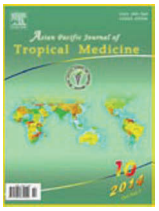
Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm



Document heading doi: 10.1016/S1995-7645(14)60141-3

# Nerve protective effect of Baicalin on newborn HIBD rats

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ARTICLE INFO

*Article history:*
Received 10 April 2014
Received in revised form 15 May 2014
Accepted 15 July 2014
Available online 20 October 2014

*Keywords:*
Newborn rats
Hypoxic ischemic brain damage
Baicalin
Nerve protection

ABSTRACT

**Objective:** To investigate the nerve protective effect and mechanism of baicalin on newborn rats with hypoxic ischemic brain damage (HIBD). **Methods:** A total of 64 SD newborn rats were randomly divided into control group, model group, nerve growth factor group and baicalin group, with 16 in each group. Left carotid artery ligation method was adopted to establish the HIBD model except for in control group, which was treated with intraperitoneal injection of salin e10 mL/kg for 3 d. After oxygen recovery on hypoxia ischemia rats, intraperitoneal injection of saline 10 mL/kg was adopted in model group for 3 d. Intraperitoneal injection of nerve growth factor injection 50 μg/kg per day was adopted in nerve growth factor group for 3 d; intraperitoneal injection of radix scutellariae 16 mg/kg per day was adopted in baicalin group for 3 d after modeling. Four rats of each group were sacrificed at Day 1, 2, 3, 7 for microscopic observation of pathological morphological changes in brain tissue after HE staining, S-P immunohistochemical method was used for observation of Fas and FasL expression in brain cells. **Results:** Neat structure of cells was observed in control group; edema cells in disordered arrangement was observed in model group, with some cells necrosis and cavity change; tissue injury in nerve growth factor group and baicalin group was significantly lighter than that in model group; Fas and FasL expression in model group, nerve growth factor group and baicalin group were significantly higher than that in control group at different time points ($P<0.05$); Fas and FasL expression in nerve growth factor group and baicalin group were significantly lower than that in model group at different time points ($P<0.05$); There was no statistical difference of Fas, FasL expression at each time point between nerve growth factor group and baicalin group ($P>0.05$). **Conclusions:** Baicalin can reduce expression of Fas and FasL in HIBD rats, inhibit apoptosis of nerve cells, thus achieve the protective effect on HIBD rat nerves.

## 1. Introduction

Neonatal hypoxic ischemic brain damage (HIBD) is relatively common clinically, mainly caused by perinatal asphyxia and hypoxia. Neuron apoptosis is important in the pathological change process of HIBD, can lead to neonatal death or permanent neurological damage[1-3]. Although significant progress on neonatal asphyxia recovery has made, the morbidity and mortality of HIBD have no obvious improvement[4]. Studies have found that[5], neurons apoptosis caused by ischemia anoxic is the main cause of death. As a result, timely intervention of apoptosis cascade can significantly reduce the neuron apoptosis. Another study reported that[6], protein expression of Fas, FasL in hippocampus of newborn HIBD rats increased significantly, and was positively associated with the degree of neuronal apoptosis, showed that Fas/FasL signal pathway is important in the brain damage of HIBD process. Nerve growth factor (NGF) is a neurotrophic factor, maintain the neuron growth and differentiation, and protect neurons of the newborn rat during HIBD acute stage[8]. Baicalin is a flavonoids monomer material extracted from Scutellaria baicalensis Georgi, serving for anti-inflammatory, anti-allergy and the immune regulation[9]. Studies have shown that[10], baicalin have certain curative effect for brain injury disease by

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Foundation project: It is supported by Yantai Science and Technology Projects (Grant No: 2004221).

adjusting the 24 kinds of ischemia-reperfusion related proteins.

To observe the neural protection mechanism of baicalin in newborn rats with HIBD, left common carotid artery ligation was adopted for launching the HIBD model in newborn rats, followed by intraperitoneal injection of nerve growth factor and baicalin in order to compare their nerve protective effects.

## 2. Materials and methods

### 2.1. Experimental animals

A total of 64 SD rats in grade clean were provided by Experimental Animal Center, male and female unlimited, aged 7 d, weighting (14±2.5) g. Free access to water and food were provided during class Ⅱ raising, administration of experimental animals was strictly performed during the experiments on animals.

### 2.2. Instrument and reagent

CY-12C digital oxygen meter (Jianse Meicheng Electrochemical Analysis Instrument Factory); BX40-12J02 system biological microscope (Olympus, Japan); BI-2000 medical image analysis system (Western Science and Technology Co., LTD. Wuhan). Inhaled-typed Sevoflurane (Jiangsu Hengrui Pharmaceutical Co., LTD.); Mouse nerve growth factor and baicalin were purchased from Xiamen North Road Biological Engineering Co., LTD.; Immunohistochemical staining kits, DAB chromogenic reagent kit, Hematoxylin and Eosin kits (Beijing Zhongshan Golden Bridge Biotechnology Co., LTD.); 0.01 mol/L sodium citrate buffer solution was provided by Research Extension Biological Technology Co., LTD. (Shanghai); Leucocyte diluent and platelet diluent were purchased from Shanghai West Don Biotechnology Limited Company.

9 μg mouse nerve growth factor was added to 1 mL saline (9 μg/mL), 0.8 mL saline was then added to mix concentration of 5 μg/mL of nerve growth factor injection.

Baicalin (40 mg) was added to 25 mL saline for preparing baicalin injection (1.6 mg/mL).

### 2.3. Model preparation

HIBD model was prepared using left common carotid artery ligation. Routine disinfection of neck skin was performed after inhaling sevoflurane anesthesia in supine position. Longitudinal incision was made in 2 mm on the left of the neck center to expose the left common carotid artery, then incision was sealed with sterile line, after 2 h of recovery. Mixture gas with 8% oxygen and 92% nitrogen was added to seal box with speed of 1.5 to 2.5 L/min for 2 h. HIBD rat modeling criteria were as follows: 30 min after hypoxia ischemia, brain injury symptoms such as drowsiness, burnout and abnormal muscle tone were observed in rats. Then rats were turned to the ligation side.

### 2.4. Animal groups

A total of 64 SD newborn rats were randomly divided into control group, model group, nerve growth factor group and baicalin group, with 16 in each group. Left carotid artery ligation method was adopted to establish the HIBD model except for in control group, which was treated with intraperitoneal injection of saline 10 mL/kg for 3 d. After oxygen recovery on hypoxia ischemia rats, intraperitoneal injection of saline 10 mL/kg was adopted in model group for 3 d. Intraperitoneal injection of nerve growth factor 50 μg/kg per day was adopted in nerve growth factor group for 3 d; intraperitoneal injection of radix scutellariae 16 mg/kg per day was adopted in baicalin group for 3 d after modeling.

### 2.5. Indexes observation

Four rats of each group were sacrificed at Day 1, 2, 3, 7 for microscopic observation of pathological morphological changes in brain tissue after HE staining, S-P immunohistochemical method was used for observation of Fas and FasL expression in brain cells.

#### 2.5.1 Specimen preparation

Ether sevoflurane inhalation was used for anesthesia. Chest was open to expose the heart. After bilateral jugular vein was clipped, all rats had left ventricular injection of saline. Treated rats jugular end effluent was clear, the lungs become white. They were beheaded in brain tissue. Brain tissue specimens were rinsed with distilled water, dried, and fixed with 4% paraformaldehyde inside the bottle, at 4 ℃. After 48 hours, all rats underwent conventional gradient ethanol dehydration, transparent, xylene paraffin embedding, dressing wax block for coronary slice in 4 microns thickness and HE staining for histological observation.

#### 2.5.2. HE staining method

Biopsy was soaked in xylene. Roasted dewax gradient ethanol dehydration and hematoxylin staining were

performed. They were rinsed with water until slices became blue. Color separation was carried out using 70% alcohol, followed by water rinsing. They were stained with 1% eosin for 5-10 min, rinsed with distilled water. Then 70%, 80%, 90% ethanol dehydration was performed. They were sealed with xylene neutral resin. BX40-12 j02 system biological microscope was used for observation of histological changes.

#### 2.5.3. Determination of Fas and FasL

Biopsy dimethyl benzene went through dewaxing, gradient and ethanol dehydration. They were rinsed with distilled water for three times; added with 3% $H_2O_2$ for 10 min to inactivate endogenous peroxidase. Then they were rinsed with PBS liquid for three times. Sample was placed into 0.01 mol/L sodium buffer (pH 6.0), heated in microwave 10 min for two times. They were washed with PBS for three times, and normal goat serum was used for 30 min at room temperature. 0.05 mL of primary antibody (CD95 antibody and FasL antibody) was added to each slice overnight at 4 ℃. They were rinsed with PBS for 3 times, added with 0.05 mL sheep anti mouse IgG. They were rinsed with 0.01 mmol/L PBS for 3 times after 15 min, followed by DAB chromogenic process. They were rinsed with distilled water, redyed with wood grain for 1 min; rinsed with water again, immersed in $Na_2HPO_4$ for 2 min until the specimens became blue, followed by conventional dehydration, transparent. Then they were sealed with neutral resin.

#### 2.5.4. Result judgment

The images were acquired from 5 positive expression sites of each vision field, three of which were randomly selected for each slice. BI-2000 system wasused for quantitative analysis of Fas and FasL, determination of positive expression area, integral optical density (IOD) and IOD/unit area.

### 2.6. Statistical analysis

SPSS19.0 statistical software was used to analyze data, and all data were expressed as mean±sd, and analyzed by *t* test. $P<0.05$ was regarded as statistically significant difference.

## 3. Results

### 3.1. Pathological changes in brain tissue of rats at each time point

Neat structure of cells was observed in control group, without edema or hemorrhage; edema cells in disordered arrangement was observed in model group at Day 1, some cells necrosis and nucleolus disappeared at Day 2, cavity change; tissue injury in nerve growth factor group and baicalin group was significantly lighter than that in model group; cells necrosis aggravated, neuron cells reduced obviously, with relieving edema at Day 7, at the same time point brain injury in nerve growth factor group and baicalin group was significantly lighter than that in model group, with mild cell edema and most normal neurons structure; Brain cell damage is roughly similar in both groups as shown in Figure 1.

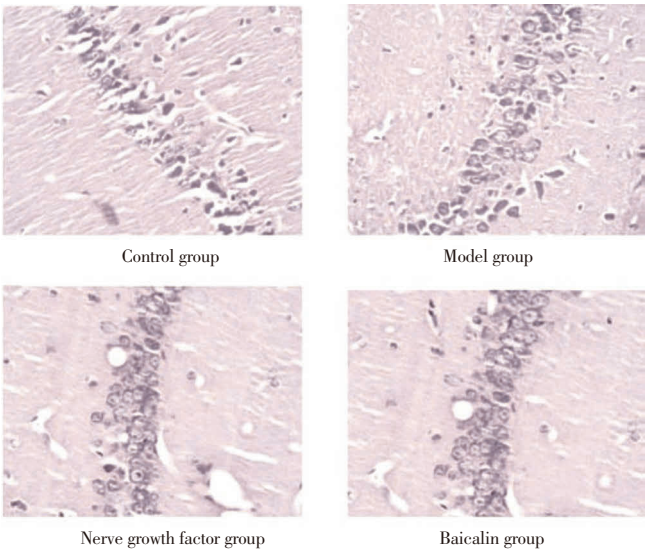


**Figure 1.** Pathological morphological changes at Day 2 after modeling (×400, HE staining).

### 3.2. Fas expression results

Fas expression in model group, nerve growth factor group and baicalin group were significantly higher than that in control group at different time points ($P<0.05$); Fas expression in nerve growth factor group and baicalin group were significantly lower than that in model group at different time points ($P<0.05$); There was no statistical difference of Fas expression at each time point between nerve growth factor group and baicalin group ($P>0.05$) as shown in Table 1.

### 3.3. Fas expression results

There was no statistical difference of FasL expression changes between different time points in control group ($P>0.05$). FasL expression in model group, nerve growth factor group and baicalin group were significantly higher than that in control group at different time points ($P<0.05$);

FasL expression in nerve growth factor group and baicalin group were significantly lower than that in model group at different time points ($P$<0.05); There was no statistical difference of FasL expression at each time point between nerve growth factor group and baicalin group, as shown in Table 2.

## 4. Discussion

Neonatal HIBD is common clinically, caused by suffocation in the perinatal period, which can lead to permanent neurological damage or death. Pathogenesis of HIBD is a multi-level and complicated pathophysiologic process, neuron apoptosis due to ischemia and hypoxia after blood supply restricted in brain[11-13]. Studies have confirmed that[14], dexamethasone pretreatment can effectively inhibit nerve cell apoptosis in HIBD rats[14],parts of pharmacological effects of baicalinsimilar to dexamethasone, therefore we hypothesized it also has the inhibitory effect of nerve cell apoptosis, thus reduce nerve injury after HIBD[10]. As such, we observed nerve protective effect of baicalin in newborn HIBD rats, in order to provide theoretical basis of baicalin in the treatment of neonatal HIBD.

Baicalin is a flavonoids monomer material extracted from Scutellaria baicalensis Georgi, taste bitter and cool, have effect of purging heat and detoxification, serving for anti-inflammatory, anti-allergy and the immune regulation[9]. Studies have shown that[10], baicalin have certain curative effect for brain injury disease by adjusting many kinds of ischemia-reperfusion related proteins[15]. Because of its effect of clearing superoxide anion, it is often used in treatment of upper respiratory tract infection and autoimmune diseases such as hepatitis and rheumatic fever.

Nerve growth factor (NGF) is a neurotrophic factor, protect neurons of the newborn rat during HIBD acute stage[16]. In this study, cells injuries of nerve growth factor group and baicalin group were significantly lighter than that of model group, with mild cell edema in normal neurons structure, suggesting that alike nerve growth factor, baicalin can also play a nerve protective effect in newborn HIBD rats.

Neuronal necrosis is a major cause of HIBD leads to cell death, in ischemia half dark band around HIBD brain damaged tissue, obvious changes of apoptosis related proteins Caspase 3 and Fas, and anti-apoptosis protein Bcl-2 were observed before and after cerebral ischemia[17,18]. Some scholars study found[19], baicalin can inhibit or activate apoptosis related proteins to suppress the neurons damage, and have certain antioxidant effect to achieve maximum protection of brain function through multiple protection mechanisms. Other studies confirm that[20], Fas/FasL signaling pathway is an important factor of HIBD, there is no definitive understanding about its expression in cell apoptosis, some animal experiments confirmed that[21], Fas/FasL signal pathways was activated 24 h after brain injury, and began to decline after the peak until Day 7. In this study, the model group rats had highest positive expression of Fas and FasL after 2 d of modeling, and then began to decline, until the seventh day, showing that Fas and FasL in HIBD rats expressed to peak after 48 h of modeling, and then gradually decline until the seventh day. And the expression of Fas and FasL and cranial nerve damage degree were positively correlated ($P$<0.05), highest level of nerve cell apoptosis was observed at the peak expression of Fas and FasL. Other scholars reported [22], intraperitoneal injection of nerve growth factor after HIBD can significantly reduce the expression of Fas and FasL. This study showed that the expression of Fas and FasL in nerve growth factor

**Table 1**

Expression of Fas in each group at different time points ($n$=4, IOD/unit area).

| Groups | Day 1 | Day 2 | Day 3 | Day 7 |
|---|---|---|---|---|
| Control group | 1.54±0.32 | 1.62±0.24 | 1.57±0.22 | 1.51±0.20 |
| Model group | 4.11±0.11* | 4.51±0.31* | 3.20±0.15* | 2.98±0.15* |
| Nerve growth factor group | 2.66±0.34*# | 2.99±0.19*# | 2.36±0.22*# | 2.09±0.17*# |
| Baicalin group | 2.89±0.39*# | 3.21±0.22*# | 2.55±0.31*# | 2.22±0.19*# |

* $P$<0.05 compared with control group; # $P$<0.05 compared with model group.

**Table 2**

FasL expression in each group at different time points ($n$=4, IOD/unit area).

| Groups | Day 1 | Day 2 | Day 3 | Day 7 |
|---|---|---|---|---|
| Control group | 1.55±0.19 | 1.64±0.18 | 1.67±0.21 | 1.52±0.25 |
| Model group | 4.21±0.22* | 4.85±0.41* | 3.61±0.52* | 3.11±0.39* |
| Nerve growth factor group | 2.82±0.36*# | 3.11±0.19*# | 2.51±0.20*# | 2.19±0.15*# |
| Baicalin group | 2.93±0.45*# | 3.35±0.21*# | 2.54±0.37*# | 2.14±0.26*# |

* $P$<0.05 compared with control group; # $P$<0.05 compared with model group.

group and baicalin gorup at each time point after modeling were significantly lower than that in control group ($P<0.05$), suggesting that baicalin have the same treatment effect of reducing of the expression of Fas and FasL in newborn HIBD rats as the nerve growth factor do.

The results showed that baicalin can reduce expression of Fas and FasL in HIBD rats, inhibit apoptosis of nerve cells, thus achieve the protective effect on HIBD rat nerves.

## Conflict of interest statement

We declare that we have no conflict of interest.

## References


[1] Liu L, Johnson HL, Cousens S, Perin J, Scott S, Lawn JE, et al. Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000. *Lancet* 2012. [Epub ahead of print].

[2] Chen HJ, Wei KL, Zhou CL, Zhou CL, Yao YJ, Yang YJ, et al. China′s seven big cities multicenter study of premature brain injury incidence. *J Clin Pediatrics* 2011; **29** (11): 1001-1011.

[3] Zhang SF, Yin Q, Chang XS, Xia L, Wu SR, Yin CJ, et al. Influence of nerve growth factor on Fas/FasL expression after hypoxic ischemic brain damage in neonatal rats. *J Chin Med Clin* 2010; **10** (4): 391-394.

[4] Zhao YM, Yu SM, Zhou QK. Function of baicalin on GAT-1 and GABAAR in cerebral hemorrhage. *Chin J New Drugs* 2010; **12** (11): 970-974.

[5] Guo MF, Yu JZ, Ma CG. Mechanisms related to neuron injury and death in cerebral hypoxic ischaemia. *Folia Neuropathol* 2011; **49** (2): 79-87.

[6] Northington FJ, Chavez-Valdez R, Martin LJ. Neuronal cell death in neonatal hypoxia-ischemia. *Ann Neurol* 2011; **69**(5): 743-758.

[7] Carlsson Y, Schwendimann L, Vontell R, Rousset CI, Wang X, Lebon S, et al. Genetic inhibition of caspase-2 reduces hypoxic-ischemic and excitotoxic neonatal brain injury. *Ann Neurol* 2011; **70**(5): 781-789.

[8] Liu C, Jia YJ, Jiang XQ, Zhang Z, Cheng X, Min LQ, et al. The influence of ceftriaxone sodium on expression of Fas and nf-κB in focal cerebral ischemia rats. *Chin J Gerontol* 2012; **32** (19): 4210-4212.

[9] Zhou QK, Yu SM, Liu P, Ma SM, Zhu QJ, Zhao YM, et al. Effects of baicalin on the content of amino acid neurotransmitter in cerebral hemorrhage rats. *Chin J Chin Med Information* 2009; (4): 35-37.

[10] Lin XJ, Yang YJ, Qi BX, Wang X, Song JH. Baicalin pretreatment protection of hypoxic ischemic brain damage in newborn rat. *J Contemporary Chin J Pediatrics* 2006; **8** (3): 221-224.

[11] Fan YY, Hu WW, Dai HB, Zhang JX, Zhang LY, He P, et al. Activation of the central histaminergic system is involved in hypoxia-induced stroke tolerance in adult mice. *J Cereb Blood Flow Metab* 2011; **31**(l): 305-314.

[12] Lin X, Li M, Hu YZ, Han ZT, Zhang HH, Shang AJ, et al. The expression changes of brain red protein after craniocerebral trauma and its relationship with neuron apoptosis. *Chin J Appl Physiol* 2010; **26** (1): 39-44.

[13] Li W, Wu Y, Ren C, Lu Y, Gao Y, Zheng X, et al. The activity of recombinant human neuroglobin as an antioxidant and free radical scavenger. **Proteins** 2011; **79**(1): 115-125.

[14] Zhao H, Zhang QR, Lu XM, Chen XX, Zhang HP. Influence of hyperbaric oxygen on expression of fossa protein-1 and matrix metalloproteinase-9 in focal cerebral ischemia reperfusion rats. *Chin J Reh Med* 2011; **26** (6): 550-552.

[15] Zhou J, Li J, Rosenbaum DM, Barone FC. Thrombopoietin protects the brain and improves sensorimotor functions: reduction of stroke-induced MMP-9 upregulation and blood-brain barrier injury. *J Cerebral Blood Flow Metabolism: Official J Int Society Cerebral Blood Flow Metabolism* 2011; **31**(3): 924-933.

[16] Acioly MA, Carvalho CH, Koerbel A, Lwenheim H, Tatagiba M, Gharabaghi A. Intraoperative brainstem auditory evoked potential observations after trigeminocardiac reflex during cerebellopontine angle surgery. *J Neurosurg Anesthesiol* 2010; **22**(4): 347-353.

[17] Niu Z, Tang XJ, Li LL, Chen LX, Yao XL. after Dynamic expression study of TNF-α and SOCS-3 in reperfusion after focal cerebral ischemia in rats. *J Pract Cardio-cerebral Pulmonary Vascular Dis Mag* 2011; **12** (1): 3-5.

[18] Jia J, Hu YS, Wu Y, Liu G, Yu HX. Curative effect of nerve growth factor in study the ischemic brain injury in rats with acute stage. *J Nerve Damage Function Reconstruction* 2011; **6** (1): 1-5.

[19] Lavrik IN, influenza PH. Regulation of CD95/Fas signaling at the DISC. *J Cell Death Differ* 2012; **12**(1): 36-41.

[20] Jing ZW, Yan ZL, CX. Comparison of the network structural characteristics ofcalcium signaling pathway in cerebral ischemia after intervention by marketers components of Chinese medicine. *J Tradit Chin Med* 2011; **31**(3): 251-255.

[21] Cao Y, Mao X, Sun C, Zheng P, Gao J, Wang X, Min D, et al., Baicalin attenuates global cerebral ischemia/reperfusion injury in gerbils via anti - oxidative and anti - apoptotic pathways. *Brian Res Bull* 2011; **30**(6): 396-402.

[22] Zhang Z, Zhou W, Lu WN, Rong X, Chen XW. Clinical study of exogenous nerve growth factor early intervention to reduce brain damage degree in severe asphyxia newborn. *Chin J Pract Pediatrics* 2011; **26**(1): 33-36.