the respective kits according to the manufacturer's specifications.

Cell culture and treatment

PC12 human neuroblastoma cells were grown in RPMI-1640 supplemented with 15% FBS. Cells were maintained at 37°C in an incubator with a saturated humid atmosphere containing 95% air and 5% CO₂. Cells were passaged once every three days. All experiments were conducted on cells between passages 10-20 after cells were purchased from National Infrastructure of Cell Resource (Beijing, China).

Cell viability analysis

Cytotoxicity was assayed in PC12 cells grown in 96-well plates. Cells (1.5×10^5 cells/ml; 0.1 ml per well) were seeded into plates and allowed to grow overnight before replacement of the medium with serum-free medium supplemented with CoCl_2 1mM co-treated with drug for 24h. Subsequently, 10 μl CCK-8 was added into each well and incubated for 1-4 h. The absorbance was read at 450 nm with a PerkinElmer Victor X Microplate Reader (PerkinElmer, Inc., Waltham, MA, USA). Reductions in optical density (OD) due to drug treatment were used to assess cell viability and normalized against control incubated in medium (100% viability).

Measurement of the mitochondrial membrane potential (MMP)

MMP was measured using flow cytometry, and the mitochondrial-specific cationic dye, JC-1. PC12 cells (1.5×10^5 cells/ml) were plated in 12-well plates CoCl_2 1mM co-treated with drug for 24h. Cells were harvested, washed twice with PBS, and incubated with 0.5 mL JC-1 (25 μM) for 20 min at 37°C. MMP was assayed, and green (JC-1 monomer) and red (JC-1 aggregate) fluorescence were monitored at emission wavelengths of 525 and 595 nm, respectively. Changes in the ratio between measurements were indicative of changes in the MMP.

Measurements of intracellular ROS and Ca^{2+}

PC12 cells (1.5×10^5 cells/ml) were plated in 12-well plates with CoCl_2 1mM co-treated with drug for 24h, and Intracellular ROS and cytosolic Ca^{2+} were measured using the fluorescent probes DCFH-DA and Fluo-3-AM, respectively, and a fluorescence-activated cell sorter. DCFH-DA is converted into a fluorescent compound in the presence of ROS. Fluo-3-AM was added to treated cells to measure Ca^{2+} . After treatment with the indicated drugs, cells were incubated with DCFH-DA (10 μM) for 20 min at 37°C in the dark (for the ROS assay) or Fluo-3/AM (5 $\mu\text{mol/l}$) for 30 min at 37°C (the Ca^{2+} assay), and the cells were then harvested and suspended in 500 μl HBSS. Intracellular ROS and Ca^{2+} were measured using a flow cytometer (excitation wavelength, 488 nm; emission wavelength, 535 nm).

Cell apoptosis

Apoptosis was also measured using annexin V-FITC and PI. PC12 cells were plated (1.5×10^5 cells/ml; 0.1 ml) in 12-well plates with CoCl_2 1mM co-treated with drug for 24h. Cells were harvested, washed twice with ice-cold PBS, and then suspended in 200 μl ice-cold binding buffer. Subsequently, 10 μl HRP FITC-labeled annexin V and 5 μL PI were added to the cells. The cell suspension was gently mixed, and incubated for 15 min at room temperature in the dark. Apoptosis was monitored using flow cytometry (488 nm excitation wavelength), and the fluorescence intensity was measured at 530 nm (emission wavelength). Annexin V+/PI- was used to document early apoptosis, whereas AnnexinV+/PI+ was used to assess the late apoptotic stages or necrotic cells.

Quantitative real-time PCR

Total RNA was extracted from brain tissue and PC12 cells using Invitrogen® TRIzol reagent according to the manufacturer's instruction (Thermo Fisher Scientific, Inc.). PC12 cells were maintained in 6-well plates until 80% confluence and then treated with Tan IIA, SAS and SE in serum-free medium for various periods of time to assess the specific induction of Ngf expression. CoCl₂ (1 mM) and dimethyl sulfoxide (DMSO) was used as the control. The quality of the RNA was confirmed by an A260/A280 ratio of ≥ 1.8 and an RNA integrity number ≥ 6.5 . Complementary DNA (cDNA) was synthesized using a Transcriptor™ first-strand cDNA synthesis kit at 42°C for 30 min followed by 70°C for 5 min. qRT-PCR reactions were performed on an ABI StepOne Plus™ Real Time PCR instrument using SYBR Green PCR Master Mix. The amplification reactions were performed as follows: 20 seconds at 95°C, and 40 cycles at 95°C for 3 seconds, and 60°C for 30 seconds. The primers used in the current study are listed in Table 2. The quantity of each transcript was calculated as described in the instrument manual and was normalized to the amount of the housekeeping gene β -actin.

Preparation of total protein and Western blotting

Total protein was extracted from brain tissue and PC12 cells according to the instruction. PC12 cells (1.5×10^5 cells/ml) were plated in 6-well plates with CoCl₂ 1mM for 24h, and then treated with SE(1g/ml), SAS(50mM), Tan IIA(40mM) and LY294002(10mM) co-treated with SE(1g/ml), SAS(50mM), Tan IIA(40mM) for 24h. The protein expression of Ngf, Hif-1 α , Akt and P-Akt were determined by Western blotting. Protein of brain and PC12 cells were extracted with ice-cold lysis buffer and centrifuged at $12,000 \times g$ for 10 min., and the resultant supernatant assayed using BCA protein assay kit standardized to BSA. Equal amounts of total protein (40 μ g) were loaded, separated by 12% SDS-PAGE, and transferred to polyvinylidene fluoride (PVDF) membrane. The membranes were blocked in 5% TBST fat free milk (blocking buffer) for 2 hrs, briefly washed, and then incubated overnight at 4°C with specific primary antibodies against human Ngf (PA5-76950; dilution 1:500), Hif-1 α (dilution 1:500), Akt (YT0175; dilution 1:1000), P-Akt (YP0006; dilution 1:1000). The samples were subsequently incubated with monoclonal IgG (1:2,000) secondary antibody for 2 hours, and visualized on film using a Santa Cruz ECL detection system. β -actin served as a loading control. The blank control group of mice and the DMSO solvent control group of PC12 cells served as the negative controls respectively. Densitometric analyses were performed to semiquantify protein expression.

Results

Pharmacodynamic evaluation

During the drug administration, the rats of the control group were noted to be livelier and more energetic, with bright hair color and normal gains of weight. By contrast, the mice in entering the low pressure and low oxygen animal test cabin were mentally depressed, had dark hair color, and consumed less food. However, upon treatment of SE and Tan IIA, the mental state of the rats was gradually restored.

No significant differences in the weight of the rats were observed before entering the low pressure and low oxygen animal test cabin. However, the body weight of the mice in the model group were found to be lower than that of the control ($P < 0.05$). By the end of the treatment cycle, the body weights of the SE and Tan IIA groups mice were significantly higher compared with those of the model group ($P < 0.05$). The body weights in the ACZ group were lower compared with that of the model group, these results suggested that SE and Tan IIA enabled the mice to retain their weights, whereas the ACZ group had no improvement. (Figure. 1, Table 3)

Histological analysis

The control group appeared to have no observable nerve injury, whereas the other groups exhibited different degrees of lesions, especially in the model group. The Nissl body of the neuronal cells were turbid, the neurons were shrunken, and the coloring power decreased. And it can be seen the vasodilatation and congestion, cerebral edema. Cerebral softening and hemorrhage with microglia proliferation, and occasional neurophagic phenomenon. Although the SE, Tan IIA, and SAS groups also had similar pathological changes, these were significantly less severe compared with those of the model groups. The pathological HE staining results are shown in Figure 2A, and the Nissl stain results are shown in Figure 2B. The neurofrontal cortex image (indicated by the arrow) is seen in the frontal cortex of the brain, blood vessels are congested, and the peripheral space is widened. Neuron cytoplasmic nuclei are blurred, vertebral cells are reduced, and tinting is poor. The results showed the cerebral parietal cortex vascular congestion around the edema (indicated by the black arrow) and nerve phagocytosis (indicated by the green arrow).

Effects of the drug on the MDA level and activity of T-SOD, GSH-Px, and CAT

In this experiment, the effects of SE, Tan IIA, and SAS on the activities of T-SOD, CAT, GSH-PX, and the content of MDA in brain tissue, and serum of mice were studied. As shown in Figure 3, the results showed that ACZ could increase the activity of CAT and T-SOD and decrease the content of MDA in serum and brain tissue compared with the model group. Also, SE, Tan IIA SAS could increase the content of CAT, T-SOD, and GSH-Px, decrease the content of MDA in serum and brain tissue.

Effect of SE, SAS, and Tan IIA on the HIF-1 α level

It has been shown that HIF-1 α is a key factor in the occurrence of hypoxia injury. In this study, The effects of SE, Tan IIA, and SAS on the content of HIF-1 α in serum and brain tissue of mice were detected by ELISA. As shown in Figure 4, low-pressure and low-oxygen chamber led to the increase of HIF-1 α level in blood and brain tissue of mice. The content of HIF-1 α in the serum and brain tissue of the model group was higher than that in the control group ($P < 0.05$). The content of HIF-1 α of the ACZ, SE, Tan IIA and SAS groups were lower than that in the model group in the serum and brain tissue ($P < 0.05$).

Ngb expression in mouse brain

In this study, we investigated the effect of SE, Tan α A, and SAS on the relative expression of Ngf mRNA and protein in mouse brain. As shown in Figure 5, the results showed that the relative expression of Ngf mRNA and protein in the control group was less than that in the model group ($P < 0.05$). Compared with the model group, the relative expression of Ngf mRNA and protein in ACZ, SE, and SAS groups were greater than that in the model group ($P < 0.05$).

CoCl₂-induced PC12 cell hypoxia model

The experimental results show that hypoxia has a significant inhibitory effect on PC12 cells. Different concentrations of CoCl₂ have hypoxia damage on PC12 cells. IC₅₀ is about 1 mM, as shown in Figure 6A. Therefore, in this experiment, 1 mM concentration of CoCl₂ is selected as the condition of PC12 cell model. The model of hypoxia injury was successfully constructed.

Under the microscope, the morphology of PC12 cells was observed: in the normal condition, the process of PC12 cells was longer, cross-linked into a network, and the cells were prismatic and polygonal. After 24 h of culture, the cell membrane was smooth, round, and full, and synaptic connections were established between cells. Under the condition of hypoxia, PC12 cells shrunk into a round shape, the synapses between cells disappeared, and the membrane was folded, which was easy to fall off, as shown in Figure 6B.

Influence of SE, SAS, and Tan α A on PC12 cell function under hypoxic conditions

CoCl₂ is able to cause hypoxia injury to cells. In order to evaluate the effects of SE, Tan α A, and SAS on the CoCl₂-induced cell hypoxia model, the viability of the PC12 cells by the CCK-8 assay was first investigated, and it was identified that SE, Tan α A, and SAS led to a significant improvement in the PC12 cells' viability compared with the CoCl₂ model group ($P < 0.05$; Figure 7A).

In addition, annexin-V and PI staining were performed, and flow cytometry was used to distinguish living cells from apoptotic and necrotic cells. These experiments demonstrated that the number of apoptotic cells in the CoCl₂ group was significantly higher compared with those in the control group ($P < 0.05$), whereas the apoptosis of PC12 cells in the SE, Tan α A, and SAS groups was significantly lower compared with that in the CoCl₂ group ($P < 0.05$). These results revealed that treatment with SE, Tan α A, and SAS was able to protect the PC12 cells from CoCl₂-induced apoptosis (Figure 7B).

As a marker of oxidative stress, ROS participated in renal injury through oxidative stress and inflammatory reactions, and the levels of ROS are increased markedly in hypoxia injury. In the present study, the levels of intracellular ROS in the CoCl₂ group were found to be significantly higher compared with those of the control group ($P < 0.05$; Figure 7C). In addition, the ROS level in the SE, Tan α A, and SAS treatment group was also significantly lower compared with that in the CoCl₂ group ($P < 0.05$). Therefore, ROS production was shown to be effectively inhibited by SE, Tan α A, and SAS.

When cells are stimulated by external stress, the mitochondrial function is compromised, with a decrease in the MMP, and an increase in the levels of ROS and the intracellular Ca^{2+} concentration. In this study, after PC12 cells were stimulated by CoCl_2 , the intracellular Ca^{2+} level increased significantly, whereas the MMP level decreased significantly ($P < 0.05$, respectively). After administration of SE, Tan IIA , and SAS, the Ca^{2+} concentration was reduced markedly, and the MMP level was elevated to an appreciable extent compared with that in the CoCl_2 group ($P < 0.05$). The decreases in Ca^{2+} and MMP caused by cell damage were also found to be alleviated following treatment with SE, Tan IIA , and SAS. (Figure 7D).

SE, SAS, and Tan IIA ameliorated hypoxic-induced nerve apoptosis via the Ngb/Akt intracellular signaling pathway

The mRNA expression level of Ngb was assessed by RT-qPCR in the CoCl_2 induced PC12 cells. In these experiments, the relative expression of Ngb mRNA in the hypoxia model group was higher than that in the control group ($P < 0.05$). The relative expression of Ngb mRNA in the drug group was significantly higher than that in the hypoxia model group ($P < 0.05$). To further investigate the correlation between Ngb/Akt intracellular signaling pathway and hypoxia, we introduced LY294002, LY294002 can penetrate cells, and specifically inhibit PI3K/Akt signaling pathways, including the common inhibition of Akt phosphorylation. The PC12 cells were divided into 9 subgroups, including control groups, drug treated groups and LY294002 co-treated groups. Protein expression of Ngb, Hif-1 α , Akt and P-Akt were determined by quantitative Western blot analysis. The results showed that CoCl_2 induced cell hypoxic injury and mechanism of hypoxia protection in PC12 cell reflected in the rise of Hif-1 α and Ngb. SE, Tan IIA , and SAS effectively improve the protein expression level of Ngb in PC12 cells, and LY294002 co-treated groups substantially attenuated the effects of drug-induced upregulation of Ngb. Akt expression was not significantly changed, but phospho-Akt expression was increased by SE, Tan IIA , and SAS groups. Moreover, LY294002, an inhibitor of the AKT signaling pathway, could reverse this result. The phospho-Akt protein levels are compared with total Akt kinase protein, LY294002 co-treated groups decreased the P-Akt protein expression level of CoCl_2 induced PC12 cell hypoxic injury (Figure 8). These results indicated that SE, Tan IIA , and SAS can protect hypoxia induced PC12 from apoptosis via the NGB-AKT pathway

Conclusion

Hypoxia research plays an important role in military medicine and high altitude medicine. Safe and reliable traditional Chinese medicine research and development are of great significance to the prevention and treatment of diseases caused by high altitude hypoxia[18]. This paper confirmed the protective effects of SE, Tan IIA , and SAS on hypoxia mice model at high altitudes by in vitro and in vivo experiments. The results showed that *S. miltiorrhiza* and its active monomers can protect neurons by activating Ngb/Akt intracellular signaling pathway, and attenuate cerebral anoxia and neuronal damage caused by hypoxia at high altitude, and it can be used to prevent and treat the injury of hypoxia to nerve tissue and the occurrence of hypoxia-related diseases.

Discussion

S. miltiorrhiza, a traditional Chinese herbal medicine, is widely used in mainland China for the treatment of cardio/cerebrovascular disorders. Tan IIA and SAS are the main natural active ingredients purified from *S. miltiorrhiza* radix. The protective effects of Tan IIA and SAS have been well proven in various models of cerebral ischemia-reperfusion injury [35]. Its possible mechanism of the protective effect on the central nervous system involves calcium channel blockade, estrogen-like action, and antiperoxidation, which may inhibit cerebral nerve cell apoptosis and ameliorate mitochondrial dysfunction, etc. Hence, we hypothesized that treatment with *S. miltiorrhiza* and its active monomers might present beneficial effects against hypoxia response at high altitudes, and to bring insights into their mechanisms of action.

Body weight is an important non-specific observation index in animal experiments, which mainly reflects the comprehensive metabolism and physical fitness of animal body. ACZ is the first choice for prevention of acute high-altitude reaction in foreign countries. But its side effects are significant, which can cause numbness, polyuria, thirst, gastrointestinal discomfort, and even lead to renal failure and toxic epidermal necrolysis[19]. The experiment also showed some adverse reactions of ACZ group mice; however, *S. miltiorrhiza* has significant efficacy and high safety compared with ACZ. Therefore, this experiment proves that *S. miltiorrhiza* can be used as a drug to prevent altitude hypoxia disease and provide experimental basis for clinical medication.

CAT, SOD, and GSH-Px play an important role in the balance of oxidation and antioxidation[20]. By producing superoxide, the body induces lipid peroxidation and forms lipid peroxides such as MDA, which indirectly reflects the degree of cell damage. ACZ, SE, Tan IIA, and SAS may increase the activity of CAT, T-SOD, GSH-Px and decrease the content of MDA to achieve the protective effect of hypoxia mice model at high altitudes.

Constructing the cell model of hypoxia by subjecting cells to CoCl_2 treatment is currently recognized and widely used. When the body is under oxidative stress conditions, the balance between oxidation and anti-oxidation is disrupted. The increases in ROS levels in the cells exceed their scavenging capacity. Mitochondria are the main site for the production of ROS, and also the main target of ROS. Numerous studies have shown that excessive ROS can cause oxidative damage to mitochondria, resulting in their abnormal function[21, 22]. The abnormalities of this function are mainly manifested in a reduction of MMP, extracellular Ca^{2+} influx, mitochondrial DNA mutation, disruption of the mitochondrial respiratory chain, and so on. and they may therefore be convenient signal markers in oxidative damage, abnormal protein expression, and cell damage [23, 24]. The present study has shown that SE, Tan IIA and SAS are able to inhibit CoCl_2 induced apoptosis of the PC12 cells. Although the intracellular ROS of PC12 cells were markedly increased upon CoCl_2 treatment, while the increase in Ca^{2+} concentration, and concomitant decrease in MMP, caused by ROS were inhibited by SE, Tan IIA and SAS, indicating that SE, Tan IIA and SAS are able to inhibit cell injury caused by CoCl_2 .

Ngb, a novel neuroprotective protein, which has neuroprotective effect on hypoxia/ischemia injury in vivo and in vitro, and may participate in the pathophysiological process of ischemia and hypoxia injury. Increasing the content of Ngb in neurons can prevent and treat the injury of brain tissue caused by hypoxia and the occurrence of hypoxia-related diseases, which is helpful to reduce the occurrence of hypoxia symptoms at high altitudes [25, 26]. Previous data have implied that the upregulated expression of Ngb could be an endogenous compensatory or protective mechanism in response to sublethal hypoxic/ischemic insults to brain neurons. At the cell level, inhibition of the expression of Ngb can reduce the survival rate of cells under hypoxia, on the contrary, increasing the expression of Ngb can enhance the survival rate of cells under hypoxia. At the animal level, the expression of Ngb was increased by intraventricular injection of adenovirus vector fused with Ngb gene and construction of transgenic mice[38]. After cerebral ischemia-reperfusion, the area of cerebral infarction in these animals was significantly smaller than that in normal animals. It is suggested that NGB can inhibit the brain injury caused by ischemia / hypoxia and has potential neuroprotective function. A large number of basic and clinical studies have shown that Ngb can stabilize MMP, affects mitochondrial related functions, including ATP and ROS production, play a neuroprotective role and reduce the number of toxic stimulation. In addition, Ngb can regulate apoptosis through direct interaction with VDAC, Gα and Cyt-C proteins, eventually trigger the downstream part of the apoptotic cascade[27–29]. In our study, *S. miltiorrhiza* and its active monomers were able to improve the expression of Ngb in brain neurons, eliminate superoxide, play the role of antioxidative stress. The regulation of SE, Tan α A, and SAS on Ngb is fully demonstrated the certain correlation with their protection against hypoxia injury.

Ngb is favorably linked to HIF-1 α and phosphorylated AKT. HIF-1 α has emerged as a critical oxygen-sensitive transcription factor that orchestrates the body's protective response to hypoxia. The expression of HIF-1 α under hypoxia has protective effects on astrocytes, thus playing an important role in cerebral protection [30]. Recent study has demonstrated that The role of HIF-1 α in the regulation of oxygen homeostasis in tissue may be correlated with Ngb expression [31]. HIF-1 α contributes to the upregulation of Ngb expression under hypoxic conditions in mice [32]. PI3K / Akt signal is involved in the regulation of promotion, differentiation, apoptosis and glucose transport. High concentration of PI3K inhibitor can block the phosphorylation of Akt, promote a series of tandem reactions caused by downstream molecules, accelerate the process of apoptosis, and inhibit the survival of neural stem cells. It reports that Ngb increases Akt phosphorylation, which can be antagonized by LY294002, the common inhibition of Akt phosphorylation. PI3K/Akt pathway is involved in Ngb inhibiting apoptosis and promoting cell growth. [33, 34] Therefore, we suggest that *S. miltiorrhiza* and its active monomers is an inducer of Ngb /Akt intracellular signaling pathway, *S. miltiorrhiza* and its active monomers may protect neurons by activating Ngb/Akt intracellular signaling pathway and inhibiting the activity of downstream target gene. In this experiment. The results showed that SE, Tan α a, and SAS could significantly inhibit the decrease of MMP and apoptosis induced by CoCl₂, and significantly improved the survival rate compared with the control group. Western blotting showed that SE, Tan α A, and SAS could not only induce the expression of Ngb protein, but also promote the expression of Akt phosphorylation level, which can be inhibited by LY294002, a specific inhibitor of PI3K / Akt signaling pathway, which indicates that SE, Tan α A, and SA

can significantly inhibit CoCl_2 induced PC12 cell injury, and the neuroprotective effects may be achieved by activating Ngb / Akt intracellular signaling pathway.

As shown in our study, SE, Tan β A, and SAS are able to increase the activity of CAT, T-SOD, GSH-Px, and decrease the content of MDA, HIF-1 α in high altitude hypoxia mouse model, Also, they are able to Improve the response to hypoxia damage at high altitudes by increasing the expression of Ngb in brain neurons, increase in Ca^{2+} concentration, and decrease in MMP, inhibit apoptosis in CoCl_2 -induced PC12 cell hypoxia model by activating Akt signaling pathway,protecting neurons, thus improving the oxygen carrying capacity of brain tissue. This study confirms the efficacy of SE, Tan β A, and SAS with respect to therapeutic treatment of hypoxia, shown that *S. miltiorrhiza* and its active monomers can protect neurons by activating Ngb/Akt intracellular signaling pathway, and attenuate cerebral anoxia and neuronal damage, subsequently nerve injury caused by hypoxia at high altitude. Finally, providing important information for the clinical treatment of nerve injury caused by hypoxia at high altitude

Declarations

Ethical Approval and Consent to participate:

Ethical approval was obtained from the Ethics Committee of the Air Force Medical Center of PLA.

Consent for publication:

Publication consent was obtained from all authors.

Data and materials availability:

All data needed to evaluate the conclusions in the paper are present in the paper.

Competing interests:

The author declares that he has no competing interests.

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Author contribution:

Zhang xianxie was involved in experimental strategy, performed the experiments and analyzed the data. Haiying Qiu and Kong Jian designed and executed experiments, Yan Wu and Hailong Yuan analysed the data and contributed to the writing of the manuscript.

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Tables

Table 1. Group assignment and drug administration

Groups (n=8)	Normoxia / Hypoxia	Drug	Dosage	Administration
Control	Normoxic	physiological saline	-	-
Model	Hypoxia	physiological saline	-	-
Acetazolamide (ACZ)	Hypoxia	ACZ	60 mg/mL	Oral administration of gastric lavage
<i>S. miltiorrhiza</i> radix extract (SE)	Hypoxia	<i>S. miltiorrhiza</i> radix extract	200 mg/mL	Oral administration of gastric lavage
Tanshinone IIA (Tan A)	Hypoxia	Tanshinone IIA (Tan A)	6 mg/mL	intraperitoneal injection
Salvianic acid A sodium (SAS)	Hypoxia	Salvianic acid A sodium (SAS)	12 mg/mL	intraperitoneal injection

Table 2. Primer sequences for RT-PCR analysis

<i>Primer name</i>	<i>Primer sequences</i> 5'-3'
β- Actin - mouse	F:ATCTACGAGGGCTATGCTCTCC R:CTGATCCACATCTGCTGGAAGG
Ngf - mouse	F:ACTGGCCAGCCATAATGGAG R:GTGGGGAGACAGGATTCAGC
β- Actin - PC12 cell	F:CCCGCGAGTACAACCTTCTT R:CGCAGCGATATCGTCATCCA
Ngf - PC12 cell	F:CTTTACGCCGGCTACAAGGA R:TCTCTTACTCCCCGTCCCAG

Table 3. The weight gain of mice in each groups

Groups n=8	Weight gain(g)
Control	9.42±0.03
Model	3.31±0.41 [#]
ACZ	1.63±0.48
SE	4.93±0.04 [*]
Tan A	4.22±0.08 [*]
SAS	3.56±0.71

Figures

Figure 1

Effect of *Salvia miltiorrhiza* extract and its main components on weight gain of mice (n=8) # Compared with the blank group, $p < 0.05$; * compared with the model group, $p < 0.05$

Fig 2A

Fig 2B

Figure 2

Histopathological changes in brain and heart tissue obtained from hypoxia mouse. (A) H&E staining; A. Control group: In the parietal lobe, neuron cells in the DG region can be seen under the high magnification of the hippocampus. The cell body is full and neatly arranged. The glial cells phagocytic neurons (black arrows), and cerebral blood vessels are basically normal. B. Model group: Edema around the parietal cortex of the brain (black arrow), Nerve phagocytosis (green arrow), vascular congestion in the frontal

cortex of the brain (black arrow) shows blood vessel congestion, and the peripheral space widens. Neuron cytoplasmic nucleus is blurred, vertebral body cells are reduced, and tinting strength is poor. C. ACZ group: Hippocampal reticulum can be seen in the parietal lobe. An increase in the perivascular hyperemic space (black arrow) and nerve phagocytosis (green arrow) in the parietal lobe of the brain D. SE group: Edema around the frontal cortex of the brain (black arrow), neurons in the hippocampal DG region of the parietal lobe are normal E. Tan IIA group: Occurrence of microfocal glial cell proliferation in the frontal cortex of the brain (black arrow), edema occasionally under the parietal cortex of the cerebral cortex, and loose reticular structure (black arrow). F. SAS group: Cerebral vascular congestion in the parietal lobe of the brain (left black arrow), nerve phagocytosis (right black arrow). (B) Nissl staining (magnification, x100). The black arrows represent typical injuries, A: Control, B: Model, C: ACZ, D: SE, E: Tan IIA, F: SAS. For further details concerning the establishment of the model groups, see the Materials and methods section.

Fig 3.

Figure 3

Effects of *Salvia miltiorrhiza* extract and its main components on hypoxia mouse model. The levels of MDA, T-SOD, CAT, GSH-PX were investigated: (A) Serum; (B) Brain tissue, Each value is expressed as the mean $\pm$ S.E.M (n=8). [#] Compared with the blank group, $p < 0.05$; ^{*} compared with the model group, $p < 0.05$ For further details concerning the establishment of the model groups, see the Materials and methods section.

Fig 4.

Figure 4

The content of hypoxia inducible factor-1 α (HIF-1 α) of *Salvia miltiorrhiza* extract and its main components on hypoxia mouse model. The levels of HIF-1 α were investigated by EISA in serum and brain. (n = 8)(compared with the blank group, $P < 0.05$; * compared with the model group, $P < 0.05$)

Fig 5

Figure 5

Effect of *Salvia miltiorrhiza* and its main components on the relative expression of Ngb mRNA and protein in brain on hypoxia mouse model ($n = 8$) (compared with the blank group, $P < 0.05$; * compared with the model group, $P < 0.05$) Shown are (A) mRNA expression of Ngb; (B) western blot analysis of Ngb. Each value is expressed as the mean $\pm$ S.E.M ($n=8$). * $P<0.05$ compared with the model group.

Fig 6

A

B

Figure 6

A:The cell viability of PC12 cells with different concentrations of CoCl₂. B: Cell morphogram of the CoCl₂ induced hypoxia model in PC12 cell: (a) normal state cell, (b) hypoxia state cell.

Fig 7

A

C

B

D

Figure 7

Effects of *Salvia miltiorrhiza* and its main components on CoCl₂ induced hypoxia model in PC12 cells. Shown are the results of experiments that investigated (A) The level of cell viability, (B) the extent of apoptosis; (C) level of ROS; (D) level of MMP. [(a) Control group; (b) CoCl₂ group; (c) SE group; (d) Tan IIA group; (e) SAS group]. For further details concerning the establishment of the model groups, see the

Materials and methods section. #P<0.05 compared with the control group; *P<0.05 compared with the CoCl2 group.

Fig. 8

A

B

C

Figure 8

Effect of Salvia miltiorrhiza and its main components on the relative expression of Ngb mRNA and protein in CoCl2 induced hypoxia model in PC12 cells. Shown are the results of experiments that investigated (A) mRNA expression of Ngb; (B) western blot analysis of Ngb, Hif-1α, Akt and P-Akt (C)

Statistical analysis of p-Akt/Akt and Ngf protein expression levels in PC12 cells. Each value is expressed as the mean $\pm$ S.E.M (n=8). *P<0.05; **P<0.01 compared with the model group.