NEURAL REGENERATION RESEARCH
Volume 8, Issue 2, January 2013

www.nrronline.org

doi:10.3969/j.issn.1673-5374.2013.02.005 [http://www.nrronline.org; http://www.sjzsyj.org]
Mao ZL, Song ZQ, Li G, Lv W, Zhao X, Li B, Feng XL, Chen YL. 8-hydroxy-2-(di-n-propylamino)tetralin intervenes with neural cell apoptosis following diffuse axonal injury. Neural Regen Res. 2013;8(2):133-142.

8-hydroxy-2-(di-n-propylamino)tetralin intervenes with neural cell apoptosis following diffuse axonal injury★

Zhenli Mao[1, 2], Zhenquan Song[1], Gang Li[3], Wei Lv[1, 2], Xu Zhao[1, 2], Bin Li[1, 2], Xinli Feng[1], Youli Chen[1]

1 *Department of Neurosurgery, General Hospital of Shenyang Military Region, Shenyang 110016, Liaoning Province, China*
2 *Postgraduate School, Liaoning Medical University, Jinzhou 121001, Liaoning Province, China*
3 *Jilin Brain Hospital, Siping 136000, Jilin Province, China*

Abstract

Previous studies have reported a neuroprotective effect of 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) against traumatic brain injury. In accordance with the Marmarou method, rat models of diffuse axonal injury were established. 8-OH-DPAT was intraperitoneally injected into model rats. 8-OH-DPAT treated rats maintained at constant temperature served as normal temperature controls. TUNEL results revealed that neural cell swelling, brain tissue necrosis and cell apoptosis occurred around the injured tissue. Moreover, the number of Bax-, Bcl-2- and caspase-3-positive cells increased at 6 hours after diffuse axonal injury, and peaked at 24 hours. However, brain injury was attenuated, the number of apoptotic cells reduced, Bax and caspase-3 expression decreased, and Bcl-2 expression increased at 6, 12, 24, 72 and 168 hours after diffuse axonal injury in normal temperature control and in 8-OH-DPAT-intervention rats. The difference was most significant at 24 hours. All indices in 8-OH-DPAT-intervention rats were better than those in the constant temperature group. These results suggest that 8-OH-DPAT inhibits Bax and caspase-3 expression, increases Bcl-2 expression, and reduces neural cell apoptosis, resulting in neuroprotection against diffuse axonal injury. This effect is associated with a decrease in brain temperature.

Zhenli Mao★, Studying for master's degree.

Corresponding author: Zhenquan Song, M.D., Professor, Chief physician, Master's supervisor, Department of Neurosurgery, General Hospital of Shenyang Military Region, Shenyang 110016, Liaoning Province, China, szqlz@163.com.

Received: 2012-09-12
Accepted: 2012-12-16
(N20120730003/WJ)

Key Words

neural regeneration; brain injury; 8-hydroxy-2-(di-n-propylamino)tetralin; diffuse axonal injury; mild hypothermia; cell apoptosis; Bcl-2; Bax; caspase-3; neuroprotection; grant-supported paper; photographs-containing paper; neuroregeneration

Research Highlights

(1) This study observed the neuroprotective effects of 8-hydroxy-2-(di-n-propylamino)tetralin against diffuse axonal injury, and explored its mechanism of action with respect to apoptosis.
(2) 8-hydroxy-2-(di-n-propylamino)tetralin reduced brain tissue injury in rats, suppressed caspase-3 and Bax protein expression, enhanced Bcl-2 protein expression and reduced neural cell apoptosis.
(3) The neuroprotective effect of 8-hydroxy-2-(di-n-propylamino)tetralin was associated with mild hypothermia in rats with diffuse axonal injury.

Abbreviations

8-OH-DPAT, 8-hydroxy-2-(di-n-propylamino)tetralin; 5-HT1A, 5-hydroxytrypt-amine 1A

INTRODUCTION

Diffuse axonal injury is a distinct form of head injury that has a high disability and death rate. At present, conservative treatments are mainly used in clinic to treat diffuse axonal injury so as to control brain edema, decrease intracranial pressure, avoid cerebral anoxia and prevent complications, including rehabilitation therapy and drug treatment for recovery of brain function[1-3]. However, to date, there are no specific drug treatments available.

Present therapies for diffuse axonal injury are as follows: (1) Mild hypothermia: early mild hypothermia therapy decreases oxygen consumption, maintains normal cerebral blood flow and cell energy metabolism, relieves lactic acid accumulation, lessens cerebral edema, contributes to the recovery of brain cell structure and function, reduces intracranial pressure, lessens axonal injury, diminishes pathological injury after ischemia, and improves the recovery of neural function after cerebral ischemia.

(2) Ca^{2+} antagonists and other drugs: these drugs relieve intracellular calcium overload-induced axonal swelling, relieve vasospasm, improve cerebral microcirculation, prevent cerebral edema, protect brain function and improve prognosis. Exogenous gangliosides can be directly inserted into the damaged neuronal membrane to maintain membrane integrity. Early application of free radical scavengers can remove free radicals, protect the neuron membrane and decrease cerebral edema.

(3) Magnesium: after brain injury, magnesium preparation can improve neural cell energy metabolism and promote the recovery of neural function after cerebral trauma.

(4) Inactivation of axonal growth inhibitors: X-ray irradiation; monoclonal antibody neutralization method.

(5) Scalp acupuncture: diffuse axonal injury in the stationary phase can be treated with scalp acupuncture, which can obtain good outcomes. Acupuncture points include the bilateral central zone and language zone.

Previous studies have verified that neural cell apoptosis can result from diffuse axonal injury[4-5]. Apoptosis refers to a metabolic program that when activated causes a cell to commit suicide, which is characterized by cell detachment and ingestion by phagocytes without eliciting a widespread inflammatory response. Apoptosis is a part of normal cell turnover and tissue homeostasis.

8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT), a 5-hydroxytrypt-amine 1A (5-HT1A) receptor agonist, is mainly used for treatment of depression and anxiety[6-7]. Previous studies confirmed that 8-OH-DPAT had a neuroprotective effect on animal models of cerebral ischemia[8-11] and Parkinson's disease[12], but there were no reports concerning the protective effect of 8-OH-DPAT on diffuse axonal injury.

Following traumatic brain injury, the main pathological changes in brain tissue are necrosis and apoptosis of neural cells. Caspases are a family of cysteine proteases that play essential roles in apoptosis. Caspases are constitutively expressed in cells as inactive proenzymes and require proteolytic cleavage to be active. Present studies showed that neural cell apoptosis associates with the Bcl-2 protein family following craniocerebral injury.

Numerous studies demonstrate that the 5-HT1A receptor agonist including 8-OH-DPAT can decrease brain temperature, and this effect is probably one of the potential mechanisms of its neuroprotective effect[13-14]. Present studies addressing models of traumatic brain injury have verified that the neuroprotective effect of 8-OH-DPAT is associated with its effect on reducing brain temperature[15-16]. However, another study revealed that its effect on improving learning and memory was not associated with the induction of mild hypothermia after traumatic brain injury[17]. It remains unknown whether 8-OH-DPAT can reduce brain temperature in models of diffuse axonal injury, and whether 8-OH-DPAT exerts a protective effect against diffuse axonal injury by decreasing brain temperature.

Neural cell apoptosis is an important pathological change during brain injury. Therefore, this study investigated the neuroprotective effect of 8-OH-DPAT in rats with diffuse axonal injury, particularly focusing on the effects of mild hypothermia on cell apoptosis.

RESULTS

Quantitative analysis of experimental animals

Rats (n = 112) were randomly assigned into four groups: model group (n = 35), constant temperature group (n = 35), 8-OH-DPAT group (n = 35) and normal group (n = 7). Excepting the normal group, rat models of diffuse axonal injury were established according to a previously

published method[18]. Rectal temperature was kept constant using a blanket. Rat models in the constant temperature and 8-OH-DPAT were intraperitoneally injected with 8-OH-DPAT, but those in the model and normal groups were intraperitoneally injected with physiological saline. Excepting the constant temperature group, the blanket was removed in other groups after diffuse axonal injury. The body temperature of rats in the constant temperature group was maintained at 37.0 ± 0.5°C using the blanket. All 112 rats were included in the final analysis.

Changes in brain temperature of rats with diffuse axonal injury

Compared with the constant temperature and model groups, brain temperature was significantly lower in the 8-OH-DPAT group at 1 hour following model establishment ($P < 0.05$), became lowest at 2 hours ($P < 0.05$), and then gradually increased. However, brain temperature changes were not significant in the constant temperature and model groups ($P > 0.05$; Figure 1). The above-mentioned results indicated that 8-OH-DPAT could decrease rat brain temperature.

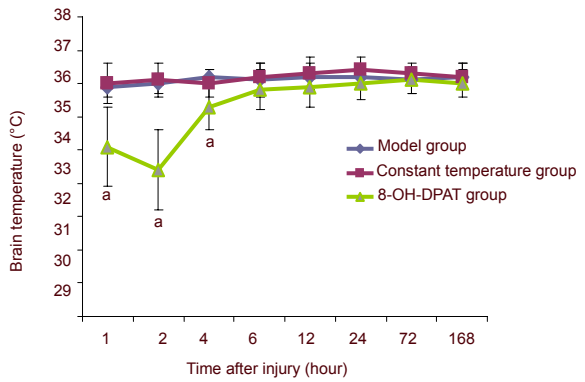

Figure 1 Changes in brain temperature of rats with diffuse axonal injury.

Brain temperature was lowest in the 8-OH-DPAT group. [a]$P < 0.05$, *vs*. model group. Data are expressed as mean ± SD, $n = 7$, two-way analysis of variance; Student-Newman-Keuls test was used for intergroup comparisons. 8-OH-DPAT: 8-hydroxy-2-(di-n-propylamino)tetralin.

Pathological changes in brain tissues of diffuse axonal injury rats

Light microscopy revealed that at 24 hours following injury, neural cell swelling, brain tissue necrosis (satellite cells) and blood capillary engorgement and inflammatory cell infiltration were detectable in brain tissues surrounding the injured region in each group. Compared with the model group, brain tissue injury was mild in the constant temperature and 8-OH-DPAT groups (Figure 2).

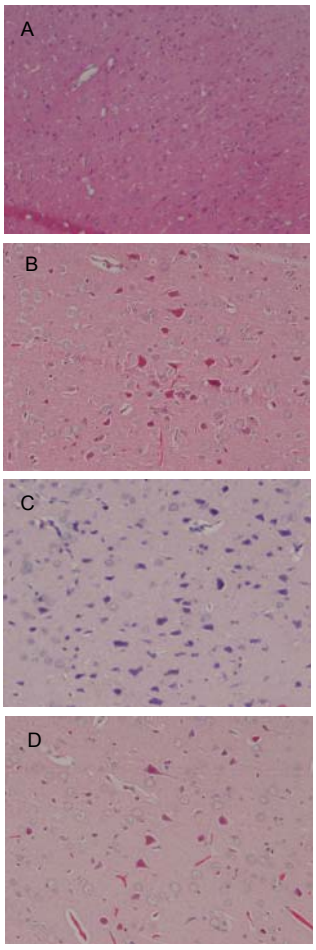

Figure 2 Pathological changes in brain tissues surrounding the injured region of rats at 24 hours following diffuse axonal injury (hematoxylin-eosin staining, light microscopy, × 400).

(A) Normal neural cells were visible in the normal group. (B) Cellular swelling, brain tissue necrosis, and satellite cells were detected in the model group. Injury was reduced in the constant temperature group (C) and 8-OH-DPAT group (D) after diffuse axonal injury. 8-OH-DPAT: 8-hydroxy-2-(di-n-propylamino)tetralin.

Apoptosis in brain tissues surrounding the injured region of rats with diffuse axonal injury

At 24 hours following model induction, TUNEL revealed few apoptotic cells in the normal group, with an apoptotic index of 1.32 ± 0.34 %. In the model group, apoptotic cells were visible at 6 hours, increased at 12 hours, and peaked at 24 hours following diffuse axonal injury. Small apoptotic cell bodies, nuclear membrane shrinkage, chromatin condensation and chromatin accumulated near the nuclear membrane were observed. The number of apoptotic cells reduced at 168 hours ($P < 0.01$). Compared with the model group, the number of apoptotic cells was lower in the constant temperature and 8-OH-DPAT groups ($P < 0.05$ or $P < 0.01$) at various time points. The number of apoptotic cells was smaller in the

8-OH-DPAT group than that in the constant temperature group at various time points ($P < 0.05$ or $P < 0.01$; Table 1, Figure 3).

Table 1 Effect of 8-OH-DPAT on apoptotic neural cells (apopototic index) in brain tissues surrounding the injured region after diffuse axonal injury (%)

Group	Time after injury (hour)		
	6	12	24
Model	22.5±3.2	45.2±4.4	86.5±10.6
Constant temperature	18.9±3.2[a]	38.9±4.5[b]	69.3±9.5[b]
8-OH-DPAT	14.3±2.1[bc]	28.9±3.7[bd]	53.1±7.3[bd]

Group	Time after injury (hour)	
	72	168
Model	60.7±8.8	54.4±5.4
Constant temperature	54.3±6.4[b]	39.9±3.1[b]
8-OH-DPAT	40.2±8.3[bd]	37.2±4.3[bd]

[a]$P < 0.05$, [b]$P < 0.01$, *vs.* model group; [c]$P < 0.05$, [d]$P < 0.01$, *vs.* constant temperature group. Data are expressed as mean ± SD. Seven rats at each time point, two-way analysis of variance; Student-Newman-Keuls test was used for intergroup comparisons. Apoptotic index was equal to the number of apoptotic cells/(number of apoptotic cells + number of normal cells) × 100%. The apoptotic index in the normal group was 2.1 ± 0.5.

8-OH-DPAT: 8-hydroxy-2-(di-n-propylamino)tetralin.

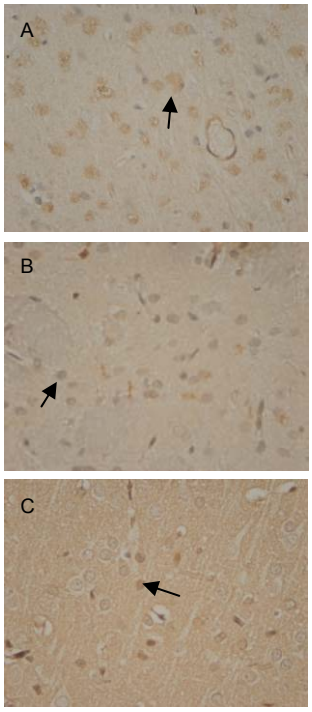

Figure 3 Neuronal apoptosis in brain tissues surrounding the injured region of rats at 24 hours following diffuse axonal injury (TUNEL staining, × 400).

TUNEL-positive cells presented brown in color (arrows). The number of apoptotic cells that were dark in color was more in the model group (A). Cells in the constant temperature group (B) and 8-OH-DPAT group (C) exhibited weak staining.

8-OH-DPAT: 8-hydroxy-2-(di-n-propylamino)tetralin.

Caspase-3 expression in brain tissues surrounding the injured region in rats with diffuse axonal injury

Immunohistochemical results revealed that caspase-3 expression was low in the normal group. Caspase-3 expression increased at 6 hours following diffuse axonal injury, peaked at 24 hours, and then gradually reduced in the model group. Compared with the model group, caspase-3 expression was significantly lower in the constant temperature and 8-OH-DPAT groups at the corresponding time points ($P < 0.05$ or $P < 0.01$).

In particular, the decrease was most significant at 24 hours following model induction ($P < 0.01$). Caspase-3 expression was lower in the 8-OH-DPAT group than that in the constant temperature group at each time point ($P < 0.05$ or $P < 0.01$). High expression of caspase-3 appeared later in the 8-OH-DPAT group compared with the constant temperature group at each time point (Table 2, Figure 4).

Table 2 Effect of 8-OH-DPAT on caspase-3 expression (absorbance value) in brain tissues surrounding the injured region of rats following diffuse axonal injury

Group	Time after injury (hour)		
	6	12	24
Model	24.7±3.3	58.4±5.4	60.3±6.2
Constant temperature	16.8±3.7[a]	46.2±4.8[a]	26.9±2.9[b]
8-OH-DPAT	7.3±2.6[bd]	32.1±3.9[bd]	24.7±3.4[bc]

Group	Time after injury (hour)	
	72	168
Model	50.2±3.6	34.3±3.3
Constant temperature	36.6±3.9[b]	17.9±3.1[b]
8-OH-DPAT	26.7±3.7[bc]	16.3±3.1[b]

[a]$P < 0.05$, [b]$P < 0.01$, *vs.* model group; [c]$P < 0.05$, [d]$P < 0.01$, *vs.* constant temperature group. Data are expressed as mean ± SD. Seven rats at each time point, using two-way analysis of variance; Student-Newman-Keuls test was used for intergroup comparisons. Mean absorbance in the normal group was 2.3 ± 0.5.

8-OH-DPAT: 8-hydroxy-2-(di-n-propylamino)tetralin.

Bcl-2 expression in brain tissues surrounding the injured region of rats following diffuse axonal injury

Immunohistochemical results revealed that Bcl-2 expression was visible in the brain tissue of the normal group. Bcl-2 expression increased at 6 hours following diffuse axonal injury, peaked at 24 hours, and then gradually decreased in the model group. Compared with the model group, Bcl-2 expression significantly increased at the corresponding time points in the constant temperature and 8-OH-DPAT groups ($P < 0.05$ or $P < 0.01$). Moreover, Bcl-2 expression was higher in the 8-OH-DPAT than that in the constant temperature group ($P < 0.05$ or $P < 0.01$; Table 3, Figure 5).

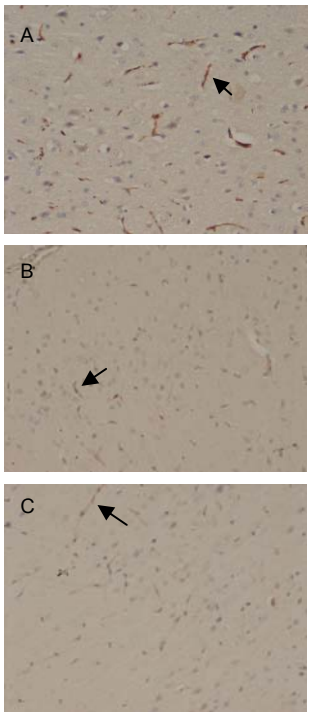

Figure 4 Caspase-3 expression in brain tissue surrounding the injured region at 24 hours following diffuse axonal injury (immunohistochemical staining, × 400).

Brown caspase-3-positive cells (arrows) were visible. Dark staining was observed in the model group (A). Caspase-3 expression was low and weak staining was visible in the constant temperature group (B) and 8-OH-DPAT group (C).

8-OH-DPAT: 8-hydroxy-2-(di-n-propylamino)tetralin.

Table 3 Effect of 8-OH-DPAT on Bcl-2 expression (absorbance value) in brain tissues surrounding the injured region of rats following diffuse axonal injury

Group	Time after injury (hour)		
	6	12	24
Model	10.0±2.8	15.8±2.2	22.8±3.3
Constant temperature	11.1±2.4	17.6±2.7[a]	25.2±3.0[a]
8-OH-DPAT	17.9±2.8[bd]	18.9±2.3[ac]	29.2±3.7[bc]

Group	Time after injury (hour)	
	72	168
Model	13.2±2.6	9.2±2.0
Constant temperature	17.7±2.8[a]	14.3±2.4[b]
8-OH-DPAT	19.0±2.4[bc]	17.5±2.8[bd]

[a]$P < 0.05$, [b]$P < 0.01$, *vs.* model group; [c]$P < 0.05$, [d]$P < 0.01$, *vs.* constant temperature group. Data are expressed as mean ± SD. Seven rats at each time point, using two-way analysis of variance; Student-Newman-Keuls test was used for intergroup comparisons. Absorbance value in the normal group was 7.8 ± 1.6.

8-OH-DPAT: 8-hydroxy-2-(di-n-propylamino)tetralin.

Bax expression in brain tissue surrounding the injured region in rats from each group

Immunohistochemical results revealed that Bax expression was low in the normal group. Bax expression rapidly increased at 6 hours following diffuse axonal injury, peaked at 12 hours, and then gradually decreased in the model group. Compared with the model group, Bax expression significantly decreased at the corresponding time points in the constant temperature and 8-OH-DPAT groups ($P < 0.05$ or $P < 0.01$). In particular, Bax expression was lowest at 24 hours following model induction ($P < 0.01$). Moreover, Bax expression was higher in the 8-OH-DPAT group than that in the constant temperature group ($P < 0.05$ or $P < 0.01$; Table 4, Figure 6).

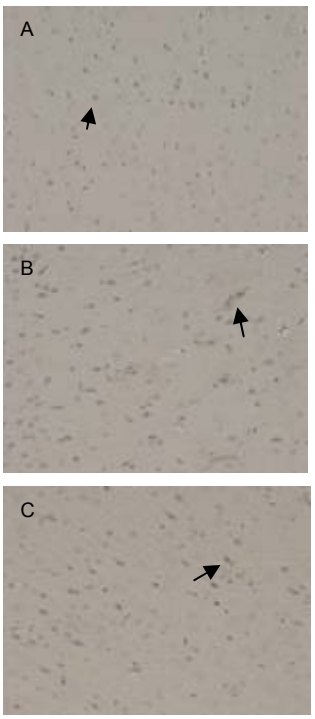

Figure 5 Bcl-2 expression in brain tissue surrounding the injured region at 24 hours following diffuse axonal injury (immunohistochemical staining, × 400).

Brown Bcl-2-positive cells (arrows) were visible. Bcl-2 expression was low in the model group (A). Bcl-2 expression was high and dark staining was visible in the constant temperature group (B) and 8-OH-DPAT group (C).

8-OH-DPAT: 8-hydroxy-2-(di-n-propylamino)tetralin.

DISCUSSION

5-hydroxytryptamine (5-HT) has many types of receptors[19-20]. Of them, the 5-HT1A receptor has been linked to anxiety, emotion and cognitive function[21]. The study sought to investigate the effects of the 5-HT1A receptor agonist 8-OH-DPAT on neural cell apoptosis in rats with diffuse axonal injury.

Table 4 Effect of 8-OH-DPAT on Bax expression (absorbance value) in brain tissues surrounding the injured region of rats following diffuse axonal injury

Group	Time after injury (hour)		
	6	12	24
Model	38.4±4.0	43.7±5.7	38.5±4.8
Constant temperature	19.1±3.5[b]	14.0±3.1[b]	9.5±1.8[b]
8-OH-DPAT	11.5±2.6[bd]	10.8±3.0[bd]	8.9±1.9[bc]

Group	Time after injury (hour)	
	72	168
Model	32.9±2.6	28.5±2.6
Constant temperature	24.1±3.9[b]	22.6±2.4[b]
8-OH-DPAT	20.5±2.3[bd]	18.6±2.1[bd]

[a]$P < 0.05$, [b]$P < 0.01$, *vs*. model group; [c]$P < 0.05$, [d]$P < 0.01$, *vs*. constant temperature group. Data are expressed as mean ± SD. Seven rats at each time point, using two-way analysis of variance; Student-Newman-Keuls test was used for intergroup comparisons. Absorbance value in the normal group was 3.7 ± 1.0.

8-OH-DPAT: 8-hydroxy-2-(di-n-propylamino)tetralin.

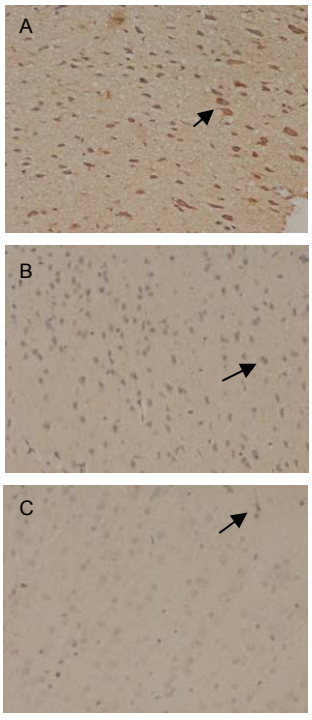

Figure 6 Bax expression in brain tissue surrounding the injured region at 24 hours following diffuse axonal injury (immunohistochemical staining, × 400).

Brown Bax-positive cells (arrows) were visible. Dark staining was observed in the model group (A). Bax expression was low and weak staining was visible in the constant temperature group (B) and 8-OH-DPAT group (C).

8-OH-DPAT: 8-hydroxy-2-(di-n-propylamino)tetralin.

Rat models of diffuse axonal injury were established according to the Marmarou method[18]. Rats with diffuse axonal injury were affected with apnea, primary coma and spasm. After regaining consciousness, their activities were reduced, and the rats were unstable when walking, and were unresponsive. Hematoxylin-eosin staining results exhibited hemorrhage, edema and cell necrosis, which was consistent with a previous study[22]. After administration of 8-OH-DPAT, brain tissue damage was reduced, which suggested that 8-OH-DPAT had a neuroprotective effect.

Previous studies confirmed that neural cell apoptosis induced by inflammatory mediators, cytokines, excitatory transmitters, Ca^{2+} overload and oxygen free radicals is an important mechanism during diffuse axonal injury[22-24]. Therefore, studies on the effects of drugs on cell apoptosis following diffuse axonal injury can provide insights on potential treatments for diffuse axonal injury in a clinical setting. Caspases exerts an important effect on cell apoptosis, especially caspase-3, which plays an important role in the death receptor pathway, endoplasmic reticulum pathway and mitochondrial pathway, and is necessary for the apoptotic protease cascade[25-28]. Results demonstrated that neural cell apoptosis was reduced and caspase-3 expression decreased in rats with diffuse axonal injury after administration of 8-OH-DPAT, which indicated that 8-OH-DPAT could inhibit apoptosis after diffuse axonal injury by reducing caspase-3 expression.

Bcl-2 is an antiapoptotic protein and Bax is a proapoptotic protein. Bcl-2 inhibits apoptosis by stabilizing the function of the mitochondrial membrane, and preventing mitochondrial release of caspases, apoptosis-inducing factors and cytochrome C[29]. Bax can induce apoptosis by suppressing Bcl-2 activity[30]. Simultaneously, Bax promotes cytochrome C release, activates caspases, and induces apoptosis. Taken together, high expression of Bax is probably the main reason for increased apoptosis following craniocerebral injury. Because the regulatory effects of Bax and Bcl-2 on apoptosis are contrary, it is believed that the ratio of these pro- and anti-apoptotic proteins, respectively, is important for the apoptotic process[31]. Hsiung *et al* [32] confirmed that 8-OH-DPAT suppressed the adenylate cyclase→cyclic adenosine monophosphate/protein kinase A→protein phosphatase 2A→Bax/Bcl-2 binding→cytochrome c release→caspase-3 pathway by inhibiting adenylate cyclase. The possible mechanisms are shown in Figure 7.

The present study observed the effect of 8-OH-DPAT on Bcl-2 and Bax expression in rat brain tissue surrounding the injured region after diffuse axonal injury, and found

that 8-OH-DPAT elevated Bcl-2 expression, and suppressed Bax expression to a certain extent. Moreover, its effect was strongest at 24 hours following diffuse axonal injury. The above-described results indicated that 8-OH-DPAT could inhibit apoptosis by controlling Bcl-2/Bax expression and exerting a neuroprotective effect against diffuse axonal injury.

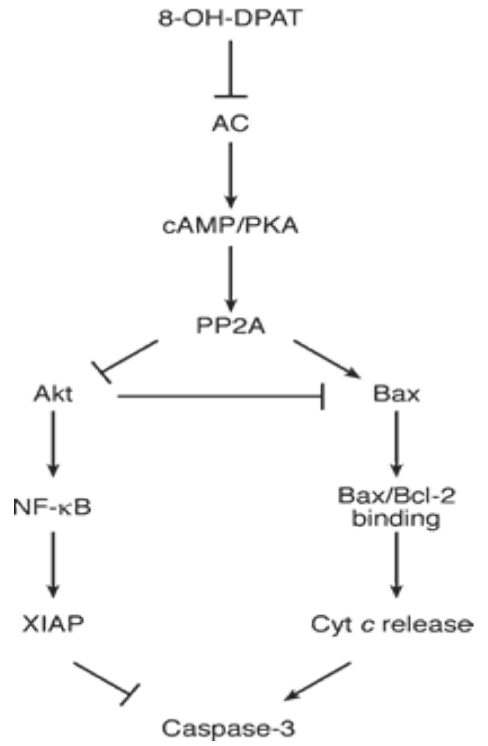

Figure 7 Possible mechanism of Bax/Bcl-2 inhibition by 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT).

AC: Adenylate cyclase; cAMP: cyclic adenosine monophosphate; PKA: protein kinase A; PP2A: protein phosphatase 2A; NF-κB: nuclear factor-κB; XIAP: X-linked inhibitor of apoptosis; Cyt: cytochrome.

Body temperature has been shown to reduce when 8-OH-DPAT binds to the 5-HT1A receptor in the central nervous system[33-35]. To verify the neuroprotective effect of 8-OH-DPAT on diffuse axonal injury associated with mild hypothermia, this study set up a constant temperature group and showed that neural cell apoptosis was reduced in the 8-OH-DPAT when compared with the constant temperature group, and that caspase-3 and Bax expression was relatively low, but that Bcl-2 expression was relatively high. Thus, we concluded that the neuroprotective effects of 8-OH-DPAT against diffuse axonal injury are associated with a decrease in brain temperature. Overall, our study and previous studies have shown that 8-OH-DPAT reduces brain temperature, decreases oxygen consumption, maintains cerebral blood flow and cell energy metabolism, reduces lactic acid accumulation, relieves cerebral edema, promotes the recovery of brain cell structure and function, reduces intracranial pressure, reduces axonal injury, decreases pathological injury after ischemia, improves recovery of neurological function after cerebral ischemia, reduces harmful transmitter release after brain trauma, improves hypoxia- and oxidative stress-induced apoptosis, protects blood-brain barrier, and reduces post- trauma cerebral edema, all of which results in neuroprotection[19-21, 36].

In summary, 8-OH-DPAT decreased Bax expression, and increased Bcl-2 expression by reducing brain temperature and inhibiting caspase-3 protein expression. 8-OH-DPAT exerts a neuroprotective effect by inhibiting apoptosis, but its precise protective mechanisms require further investigation.

MATERIALS AND METHODS

Design

A randomized controlled animal study.

Time and setting

Experiments were performed at the Institute of Neurosurgery, General Hospital of Shenyang Military Region, China from October 2011 to July 2012.

Materials

Healthy male Wistar rats (*n* = 112), weighing 240–330 g, were provided by the Animal Experimental Center, General Hospital of Shenyang Military Region (Experimental Center license No. SYXK (Jun) 2002-019) (Animal license No. SCXK (Jun) 2002-0174). The rats were allowed free access to food and water and were maintained at 24–26°C. The protocols 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[37].

Methods

Establishment and 8-OH-DPAT intervention of the diffuse axonal injury rat model

With the exception of the normal group, rat models of diffuse axonal injury were established using the Marmarou method[18]. Rats were intraperitoneally anesthetized with 10% (w/v) chloral hydrate. The rats were fixed on a sponge bed in the prone position and hit with a free falling 450 g iron rod from a height of 1.3 m

through an empty steel pipe. The iron rod hit the rat median calvaria (between the coronal suture and lambdoid suture) to induce diffuse axonal injury. The normal group did not receive any hit. The probe head of a thermometer (Taimeng Technology Co., Ltd., Chengdu, Sichuan Province, China) connected to an intellectual thermostatic controller (Taimeng Technology Co., Ltd.) was inserted into the rat anal tube to maintain body temperature at 37.0 ± 0.5°C (constant temperature was maintained using a blanket). At 15 minutes following diffuse axonal injury, 8-OH-DPAT was intraperitoneally administered (0.5 mg/kg) (5 mg/tablet; Sigma, St. Louis, MO, USA) in the constant temperature and 8-OH-DPAT. An equal volume of physiological saline was intraperitoneally injected in the model and normal groups. Excepting the constant temperature group, the blanket was removed in all other groups. Body temperature (37.0 ± 0.5°C) was maintained using a constant temperature blanket (Chengdu Kangyu Medical Equipment Engineering Co., Ltd., Chengdu, Sichuan Province, China) for 168 hours.

Determination of rat brain temperature

The head of the rat was fixed using a stereo-directional fixation system, which was supplied by the Laboratory of Neurosurgery, General Hospital of Shenyang Military Region, China. Approximately 1.5 cm skin from the median head was incised and cooled using physiological saline at room temperature under a light microscope (Nikon, Tokyo, Japan). Simultaneously, the bilateral frontal bone-parietal bone was drilled using a dental high speed drill, with a bone window of about 5 mm × 5 mm. The probe of the electrode (Testo 735 thermodetector; Testo, Shanghai, China) was placed 3 mm below the right frontal cortex of rats to monitor brain temperature.

Specimen collection

At 6, 12, 24, 72 and 168 hours following diffuse axonal injury, seven rats were collected from each group. Rats from the normal group were sacrificed at 24 hours following model induction. After measuring brain temperature, the rats were subjected to anesthesia overdose. Injured brain tissues were obtained at 24 hours following model establishment in the normal group. The heart was exposed by opening the chest. Intubation was performed in the left heart apex, and the right auricle was cut. The specimen was washed using 100 mL physiological saline, and perfused using 4% (w/v) paraformaldehyde buffer at 4°C, pH 7.4, for 0.5 hours. Subsequently, approximately 3 mm^3 brain tissues at the edge of the wound area of the left frontal lobe was collected, fixed in 4% (w/v) paraformaldehyde buffer, and embedded in paraffin.

Hematoxylin-eosin staining for pathological changes in injured brain tissues following diffuse axonal injury

Paraffin-embedded specimens were sliced into 2 μm sections, and unfolded in warm water and hot water. The slide was baked in an oven (Yaguang Medical Electrical Technique Institute, Xiaogan, Hubei Province, China) for 24 hours. The specimen was rapidly dewaxed in xylene (I, II), immersed in dehydrated alcohol and gradient alcohol (95%, 90%, 80%), stained with hematoxylin and 2% (w/v) eosin for 2–3 minutes, washed with running water, 95% (v/v) and 80%(v/v) alcohol, and mounted in neutral gum.

Immunohistochemical staining for Bcl-2, Bax and caspase-3 expression in injured brain tissues

Sections were dewaxed and incubated with methanol containing 3% (v/v) hydrogen peroxide to inactivate endogenous peroxidase for 10 minutes at room temperature. Antigen was retrieved using a microwave oven (WD750S; Galanz, Zhongshan, Guangdong Province, China). Sections were blocked with confining liquid for 20 minutes, incubated with rabbit anti-Bcl-2, Bax, caspase-3 polyclonal antibodies (1: 100; Boster, Wuhan, Hubei Province, China) at 37°C for 1 hour, at 4°C overnight, treated with biotinylated goat anti-rabbit IgG (1:1 000) at 37°C for 20 minutes, developed with 3,3'-diaminobenzidine, and then mounted with neutral gum.

TUNEL for apoptosis in injured brain tissues

The *in situ* cell apoptosis detection kit was purchased from Boster. Sections were dewaxed with xylene, hydrated with gradient alcohol (95%, 90%, 80% (v/v)), digested with 20 μg/mL proteinase K for 20 minutes, and incubated with methanol containing 0.3% (v/v) hydrogen peroxide to inactivate endogenous peroxidase for 30 minutes. After being washed with sodium citrate buffer containing 0.1% (v/v) Triton X-100 for 5 minutes, sections were incubated with TUNEL 50 μL reaction mixture at 37°C for 1 hour, washed with PBS, incubated with 50 μL converter peroxidase at 37°C for 30 minutes, washed with PBS, and then developed with 3,3'-diaminobenzidine for 5–10 minutes.

Assessment of results

Three specimens were randomly collected from each rat at each time point in each group for measuring apoptosis, caspase-3, Bcl-2 and Bax expression. Five fields from each section were observed by two observers under a 400 × light microscope (Olympus, Tokyo, Japan), and the

number of apoptotic cells was quantified. Absorbance values of caspase-3, Bcl-2 and Bax expression were obtained in each group using Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA), and the average value was calculated and compared. Apoptosis is presented as the apoptotic index:

> Apopototic index = number of apoptotic cells/(the number of apoptotic cells + the number of normal cells) × 100%.

Statistical analysis

Measurement data are expressed as mean ± SD, and were analyzed using SPSS 17.0 software (SPSS, Chicago, IL, USA). The mean difference among groups was compared using two-way analysis of variance; Student-Newman-Keuls test was used for intergroup comparisons. A value of $P < 0.05$ was considered statistically significant.

Acknowledgments: We thank Teacher Mingguang Zhao from the Department of Neurosurgery, General Hospital of Shenyang Military Region in China for his technical assistance.

Funding: This project was funded by the Natural Science Foundation of Technology Department of Liaoning Province, No. 20032047.

Author contributions: Zhenquan Song was in charge of study design and obtained the funding. Zhenli Mao performed the experiments and data processing, and wrote the manuscript. Gang Li collected the data. Wei Lv, Xu Zhao and Bin Li participated in experiment implementation. Xinli Feng and Youli Chen assisted with animal experiments and immunohistochemical staining. All authors have read and agreed to the manuscript as written.

Conflicts of interest: None declared.

Ethical approval: This study was approved by the Animal Ethics Committee, General Hospital of Shenyang Military Region in China.

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

REFERENCES

[1] Reynolds CA, Kallakuri S, Bagchi M, et al. Endothelin receptor A antagonism reduces the extent of diffuse axonal injury in a rodent model of traumatic brain injury. Neurol Res. 2011;33(2):192-196.

[2] Matsukawa H, Shinoda M, Fujii M, et al. Genu of corpus callosum in diffuse axonal injury induces a worse 1-year outcome in patients with traumatic brain injury. Acta Neurochir (Wien). 2011;153(8):1687-1693; discussion 1693-1694.

[3] Zhao CP, Qin JZ, Zhao GL, et al. Recent advances in the diagnosis and treatment of diffuse axonal injury. Zhonghua Shenjing Yixue Zazhi. 2009;8(10):1078-1080.

[4] Haynes RL, Billiards SS, Borenstein NS, et al. Diffuse axonal injury in periventricular leukomalacia as determined by apoptotic marker fractin. Pediatr Res. 2008;63(6):656-661.

[5] Park CO, Hyun DK. Apoptotic change in response to magnesium therapy after moderate diffuse axonal injury in rats. Yonsei Med J. 2004;45(5):908-916.

[6] Burke TF, Advani T, Adachi M, et al. Sensitivity of hippocampal 5-HT1A receptors to mild stress in BDNF-deficient mice. Int J Neuropsychopharmacol. 2012.

[7] Singh M, Schwartz TL. Clinical utility of vilazodone for the treatment of adults with major depressive disorder and theoretical implications for future clinical use. Neuropsychiatr Dis Treat. 2012;8:123-130.

[8] Ramos AJ, Rubio MD, Defagot C, et al. The 5HT1A receptor agonist, 8-OH-DPAT, protects neurons and reduces astroglial reaction after ischemic damage caused by cortical devascularization. Brain Res. 2004;1030(2): 201-220.

[9] Salazar-Colocho P, Del Río J, Frechilla D. Neuroprotective effects of serotonin 5-HT 1A receptor activation against ischemic cell damage in gerbil hippocampus: Involvement of NMDA receptor NR1 subunit and BDNF. Brain Res. 2008;1199:159-166.

[10] Alessandri B, Tsuchida E, Bullock RM. The neuroprotective effect of a new serotonin receptor agonist, BAY X3702, upon focal ischemic brain damage caused by acute subdural hematoma in the rat. Brain Res. 1999; 845(2):232-235.

[11] Mauler F, Fahrig T, Horváth E, et al. Inhibition of evoked glutamate release by the neuroprotective 5-HT(1A) receptor agonist BAY x 3702 in vitro and in vivo. Brain Res. 2001;888(1):150-157.

[12] Bezard E, Gerlach I, Moratalla R, et al. 5-HT1A receptor agonist-mediated protection from MPTP toxicity in mouse and macaque models of Parkinson's disease. Neurobiol Dis. 2006;23(1):77-86.

[13] Audero E, Coppi E, Mlinar B, et al. Sporadic autonomic dysregulation and death associated with excessive serotonin autoinhibition. Science. 2008;321(5885):130-133.

[14] Lo Iacono L, Gross C. Alpha-Ca^{2+}/calmodulin-dependent protein kinase II contributes to the developmental programming of anxiety in serotonin receptor 1A knock-out mice. J Neurosci. 2008;28(24):6250-6257.

[15] Yelleswarapu NK, Tay JK, Fryer WM, et al. Elucidating the role of 5-HT(1A) and 5-HT(7) receptors on 8-OH-DPAT-induced behavioral recovery after experimental traumatic brain injury. Neurosci Lett. 2012; 515(2):153-156.

[16] Schneider A, Teschendorf P, Vogel P, et al. Facilitation of hypothermia by quinpirole and 8-OH-DPAT in a rat model of cardiac arrest. Resuscitation. 2012;83(2):232-237.
[17] Kline AE, Massucci JL, Dixon CE, et al. The therapeutic efficacy conferred by the 5-HT(1A) receptor agonist 8-Hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) after experimental traumatic brain injury is not mediated by concomitant hypothermia. J Neurotrauma. 2004;21(2): 175-185.
[18] Marmarou A, Foda MA, van den Brink W, et al. A new model of diffuse brain injury in rats. Part I: Pathophysiology and biomechanics. J Neurosurg. 1994; 80(2):291-300.
[19] Marazziti D, Marracci S, Palego L, et al. Localization and gene expression of serotonin 1A (5HT1A) receptors in human brain postmortem. Brain Res. 1994;658(1-2): 55-59.
[20] Blackshear MA, Steranka LR, Sanders-Bush E. Multiple serotonin receptors: regional distribution and effect of Raphe lesions. Eur J Pharmacol. 1981;76(4):325-334.
[21] Polter AM, Li X. 5-HT1A receptor-regulated signal transduction pathways in brain. Cell Signal. 2010; 22(10):1406-1412.
[22] Rink A, Fung KM, Trojanowski JQ, et al. Evidence of apoptotic cell death after experimental traumatic brain injury in the rat. Am J Pathol. 1995;147:1575-1583.
[23] Yang XF, Wang H, Wen L. From myelin debris to inflammatory responses: a vicious circle in diffuse axonal injury. Med Hypotheses. 2011;77(1):60-62.
[24] Lima RR, Guimaraes-Silva J, Oliveira JL, et al. Diffuse axonal damage, myelin impairment, astrocytosis and inflammatory response following microinjections of NMDA into the rat striatum. Inflammation. 2008;31(1):24-35.
[25] YuanJ, Yankner BA. Apoptosis in the nervous system. Nature. 2000;407(6805):802-809.
[26] Yu H, Liu WG. Caspase-3 and traumatic brain injury. Guoji Shenjing bingxue Shenjing Waike Xue Zazhi. 2005;32(4): 377-380.
[27] Clark RS, Kochanek PM, Watkins SC, et al. Caspase-3-medeiated neuronal death after traumatic brain injury in rats. J Neurochem. 2000;74(2):740-753.
[28] Adayev T, Ray I, Sondhi R, et al. The G protein-coupled 5-HT1A receptor causes suppression of caspase-3 through MAPK and protein kinase Calpha. Biochim Biophys Acta. 2003;1640(1):85-96.
[29] Fiskum G. Mitochondrial participation in ischemic and traumatic neural cell death. J Neurotrauma. 2000;17(10): 843-855.
[30] Chen CJ, Cheng FC, Liao SL, et al. Effects of naloxone on lactate, pyruvate metabolism and antioxidant enzyme activity in rat cerebral ischemia/reperfusion. Neurosci Lett. 2000;287(2):113-116.
[31] Liu WG, Yang XF, Li G. Study on effect of Naloxone on apoptosis of neuron and expression of Bcl-2 Bax gene after severe brain injury. Zhonghua Shenjing Waike Zazhi. 2004;20(2):170-172.
[32] Hsiung SC, Tin A, Tamir H, et al. Inhibition of 5-HT1A receptor-dependent cell survival by cAMP/protein kinase A: role of protein phosphatase 2A and Bax. J Neurosci Res. 2008;86(10):2326-2338.
[33] Ox B, Kerwin RW, Lee TF, et al. A dopamine-5-hydroxytryptamine link in the hypothalamic pathways which mediate heat loss in the rat. J Physiol. 1980;303(6): 9-21.
[34] Duncan WC, Johnson KA, Wehr TA. Antidepressant drug-induced hypothalamic cooling in Syrian hamsters. Neuropsychopharmacology. 1995;12(1):17-37.
[35] Gudelsky GA, Koenig JI, Meltzer HY. Thermoregulatory responses to serotonin (5-HT) receptor stimulation in the rat. Neuropharmacology. 1986;25(12):1307-1313.
[36] Gudelsky GA, Koenig JI, Meltzer HY. Thermoregulatory responses to serotonin (5-HT) receptor stimulation in the rat. Evidence for opposing roles of 5-HT2 and 5-HT1A receptors. Neuropharmacology. 1986;25(12):1307-1313.
[37] 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.

(Edited by Pan Y, He XJ/Qiu Y/Song LP)