**NEURAL REGENERATION RESEARCH**
Volume 7, Issue 27, September 2012
www.nrronline.org

*doi:10.3969/j.issn.1673-5374.2012.27.003 [http://www.crter.org/nrr-2012-qkquanwen.html]*
*Yan WH, Liu JZ. Effects of Chinese herbal monomers on oxidative phosphorylation and membrane potential in cerebral mitochondria isolated from hypoxia-exposed rats in vitro. Neural Regen Res. 2012;7(27):2099-2106.*

# Effects of Chinese herbal monomers on oxidative phosphorylation and membrane potential in cerebral mitochondria isolated from hypoxia-exposed rats *in vitro*★

**Weihua Yan[1, 2, 3], Junze Liu[1, 2, 3]**

1 *Department of Pathophysiology and High Altitude Physiology, College of High Altitude Military Medicine, the Third Military Medical University of Chinese PLA, Chongqing 400038, China*
2 *Key Laboratory of High Altitude Medicine, Ministry of Education, Chongqing 400038, China*
3 *Key Laboratory of High Altitude Physiology and High Altitude Disease of Chinese PLA, Chongqing 400038, China*

## Abstract
Mitochondrial dysfunction is the key pathogenic mechanism of cerebral injury induced by high-altitude hypoxia. Some Chinese herbal monomers may exert anti-hypoxic effects through enhancing the efficiency of oxidative phosphorylation. In this study, effects of 10 kinds of Chinese herbal monomers on mitochondrial respiration and membrane potential of cerebral mitochondria isolated from hypoxia-exposed rats *in vitro* were investigated to screen anti-hypoxic drugs. Rats were exposed to a low-pressure environment of 405.35 mm Hg (54.04 kPa) for 3 days to establish high-altitude hypoxic models. Cerebral mitochondria were isolated and treated with different concentrations of Chinese herbal monomers (sinomenine, silymarin, glycyrrhizic acid, baicalin, quercetin, ginkgolide B, saffron, piperine, ginsenoside Rg1 and oxymatrine) for 5 minutes *in vitro*. Mitochondrial oxygen consumption and membrane potential were measured using a Clark oxygen electrode and the rhodamine 123 fluorescence analysis method, respectively. Hypoxic exposure significantly decreased the state 3 respiratory rate, respiratory control rate and mitochondrial membrane potential, and significantly increased the state 4 respiratory rate. Treatment with saffron, ginsenoside Rg1 and oxymatrine increased the respiratory control rate in cerebral mitochondria isolated from hypoxia-exposed rats in dose-dependent manners *in vitro*, while ginsenoside Rg1, piperine and oxymatrine significantly increased the mitochondrial membrane potential in cerebral mitochondria from hypoxia-exposed rats. The Chinese herbal monomers saffron, ginsenoside Rg1, piperine and oxymatrine could thus improve cerebral mitochondrial disorders in oxidative phosphorylation induced by hypobaric hypoxia exposure *in vitro*.

## Key Words
high-altitude hypoxia; oxidative phosphorylation; Chinese herbal medicine; mitochondria; brain; neural regeneration

## Research Highlights
(1) Saffron, ginsenoside Rg1 and oxymatrine significantly and dose-dependently increased the respiratory control rate in cerebral mitochondria isolated from hypoxia-exposed rats.
(2) Ginsenoside Rg1, piperine and oxymatrine *in vitro* significantly increased the membrane potential of isolated cerebral mitochondria from hypoxia-exposed rats.
(3) Saffron, ginsenoside Rg1 and oxymatrine may play roles in increasing the tolerance of organisms to hypoxia and thus decreasing the incidence of high-altitude disease.

Weihua Yan★, Master, Department of Pathophysiology and High Altitude Physiology, College of High Altitude Military Medicine, the Third Military Medical University of Chinese PLA, Chongqing 400038, China; Key Laboratory of High Altitude Medicine, Ministry of Education, Chongqing 400038, China; Key Laboratory of High Altitude Physiology and High Altitude Disease of Chinese PLA, Chongqing 400038, China

Corresponding author: Junze Liu, Master, Professor, Department of Pathophysiology and High Altitude Physiology, College of High Altitude Military Medicine, the Third Military Medical University of Chinese PLA, Chongqing 400038, China; Key Laboratory of High Altitude Medicine, Ministry of Education, Chongqing 400038, China; Key Laboratory of High Altitude Physiology and High Altitude Disease of Chinese PLA, Chongqing 400038, China
liuliu@tmmu.edu.cn

Received: 2012-03-30
Accepted: 2012-07-18
(N20111117004/YJ)

### Abbreviations

ST3, state 3 respiration rate; ST4, state 4 respiration rate; RCR, respiratory control rate

## INTRODUCTION

Over 90% of oxygen is consumed in mitochondria, which represent the main site of energy (ATP) production[1]. Electrons from the oxidative substrate are delivered to oxygen *via* the respiratory chain in the inner mitochondrial membrane. The oxidative energy is converted to phosphorylated energy and stored in ATP by ATP synthetic enzymes. The utilization efficiency of oxygen depends on the coupling level of mitochondrial oxidation and phosphorylation.

Mitochondrial state 3 respiration rate (ST3, oxygen consumption in the presence of ADP), state 4 respiration rate (ST4, oxygen consumption in the absence of ADP), respiratory control rate (RCR, reflecting the coupling level between oxidative and phosphorylative processes) and mitochondrial membrane potential are the major indexes reflecting mitochondrial function, which is a primary target of hypobaric hypoxic insult after exposure to high-altitude or ischemia[2-3]. Previous studies found that ST3 and RCR were reduced, while ST4 was increased in skeletal muscle[4] and brain[5-6] in acute hypoxia-exposed mice and rats. These results indicated that hypoxic exposure could induce the uncoupling of mitochondrial oxidation and phosphorylation, thus reducing ATP production and oxygen utilization efficiency. Decreased ATP production during hypoxia may disturb cellular structure, function and metabolism, which form the bases of many other hypoxic responses in organs, systems and organisms, such as maladaptation to high altitude or mountain sickness[7-8]. New measures are therefore needed to improve the energy metabolism of cells or bodies exposed to hypoxia, to relieve the distress induced by low oxygen concentrations[9-10].

The results of our previous study demonstrated that guanine nucleotides decreased the uncoupling of respiration and increased RCR and mitochondrial membrane potential in brain mitochondria from hypoxia-exposed rats *in vitro* [11]. However, their rapid decomposition makes guanine nucleotides unsuitable as an anti-hypoxia medicine. To the best of our knowledge, no effective agents able to improve the coupling of mitochondrial oxidative phosphorylation impaired by acute and severe simulated high-altitude hypoxia exposure have yet been reported.

Many Chinese herbal agents are known to have organs protecting effects against ischemia or ischemia-reperfusion injury, including *Salvia miltiorrhiza* [12], *Rhodiola rosea* [13] and ginseng[14-15]. However, their chemical compositions are complex and it is hard to identify the active ingredients of these Chinese herbal agents and thus investigate the mechanisms of these components. Based on the findings discussed above, it is reasonable to hypothesize that there are some kinds of Chinese herbal monomers would have effects on mitochondrial respiration of hypoxia-exposed rat. In this study, we therefore investigated the effects of herbal monomers with previously demonstrated efficacies against hypoxic, ischemic or ischemia-reperfusion diseases of the heart, brain or other organs[12-15], on respiratory activity and membrane potential in hypoxic-exposed rat brain mitochondria *in vitro*.

## RESULTS

### Quantitative analysis of experimental animals

Ninety-six male Sprague-Dawley rats were initially included in the study and randomly divided into normal , hypoxic, sinomenine, silymarin, glycyrrhizic acid, baicalin, quercetin, ginkgolide B, saffron, piperine, ginsenoside Rg1 and oxymatrine intervention groups ($n$ = 8 each). All rats were included in the final analysis of results.

### Effects of Chinese herbal monomers on RCR in brain mitochondria isolated from hypoxia-exposed rats *in vitro*

Hypoxic exposure induced decreases of 22.19% and 42.06% in ST3 and RCR, respectively, and an increase of 40.86% in ST4 ($P$ < 0.01; Figure 1), consistent with the results of a previous report[5]. *In vitro* treatment with glycyrrhizic acid, baicalin, silymarin, and ginkgolide B resulted in further decreases in ST3 and RCR ($P$ < 0.05 or 0.01; Figures 2A–D), while saffron markedly increased ST3 from 8.05% (0.2 mM) to 25.29% in a dose-dependent manner (1.5 mM) ($P$ < 0.01; Figure 2E) compared with the 0-mM intervention group. Treatment with ginsenoside Rg1, oxymatrine, sinomenine and piperine up to 1.5 mM decreased ST4 in hypoxic mitochondria by 28.75%, 25.58%, 23.38% and 21.95%, respectively ($P$ < 0.01; Figures 2F–I). Because sinomenine, piperine and quercetin depressed both ST3 and ST4 simultaneously, they had no significant effect on RCR (Figures 2H–J). Compared with the 0-mM

intervention group, the maximal increases in RCR in mitochondria treated with saffron, ginsenoside Rg1 and oxymatrine were 32.06%, 34.25% and 33.04%, respectively ($P < 0.01$; Figures 2E–G).

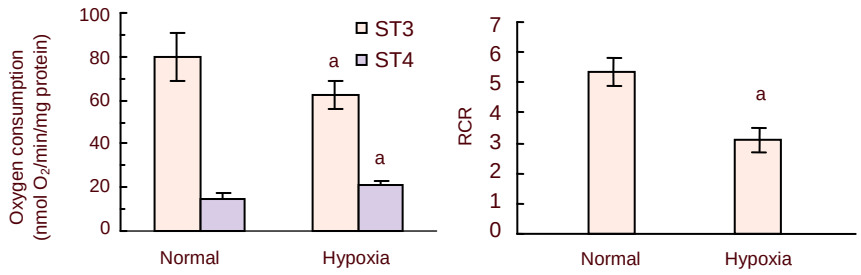



Figure 1 Effect of hypoxia on mitochondrial respiration rates in rat brain mitochondria.

All data are shown as mean ± SD based on eight separate experiments. [a]$P < 0.01$, *vs.* normal group by unpaired Student's *t*-test. ST3: State 3 respiration rate; ST4: state 4 respiration rate; RCR: respiratory control rate.

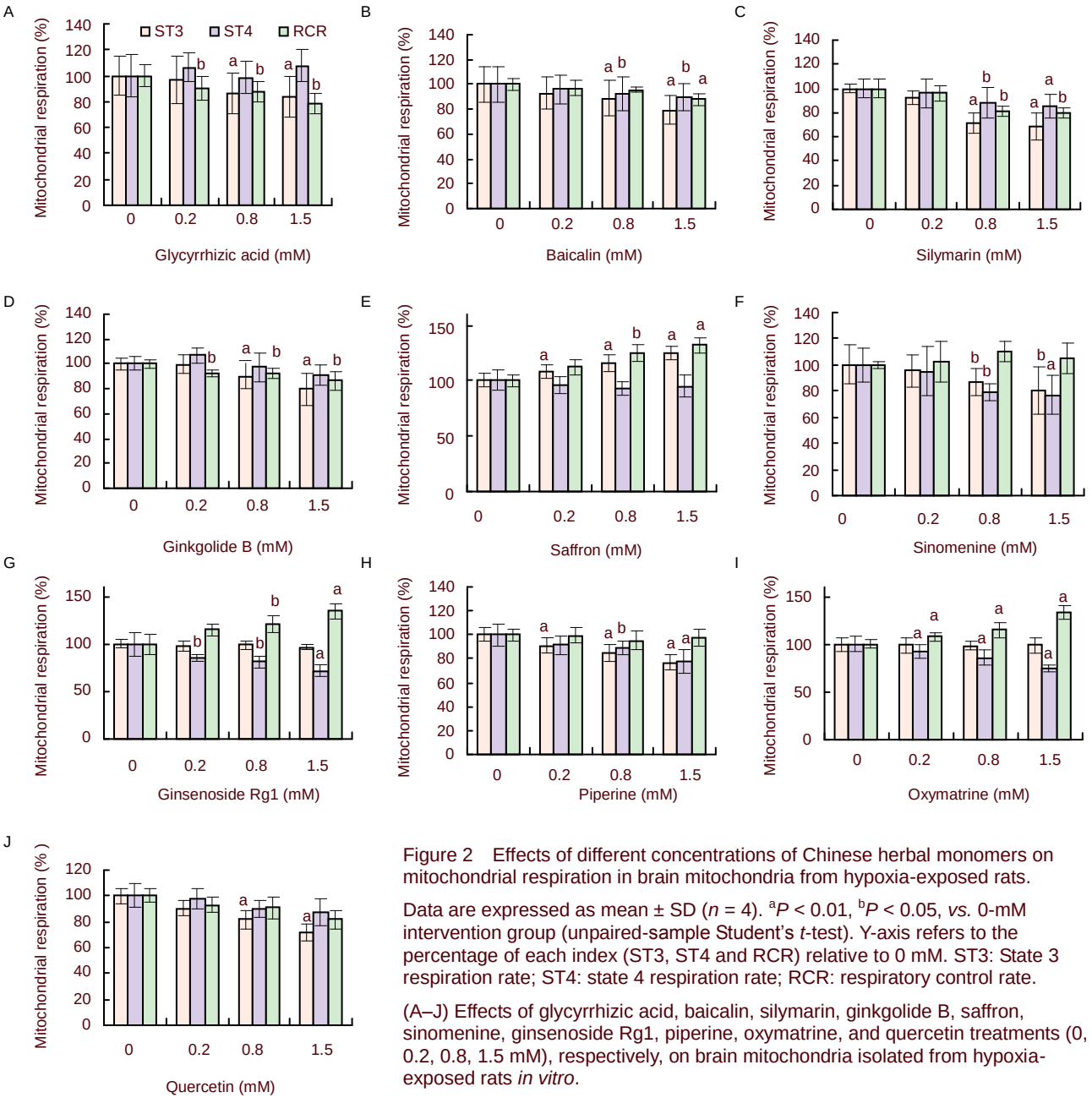



Figure 2 Effects of different concentrations of Chinese herbal monomers on mitochondrial respiration in brain mitochondria from hypoxia-exposed rats.

Data are expressed as mean ± SD ($n = 4$). [a]$P < 0.01$, [b]$P < 0.05$, *vs.* 0-mM intervention group (unpaired-sample Student's *t*-test). Y-axis refers to the percentage of each index (ST3, ST4 and RCR) relative to 0 mM. ST3: State 3 respiration rate; ST4: state 4 respiration rate; RCR: respiratory control rate.

(A–J) Effects of glycyrrhizic acid, baicalin, silymarin, ginkgolide B, saffron, sinomenine, ginsenoside Rg1, piperine, oxymatrine, and quercetin treatments (0, 0.2, 0.8, 1.5 mM), respectively, on brain mitochondria isolated from hypoxia-exposed rats *in vitro*.

**Effects of Chinese herbal monomers on membrane potential of brain mitochondria isolated from hypoxia-exposed rats *in vitro*** (Figure 3)

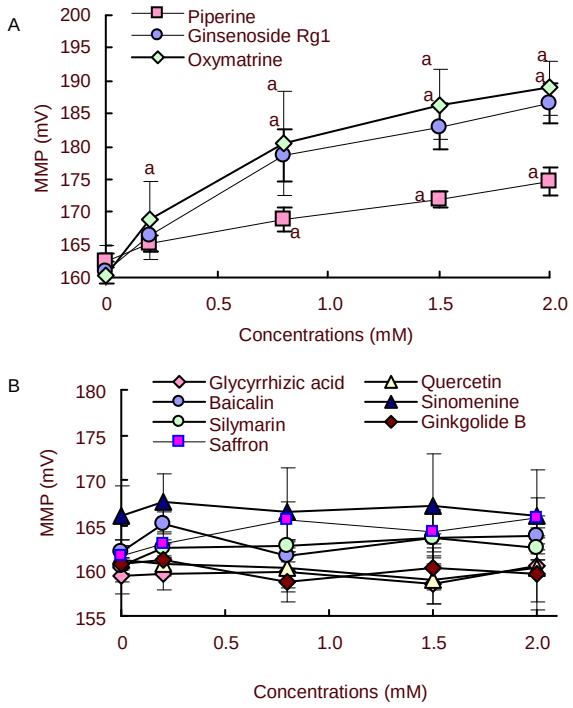



Figure 3 Effects of different concentrations (0, 0.2, 0.8, 1.5, 2 mM) of Chinese herbal monomers on mitochondrial membrane potential (MMP) in brain mitochondria from hypoxia-exposed rats *in vitro*.

Piperine, ginsenoside Rg1 and oxymatrine protected mitochondria from hypoxic insult (A), while other monomers had no significant influence on brain MMP (B).

Data are shown as mean ± SD ($n$ = 4). [a]$P$ < 0.01, *vs.* 0-mM intervention group; [b]$P$ < 0.01, *vs*. piperine intervention group (unpaired-sample Student's *t*-test).

Piperine, ginsenoside Rg1 and oxymatrine prevented the decrease in mitochondrial membrane potential induced by hypobaric hypoxia in dose-dependent manners (Figure 3A). Treatment with 2 mM piperine increased the mitochondrial membrane potential in mitochondria from hypoxia-exposed rat brain by 8.02%. Compared with piperine, ginsenoside Rg1 and oxymatrine enhanced mitochondrial membrane potential more effectively by 16.15% and 18.13% at the same concentrations ($P$ < 0.01; Figure 3A). The other tested drugs had no significant influence on mitochondrial membrane potential in cerebral mitochondria from hypoxia-exposed rats compared with the 0-mM group (Figure 3B). Furthermore, oxymatrine had no significant influence on mitochondrial membrane potential in cerebral mitochondria from normal rats, despite its protective effect on mitochondria from hypoxia-exposed rat brains (Figure 4).

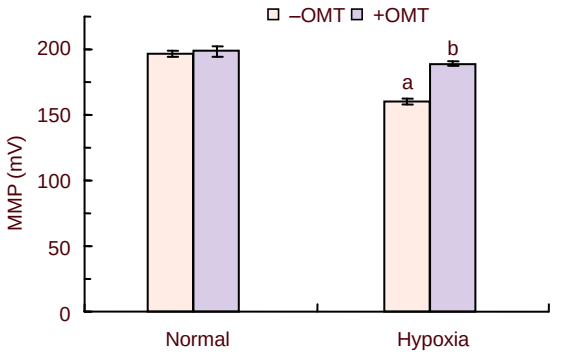



Figure 4 Protective effect of 2 mM oxymatrine (OMT) on mitochondrial membrane potential (MMP) in brain mitochondria from hypoxia-exposed rats *in vitro*.

Data are shown as mean ± SD ($n$ = 8). [a]$P$ < 0.01, *vs*. normal group; [b]$P$ < 0.01, *vs*. OMT group (unpaired-sample Student's *t*-test).

## DISCUSSION

In normal cerebral mitochondria, protons from nicotinamide adenine dinucleotide or flavin adenine dinucleotide are transported from the inside to the outside of mitochondria by the electron tr ansport chain. Meanwhile, electrons are transferred to oxygen and a transmembrane proton gradient ($\Delta\psi$) is established (oxidation). The mitochondrial membrane potential mainly reflects the $\Delta\psi$, and is the driving force behind energy production by $F_0F_1$-ATP synthase (phosphorylation)[16]. These processes of ATP production represent "coupling" between oxidation and phosphorylation[17]. $\Delta\psi$ can be dissipated by some transmembrane proton-channel proteins (such as uncoupling proteins), resulting in the "uncoupling" of oxidation and phosphorylation[18-19].

Reactive oxygen species are by-products of complexes I and III of the mitochondrial respiratory chain and increase rapidly during hypoxia[20-21]. Mitochondria can be attacked by the increased oxidative stress in a variety of ways, including reduced availability of reduced substrates for the electron transport chain[22] and damage to the mitochondrial inner membranes[23]. Disruption of the respiratory chain results in decreased oxygen consumption of ST3, while high proton penetrability of the membrane promotes ST4, which is also increased by the higher expression or activity of some proteins mediating proton leak during hypoxia exposure. The decrease in ST3 and increase in ST4 lead to reductions in mitochondrial membrane potential and

RCR, which reflect the coupling states of oxidative phosphorylation. All these alterations contribute to metabolic depression and lowered efficiency of mitochondrial oxidative phosphorylation.

The results of the current study showed that saffron, ginsenoside Rg1 and oxymatrine increased RCR in brain mitochondria in hypoxia-exposed rats. Saffron elevated ST3 and RCR, but had no effect on ST4 or mitochondrial membrane potential. Elevation of malondialdehyde and decrease of superoxide dismutase and catalase in rat brains induced by middle cerebral artery occlusion were reportedly significantly attenuated by pretreatment with saffron[24], which was also able to inhibit lipid peroxidation and preserve mitochondrial function in synaptosomal fractions from rats treated with the mitochondrial toxin 3-nitropropionic acid[25]. These results suggest that saffron acts as an anti-oxidative substance, relieving the oxidative insult to the electron transport chain and suppressing mitochondrial membrane impairment. In contrast to saffron, ginsenoside Rg1 increased RCR based on its ability to depress ST4. Some investigators have reported that ginsenoside Rg1 inhibited $Ca^{2+}$ over-influx into mitochondria in mesencephalic dopaminergic cells[26-27], and acted as a calcium channel blocker in ischemia/reperfusion-injured rat cardiomyocytes[28]. This suggests that ginsenoside Rg1 modulates the activity of mitochondrial membrane-channel proteins. The effects of oxymatrine on mitochondria from hypoxia-exposed rats were similar but more effective than those of ginsenoside Rg1. Oxymatrine was previously found to down-regulate many genes and proteins over-expressed in rats subjected to middle cerebral artery occlusion, such as nuclear factor κB[29] and phosphorylated p38 mitogen-activated protein kinases[30], thereby protecting rats from ischemic insult. The current study showed that oxymatrine inhibited mitochondrial membrane potential in hypoxia-exposed but not normal rats, also indicating that its protective effect might be associated with genes or proteins related to hypoxic damage. All three monomers inhibited the uncoupling between oxidation and phosphorylation induced by hypoxia, suggesting their protective effects on mitochondria. Piperine decreased ST4 and increased mitochondrial membrane potential, but had no significant effect on RCR because it further decreased ST3. Piperine has also been reported to improve ATPase activity in liver mitochondria, and inhibit mitochondrial oxidative phosphorylation at the level of the respiratory chain[31]. Piperine at low concentrations could reduce 1-methyl-4-phenylpyridinium ($MPP^+$, cytotoxin)-induced death in PC12 cells by suppressing the decrease in mitochondrial membrane permeability, but was cytotoxic at high concentrations[32]. These apparent discrepancies suggest that the mechanism of piperine in mitochondria might be more complex than those of other monomers. In summary, saffron, ginsenoside Rg1, piperine and oxymatrine attenuated mitochondrial dysfunction caused by hypobaric hypoxia exposure.

## MATERIALS AND METHODS

### Design

A randomized, controlled animal experiment.

### Time and setting

The experiments were performed at the Department of Pathophysiology and Altitude Physiology, the Third Military Medical University of Chinese PLA, Chongqing, China from May to October 2010.

### Materials

#### *Animals*

A total of 96 healthy male Sprague-Dawley rats, specific-pathogen-free grade, aged 11–14 weeks, weighing 200 ± 20 g, were provided by the Animal Center of the Third Military Medical University of Chinese PLA, Chongqing, China (certificate No. SCXK (Yu) 2007-0003). Rats were housed at 23.0 ± 0.5°C at a relative humidity of 50.0 ± 0.5%, under a 12-hour light/dark cycle, with free access to food and water. All experimental procedures were conducted in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[33].

#### *Drugs*

Ten Chinese herbal monomers, including sinomenine, silymarin, glycyrrhizic acid, baicalin, quercetin, ginkgolide B, saffron, piperine, ginsenoside Rg1 and oxymatrine (molecular and structural formulae are shown in Table 1) were provided by Push Biotechnology Company (Chengdu, Sichuan Province, China). Water-soluble monomers (saffron, ginsenoside Rg1 and oxymatrine) were dissolved in distilled water, while the fat-soluble drugs (sinomenine, silymarin, glycyrrhizic acid, baicalin, quercetin, ginkgolide B and piperine) were dissolved in dimethylsulfoxide (supplementary Figure 1 online).

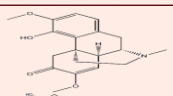
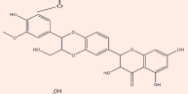
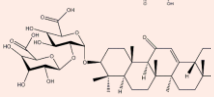
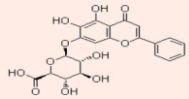
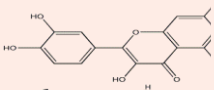
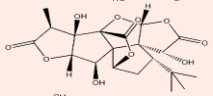
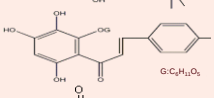
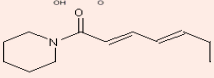
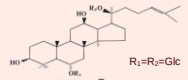
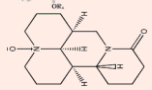
### Methods

#### *Establishment of hypoxic models in rats*

After receiving food and water *ad libitum* for 3 days of acclimation, animals in the hypoxic and drug-intervention groups were placed in a hypobaric chamber at a

simulated atmospheric pressure of 405.35 mm Hg (equal to 54.04 kPa), equivalent to an altitude of 5 000 m, for 23 hours per day, for 3 days. The chamber was kept at constant temperature (22–25°C) and relative humidity (70%) with artificial illumination from 7 a.m. to 9 p.m.[5, 11]. Rats in the normal group were fed at sea-level pressure under the same conditions. All rats continued to be provided with water and food *ad libitum* during the experiments.

Table 1 Chemical information of ten Chinese herbal monomers

| Name | Molecular formula | Molecular weight | Structural formula |
|---|---|---|---|
| Sinomenine | $C_{19}H_{23}NO_4$ | 329.38 |  |
| Silymarin | $C_{25}H_{22}O_{10}$ | 482.44 |  |
| Glycyrrhizic acid | $C_{42}H_{62}O_{16}$ | 822.93 |  |
| Baicalin | $C_{21}H_{18}O_{11}$ | 446.36 |  |
| Quercetin | $C_{15}H_{10}O_7$ | 302.24 |  |
| Ginkgolide B | $C_{20}H_{24}O_{10}$ | 424.40 |  |
| Saffron | $C_{21}H_{22}NO_{11}$ | 450.39 |  |
| Piperine | $C_{17}H_{19}NO_3$ | 285.34 |  |
| Ginsenoside Rg1 | $C_{42}H_{72}NO_{14}$ | 801.03 |  |
| Oxymatrine | $C_{15}H_{24}N_2O_2$ | 264.36 |  |

### *Preparation of brain mitochondria*

Brain mitochondria were prepared by conventional methods using differential centrifugation, as described previously[34-35]. In brief, rats were killed by decapitation and the cerebral hemispheres were rapidly isolated and removed into ice-cold isolation medium containing 0.25 M sucrose, 0.5 mM EDTA-2K and 10 mM Tris-HCl (pH 7.4). The above procedure was usually accomplished within 30 seconds in a hypobaric chamber with the atmospheric pressure maintained at 405.35 mm Hg (54.04 kPa). Tissues were chopped finely with scissors after washing three times in medium. The resulting tissue pieces were placed in a Teflon homogenizer with 8 mL medium and homogenized by hand using 15 up-and-down strokes with a pestle. The homogenate was fractionated at 2 000 × *g* for 3 minutes. The resulting pellet was removed, and the supernatant suspension was re-centrifuged. The supernatant suspension was then centrifuged at 12 500 × *g* for 10 minutes, the supernatant was decanted, and the crude mitochondrial pellet was resuspended in 0.8 mL 3% Ficoll medium (3% Ficoll, 120 mM mannitol, 30 mM sucrose, 25 μM EDTA-2K, pH 7.4). This suspension was carefully layered onto 3.2 mL of 6% Ficoll medium (6% Ficoll, 240 mM mannitol, 60 mM sucrose, 50 μM EDTA-2K, pH 7.4) and centrifuged at 11 500 × *g* for 30 minutes. The pellet was resuspended in 4 mL isolate medium and centrifuged at 12 000 × *g* for 8 minutes. The mitochondria were made up to a concentration of 10–20 mg of protein per mL in the appropriate isolation medium. All the isolation procedures were performed at 0–4°C within 90 minutes. Mitochondrial suspensions were maintained on ice and used within 4 hours after death of the animal.

### *Establishment of drug intervention models in isolated mitochondria in vitro*

The mitochondrial protein concentration was assessed using the bicinchoninic acid method[36] with bovine serum albumin (Beyotime, Haimen, China) as a standard, and then adjusted to 10 mg of protein per 1 mL in isolation medium. The mitochondrial suspension isolated from the brains of hypoxia-exposed rats was divided into several groups, each containing 100 mL of suspension and 1 mg of mitochondrial protein. Different volumes of the tested Chinese herbal monomers (as above) were added to 100 mL mitochondrial suspensions in each group, to final concentrations of 0, 0.2, 0.8 and 1.5 mM. The suspensions were then incubated at 37°C for 5 minutes, followed by measurement of mitochondrial respiratory activity and mitochondrial membrane potential.

### *Measurement of mitochondrial respiratory activity*

Mitochondrial respiratory function was determined polarographically at 28°C using a Clark-type oxygen electrode (Strathkelvin Instruments, North Lanarkshire, ML1 5RX, Scotland) as described previously[37-38]. Reactions were conducted in a 1.5-mL closed thermostatic glass cell (28°C) with a magnetic stirrer. One hundred microliters of mitochondrial suspension containing 1 mg of mitochondrial protein was added to buffer containing 225 mM mannitol, 75 mM sucrose, 100 mM KCl, 5 mM $K_2HPO_4$, 50 μM EDTA-2K, and 10 mM Tris (pH 7.4) in a final volume of 0.8 mL. After a 1-minute equilibration period, mitochondrial respiration

was initiated by adding pyruvate (6 mM) and malate (6 mM). Oxygen consumption (nmol $O_2$/min/mg) was measured in the presence of 625 μM ADP (ST3) and after depletion of ADP (ST4). RCR (the ratio of ST3 and ST4) was calculated[39] (supplementary Figure 2 online).

### *Measurement of mitochondrial membrane potential*

Mitochondrial membrane potential was estimated spectrophotofluorometrically using the rhodamine 123 method established by Arrighi *et al* [40-41]. Rat brain mitochondria containing 1 mg protein were added to 1 mL reactive buffer (150 mM sucrose, 5 mM $MgCl_2$, 5 mM succinate, 2.5 μM rotenone, 5 mM $K_3PO_4$, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 1 μM rhodamine123, pH 7.4) and incubated at 37°C for 5 minutes. Following centrifugation at 5 000 × *g* for 5 minutes, 200 μL supernatant was removed to a 96-well plate. The rhodamine123 concentration outside the mitochondria ($[X]_{out}$) was determined according to the fluorescence value assessed using a microplate reader (BioTek Instruments, Winooski, VT, USA) at 488 nm excitation wavelength and 525 nm emission wavelength. Based on the volume of mitochondria to 1 μL/mg, the concentration of rhodamine123 inside the mitochondria ($[X]_{in}$) calculated by the following formula:

$[X]_{in} = (1–1.1 \times [X]_{out}) \times 10\,000/[C]$,

where [C] is the mean mitochondrial protein concentration. Mitochondrial membrane potential (mV) was then equal to 59 lg ($[X]_{in}/[X]_{out}$).

### *Statistical analysis*

The Statistical Package for the Social Sciences (SPSS version 10.0, Chicago, IL, USA) statistical software was used for all analyses. Statistical values were given as mean ± SD. Comparisons between normal control and hypoxic groups were made using unpaired-sample Student's *t*-tests, while comparisons among monomer-intervention groups were made using analysis of variance. A level of $P < 0.05$ was considered statistically significant.

**Funding:** This work was supported by the Natural Science Foundation of China, No. 81171875.

**Author contributions:** Weihua Yan completed the majority of the experiments, wrote the manuscript, provided data and performed data analysis. Junze Liu was responsible for the study proposal and design. All authors read and approved the final manuscript.

**Conflicts of interest:** None declared.

**Ethical approval:** All animal protocols were approved by the Animal Ethics Committee of the Third Military Medical University of the Chinese PLA in China.

**Supplementary information:** Supplementary data associated with this article can be found in the online version, by visiting www.nrronline.org.

## REFERENCES

[1] Xu JX. Radical metabolism is partner to energy metabolism in mitochondria. Ann N Y Acad Sci. 2004;1011: 57-60.

[2] Schild L, Reinheckel T, Reiser M, et al. Nitric oxide produced in rat liver mitochondria causes oxidative stress and impairment of respiration after transient hypoxia. FASEB J. 2003;17(15):2194-2201.

[3] Liu JZ, Gao WX, Cai MC, et al. Effects of ATP concentration and hypoxic exposure on RNA and protein synthesis activity in isolated mitochondria from rat brain. Acta Physiologica Sinica. 2002;54(6):485-489.

[4] Magalhães J, Ascensão A, Soares JM, et al. Acute and severe hypobaric hypoxia increases oxidative stress and impairs mitochondrial function in mouse skeletal muscle. J Appl Physiol. 2005;99(4):1247-1253.

[5] Gao P, Liu JZ, Xia C. Effects of brain mitochondrial uncoupling protein content and activity in rat exposed to hypoxia simulating high altitude on mitochondrial energy synthesis. Disan Junyi Daxue Xuebao. 2006;28(21): 2127-2129.

[6] Chávez JC, Pichiule P, Boero J, et al. Reduced mitochondrial respiration in mouse cerebral cortex during chronic hypoxia. Neurosci Lett. 1995;193(3):169-172.

[7] Liu JZ. The Mechanism of High Altitude Acclimatization and Adaptation on Tissue and Cellular Level. Beijing: Environment Scientific Press. 2002.

[8] Woods DR, Stacey M, Hill N, et al. Endocrine aspects of high altitude acclimatization and acute mountain sickness. J R Army Med Corps. 2011;157(1):33-37.

[9] Palmer BF. Physiology and pathophysiology with ascent to altitude. Am J Med Sci. 2010;340(1):69-77.

[10] Singer D. Metabolic adaptation to hypoxia: cost and benefit of being small. Respir Physiol Neurobiol. 2004; 141(3):215-228.

[11] Xia C, Liu JZ, Xu Y. Inhibition of GDP on mitochondrial respiratory oxygen consumption and its effect on the mitochondrial membrane potential of rat brain. Disan Junyi Daxue Xuebao. 2007;29(5):290-293.

[12] Han JY, Fan JY, Horie Y, et al. Ameliorating effects of compounds derived from Salvia miltiorrhiza root extract on microcirculatory disturbance and target organ injury by ischemia and reperfusion. Pharmacol Ther. 2008;117(2): 280-295.

[13] Ip SP, Che CT, Leung PS. Association of free radicals and the tissue renin-angiotensin system: prospective effects of Rhodiola, a genus of Chinese herb, on hypoxia-induced pancreatic injury. JOP. 2001;2(1):16-25.

[14] Seong YH, Kim HS. Inhibitory effects of ginseng total saponins on hypoxia-induced dysfunction and injuries of cultured astrocytes. Arch Pharm Res. 1997;20(2):103-109.
[15] Li XT, Chen R, Jin LM, et al. Regulation on energy metabolism and protection on mitochondria of Panax ginseng polysaccharide. Am J Chin Med. 2009;37(6): 1139-1152.
[16] Mitchell P. Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. 1966. Biochim Biophys Acta. 2011;1807(12):1507-1538.
[17] Dragicevic N, Bradshaw PC, Mamcarz M, et al. Long-term electromagnetic field treatment enhances brain mitochondrial function of both Alzheimer's transgenic mice and normal mice: a mechanism for electromagnetic field-induced cognitive benefit? Neuroscience. 2011;185: 135-149.
[18] Divakaruni AS, Brand MD. The regulation and physiology of mitochondrial proton leak. Physiology (Bethesda). 2011; 26(3):192-205.
[19] Zhang CM, Gu Y, Qing DN, et al. Tissue-specific distribution of uncoupling proteins in normal rats and rats with high-fat-diet-induced obesity. Mol Biol Rep. 2010;37 (7):3177-3182.
[20] Duranteau J, Chandel NS, Kulisz A, et al. Intracellular signaling by reactive oxygen species during hypoxia in cardiomyocytes. J Biol Chem. 1998;273(19):11619-11624.
[21] Fato R, Bergamini C, Bortolus M, et al. Differential effects of mitochondrial Complex I inhibitors on production of reactive oxygen species. Biochim Biophys Acta. 2009; 1787(5):384-392.
[22] Nulton-Persson AC, Szweda LI. Modulation of mitochondrial function by hydrogen peroxide. J Biol Chem. 2001;276(26):23357-23361.
[23] Ilavazhagan G, Bansal A, Prasad D, et al. Effect of vitamin E supplementation on hypoxia-induced oxidative damage in male albino rats. Aviat Space Environ Med. 2001; 72(10):899-903.
[24] Saleem S, Ahmad M, Ahmad AS, et al. Effect of Saffron (Crocus sativus) on neurobehavioral and neurochemical changes in cerebral ischemia in rats. J Med Food. 2006; 9(2):246-253.
[25] Del-Angel DS, Martínez NL, Cruz ME, et al. Saffron extract ameliorates oxidative damage and mitochondrial dysfunction in the rat brain. Acta Hortic. 2006:e739.
[26] Tang B, Qu Y, Wang D, et al. Targeting hypoxia inducible factor-1α: a novel mechanism of ginsenoside Rg1 for brain repair after hypoxia/ischemia brain damage. CNS Neurol Disord Drug Targets. 2011;10(2):235-238.
[27] Wang D, Huang Y, Li Q, et al. Anti-apoptotic effect of ginsenoside Rg1 on neuron after neonatal hypoxia ischemia brain damage. Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi. 2010;24(9):1107-1112.
[28] Zhu D, Wu L, Li CR, et al. Ginsenoside Rg1 protects rat cardiomyocyte from hypoxia/reoxygenation oxidative injury via antioxidant and intracellular calcium homeostasis. J Cell Biochem. 2009;108(1):117-124.
[29] Fan H, Li L, Zhang X, et al. Oxymatrine downregulates TLR4, TLR2, MyD88, and NF-kappaB and protects rat brains against focal ischemia. Mediators Inflamm. 2009; 2009:704706.
[30] Cui L, Zhang X, Yang R, et al. Neuroprotection and underlying mechanisms of oxymatrine in cerebral ischemia of rats. Neurol Res. 2011;33(3):319-324.
[31] Reanmongkol W, Janthasoot W, Wattanatorn W, et al. Effects of piperine on bioenergetic functions of isolated rat liver mitochondria. Biochem Pharmacol. 1988;37(4): 753-757.
[32] Lee CS, Han ES, Kim YK. Piperine inhibition of 1-methyl-4-phenylpyridinium-induced mitochondrial dysfunction and cell death in PC12 cells. Eur J Pharmacol. 2006; 537(1-3):37-44.
[33] The Ministry of Science and Technology of the People's Republic of China. Guidance Suggestions for the Care and Use of Laboratory Animals. 2006-09-30.
[34] Nicklas WJ, Clark JB, Williamson JR. Metabolism of rat brain mitochondria. Studies on the potassium ion- stimulated oxidation of pyruvate. Biochem J. 1971;123(1): 83-95.
[35] Bak LK, Ziemińska E, Waagepetersen HS, et al. Metabolism of [U-13C]glutamine and [U-13C]glutamate in isolated rat brain mitochondria suggests functionaion of mitochol phosphate-activated glutaminase activity in matrix. Neurochem Res. 2008;33(2):273-278.
[36] Smith PK, Krohn RI, Hermanson GT. Measurement of protein using bicinchoninic acid. Anal Biochem. 1985; 150(1):76-85.
[37] Sims NR. Rapid isolation of metabolically active mitochondria from rat brain and subregions using Percoll density gradient centrifugation. J Neurochem. 1990;55(2): 698-707.
[38] Sims NR, Anderson MF. Isolation of mitochondria from rat brain using Percoll density gradient centrifugation. Nat Protoc. 2008;3(7):1228-1239.
[39] Murray AJ, Cole MA, Lygate CA, et al. Increased mitochondrial uncoupling proteins, respiratory uncoupling and decreased efficiency in the chronically infarcted rat heart. J Mol Cell Cardiol. 2008;44(4):694-700.
[40] de Vantéry Arrighi C, Lucas H, Chardonnens D, et al. Removal of spermatozoa with externalized phosphatidylserine from sperm preparation in human assisted medical procreation: effects on viability, motility and mitochondrial membrane potential. Reprod Biol Endocrinol. 2009;7:1.
[41] Distelmaier F, Koopman WJ, Testa ER, et al. Life cell quantification of mitochondrial membrane potential at the single organelle level. Cytometry A. 2008;73(2):129-138.

(Edited by Wang MY, Chen JX/Yang Y/Wang L)