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

Zhenli Mao1,2, Zhenquan Song1, Gang Li3, Wei Lv1,2, Xu Zhao1,2, Bin Li1,2, Xinli Feng1, Youli Chen1

1 Department of Neurosurgery, General Hospital of Shenyang Military Region, Shenyang 110016, Liaoning Province, China
2 Postgraduate School, Liaoning Medical University, Jinhzhou 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.

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
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\textsuperscript{[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](image1.png)

**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](image2.png)

**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, \(\times 400\)).

(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).

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

<table>
<thead>
<tr>
<th>Group</th>
<th>Time after injury (hour)</th>
<th>6</th>
<th>12</th>
<th>24</th>
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<tbody>
<tr>
<td>Model</td>
<td>22.5±3.2</td>
<td>45.2±4.4</td>
<td>86.5±10.6</td>
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<tr>
<td>Constant temperature</td>
<td>18.9±2.2</td>
<td>38.9±4.5</td>
<td>69.3±9.5</td>
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<tr>
<td>8-OH-DPAT</td>
<td>14.3±2.1</td>
<td>28.9±3.7</td>
<td>53.1±7.3</td>
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</table>

**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

<table>
<thead>
<tr>
<th>Group</th>
<th>Time after injury (hour)</th>
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<td>Model</td>
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<tr>
<td>8-OH-DPAT</td>
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<td>32.1±3.9</td>
<td>24.7±3.4</td>
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</table>

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 group than in the constant temperature group (P < 0.05 or P < 0.01; Table 3, Figure 5).

**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.

**Table 3** Effect of 8-OH-DPAT on apoptotic neural cells (apoptotic index) in brain tissues surrounding the injured region after diffuse axonal injury (%)

<table>
<thead>
<tr>
<th>Group</th>
<th>Time after injury (hour)</th>
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<tbody>
<tr>
<td>Model</td>
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<tr>
<td>8-OH-DPAT</td>
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**Figure 4** 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.

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).

**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.
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

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<th>Model</th>
<th>Constant temperature</th>
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<tr>
<td>6</td>
<td>38.4±4.0</td>
<td>43.7±5.7</td>
<td>38.5±4.8</td>
<td>19.1±3.5</td>
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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

\*P < 0.05, \(\dagger\)P < 0.01, vs. model group; \(\ddagger\)P < 0.05, \(\ddagger\)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.

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<tr>
<th>Time after injury (hour)</th>
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<td>72</td>
<td>32.9±2.6</td>
<td>28.5±2.6</td>
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<tr>
<td>Constant temperature</td>
<td>24.1±3.6</td>
<td>22.6±2.4</td>
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<tr>
<td>8-OH-DPAT</td>
<td>20.5±2.3</td>
<td>18.6±2.1</td>
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<td>24</td>
<td>32.9±2.6</td>
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<tr>
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<td>24.1±3.6</td>
<td>22.6±2.4</td>
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<tr>
<td>8-OH-DPAT</td>
<td>20.5±2.3</td>
<td>18.6±2.1</td>
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</table>

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.
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.

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. Excluding 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 mm³ 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:

\[
\text{Apoptotic index} = \frac{\text{number of apoptotic cells}}{\text{(the number of apoptotic cells + the number of normal cells)}} \times 100\%.
\]

**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.

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Chen assisted with animal experiments and
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