

Original Research Article

Effect of oxymatrine on hypoxic-ischemic brain injury in neonatal rats

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Abstract

Purpose: To study the influence of oxymatrine on hypoxic-ischemic brain injury (HIBI) in neonatal rats.

Methods: Newborn SPF Sprague-Dawley (SD) rats were randomly assigned to 3 groups (10 rats/group): control, HIBI and oxymatrine groups. Neurobehavioral latency of each rat was determined after 48 h of treatment, and pathological changes in rat cerebral cortex were evaluated using H&E staining. Hippocampal neurons prepared from rat brain tissue were grouped and treated as per the above in vivo study. Cell survival and neuronal apoptotic changes were measured with CCK-8 and flow cytometric analysis, respectively, while protein expressions of bcl-2, mcl-1, bax, caspase-3, PI3K, p-PI3K, Akt, p-Akt, GSK3 β and p-GSK3 β were determined with Western blotting.

Results: Treatment of HIBI rats with oxymatrine significantly reduced their neurobehavioral latencies (reflex, cliff avoidance reflex, and negative reflex (latencies), but repaired HIBI-induced histological damage in rat cerebral cortex ($p < 0.05$). It also significantly enhanced the survival of rat hippocampal neurons, while reducing neuronal apoptosis ($p < 0.05$). Moreover, oxymatrine significantly upregulated bcl-2, mcl-1, p-PI3K, AKT, p-AKT, GSK3 β and p-GSK3 β protein expressions, but significantly downregulated the protein expressions of bax and caspase-3 in cerebral cortex of HIBI rat ($p < 0.05$).

Conclusion: These results indicate that oxymatrine reduces neuronal apoptosis and alleviates HIBI in rats via the regulation of proteins associated with PI3K/Akt/GSK3 β signal pathway. This finding provides a new research direction on novel botanical monomers for treating HIBI.

Keywords: Cell survival, Hypoxic-ischemic brain injury, Neurobehavioral latency, Neuronal apoptosis, Oxymatrine

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INTRODUCTION

Hypoxic-ischemic brain injury (HIBI) or hypoxic-ischemic encephalopathy (HIE), refers to

newborn brain damage caused by oxygen deprivation and limited cerebral blood flow (CBF). The disease is a neurological disability caused by perinatal hypoxia, and a major cause

of death in infants [1]. Fatality caused by HIBI may get as high as 20 %, while survivors may develop epilepsy, cerebral palsy, developmental delay, visual and auditory impairments, and other sequelae [2]. Statistics show that 13.6 % of neonates in China suffer from asphyxia, while 15.6 % of them have varied degrees of disability [3]. The condition endangers the life and health of neonates in China [3]. Since there is presently no specific treatment for HIBI, an in-depth understanding of its molecular mechanism and attendant neurological dysfunction may provide insight on possible treatment strategies for the disease.

Traditional Chinese Medicine (TCM) has shown promising potential in the treatment of asphyxia. The herbs are readily available and inexpensive, and they are relatively of low toxicity [4]. Oxymatrine is a quinolizidine alkaloid isolated from the root of *Sophora alopecuroides*. The compound has been reported to possess antioxidant, anti-inflammatory, antiviral, anti-apoptotic, antitumor, immunomodulatory and hepatoprotective properties [5]. Although studies have shown that oxymatrine exerts a variety of effects *in vitro* and in animal models, the precise molecular mechanism remains largely unknown [6]. This investigation was aimed at determination of the influence of oxymatrine on HIBI in neonatal rats.

EXPERIMENTAL

Rats

Newborn, specific-pathogen-free SPF rats were supplied by Guangdong Medical Experimental Animal Centre. The rats were maintained at a temperature of 25 °C and 55 % humidity under equal light/dark photoperiod, and allowed *ad libitum* access to feed and water. The study received approval from the Animal Ethical Committee of Hudson International Peace Hospital affiliated to Hebei Medical University (approval no. 2019110304), and was conducted according to "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985) [7].

Three groups of rats (10 rats/group) were used: control, HIBI and oxymatrine groups. The HIBI was induced in the rats via standard procedures. The rats were placed in an anoxic chamber saturated with a mixture of 8 % CO₂ and 92 % N₂ at 37 °C and constant speed of 4 L/min. Timing of anoxic episodes started after attainment of stable gas flow. The procedure lasted 2.5 h. Rats in

oxymatrine group were treated with oxymatrine injection (*i.p.*) at a single dose of 120 mg/kg bwt 2 h after induction, while those in control group received equivalent volume of normal saline intraperitoneally. Neurobehavioral latency of each rat was determined after 48 h of treatment.

Neuronal cell culture

The cortices of rats were freed from the meninges under sterile conditions, and mechanically dissociated to single-cell suspensions using flame-polished Pasteur pipettes of progressively smaller diameters. A given tissue sample was passed through a pipette 3 - 4 times and the final diameter of the pipette was approximately 0.5 - 1.0 mm. The pooled, dissected cortices were put in 2 - 5 mL of DMEM containing 1 % (*w/v*) L-glutamine at room temperature until all the tissues were dissected. The dissociation of rat cortex was done in a 15 mL sterile polystyrene centrifuge tube lightly coated with 5 % (*v/v*) heat-inactivated fetal bovine serum (FBS). Three dissociation cycles were carried out to obtain enough cell suspension. Three groups of neuronal cells were used: control, HIBI and oxymatrine groups. Cells in oxymatrine group were treated with 3 µg/mL oxymatrine. The cells were also treated with Earle's Balanced Salt Solution (EBSS) of the same volume as the original culture, and the petri dishes were incubated for 2 h at 37 °C in pre-heated anoxic chamber.

Measurement of neurobehavioral latency

Righting reflex, cliff avoidance reflex and negative geotactic reflex latencies of rats were measured 48 h after treatment.

Reverse reflex

Newborn rats were placed in supine position on the test bench, and the time taken by a rat to recover from the supine position was recorded.

Cliff avoidance time

Neonatal rats were placed on the test bed 1.5 cm away from the edge, and the time of falling from the edge was recorded.

Negative geotactic reflex

The head of each rat was placed downward in the centre of an inclined plane at 45°, and the time taken to turn the head upward was recorded.

Histopathological examination of rat cerebral cortex

Hematoxylin and eosin (H & E) staining was used to evaluate pathological changes in cerebral cortex of each rat. A part of the excised brain tissue was fixed in 10 % normal saline and paraffinized. Using a microtome, 5- μ m slices were generated. Then, the sections were stained with H & E, and observed under a light microscope. Pathological changes in rat cerebral cortex were recorded and analysed with ImageJ analysis software.

Assessment of cell survival

Neuronal cell survival was measured with CCK-8 assay. Hippocampal neurons were cultured in 96-well plates (4×10^3 cells per well; 100 μ L/well) for 20 min at 37 °C in a 5 % CO₂ atmosphere. After oxygen and glucose deprivation/reperfusion, CCK-8 was put into every well, and the mixture was cultured for 1 h, followed by absorbance reading at 450 nm.

Apoptosis assay

Hippocampal neurons were cultured for 24 h. The resultant cell suspension was trypsinized with 0.25 % trypsin, rinsed two times with PBS, and vortexed with 400 μ L binding buffer, stained using 5 μ L of Annexin V-FITC and PI (10 μ L) away from light within 15 min. Neuronal apoptotic changes were measured flow cytometrically.

Western blotting

Rat brain tissue was homogenized in PBS and subjected to lysis with RIPA buffer mixed with anti-protease. The protein content of the lysate was measured with BCA method, followed by SDS-polyacrylamide gel electrophoresis and electro-transfer to PVDF membrane which was sealed by incubation with 5 % fat-free milk solution. Thereafter, the blots were subjected to incubation for 12 h at 4 °C with 1:800 diluted 1° antibodies for mcl-1, bcl-2, bax, caspase-3, PI3K, p-PI3K, Akt, p-Akt, GSK3 β , p-GSK3 β and glyceraldehyde 3-phosphate dehydrogenase

(GADPH). Then, the membrane was rinsed three times with TBS-T, followed by incubation with HRP-linked goat anti-rabbit IgG 2° antibody for 60 min at laboratory temperature. Blot development was done with X-ray film, while ImageJ Launcher software was employed for Grayscale analysis. Protein levels were calculated relative to GADPH which was used as internal reference.

Statistics

Measurement data are presented as mean \pm SEM, and were statistically analyzed using SPSS (20.0). Group comparisons were done with SNK-q test and χ^2 test. Statistical significance was assumed at $p < 0.05$.

RESULTS

Effect of oxymatrine on neurobehavioral latencies

Treatment of HIBI rats with oxymatrine led to significant reductions in their neurobehavioral latencies (reflex, cliff avoidance reflex, and negative reflex latencies) ($p < 0.05$; Table 1).

Effect of oxymatrine on histology of rat cerebral cortex

The results of H & E staining showed that the cerebral cortices of rats in control group were complete, with uniformly arranged neurons, while those of HIBI group were highly disorganized, with reduced number of neurons characterized by pyknotic nuclei. However, the number of neurons in cerebral cortices of oxymatrine group rats was significantly increased, with closely packed neurons, and the nuclear pyknotic nuclei was markedly reduced. These results are shown in Figure 1.

Effect of oxymatrine on neuronal cell survival

Oxymatrine markedly enhanced the survival of the hippocampal neurons of HIBI rats (Figure 2).

Table 1: Comparison of neurobehavioral latencies amongst rats in the groups

Group	Reflex latency time (s)	Cliff avoidance reflex latency time (s)	Negative reflex latency time (s)
Control	1.26 \pm 0.41	4.35 \pm 1.59	18.56 \pm 6.58
HIBI	12.86 \pm 3.46 ^a	13.46 \pm 2.41 ^a	56.41 \pm 5.16 ^a
Oxymatrine	4.26 \pm 1.74 ^{ab}	5.36 \pm 2.46 ^b	28.19 \pm 9.56 ^{ab}
F	143.410	103.990	143.920
P-value	< 0.001	< 0.001	< 0.001

^a $P < 0.05$, vs control; ^b $p < 0.05$, vs with HIBI group

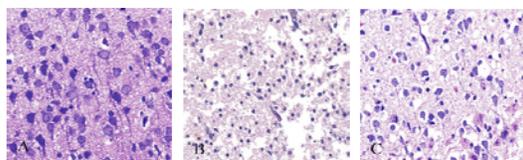


Figure 1: Comparison of pathological changes in cerebral cortices of rats. **(A):** H&E staining of cerebral cortices of rats in control group; **(B):** Result of H&E staining of cerebral cortices of rats in HIBI group; and **(C):** H&E staining map of cerebral cortices of rats in oxymatrine group

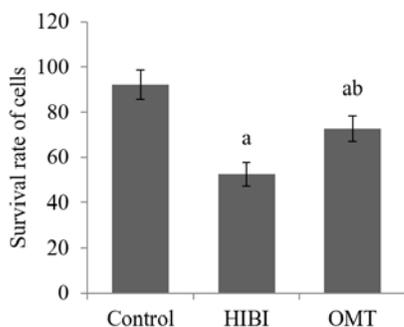


Figure 2: Comparison of cell survival among the groups. ^a*P* < 0.05, vs control; ^b*p* < 0.05, vs HIBI rats

Neuronal apoptosis

Neuronal apoptosis was significantly increased in HIBI rats, but it was markedly reduced by oxymatrine (Figure 3).

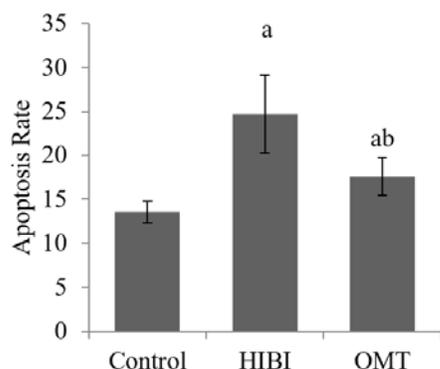


Figure 3: Comparison of neuronal apoptosis among the groups. ^a*P* < 0.05, vs control; ^b*p* < 0.05, vs HIBI rats

Influence of oxymatrine on levels of apoptosis-related proteins

Treatment of HIBI rats with oxymatrine markedly upregulated the protein expressions of bcl-2 and mcl-1, but it significantly downregulated those of caspase-3 and bax (Figure 4).

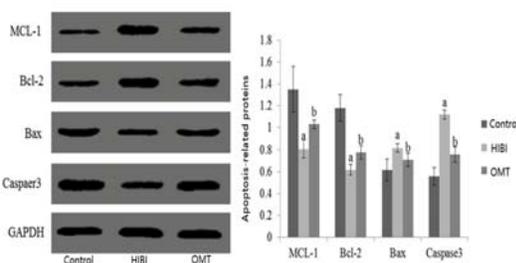


Figure 4: Comparison of expression levels of apoptosis-related proteins among the groups. **(A):** bcl-2, mcl-1, bax and caspase-3, and **(B):** Quantified Western blotting results. ^a*P* < 0.05, vs control; ^b*p* < 0.05, vs HIBI rat

Levels of proteins associated with PI3K/Akt/GSK3β signal route

Protein expressions of p-PI3K, AKT, p-AKT, GSK3β and p-GSK3β in brain tissues of HIBI rats were significantly upregulated by oxymatrine (*p* < 0.05; Figure 5).

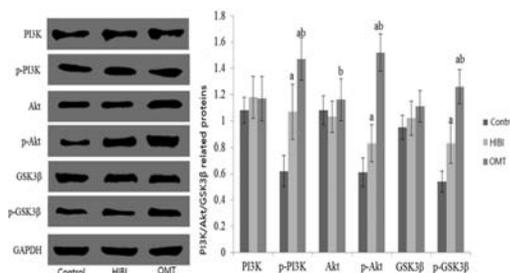


Figure 5: Levels of PI3K/Akt/GSK3β signal route proteins. **(A):** Protein expressions of PI3K, p-PI3K, AKT, p-AKT, GSK3β and p-GSK3β; and **(B):** Quantified Western blotting results. ^a*P* < 0.05, vs control; ^b*p* < 0.05, vs HIBI. Rats

DISCUSSION

Hypoxic-ischemic brain injury (HIBI), also known as HIE or perinatal asphyxia, is associated with clinical and laboratory manifestation of acute or subacute brain damage. The primary causes of HIBI are systemic hypoxemia and reduced CBF. Asphyxia causes respiratory distress syndrome, severe haemolysis, and frequent apnoea, which in turn affect oxygen supply and metabolism in newborns. With prolonged asphyxia insult and failure of compensatory mechanisms, CBF falls, leading to ischemic brain injury. Irreversible damage to nerve cells and severe necrosis may occur in HIBI due to low tolerance of the brain tissue to ischemia and hypoxia, as well as short duration of treatment [8]. Therefore, prevention and early diagnosis are of great importance in HIBI treatment.

Studies have shown that oxymatrine inhibited cell apoptosis and protected rabbits against arrhythmia caused by ischemia and hypoxia [9]. Oxymatrine has also been reported to alleviate myocardial ischemia in dogs [10]. This research has shown that treatment of HIBI rats with oxymatrine significantly reduced their neurobehavioral latencies, and also markedly reversed histological damage caused by HIBI in rat cerebral cortices. These results indicate that oxymatrine may alleviate ischemia/ hypoxia-induced neurological dysfunction, neuronal necrosis and apoptosis in neonatal rats.

The viability of neurons involves interactions amongst different signaling routes which are triggered by stress factors within cells. Changes in these signal pathways influence neuronal apoptosis or neuronal survival. In this study, treatment of HIBI rats with oxymatrine significantly enhanced the survival of their hippocampal neurons, while markedly reducing neuronal apoptosis. It is likely that oxymatrine inhibited neuronal apoptosis in oxygen-glucose deprivation/reperfusion (OGD/RP) injury, which is consistent with an earlier report [11].

The PI3K/Akt/GSK3 β signaling route is crucial for maintenance of cell survival and inhibition of neuronal apoptosis during cerebral ischemia/reperfusion injury [12]. Activation of PI3K (via phosphorylation) phosphorylates and activates Akt, which in turn phosphorylates GSK3 β , thereby inhibiting its activity. Phosphorylated GSK3 β (p-GSK3 β) activates downstream molecules/proteins involved in the regulation of cell proliferation, differentiation, migration and apoptosis [13]. It has been reported that activation of PI3K/Akt/GSK3 β signaling pathway by limb ischemic preconditioning (IPC) alleviates intrauterine distress-induced brain injury, and markedly downregulates the expression of apoptosis-related proteins in pregnant rats [14].

It is known that mcl-1 controls apoptosis and autophagy. It is a substrate for GSK3 β . Down-regulation of mcl-1 via GSK3 β activation has been shown to contribute to As₂O₃-mediated apoptosis in acute myeloid leukemia cells. Myeloid cell leukemia-1 (mcl-1) binds to pro-apoptotic protein of bcl-2 family and inhibits the activation of caspases, thereby inhibiting apoptosis [15]. Up-regulation of mcl-1 has been demonstrated to potentially modulate beclin-1-controlled autophagy in ischemic stroke in rats. The proteins bcl-2 and bax are not only upstream regulators of caspase-3, but they are also direct substrates of the enzyme, thereby forming a mutually restrictive relationship [16-18]. In this

study, treatment of HIBI rats with oxymatrine significantly upregulated protein expressions of bcl-2, mcl-1, p-PI3K, AKT, p-AKT, GSK3 β and p-GSK3 β , but significantly downregulated those of caspase-3 and bax. These results suggest that the mechanism by which oxymatrine alleviates hypoxic ischemic injury may be linked to its regulation of proteins related to PI3K/Akt/GSK3 β signaling pathway.

CONCLUSION

The results obtained in this study indicate that oxymatrine reduces neuronal apoptosis and alleviates HIBI via regulation of PI3K/Akt/GSK3 β signaling pathway-associated proteins. This finding provides a new research direction on novel botanical monomers for HIBI.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was performed by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Aihuan Li designed the study, supervised the data collection, and analyzed the data. Chao Wei interpreted the data and prepared the manuscript for publication. Shujing Zhao, Ruiqing Diao, Liang He and Weizhan Wang supervised the data collection, analyzed the data and reviewed a draft of the manuscript.

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