

## Original Article

# Combination of Salidroside and hyperbaric oxygen improves leukoaraiosis through P13K/Akt signaling

Dazhi Duo<sup>1</sup>, Yuan Feng<sup>2</sup>, Xiange Meng<sup>1</sup>, Yan Lv<sup>1</sup>, Yu Zhang<sup>1</sup>, Shuyi Pan<sup>1</sup>

<sup>1</sup>Department of Hyperbaric Oxygen, Navy General Hospital, Haidian District, 6 Fu Cheng Road, Beijing 100048, China; <sup>2</sup>Southern Medical University, Baiyun District, Guangzhou, 1023-1063 Sha Tai Road, Guangzhou 510515, China

Received August 8, 2017; Accepted January 28, 2018; Epub May 15, 2018; Published May 30, 2018

**Abstract:** Background: Leukoaraiosis (LA) is an abnormal change in appearance of white matter near the lateral ventricles. It is responsible for changes in memory, cognition and behavior. Salidroside is a drug responsible for the antidepressant and anxiolytic action. Hyperbaric oxygen therapy involves breathing pure oxygen in a pressurized room or tube. Thus, in this study the role of combination of Salidroside (Sal) and hyperbaric oxygen (HBO) on cognitive dysfunction in rats with LA was verified. Methods: Bilateral carotid artery of rat was ligated for 2 weeks in order to establish the model of cerebral ischemia and hypoxia. From the fifteenth days, the animals were given Sal or HBO or both for 2 weeks. LFB (Luxol fast blue stain) and MBP (myelin basic protein) staining were used to observe the changes of white matter myelin and oligodendrocytes, TUNEL was used to observe the apoptosis of oligodendrocytes and phosphor P13K/Akt signaling was assayed by Western blot. Results: We observed that myelin lesions as well as the number of apoptotic cells has increased and the proliferation of oligodendrocytes has decreased in the model group as compared to control group. Salidroside significantly alleviated the myelin and oligodendrocytes lesions and HBO inhibit the apoptosis of oligodendrocytes. Combination Sal and HBO had better effects on myelin and oligodendrocytes through promoting P13K/Akt (phosphoinositide-3 kinase) signaling pathway. Conclusion: Combination of Sal and HBO contributes to improvement of demyelination and survival of oligodendrocytes through P13K/Akt signaling.

**Keywords:** Leukoaraiosis, salidroside, hyperbaric oxygen, P13K/Akt

## Introduction

Leukoaraiosis (LA) is a disease of cerebral white matter. It often occurs in old age but sometimes in young adults. Pathological studies have shown that LA lesions are mainly caused by loss and deformation of the myelin sheath in white matter. In addition to this, LA is also caused by ischemia, micro-hemorrhages, gliosis, damage to small blood vessel walls, breaches of the barrier between the cerebrospinal fluid and the brain. Leukoaraiosis appears on CT as hypodense periventricular white-matter lesions and on MRI as white matter hyperintensities [1, 2]. Studies have shown that LA is a biomedical marker for brain aging and is closely related to Alzheimer [3-5]. Leukoaraiosis in Alzheimer's disease may produce significant basal ganglia, thalamic and frontal lobe dysfunction which may be associated with more severe apathy and extrapyrami-

dal signs. There is no effective therapy for LA till now so it has become challenging issue for the prevention and treatment of LA. Therefore, prevention of LA has become emerging research issue

Hyperbaric oxygen therapy (HBO) is defined as the inhalation of 100% oxygen inside a hyperbaric chamber that is pressurized to greater than 1 atmosphere. Many experiments have shown that hyperbaric oxygen (HBO) can protect against subsequent multi-organ injury to the brain, heart, or liver [6]. During LA treatment hyperbaric oxygen increases both the dissolved oxygen and the partial pressure of oxygen in blood plasma. Consequently, a large amount of oxygen-dependent reactions and signaling pathways are enhanced.

Salidroside (Sal) is a glucoside of tyrosol found in the plant roots and stems *Rhodiola rosea*.

The previous studies confirmed that Sal has pharmacological effects to reduce cerebral ischemia-reperfusion injury through the activation of the Nrf2 pathway [7]. Moreover, Sal has a protective effect against isoflurane-induced cognitive dysfunction by inhibiting excessive inflammatory responses, decreasing oxidative stress, and regulating the cholinergic system [8]. Besides, Sal improves behavioral and histological outcomes and reduces apoptosis via PI3K/Akt signaling after experimental traumatic brain injury [9]. These all facts suggest that Salidroside and hyperbaric oxygen may effectively improve leukoaraiosis. Therefore, it is hypothesized that Sal may be used as a neuro-protective agent to promote the potential of nerve repair. However, there is no literature about the protection of Sal against leukoaraiosis induced by chronic cerebral ischemia. The present study was designed to explore the effects of combination of Sal and HBO on cerebral white matter.

## Materials and methods

### *Animal models*

All animal experiments were approved by Naval General Hospital, Beijing, China. Anesthesia was induced with pentobarbital (12.5 mg/kg) and maintained with  $\alpha$ -chloralose (75 mg/kg) during surgery and preparation. Male Wistar rats (250-300 g) were categorized into Control group, model group, Sal Group, HBO group and Sal+HBO group with 10 in each group. All animals were reared in constant temperature with 12 h: 12 h natural light cycle. Bilateral arteria carotis communis were ligated for 2 weeks to establish ischemic model. Animals in control group were given the same operations without the ligation of bilateral common carotid arteries. HBO was performed from the 15<sup>th</sup> day in an animal research chamber equipped with a hyperbaric ventilator (Sechrist 500A) for 2 weeks. HBO was initiated for 60 minutes and consisted of a 25-minute descent to 2.7 atm, which was maintained for 60 minutes, followed by a 25-minute ascent. In the Sal group, the animals were intravenously injected 50 mg/kg of Sal from the 15<sup>th</sup> day for consecutive 2 weeks. The animals were killed to death by pentobarbital sodium (i.v. 100 mg/kg).

### *Luxol fast blue (LFB) staining*

After saline and Zamboni's fixative was sequentially injected into the brain, the brain tissue

was removed and then fixed and frozenly sectioned into 10  $\mu$ m. Sections ranged from fonticulus 0.2 mm to -0.3 mm, containing the entire corpus callosum. The frozen sections were mounted on the coated glass slides, toasted at 37°C for 2 h, and then prepared in a Luxol fast blue solution at a temperature of about 60°C for 6 h, followed by washing in 95% ethanol and distilled water sequentially. Next, 0.05%  $\text{Li}_2\text{CO}_3$  solution for 10 s and 70% ethanol for 10 s were sequentially used for color separation. Under a microscope, if the gray and white matter are easy to distinguish, the slides were then treated with 80%, 95% and 100% of gradient alcohol for dehydration, xylene for transparency and neutral balata for mount.

### *MBP staining*

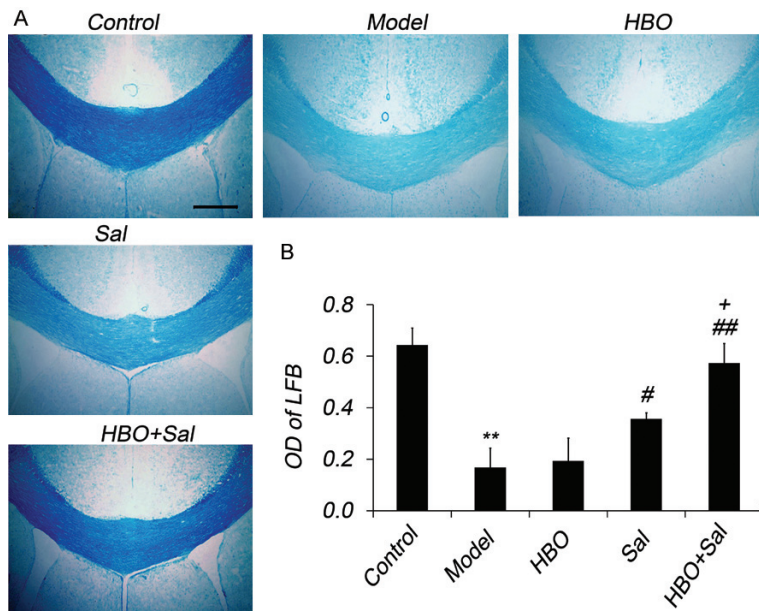
The frozen sections were sequentially treated with 3%  $\text{H}_2\text{O}_2$  at room temperature for 15 min to block endogenous peroxidase activity, 1% BSA/0.4% Triton X-100 at 37°C for 30 min to block nonspecific antibody binding, primary antibody at 4°C overnight, biotinylated secondary antibody at 37°C for 3 h, ABC compound at 37°C for 3 h and DAB 30 min for coloration, followed by washing in 0.01 M PBS for 5 min $\times$ 3 after each step. Olympus microscope was used for observation. Image Pro Plus software was used to analyze the positive results of immune reaction.

### *TUNEL*

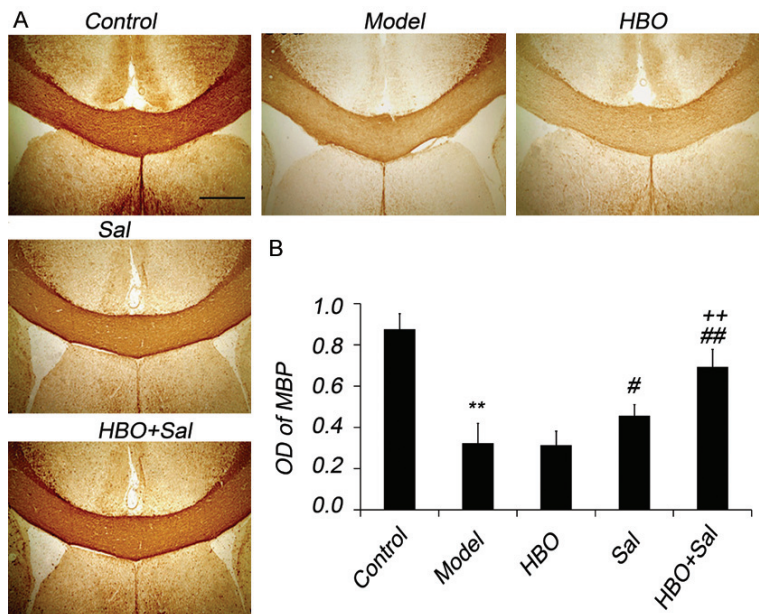
Frozen sections were sequentially treated with 3%  $\text{H}_2\text{O}_2$ -methanol at 4°C for 10 min, 0.1% Triton X-100/0.1% sodium citrate solution at 4°C for 2 min, TUNEL reaction mixture at 37°C for 60 min, converter-POD at 37°C for 30 min followed by 0.01 M PBS rinse of 3 times (each 10 min). DAB was used for colorization. After gradient alcohol dehydration, xylene treatment and neutral balata mount, Olympus microscope was used to observe the results. TUNEL mixed solution without TDT enzyme solution was used as a negative control. The number of positive cells in corpus callosum of 0.25 mm<sup>2</sup> was analyzed.

### *Western immunoblotting*

For Western blotting, protein concentrations of cell lysates were determined with a Coomassie plus protein assay (Dingguo, Beijing, China) and were resolved by 10% SDS-polyacrylamide gel



**Figure 1.** The myelin sheaths histopathology was identified by LFB staining. A: Representative images (Scale bar=500  $\mu$ m); B: Quantitative analysis. \*\* $P$ <0.01 vs. control; # $P$ <0.05 and ## $P$ <0.01 vs. Model group; + $P$ <0.05 vs. Sal group.



**Figure 2.** The oligodendrocytes were identified by MBP staining. A: Representative images (Scale bar=500  $\mu$ m). B: Quantitative analysis. \*\* $P$ <0.01 vs. control; # $P$ <0.05 and  $P$ <0.01 vs. Model group; ++ $P$ <0.01 vs. Sal group.

electrophoresis and then transferred onto polyvinylidene fluoride membrane (Invitrogen, China). Membranes were blocked with 5% skim milk and incubated with rabbit polyclonal to PI3K and AKT1 (phospho, Abcam, Shanghai,

China). After being washed, membranes were incubated with corresponding horseradish peroxidase (HRP)-conjugated IgG (1:2,000; Abcam, Shanghai, China) for 1 h at room temperature. Antibody-antigen complexes were then detected using a SuperSignal West Pico Chemiluminescent Substrate (Pierce, IL USA). GAPDH was used as a loading control. A densitometry analysis was performed using Al-phaEase software version 2200.

#### Statistical analysis

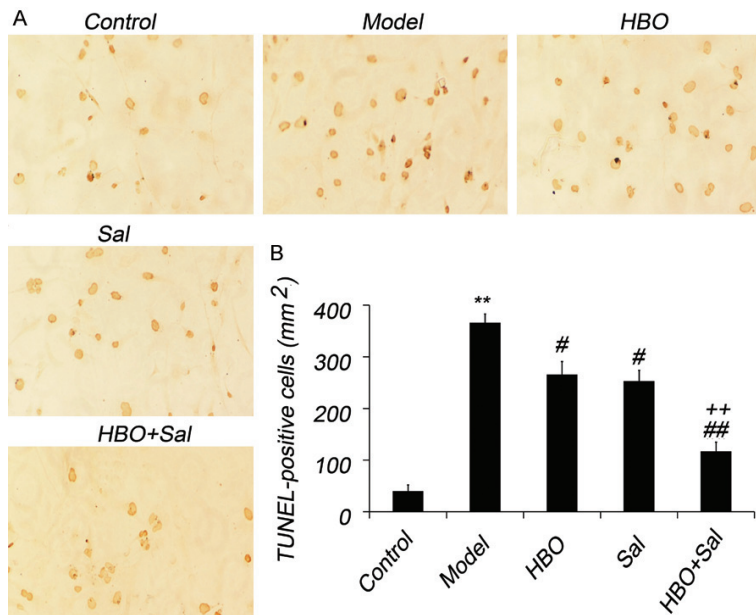
All statistical data were presented as mean  $\pm$  standard deviation (SD). The comparisons among groups were performed using one way analysis of variance (ANOVA). A  $P$ -value less than 0.05 were considered as statistically significant.

#### Results

##### Combination of Sal and HBO inhibited demyelination

In the control group, the LFB staining was clearly distributed in the corpus callosum, cingulate gyrus, caudate putamen, external capsule, anterior commissure and lateral olfactory tract. The LFB staining in these regions was strong and the myelin sheath was arranged in order without edema, stratification, fragmentation and vacuolization. After ischemia, LFB staining became light, and the lesions of myelin sheath were obvious as well as some of them were

disintegrated and vacuolated. Sal treatment alone can effectively alleviate the pathological changes and combination with Sal and HBO was verified to further reduce the lesions (Figure 1A). Quantitative analysis showed that



**Figure 3.** Apoptosis of oligodendrocyte was analyzed by TUNEL (n=10). A: Representative images (Scale bar=50  $\mu$ m). B: Quantitative analysis. \*\*P<0.01 vs. control; #P<0.05 and ##P<0.01 vs. Model group; ++P<0.01 vs. Sal group.

as that of LFB and the brown positive staining boundary was clear (**Figure 2A**). Quantitative analysis showed that the average optical density (OD) of the model group decreased, Sal treatment promoted the average OD and combination of Sal and HBO further increased the average OD when compared to Sal group (P<0.01) (**Figure 2B**).

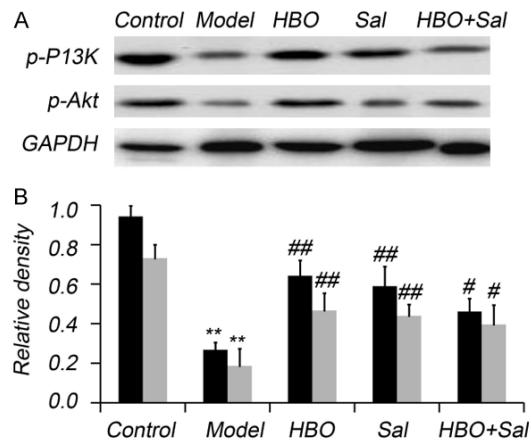
#### Combination of Sal and HBO inhibited apoptosis of oligodendrocytes

TUNEL positive apoptotic cells were observed in the corpus callosum of rats in each group. The number of TUNEL positive cells of the model group increased significantly which had significant differences with the control group.

When compared with model group, the number of TUNEL positive cells in Sal group or HBO group significantly reduced (P<0.05). Moreover, the TUNEL positive cells in Sal+HBO group decreased obviously when compared with sole Sal or HBO group (P<0.01) (**Figure 3A** and **3B**).

#### Combination of Sal and HBO promoted P13K/Akt signaling

The results showed that the phosphorylation of P13K/Akt decreased significantly in the model group while Sal or HBO promotes the phosphorylation of P13K/Akt (P<0.05); the P13K/Akt phosphorylation of Sal and HBO group compared with Sal or HBO had no obvious difference (**Figure 4**).



**Figure 4.** Phosphorylation of P13K/Akt was analyzed by Western blot (n=5). A: Representative images. B: Quantitative analysis. \*\*P<0.01 vs. control; #P<0.05 vs. Model group.

the average optical density (OD) of the model group decreased, Sal treatment promoted the average OD and combination of Sal and HBO further increased the average OD when compared to Sal group (P<0.01) (**Figure 1B**).

#### Combination of Sal and HBO promoted proliferation of oligodendrocytes

In the control group, the positive region of MBP immunohistochemical staining was the same

#### Discussion

LA is a diffuse subcortical white matter disease. It is clinical syndrome induced by various etiologies (hypertension, smoking, diabetes mellitus, hyperhomocysteinemia, cardiovascular disease) and is correlated to many factors [10]. The main pathogenesis of LA is cerebral ischemia and hypoxia injury. Structural changes in brain affecting the small intraparenchymal cerebral arteries and arterioles that altered cerebral blood flow autoregulation which results in cerebral ischemia and contribute to

the development of leukoaraiosis. The white matter of the brain connects the cortex and the subcortical area and plays a very important role in the information transmission. The white matter mainly depends on the deep perforating artery without collateral circulation as well as the artery needs a long distance to reach white matter. These characteristics make the white matter in the region most vulnerable to ischemia. As a result ischemia leads to demyelination. CT outcomes of LA are closely related to age.

During the recent clinical trials, it is found that HBO protect against focal and global cerebral ischemia as well as traumatic brain injury [11]. Moreover, HBO can promote both cerebral-protective and cardiac-protective effects as determined by biochemical markers of neuronal and myocardial injury and clinical consequences in patients experiencing on-pump coronary artery bypass-graft surgery [6]. In addition to this, HBO can significantly lessen cognitive impairment as well as considered responsible for decreases in pro-inflammatory cytokines and caspase-3 activity [12]. Thus, HBO is associated with anti-oxidants stress, anti-inflammation, anti-apoptosis, as well as increased regional cerebral blood flow distribution and improvement of blood-brain barrier integrity. In this study, HBO has effectively inhibit apoptosis of oligodendrocytes.

Salidroside can effectively reduce the cerebral edema with global cerebral ischemia-reperfusion injury, relieve the metabolism abnormality of free radical and improve the function of cognition. The results of the present study showed that Sal treatment alone can effectively alleviate the myelin sheaths lesions and combination of Sal and HBO was verified to further reduce the lesions. Moreover, Sal treatment promoted proliferation and inhibited apoptosis of oligodendrocyte and combination of Sal and HBO further promoted the effects.

P13K/Akt signaling pathway is an important signal pathway to promote cell survival which inhibit cell apoptosis by enhancing transcription of anti-apoptotic genes. Previous studies have found that the protective effect of Sal is related to the P13K/Akt signaling pathway [9]. The anti-apoptotic properties of P13K/Akt signaling pathway and its upregulation plays an important role in oligodendrocyte survival [13].

In this study,the combination of Sal and HBO have shown better effects against apoptosis through P13K/Akt signaling pathway.

In conclusion, combination of Sal and HBO contributes in remyelination and survival of oligodendrocyte through P13K/Akt signaling.

### Acknowledgements

The project was funded by Capital Public Health project (Z141100002114039) and Beijing Nova Program (Z161100004916144).

### Disclosure of conflict of interest

None.

**Address correspondence to:** Shuyi Pan, Department of Hyperbaric Oxygen, Navy General Hospital, Haidian District, 6 Fu Cheng Road, Beijing 100048, China. Tel: 86-18600310183; E-mail: syp201002@sina.com

### References

- [1] Nistri M, Mascalchi M, Moretti M, Tessa C, Politi LS, Orlandi I, Pellicanò G, Villari N. Diffusion weighted MR: principles and clinical use in selected brain diseases. *Radiol Med* 2000; 100: 470-479.
- [2] Turk M, Zaletel M, Pretnar-Oblak J. Ratio between carotid artery stiffness and blood flow - a new ultrasound index of ischemic leukoaraiosis. *Clin Interv Aging* 2016; 11: 65-71.
- [3] Kandel BM, Avants BB, Gee JC, McMillan CT, Erus G, Doshi J, Davatzikos C, Wolk DA. White matter hyperintensities are more highly associated with preclinical Alzheimer's disease than imaging and cognitive markers of neurodegeneration. *Alzheimers Dement (Amst)* 2016; 4: 18-27.
- [4] Weller RO, Hawkes CA, Kalaria RN, Werring DJ, Carare RO. White matter changes in dementia: role of impaired drainage of interstitial fluid. *Brain Pathol* 2015; 25: 63-78.
- [5] Price CC, Mitchell SM, Brumback B, Tanner JJ, Schmalfuss I, Lamar M, Giovannetti T, Heilman KM, Libon DJ. MRI-leukoaraiosis thresholds and the phenotypic expression of dementia. *Neurology* 2012; 79: 734-740.
- [6] Gao ZX, Rao J, Li YH. Hyperbaric oxygen preconditioning improves postoperative cognitive dysfunction by reducing oxidant stress and inflammation. *Neural Regen Res* 2017; 12: 329-336.
- [7] Han J, Xiao Q, Lin YH, Zheng ZZ, He ZD, Hu J, Chen LD. Neuroprotective effects of salidro-

- side on focal cerebral ischemia/reperfusion injury involve the nuclear erythroid 2-related factor 2 pathway. *Neural Regen Res* 2015; 10: 1989-1996.
- [8] Liang L, Ma Z, Dong M, Ma J, Jiang A, Sun X. Protective effects of salidroside against isoflurane-induced cognitive impairment in rats. *Hum Exp Toxicol* 2017; 36: 1295-1302.
  - [9] Chen SF, Tsai HJ, Hung TH, Chen CC, Lee CY, Wu CH, Wang PY, Liao NC. Salidroside improves behavioral and histological outcomes and reduces apoptosis via PI3K/Akt signaling after experimental traumatic brain injury. *PLoS One* 2012; 7: e45763.
  - [10] Valencia C, Martí-Fàbregas J, Belvis R, Martí-Vilalta JL. Leukoaraiosis. Clinical importance and prognosis. *Neurologia* 2003; 18: 149-157.
  - [11] Matchett GA, Martin RD, Zhang JH. Hyperbaric oxygen therapy and cerebral ischemia: neuroprotective mechanisms. *Neurol Res* 2009; 31: 114-121.
  - [12] Chen C, Huang L, Nong Z, Li Y, Chen W, Huang J, Pan X, Wu G, Lin Y. Hyperbaric oxygen prevents cognitive impairments in mice induced by D-galactose by improving cholinergic and anti-apoptotic functions. *Neurochem Res* 2017; 42: 1240-1253.
  - [13] Kumar S, Patel R, Moore S, Crawford DK, Suwanna N, Mangiardi M, Tiwari-Woodruff SK. Estrogen receptor  $\beta$  ligand therapy activates PI3K/Akt/mTOR signaling in oligodendrocytes and promotes remyelination in a mouse model of multiple sclerosis. *Neurobiol Dis* 2013; 56: 131-144.