Research Report

Neuroprotective effect of memantine combined with topiramate in hypoxic–ischemic brain injury

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ARTICLE INFO

Article history:
Accepted 20 May 2009
Available online 6 June 2009

Keywords:
Neuroprotection
Memantine
Topiramate
Pharmacology
Combination therapy
Glutamate receptor
Apoptosis

ABSTRACT

Glutamate receptor-mediated neurotoxicity is a major mechanism contributing to hypoxic–ischemic brain injury (HIBI). Memantine is a safe non-competitive NMDA receptor blocker characterized by its low affinity and fast unblocking kinetics. Topiramate is an AMPA/KA receptor blocker and use-dependent sodium channel blocker with several other neuroprotective actions and little neurotoxicity. We hypothesized that the coadministration of memantine and topiramate would be highly effective to attenuate HIBI in neonatal rats. Seven-day-old Sprague–Dawley rat pups were subjected to right common carotid artery ligation and hypoxia for 2 h, and then were randomly and blindly assigned to one of four groups: vehicle, memantine, topiramate and combination group. Brain injury was evaluated by gross damage and weight deficit of the right hemisphere at 22d after hypoxic-ischemia (HI) and by neurofunctional assessment (foot-fault test) at 21d post-HI. Acute neuronal injury was also evaluated by microscopic damage grading at 72 h post-HI. Results showed the combination of memantine and topiramate improved both pathological outcome and performance significantly. The drug-induced apoptotic neurodegeneration was assessed by TUNEL staining at 48 h post-HI and the result showed no elevated apoptosis in all observed areas. The result of the experiment indicates the combination therapy is safe and highly effective to reduce brain damage after HIBI.

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1. Introduction

The excessive glutamate release and overactivation of glutamate receptors are crucial contributors to the pathogenesis of HIBI. They cause a massive influx of sodium (Na⁺) and calcium (Ca²⁺) that triggers a cascade of biochemical events, and lead to neuronal necrosis and apoptosis in many types of cells in neonatal brain. There are three subtypes of ionotropic glutamate receptors involved, namely N-methyl-D-aspartate (NMDA) receptors, α-3-amino-hydroxy-5-methyl-4-isoxazole pro-pionic acid (AMPA) receptors and kainate (KA) receptors. Excitotoxic injury occurs secondary to glutamate-triggered Ca²⁺ influx through any of three routes: NMDA channels, voltage-sensitive Ca²⁺ channels, and Ca²⁺-permeable AMPA/KA channels (Lu et al., 1996). The excitotoxic overactivation of NMDA and AMPA/KA glutamate receptors provokes further glutamate release and further NMDA and AMPA/KA receptor stimulation, and it forms a positive feedback cycle making the condition worse (Villmann and Becker, 2007). To break this vicious cycle, researchers utilized many NMDA and/or AMPA/KA receptor antagonists, but most of them have severe side effects (Haberny et al., 2002; Puka-Sundvall et al., 2000). For example, a widely used NMDA antagonist MK-801 was found inducing widespread apoptotic neurodegeneration and impairing many normal neuronal functions in developing rat brain (Ikonomidou et al., 1999). As NMDA receptors are essential for normal...
physiological processes in brain development, including the proliferation, migration, survival and differentiation of neurons, blockade of excessive NMDA receptor activity must be achieved without affecting normal brain functioning (Kohr, 2007).

Recently, increasing evidence based on molecular studies suggests that memantine, an uncompetitive NMDA receptor blocker with fast channel unblocking kinetics to prevent it from occupying the channels and interfering with normal synaptic transmission, is a potent neuroprotectant without above-mentioned side effects (Chen et al., 1992, 1998; Chen and Lipton, 2005; Johnson and Kotermanski, 2006). In contrast to MK-801 and ketamine, memantine shows unusual clinical tolerance in the treatment of moderate-to-severe Alzheimer’s disease in adults through its low affinity and relatively fast unblocking kinetics (de Lima et al., 2000; Lipton, 2004; Lipton, 2006). As a neuroprotective agent, memantine can reduce functional as well as morphological sequelae induced by ischemia (Block and Schwarz, 1996; Chen et al., 1998). A recent study showed the NMDA receptor blockade with memantine could provide an effective pharmacological prevention of periventricular leukomalacia (PVL) in the premature infant (Manning et al., 2008).

Topiramate, a well tolerated antiepileptic drug (AED) used clinically, confers neuroprotection by blocking AMPA/KA receptors and use-dependent Na+ channel in developing rat brain without serious side effects compared to conventional anticonvulsants (Noh et al., 2006). Topiramate has antiexcitotoxic properties, because it protects against motor neuron degeneration. The other neuroprotective effects of topiramate include positive modulation of gamma-aminobutyric acid (GABA) receptors, increase of seizure threshold and so on (Pappalardo et al., 2004). Furthermore, Topiramate also protects preoligodendrocytes against excitotoxic cellular death in white matter lesions and prevents the periventricular white matter damage from the damage induced by an AMPA/KA agonist in newborn mice (Follett et al., 2004; Sfaello et al., 2005).

Due to the complex pathological mechanisms in HIBI described above, combination therapy or multimodal targeting is thought to be a key future approach to provide effective neuroprotection. Most promising combination should target different neuroprotective mechanisms, expand the therapeutic time window, and alleviate the possibility of side effects (Rogalewski et al., 2006). Studies on the mechanisms of the superfAMILY of glutamate receptors revealed that NMDA and AMPA glutamate receptors showed a fine-tuned interaction at the glutamatergic synapse: the rapid activation and brief open time of AMPA receptors facilitates unblock of NMDA receptors (Villmann and Becker, 2007). Functional interdependence of AMPA and NMDA receptors has been proven by experiments where a transient synaptic activation of NMDA receptors reliably induces a long-term potentiation phenomenon, associated with an increase in the intensity and number of synaptic AMPA-receptor clusters (Liao et al., 2001; Liu et al., 2004a). These findings suggest that it will be more effective and beneficial to block both NMDA and AMPA/KA receptors by combination of different glutamate receptor antagonists.

Based on the pharmacology and mechanism studies, we designed the experiments to evaluate the efficacy of the combination therapy by measuring gross brain damage, brain weight deficit in the right hemisphere and regional neuronal proliferation, migration, survival and differentiation. Besides the morphologic and histopathologic measurement, a neurofunctional test was performed to verify the results. To ensure therapeutic safety, the possible drug-induced apoptosis was assessed even though the two drugs were approved safe and efficient in their respective therapeutic categories (Chen et al., 1998; Glier et al., 2004).

### Table 1 – Neurologic damage score.

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>Normal=1</th>
<th>Mild=2</th>
<th>Moderate=3</th>
<th>Severe=4</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>19</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>8</td>
<td>NS</td>
</tr>
<tr>
<td>Memantine</td>
<td>24</td>
<td>10</td>
<td>7</td>
<td>4</td>
<td>3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Topiramate</td>
<td>21</td>
<td>5</td>
<td>5</td>
<td>6</td>
<td>1</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Combination</td>
<td>24</td>
<td>6</td>
<td>4</td>
<td>4</td>
<td>1</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

The number of pups receiving the designated gross damage score by a blinded observer.

* p value, memantine, topiramate, combination vs. vehicle.

### 2. Results

#### 2.1. Gross brain damage grading

The neurologic damage score was determined by an observer blind to the drug treatment of the rat pups. Table 1 shows the neurologic damage scores in each group. The neurologic damage score was significantly higher in the vehicle-treated group (2.79±1.23, n=19) than that in the combination-treated group (2.4±1.18, n=19).

Fig. 1 – The percentage of reduction in right cerebral hemisphere weight measured using the left hemisphere weight as standard. The animal numbers are as described in the result. The percentage of reduction in right hemisphere weight was significantly decreased in the combination group compared with the vehicle group (*p < 0.01 vs. vehicle). The percentage of reduction in right hemisphere weight was significantly decreased in the memantine group compared with the vehicle group (p < 0.05 vs. vehicle). Data are presented as mean ± S.D.
group (1.71±0.91, n=24, p<0.01 versus vehicle). The neurologic damage score was significantly higher in the vehicle-treated group than that in the memantine-treated group (2.00±1.06, n=24, p<0.05 versus vehicle). The neurologic damage score in the topiramate-treated group (2.57±1.17, n=21, p>0.05 versus vehicle) was lower but not statistically significant compared with the vehicle-treated group.

2.2. Brain weight deficit

Fig. 1 shows the weight deficit in the right hemisphere relative to the left hemisphere. The weight deficit in the combination-treated group (9.2±2.5%, n=24, p<0.01 versus vehicle) was significantly reduced compared with the vehicle-treated group (26.9±4.1%, n=19). The weight deficit in the memantine-treated group (16.3±3.2%, n=24, p<0.05 versus vehicle) was significantly reduced compared with the vehicle-treated group. The weight deficit in the topiramate-treated group (21.5±4.0%, n=21, p>0.05 versus vehicle) was reduced but not statistically significant compared with the vehicle-treated group. Body weights of rat pups in each group were recorded and analyzed. Results showed that the body weights of the treated groups were not significantly different from the vehicle-treated group at 1, 3, 7, 14 and 22 days after injury (data not shown). Mortality rates were not significantly different in four groups, although there was a trend toward reduced mortality in the combination group.

2.3. Microscopic brain damage grading

The microscopic brain damage score (histopathologic score) was determined by an observer blind to the drug treatment of the rat pups. Fig. 2 shows the microscopic brain damage score in each group. The histopathologic score in the memantine-treated group (2.15±0.52 and 1.51±0.47, n=12, p<0.05 and p<0.05 versus vehicle) was significantly lower compared with the vehicle-treated group (4.15±0.73 and 3.38±0.72, n=10) in the cortex and thalamus. The histopathologic score in the combination-treated group (1.91±0.51, 1.45±0.49 and 0.91±0.42, n=12, p<0.05, p<0.05 and p<0.01 versus vehicle) was significantly lower compared with the vehicle-treated group (4.15±0.73, 3.68±0.62 and 3.38±0.72, n=10) in the cortex, hippocampus and thalamus. In the striatum, the histopathologic score in the combination-treated group was lower but not statistically significant compared with the vehicle-treated group.

2.4. Foot-fault test

Fig. 3 shows the number of foot-faults in each group. The number of foot-faults per pup was significantly greater in the vehicle-treated group (8.62±1.51, n=10) than that in the
combination-treated group (4.26±0.93, n=12, p<0.05 versus vehicle). The number of foot-faults per pup was significantly greater in the vehicle-treated group than that in the memantine-treated group (4.66±1.03, n=12, p<0.05 versus vehicle). The number of foot-faults per pup was less but not statistically significant in the topiramate-treated group (6.94±1.22, n=11) compared with in the vehicle-treated group.

2.5. TUNEL-positive cell counting

The numbers of TUNEL-positive apoptotic cells of each group are presented in Fig. 4 and areas examined for drug-induced apoptosis are shown in Fig. 5. In all observed areas, the numbers of apoptotic cells in the treated group (single or combined) were not significantly increased compared with the vehicle-treated group. In the CA1 sector of the hippocampus, the numbers of apoptotic cells in the combination-treated group (31.2±20.7 and 45.5±31.2, n=12, p<0.01 and p<0.01 versus vehicle) were significantly reduced compared with the vehicle-treated group (82.1±32.6 and 175±48.2, n=12). In the CA1 sector of the hippocampus and the subcortical white matter, the numbers of apoptotic cells in the memantine-treated group (50.5±28.3 and 99.8±38.7, n=12, p<0.05 and p<0.05 versus vehicle) were significantly reduced compared with the vehicle-treated group. In other areas, no significant differences were found between any of the treated groups (single or combined) and the vehicle group. Fig. 6 shows some sample pictures of apoptotic cells in the CA1 sector of the hippocampus.

3. Discussion

The present study shows for the first time to our knowledge that the combination of memantine and topiramate exerts enhanced protection of neurons against HIBI in vivo, compared with each of these agents alone. In this study, we measured brain damage in each group by using the gross anatomic method of Palmer et al. at 22d post-HI. By delaying assessment until 22d after HI, we included very late cell death that reflects overall neuroprotective effect of the drugs in a relatively long period. We also examined the brain weight deficit presented by the loss of brain weight on the ipsilateral side relative to the contralateral side. Results showed the combination therapy significantly reduced the degree of brain injury in this model. Besides the morphologic examinations, we applied the foot-
fault test to evaluate sensorimotor function of the rat pups at 21d post-HI. Foot-faults per pup in the combination group were significantly less than that in the vehicle group. The functional outcome was consistent with the morphologic findings in the long-term perspective. The short-term effect of the combination therapy was evaluated by microscopic brain damage scoring at 72 h post-HI. Results showed that the combination therapy reduced neuronal injury significantly in the cortex, hippocampus and thalamus.

Neuronal cell death after HI has generally been attributed to either rapid necrosis or delayed apoptosis. There is no doubt that necrosis plays major role in the course. But the developing brain may have good plasticity and a high capacity for self-repair (Daval et al., 2004; Grafe, 1994). After most compensatory and reparative phrases have passed, there are at least three different end points should be taken into account in assessment: the long-term deficit of brain tissue, the functional consequences of the brain injury and the acute extent of brain injury (Bona et al., 1997). The quantitative assessment of brain weight deficit and gross brain damage used in this study can accurately evaluate neuroprotective effects of glutamate antagonists against NMDA-mediated brain injury in vivo (Andine et al., 1990; McDonald et al., 1989a). On the other hand, behavioral consequences after HIBI are essential to reveal the true functional disability and to study the effects of drug intervention. In this study, the foot-fault test was done at 21d post-HI to evaluate the long-term functional outcome. Different from other cognitive function tests (Morris water maze, etc) related mostly to the hippocampus formation, the foot-fault test correlates with brain lesion in the cerebral cortex which is the most constantly affected region in both mild and severe HIBI in this model (Bona et al., 1997). Short-term effect of the therapy was evaluated by a scoring system on neuronal injury in 4 main regions of the rat brain at 72 h post-HI. Because short-term neuronal injury in the developing brain after HI is caused by both early and delayed neurodegeneration, the onset of damage in different regions of the brain is time-dependent and progressive, and it has an uneven distribution within regions (Northington et al., 2001). However, 72h (3d) post-HI seems an appropriate time point to evaluate short-term neuronal injury after insult in this model (Feng et al., 2005, 2008; Manning et al., 2008; Zhu et al., 2004).

In our experiment, the time window and doses of memantine and topiramate were chosen according to a general purpose to achieve an application for potential clinical use. Based on published data of rat pharmacokinetics and dose–response studies, 20 mg/kg dose of memantine can provide minimal neuroprotection (Chen et al., 1998; Hesselink et al., 1999). Considering the short therapeutic time window (Culmsee et al., 2004) and the confirmed neuroprotective effects of memantine at 20 mg/kg dose in HI and PVL model, we administered the 20 mg/kg loading dose of memantine immediately after HI in the treatment. Topiramate (loading dose 50 mg/kg, maintenance dose 20 mg/kg/day) can reduce neuronal cell loss significantly but increase apoptosis in the frontal white matter in newborn piglets (Schubert et al., 2005). Furthermore, topiramate may cause neurodegeneration in the developing rat brain only at doses above 50 mg/kg (Glier et al., 2004). The reason why topiramate at doses above 50 mg/kg can protect neurons but increase apoptosis may relate to two mechanisms. The first one is the blockade of AMPA/KA receptors lack of interference with NMDA-receptor signaling (Gibbs et al., 2000). Topiramate cannot provide neuroprotection only through AMPA/KA receptor channel unless it reaches threshold dosage. The second one is the depression of the endogenous neurotrophin system in the brain which may account for the proapoptotic effect (Bittigau et al., 2002). In a gerbil model, topiramate was found reducing...
hippocampal neuronal damage in dose-dependent manner (Lee et al., 2000). Based on the dose–response studies and our preliminary experiment, we chose 40 mg/kg as the loading dose for topiramate. The dose of topiramate (loading dose 40 mg/kg; maintenance dose 20 mg/kg/day) was proven considerably safe but unlikely to be neuroprotective.

Although the mechanisms underlying the neuroprotection are not fully understood, the results demonstrate that a synergistic reduction in brain damage can be achieved effectively by memantine combined with topiramate. The neuroprotective actions and unique characteristics of these two drugs may account for the experimental outcome. It is well documented that memantine antagonizes NMDA receptor activation by inhibiting the influx of Ca²⁺ through this channel (Johnson and Kotermanski, 2006). As an open-channel blocker, memantine can provide neuroprotection without interference with the normal brain development (Parsons et al., 1999). The favorable kinetics of memantine interaction with NMDA channels may be partly responsible for its high index of therapeutic safety, and it makes memantine a candidate drug for use in many NMDA receptor-mediated human CNS disorders (Johnson and Kotermanski, 2006; Lipton, 2004). In a four-vessel-occlusion (4VO) global ischemic model, neuronal damage in the CA1 sector of the hippocampus and in the striatum produced by 4VO was significantly attenuated by 20 mg/kg memantine (Block and Schwarz, 1996). Memantine has been used clinically for excitotoxic disorders at neuroprotective doses administered up to 2 h after induction of HI in immature and adult rats. At neuroprotective concentrations, memantine results in few adverse side effects and displays virtually no effects on Morris water maze performance or on neuronal vacuolation (Chen et al., 1998). Rosi et al. found that memantine protects against LPS-induced neuroinflammation, and confers neural and cognitive protection (Rosi et al., 2006). Furthermore, NMDA receptor blockade with memantine can provide an effective pharmacological prevention of PVL in the premature infant without affecting normal myelination or cortical growth (Manning et al., 2008).

Topiramate is a novel broad spectrum antiepileptic drug (AED) used clinically in adults and children older than 2 years. Amongst new-generation AEDs examined for neurotoxicity in neonatal rats, topiramate holds promise for minimizing the risk of neuronal death without side effects such as the impairment of cognitive performance (Cha et al., 2002; Glier et al., 2004; Mellon et al., 2007). Pharmacological actions of topiramate include positive modulation of GABA receptors, inhibition of the AMPA/KA glutamate receptor subtypes and blockade of a use-dependent Na⁺ channel (Schubert et al., 2005). Noh and his coworkers reported the co-treatment of topiramate and an NMDA receptor antagonist d-AP5 greatly increased the number of viable neurons in oxygen–glucose deprived cells. The experiment determined that neuroprotective effect of topiramate was mainly mediated by the inhibition of AMPA glutamate receptors (Noh et al., 2006).

Topiramate blocks the spread of seizures caused by transient global cerebral ischemia, and reduces the abnormally high extracellular levels of glutamate in the hippocampus in the immature rat spontaneous epileptic model by blocking AMPA receptors (Koh et al., 2004). It also affects the expression of glutamate transporters (GLAST and GLT-1) which are responsible for the inactivation of glutamate as a neurotransmitter (Poulsen et al., 2006). Moreover, topiramate was found effective in attenuating seizure-induced neuronal cell death and reducing KA-induced Phospho-extracellular signal-regulated kinase-immunoreactive (p-Erk IR) in the CA3 region of the hippocampus (Park et al., 2008). In a rat pup model of PVL, topiramate has been demonstrated effective to attenuate AMPA/KA receptor-mediated cell death and Ca²⁺ influx, as well as KA-evoked currents in developing oligodendrocytes (Follett et al., 2004).

Many studies suggest that combination of drugs may produce greater toxicity than individual ones. Thus, the safety of combination therapies should be most concerned, when these animal findings are intended for extrapolating to a pediatric surgical patient population (Bittigau et al., 2002). The rat is most sensitive to NMDA receptor-mediated neurotoxicity during early neuronal pathway development, referred to as the “brain-growth spurt period” or period of synaptogenesis (Haberdy et al., 2002). Blockade of NMDA receptors up to 4 h is sufficient to trigger apoptotic neurodegeneration in the developing brain (Ikonomidou et al., 1999). In consideration of the possible neurotoxicity caused by the coadministration of drugs and the complicated interaction between NMDA receptor blocker and AMPA receptor blocker, we examined the possible drug-induced neuronal apoptosis by TUNEL staining at 48 h post-HI even through the two drugs are proven safe at the given doses respectively (Chen et al., 1998; Glier et al., 2004). The time course of apoptotic injury varies regionally because HI damage generally evolves more rapidly in the immature brain than its adult counterpart. Injury in the cortex and striatum occurs in a biphasic manner, where the early phase (by 3 h) is classified as necrosis and the later phase (by 48 h) displays signs of apoptosis (Northington et al., 2001). Nakajima et al. found that the density of caspase-3 immunoreactivity was enhanced in the frontal, parietal, and cingulate cortex and in the striatum 24 h after hypoxic ischemic injury. In the CA3 sector of the hippocampus, the dentate gyrus, medial habenula and laterodorsal thalamus, the density of apoptotic cells was highest at 24–72 h after HI and then declined. In thalamus, increased caspase-3 immunoreactivity was distributed in lateral, laterodorsal, and reticular nuclei with a peak in density at 48 h after HI. In hippocampus, intense caspase-3 immunoreactivity was present in CA1 and in the dentate gyrus at 48 h after insult but had nearly disappeared by 7d after HI injury (Nakajima et al., 2000). Based on all these results on apoptotic injury, the time point (48 h post-HI) was chosen to examine the apoptotic neurodegeneration.

In this experiment, massive cellular apoptosis was not found in all observed areas in the treated groups, and apoptosis was reduced in the CA1 sector of the hippocampus and the subcortical white matter in the combination group compared with the vehicle group. The safe dosing regimen and anti-apoptotic actions of memantine and topiramate may contribute to the results synergistically. Regional patterns of neuronal death can also be detected by expression of caspase-3, a cysteine protease involved in the execution phase of apoptosis. Immunocytochemical and Western blot analyses show increased caspase-3 expression in damaged hemispheres 24 h to 7d after HI. Reduced caspase-3 activity has
been shown to be associated with neuroprotection (Endres et al., 1998; Puka-Sundvall et al., 2000). Memantine (20 mg/kg, i.p.) can prevent isoflurane-induced caspase-3 activation and apoptosis in vivo and in vitro. The results also indicated that isoflurane-induced caspase activation and apoptosis are dependent on cytosolic calcium levels (Zhang et al., 2008). In recent years, many studies focus on the protection of white matter because the importance of PV/II pathophysiology has been realized gradually (Khwaja and Volpe, 2008; Volpe, 2008). NMDA receptor blockade with memantine acts as an effective pharmacological contributor with little side effects in attenuating white matter injury, and the protective dose of memantine does not affect normal myelination or cortical growth (Manning et al., 2008; Micu et al., 2006). In our experiment, the apoptosis in the subcortical white matter was reduced significantly in the combination group, which is consistent with the previous findings on caspase-3 activation.

The present study demonstrated that a synergistic reduction in brain damage could be achieved by combination of neuroprotective agents targeting different mechanisms. Although an evolving body of work has shown that combination therapy holds promise in the treatment of HIBI, there has been relatively little research on the combination therapy of two glutamate receptor antagonists. The combination of NMDA receptor antagonist MK-801 and AMPA receptor antagonist NBQX shows an “overadditive” effect in cell culture and focal ischemia model in mice (Lippert et al., 1994). On the other hand, several studies on memantine or topiramate have shown multitarg drug strategies are required for optimal therapeutic outcome. The combination of memantine and celecoxib shows better effects in neuroprotection and anti-inflammation in intracerebral hemorrhage treatment (Sinn et al., 2007). Combined treatment with topiramate and delayed hypothermia improves both performance and pathological outcome in P15 and P35 rats (Liu et al., 2004b).

Although the present study demonstrates the neuroprotective effect of memantine combined with topiramate, further studies are still needed in two aspects. A full dose–response experiment was not performed in the present study, so further investigation is still needed to determine the most optimal dosing regimen of memantine and topiramate. Noh et al. suggested that the pretreatment with topiramate before HI was more effective than the post-treatment after HI (Noh et al., 2006). The result implies that the pretreatment with topiramate in the combination therapy can be considered in the future.

Collectively, the present study not only shows a promising therapy for neuroprotection, but also proposes a new paradigm for multidrug development which is thought to be a promising approach in the treatment of HIBI.

4. Experimental procedures

4.1 Animal procedures

Seven-day-old rat pups of either sex, weighing between 12 g and 16 g, were used in this study. The rat pups were randomly assigned to one of the following groups: vehicle group (saline), memantine group, topiramate group, combination group (memantine and topiramate). All animal experiments followed a protocol approved by the ethical committee on animal research at our institution. The neonatal HI brain damage was induced according to the modified Levine–Rice procedure (Northington, 2006; Rice et al., 1981; Vannucci and Vannucci, 2005). For short, rat pups were anaesthetized by halothane inhalation and duration of anesthesia was less than 5 min. The right common carotid artery was dissected, and doubly ligated. One hour later, rats were then placed in a plastic chamber (37 °C) and exposed to 8% oxygen and 92% nitrogen for 2 h. After this hypoxic exposure, the pups were returned to their dams for 2 h recovery.

4.2 Drug administration

During recovery from HI, drugs were injected intraperitoneally: vehicle group received vehicle (0.5 ml 0.9% saline) immediately after HI; memantine group received 20 mg/kg loading dose immediately after HI, then 1 mg/kg maintenance dose at 12 h intervals for 48h; topiramate group received 40 mg/kg loading dose then 10 mg/kg maintenance dose on the same schedule as memantine; combination group received both memantine and topiramate, the drug doses and schedule were the same as above.

4.3 Gross brain damage grading

To quantify the severity of brain damage, rat pups were decapitated at 22d after HI and their brains were rapidly dissected and frozen (Uhm et al., 2003). Then brains were scored normal, mild, moderate or severe by a blinded observer according to the method of Palmer et al. (1990). The neurologic damage scores were given according to the following criteria. Normal (1) is no reduction in the size of the right hemisphere, mild (2) is visible reduction in right hemisphere size, moderate (3) is large reduction in hemisphere size from a visible infarct in the right parietal area and severe (4) is near total destruction of the hemisphere.

To measure the loss of hemispheric weight, the brain was divided into two hemispheres and weighed after removing the cerebellum and brainstem. Results are presented as the percent loss of hemispheric weight of the right side relative to the left [(left–right)/left×100]. The HI model used in this study results in brain damage only on the ipsilateral side, thus the loss of hemispheric weight can be used as a measure of brain damage in this model (Rice et al., 1981). Because the brain weighs approximately 1 g/ml, weight loss is equivalent to volume loss. According to the method by McDonald et al., the loss of brain weight on the ipsilateral side relative to the contralateral side is highly correlated with cellular damage (McDonald et al., 1989b). For short, weighing can assess the degree of brain damage.

4.4 Microscopic brain damage grading

Microscopic examination of the tissues was carried out to verify that the gross changes were a reflection of the expected histopathologic changes. The rat pups were anesthetized...
with pentobarbital 3 days after injury. Their brains were perfusion fixed by cardiac puncture. They were flushed with saline then fixed with 10% buffered formalin. After removal, the brains were stored in 10% buffered formalin. Sections were then embedded with paraffin. Five-micron coronal sections were cut in the parietal region aiming for the equivalent of Bregma −4.3 to −4.5 mm in the adult rat (Kruger et al., 1995) and then stained with hemotoxylin and eosin. Cerebral cortex, hippocampus, striatum, thalamus was scored from 0 to 5 by an observer blind to the treatment according to the method of Cataltepe et al. (1995), where “0” is normal, “1” is 1–5% of neurons damaged, “2” is 6 to 25% of neurons damaged, “3” is 26–50% of neurons damaged, “4” is 51–75% of neurons damaged, “5” is >75% of neurons damaged.

4.5. Neurofunctional assessment: foot-fault test

The foot-fault test was performed at 21d post-HI according to a published method (Bona et al., 1997). Rats were placed on an elevated stainless steel grid floor 50×40 cm, 1 m above the floor with 3 cm2 holes and a wire diameter of 0.4 cm. Each pup was placed on the grid and observed for 2 min. The foot-fault was defined as when the animal misplaced a fore- or hindlimb and the paw fell through between the grid bars. The excess of left (contralateral foot-faults) to right (ipsilateral foot-faults) was recorded. Only the side difference of foot-faults was used for the statistical evaluation to eliminate the influence of the extent of activity in different rats (Barth and Stanfield, 1990).

4.6. TUNEL staining and apoptotic cell counting

We applied the Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling (TUNEL) staining to detect drug-induced apoptosis at 48 h after HI. All procedures were performed following the manufacturer's instructions (In Situ Cell Apoptosis Detection Kit I, POD; Boster, Wuhan, China).

Cell counting was performed in the cortex, hippocampus, striatum and the subcortical white matter. The hippocampus was divided into the CA1, CA3 and dentate gyrus subfields. Positive cells were counted at 400× magnification (one visual field = 0.196 mm2). By use of the ImageJ software, all analyses were done by an individual who was unaware of treatment conditions. The average number of TUNEL-positive cells was calculated from at least three sections within each region for each animal. In the hippocampus subfields, counting was performed throughout the entire region. In the cortex, striatum and the subcortical white matter, three visual fields were counted as average number per visual field. Only the densely stained cells were counted as TUNEL-positive, slightly TUNEL-stained cells were not (Zhu et al., 2004).

4.7. Statistical analysis

Data are presented as mean±S.D., if not otherwise indicated. Comparisons were performed by one-way ANOVA with Fisher's post hoc test. Differences were considered significant when p<0.05.

Acknowledgments

This work was partly supported by Natural Science Foundation of Guangdong Province, China. We gratefully thank Tianhua, Huang for his technical assistance.

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