

Piracetam Improves Cognitive Deficits Caused by Chronic Cerebral Hypoperfusion in Rats

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Abstract Piracetam is the derivate of gamma-aminobutyric acid, which improves the cognition, memory, consciousness, and is widely applied in the clinical treatment of brain dysfunction. In the present experiments, we study the effects of piracetam on chronic cerebral hypoperfused rats and observe its influence on amino acids, synaptic plasticity in the Perforant path-CA3 pathway and apoptosis *in vivo*. Cerebral hypoperfusion for 30 days by occlusion of bilateral common carotid arteries induced marked amnesic effects along with neuron damage, including: (1) spatial learning and memory deficits shown by longer escape latency and shorter time spent in the target quadrant; (2) significant neuronal loss and nuclei condensation in the cortex and hippocampus especially in CA1 region; (3) lower induction rate of long term potentiation, overexpression of BAX and P53 protein, and lower content of excitatory and inhibitory amino acids in hippocampus. Oral administration of piracetam (600 mg/kg, once per day for 30 days) markedly improved the memory impairment, increased the amino acid content in hippocampus, and attenuated neuronal damage. The ability of piracetam to attenuate memory deficits and neuronal damage after hypoperfusion may be beneficial in cerebrovascular type dementia.

Keywords Piracetam · Chronic cerebral hypoperfusion · Morris water maze · LTP · BAX · P53

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Introduction

Vascular dementia is a chronic acquired syndrome of cognitive impairment caused by cerebrovascular diseases with pathologic features of ubiquitous cerebral arteriosclerosis and infarction (David, 2004). With an increasing number of elderly people in the world population, dementia, characterized by progressive loss of memory and higher cortical functions, has given rise to an enormous socioeconomic burden. Apart from Alzheimer's disease, vascular dementia (VD) was usually considered the second most common dementia produced by ischemia, hypoxia, or hemorrhagic brain lesion in Western countries and perhaps the most frequent cause of dementia in Asian countries (Gunstad et al. 2005).

It is now accepted that the pathogenic mechanisms of vascular dementia are related to the ischemia, hypoxia, or hemorrhagic damage to specific corresponding regions involved in cognition and memory (Charles, 2004). Ischemic lesion is a complicated physiopathologic process. Up date, energy failure because of decreased cerebral flow was considered the reason of cell death. Among the studies on its pathogenic mechanisms, cascade of damage because of discontinuance and reperfusion of blood flow was considered the reason of neuron death. The cascade of damage includes four steps, i.e., energy failure and excitatory amino acids toxicity, peri-infarct depolarizations, inflammation response, programmed cell death or apoptosis, which overlapped and correlated to each other, usually occurred in the period from some seconds to some weeks after ischemia (White et al. 2000; Davis et al. 2000).

Piracetam is a gamma-aminobutyric acid derivative, which improves cognition, memory, and consciousness, and is widely applied in the clinical treatment of brain dysfunction (Waegemans et al. 2002; Boiko et al. 2005; Xerri and Zennou-Azogui 2003; Karacostas et al. 1999; Tomczykiewicz and Domzal 1997). It was documented that piracetam could normalize ATP metabolism, stimulate phospholipid synthesis and ribosome function, and increase glucose utilization (Bogolepov et al. 1983; Xerri and Zennou-Azogui 2003; Kozubski 1998). And there are many reports that piracetam can exert antioxidant properties, block the entry of Ca^{2+} caused by glutamate in the ischemic region (Solntseva and Zhang 1991; Klimenko 1993), and increase the blood flow by reducing the resistance of cerebral vessels (Vernon and Sorkin 1991; Platt et al. 1993). In vitro, there is a report that piracetam exerts cytoprotective activity and prevents apoptosis of astrocytes submitted to hypoxia and reoxygenation (Gabryel et al. 2002). In this study, the vascular dementia model is produced by permanent bilateral ligation of the common carotid arteries, which can induce hypoperfusion and spatial memory impairment (Guang and Du 2006), our aim was to search for whether piracetam has beneficial effects in the cerebral ischemia model in rats and observe the effects on amino acids, synaptic plasticity in the Perforant path-CA3 pathway and apoptosis in vivo.

Materials and Methods

Chemicals

Piracetam (colorless power, purity >98%) was a product of Shanghai Huaihai Pharmaceutical Co. Rabbit anti-mouse P53 monoclonal IgG (sc-100) and rabbit

anti-mouse BAX monoclonal IgG (sc-7480) were purchased from Santa Cruz Biotechnology. Standard preparation of glutamate (GLU), aspartate (ASP), glycine (GLY), gamma-aminobutyric acid (GABA) and taurine (TAU) and *O*-phthaldialdehyde (OPA) were purchased from Sigma Chem. Co. Ltd (St. Louis, MO, USA). Sodium Dihydrogen Phosphate, dibasic sodium phosphate and borax were purchased from XINHUA Chem. Co. Ltd (Shanghai, China). Kit of total protein quantitation were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). β -mercaptoethanol (BME) were purchased from AMRESCO Inc. (Ohio, USA).

Animals

Adult male Sprague-Dawley rats weighing 200–250 g were used (Experimental Animal Center, Tongji Medical College, Huazhong University of Science and Technology). They were housed 5 per cage in a temperature and humidity-controlled room (temperature: $22 \pm 1^\circ\text{C}$, humidity: 60%) with free access to food and water. The rats were kept on a 12-h light/dark cycle. The rats were acclimatized to housing conditions for at least 4 d before being used. About 70 rats were divided into normal group, sham-operated group (this group was treated with 0.9% saline (p.o.)), hypoperfusion group (HP group) and piracetam-treated group (oral administration of piracetam 600 mg/kg after surgery daily for 30 d) according to random digit table method. Handling and experimental procedures on all animals were in accordance with the institutional and National Research Council's guideline for animal experiments.

Surgery

The rats were anesthetized with chloral hydrate (350 mg/kg, i.p.). Bilateral common carotid arteries were exposed through a midline neck incision, double ligated with 4-0 type surgical silk, and cut between ligation in ischemia rats. The sham-operated rats received the same operation except ligatures. During the surgery, their body temperature was monitored and maintained at $37.5 \pm 0.5^\circ\text{C}$ by means of a heating lamp until the rats recovered thermal homeostasis.

Water Maze Task

The spatial memory performances were evaluated using a Morris water maze 30 days after induction of hypoperfusion (Lee et al. 2003; Liao et al. 2004). The swimming pool of the Morris water maze was a circular water tank, 200 cm in diameter and 35 cm deep. It was filled to a depth of 21 cm with water at $23 \pm 2^\circ\text{C}$. A platform, 15 cm in diameter and 20 cm in height, was placed inside the tank, its top surface being 1.5 cm below the surface of the water. The pool was surrounded by many cues external to the maze. Each rat received two trials everyday for 5 consecutive days. Latency to escape onto the hidden platform was recorded. On the sixth training day, each rat was subjected to a 180 s probe trial in which the platform was removed and the time spent in Q3 was recorded. In order to examine the possibility that the difference in spatial learning between groups, if any, was associated with vision impairment, the escape latency of each rat was determined with the visible platform at 1 cm above water surface after the probe trial. Swimming activities were monitored by a video camera linked to a computer-based

image analyzer (MT-200, China). Tests were performed between 19:30 and 21:00 PM. Piracetam was administered orally 30 min before the test.

Morphology

Three rats chosen randomly from each group were anesthetized with chloral hydrate (350 mg/kg, i.p.) after behavior experiment, and then perfused transcardially with normal saline followed by 4% paraformaldehyde. All brains were then postfixed in the same fixative at 4°C, dehydrated and then embedded in paraffin blocks. Coronal sections of 8 μm were stained with hematoxylin-eosin (HE). Hippocampal cells were measured by placing the grid on the hippocampal CA1 area at the level of 4.8 mm bregma according to the atlas of Paxinos and Watson (Paxinos and Watson, 1986). The cells of the hippocampal CA1 area and cortex were counted at 100 \times magnification using a microscope rectangle grid measuring 100 \times 100 microns. Evaluation of cell loss was determined by calculating the density of cells using the Scion image system (Scion Corp., Maryland, USA). Cells within the examined area were counted on each of 3 sections per animal.

Immunohistochemistry

Tissue sections were deparaffinized and rehydrated through graded alcohol. Endogenous peroxidase activity was blocked by incubation in 10% hydrogen peroxide for 10 min. After three time rinses with PBS, the sections were blocked with 1:10 normal goat serum to suppress nonspecific background staining. Then, the primary antibody, rabbit anti-mouse P53 monoclonal antibody and rabbit anti-mouse BAX monoclonal antibody was applied at 1:50 dilution. After incubation for 20 h at 4°C, the sections were incubated with biotinylated goat anti-rabbit IgG (1:100, 37°C, 1 h), then followed by SABC kit. The sections were subsequently incubated with diaminobenzidine 0.5 g/l, observed under light microscope. For quantitative analysis of P53 and BAX immunoreactivity in the hippocampus and cortex, 15 sections per animal were randomly selected within the corresponding levels of the hippocampus and cortex. At a magnification of 100 \times , the regions were outlined on the monitor and their areas were measured. All immunoreactive structures were obtained through an Axiophot light microscope (Carl Zeiss, Germany) connected via CCD camera to a PC monitor. Video images were digitized into an array of 512 \times 512 μm pixels corresponding to a tissue area of 140 \times 140 μm (100 \times primary magnification). Each pixel resolution was of 256 gray levels. The intensity of P53 and BAX immunoreactivity was evaluated by means of a relative optical density (ROD) value. ROD was obtained after transformation of mean gray values into ROD using the formula: $\log(256/\text{mean gray})$. The values of background staining were obtained and subtracted from all immunoreactive structures ROD value before statistically processing the obtained values. ROD values were informed as ROD units.

LTP Recording

The rats were anesthetized by urethane (1.2 g/kg i.p.) and then positioned in a stereotaxic apparatus (SN-3, Narishige Japan). Body temperatures were kept 37°C via a constant temperature water cycling system. The skull landmark bregma was chosen to be the stereotaxic reference zero point. A local craniotomy was performed. Two bore holes with a diameter of 1.5 mm were made: one hole was made between 6.8 mm and

7.0 mm posterior to the bregma and 4.3–4.5 mm lateral to the midline for perforant path (PP) fibers stimulating. Another hole was made at 3.5 mm posterior to the bregma and 3.5 mm lateral to the midline for CA3 region recording. The dura was removed, and the pial surface was moistened with saline periodically throughout the experiment. Bipolar stimulating electrodes (140 μ m diameter stainless steel needles insulated with teflon except for the cut tips with an interelectrode distance of 0.5 mm) were placed in the perforant path (PP). A stainless steel needle monopolar recording electrode coated with Teflon was positioned in CA3 area. The stereotaxic coordinates of stimulating and recording sites are as follows: PP (6.8–7.0 mm posterior to bregma, 4.3–4.5 mm lateral to midline, 3.0–4.0 mm ventral to dura), CA3 (3.2–3.5 mm posterior to bregma, 3.2–3.5 mm lateral to midline, 3.0–3.5 mm ventral to dura) (Mori et al. 2001; Bliss and Collingridge 1993).

Monopolar stainless steel needle electrode coated with teflon was applied to record the extracellular field potentials in the CA3 area, first positioned as above-mentioned description, and connected to SMUP-PC biology signal analyzing system (Second Military Medical University, China) which carried out the data's on-line acquiring, amplifying, recording and analyzing. Single-pulse test stimulation consisted of monophasic square wave pulse (0.15 ms duration, 0.5 Hz, 45 V) at 2 s intervals was delivered to PP by the previous electric stimulator via stimulating electrodes. By slightly varying the depth of the stimulating and recording electrode at above-mentioned range below the cortical surface, the characteristic waveforms evoked by stimulation PP could be acquired (Fig. 1). Once the maximal typical extracellular potentials in the CA3 area were recorded, the positions of stimulating and recording electrodes were fixed. As Fig. 1 showed, the field potentials in the CA3 area evoked by stimulation PP was observed as a slow wave including a previous occurred positive peak and followed posterior negative peak, and the population spike (PS) in the CA3 region was described as the amplitudes from the first positive peak (a) to negative peak (b). Before each experiment, the whole system was required to be balanced until the field potentials keep in a steady condition at least 30 min later, then recording began. The signals were passed through SMUP-PC for on-line averaging and recording. Each stimulation could induce an evoked field potential. The recorded traces showed in the figures were averages of 10 consecutive records. The averaged PS amplitudes of 10 different time points within an hour after the steady conditions were considered as the baseline values.

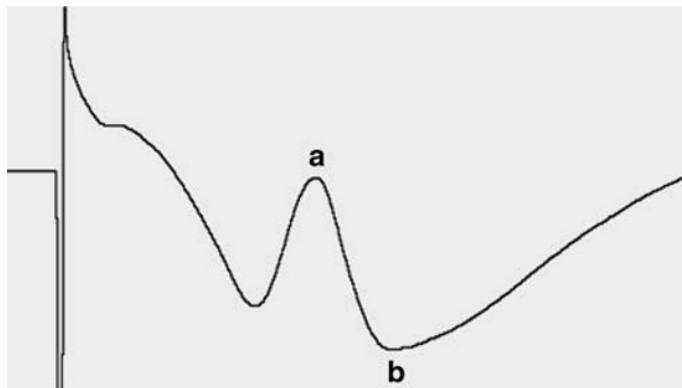


Fig. 1 A typical evoked potential recorded in hippocampus CA3 region, population spike was defined as the amplitudes from the first positive peak (a) to negative peak (b)

In the electrophysiology experiment, high-frequency stimulation (HFS) consisted of 50 trains at 0.5 Hz each composed of 4 pulses at 500 Hz. After HFS, the amplitude of the population spike was measured for 1 h, and the corresponding percentage of each PS amplitude at different time points, which were obtained by absolute PS amplitude divided by baseline value, i.e., relative PS amplitudes (%), was used to describe the PS amplitude level variation. It was defined as a successful induction of LTP if the amplitude of population spike change exceeded 20% (Gao et al. 2003).

Quantification of Protein and Five Finds of Amino Acids in the Rats Experimental Ipsilateral Hippocampus

At the end of electrophysiology experiment, each animal was immediately decapitated and the experimental ipsilateral hippocampus was carefully dissociated from the rats cerebrum, washed with ice saline solution, then frozen in -75°C . The Kit of total protein quantitation (Coomassie Brilliant Blue, Nanjing Jiancheng Bioengineering Institute, China) was used to quantify the protein concentration. According to Bianchi's methods (Bianchi et al. 1998), concentrations of the amino acids were determined by high-performance liquid chromatography (HPLC) with fluorescence detection using a spectrophotometer (excitation wavelength 330 nm, emission wavelength 420 nm, Prostar 370, VARIAN, Holland) after automatic precolumn derivatization with *o*-phthaldialdehyde. The derivatization of samples and standards was carried out in an autosampler (Prostar, VARIAN, Holland). Derivatives were separated on a Hypersil ODS2C18 column (Dalian Elite, China). The mobile phase gradient elution profile consisted of Buffer A: 0.1 M phosphate buffered solution (PBS), pH 6.8, and Buffer B: pure methanol, flow rate 1 ml/min in a three linear steps gradient (from 25% to 90% methanol). Data were collected and analyzed using the ANASTAR software. External standard method was used to quantify the concentrations of amino acid according to each peak area. The final data values of amino acids were calculated according to the corresponding hippocampus tissue protein concentration, described as $\mu\text{mol/g}$ protein.

Statistics Analysis

All results were presented as mean \pm SEM. Group differences in the escape latency, data collected from the probe trials in the Morris water maze training task and the evaluation of cell loss were analyzed with Tukey's test following a repeated ANOVA. Group differences of LTP were assessed with the Tukey's post-hoc multiple comparison test. Group differences of ROD was assessed by the Duncan's new multiple range method or Newman-Keuls test. The amino acids contents among different groups were analyzed by using one-way analysis of variance (ANOVA) followed by the Fisher LSD test (Least-significant difference) using SPSS 12.0 for windows. A value of $P < 0.05$ was considered statistically significant.

Results

Effects of Piracetam on the Water Maze Test

The rats with chronic hypoperfusion took longer to find the platform than did sham-operated rats ($P < 0.05$). This prolongation of latency was shortened by piracetam

600 mg/kg ($P < 0.05$, Fig. 2A). In the probe trials, the swimming time in Q3 was used to evaluate the retention performance. The sham-operated group and the piracetam-treated group swam longer in Q3 than HP group ($P < 0.01$, Fig. 2B). The typical swimming tracks indicated that ischemic rats often searched for the platform in an inappropriate way resulting in the longer latency to locate the platform (Fig. 2C). All rats reached the platform in a short period of less than 30 s tested with a visible platform

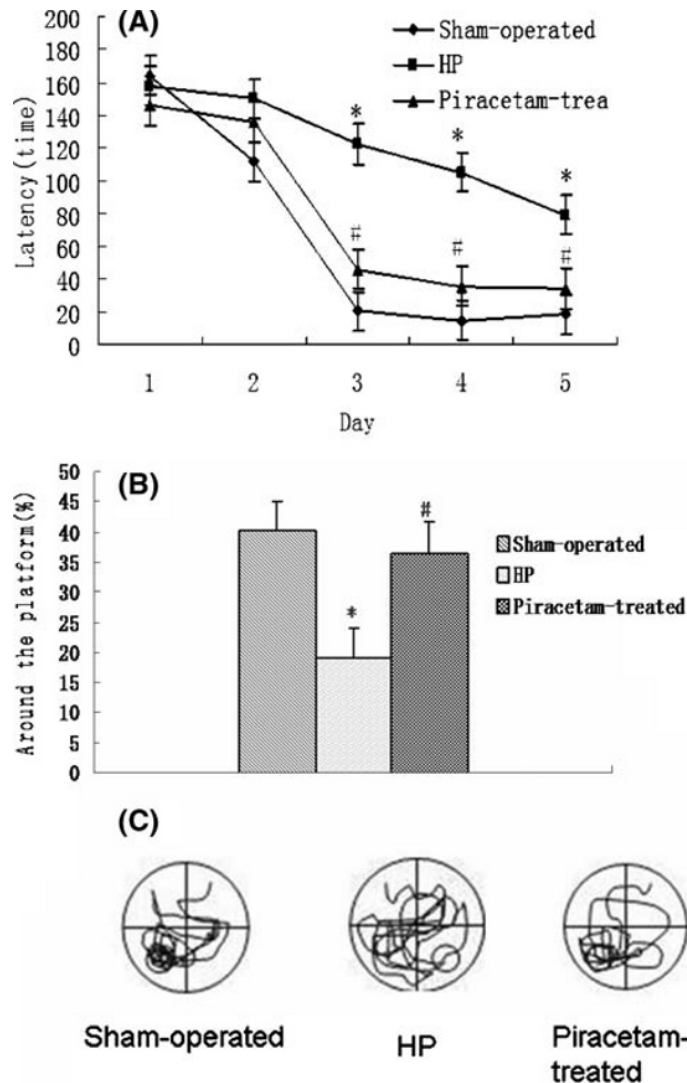


Fig. 2 Effect of piracetam on cerebral hypoperfusion-induced deficits in learning and memory in rats performing the Morris water maze. The task was performed with four trials per day during 5 days for the acquisition test (A), and performed with four trials on day 6 without the platform for retention test (B). (C) shows the typical swim-tracking path in probe trial on the sixth training day (no platform). Rats were administrated piracetam (oral administration of piracetam 600 mg/kg, once per day for 30 days, Piracetam-treated, $n = 10$) after surgery, pretreated with saline (1 ml/kg, HP, $n = 10$). The sham-operated group (Sham-operated, $n = 10$) was treated with only saline without induction of hypoperfusion. Significance with Tukey's test following a repeated ANOVA is indicated as $*P < 0.01$ versus Sham-operated and $\#P < 0.05$ versus HP. Vertical lines indicate SEM ($N = 10$)

task, and there was no difference between any two groups, indicating that the difference in spatial performance between groups is unlikely to be associated with vision impairments.

Effects of Piracetam on Hypoperfusion-induced Neuronal Damage

In the cortex and hippocampus, marked morphological changes were detected in the ischemic group: neuronal cells loss, nuclei shrinkage, and dark staining of neurons were observed in hippocampal CA1 region and cortex. Treatment with piracetam (600 mg/kg) markedly reduced these pathological changes (Figs. 3, 4).

Effects of Piracetam on the Expression of BAX and P53 Protein

Photomicrographs of immunohistochemical localization of BAX and P53 protein in cortical neuronal cells were shown in Figs. 5 and 6. Chronic hypoperfusion rats exhibited strongly increased BAX and P53 reactivity. However, in the piracetam-treated group, no obvious abnormalities in the intensity or localization of immunostaining for the two pro-apoptotic proteins could be found. No significant difference on BAX and P53 expression was observed among three treatments in hippocampus (data not shown).

Effects of Piracetam on LTP in CA3 Region of Hippocampus In Vivo

There were differences on the induction rate of LTP among the sham-operated group (100%), HP group (28.57%) and piracetam-treated group (85.71%). LTP was blocked following bilateral ligation of the common carotid arteries. Piracetam restored LTP to the similar extent as that observed in the sham-operated. When compared the percentage change of amplitude of the population spike, there were differences on it

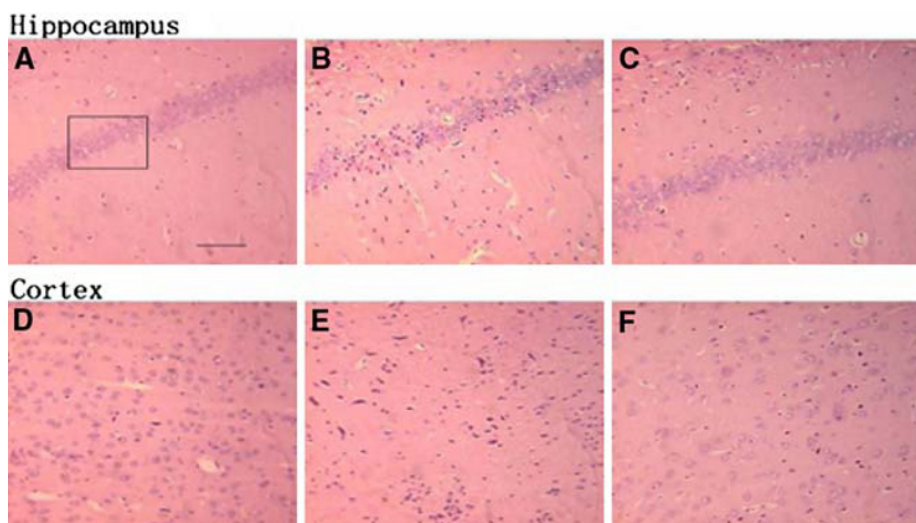


Fig. 3 Representative photographs showing the rats hippocampus and cerebral cortex. Sections from three rats in each group were examined. **A** and **D**: Sham-operated group; **B** and **E**: HP group; **C** and **F**: Piracetam-treated group. Scale bars represent 50 μ m

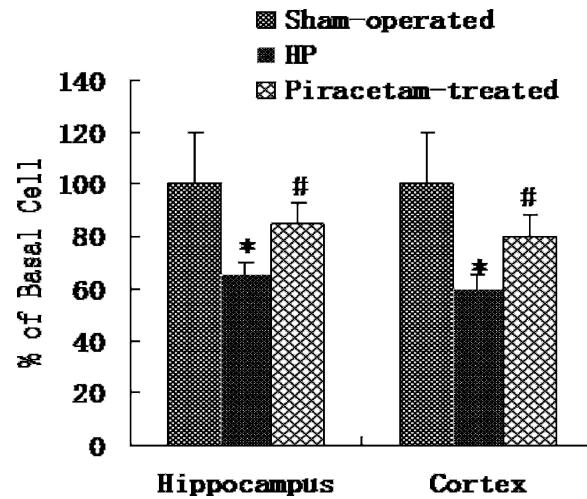


Fig. 4 Effects of piracetam on morphologic changes in rats hippocampus and cerebral cortex induced by chronic hypoperfusion. Sections from three rats in each group were examined. Rats were administrated piracetam (oral administration of piracetam 600 mg/kg, once per day for 30 days, Piracetam-treated, $n = 3$) after surgery, pretreated with saline (1 ml/kg, HP, $n = 3$). The sham-operated group (Sham-operated, $n = 3$) was treated with only saline without induction of hypoperfusion. Significance with Tukey's test following a repeated ANOVA is indicated as * $P < 0.01$ versus Sham-operated and # $P < 0.05$ versus HP. Vertical lines indicate SEM ($N = 3$)

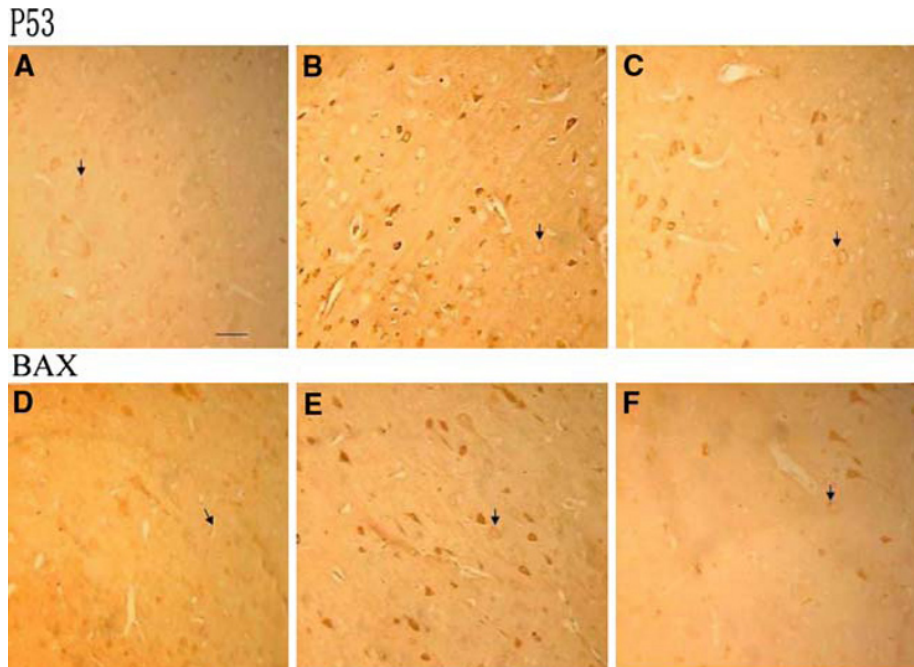


Fig. 5 Representative photographs show effects of piracetam on apoptotic related protein expression in cortical region of rats chronically hypoperfused after ligation of the bilateral carotid arteries. **A** and **D**: Sham-operated; **B** and **E**: Ischemia group; **C** and **F**: piracetam 600 mg/kg. Scale bars represent 50 μm . Arrows indicate positive immunoreactivity cells

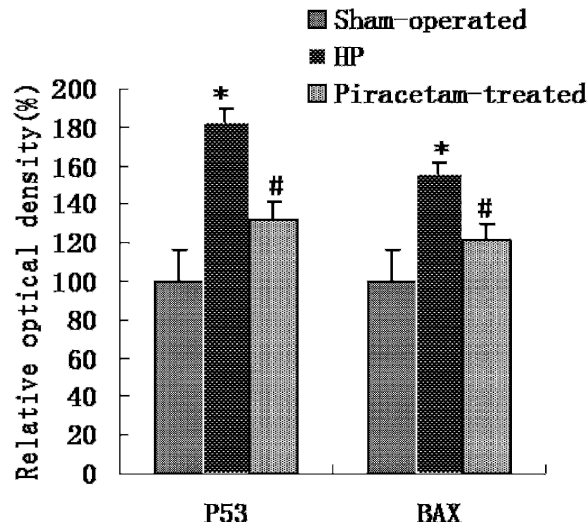


Fig. 6 Effects of piracetam on apoptotic related protein expression in cortical region of rats chronically hypoperfused after ligation of the bilateral carotid arteries. The relative optical density as % values of immunoreactivity is also represented. Rats were administrated piracetam (oral administration of piracetam 600 mg/kg, once per day for 30 days, Piracetam-treated, $n = 3$) after surgery, pretreated with saline (1 ml/kg, HP, $n = 3$). The sham-operated group (Sham-operated, $n = 3$) was treated with only saline without induction of hypoperfusion. Significance with one-way analysis of variance followed by the Duncan's new multiple range method or Newman–Keuls test is indicated as * $P < 0.01$ versus Sham-operated and # $P < 0.05$ versus HP. Vertical lines indicate SEM ($N = 3$)

among the three groups (sham-operated group (189.15 ± 9.84) %, HP group (118.06 ± 5.20) %, piracetam-treated (162.25 ± 7.54) %) (Fig. 7).

Effects of Piracetam on the Contents of GLU, ASP, GLY, GABA and TAU in Hippocampus in Different Groups in High Frequency Synaptic Transmission Evoked by Stimulating the PP Fibers

In order observe the effects of piracetam on the excitatory and inhibitory amino acids changes in hippocampus in the high-frequency synaptic transmission by stimulating PP, the total contents of GLU, ASP, GLY, GABA, and TAU in hippocampus in rats of normal, sham-operated, HP, and piracetam-treated group were determined, respectively, by HPLC. Compared with normal group (without HFS), the contents of all the five sorts of amino acids in hippocampus in sham-operated group after HFS all increased ($P < 0.05$ for ASP, GLY, GABA and TAU. For GLU, which has increasing tendency but without statistics significance), which in HP group all reduced compared with sham group, but in piracetam-treated group all of them were increased after HFS compared with HP group except GABA (Fig. 8).

Discussion

Animal models of cerebral ischemia are widely used in vascular dementia research. The 2VO model (bilateral ligation of the common carotid arteries) made by occluding the bilateral common carotid arteries permanently is frequently used. This model produces selective damage in the hippocampus similar to that caused by the 4VO model,

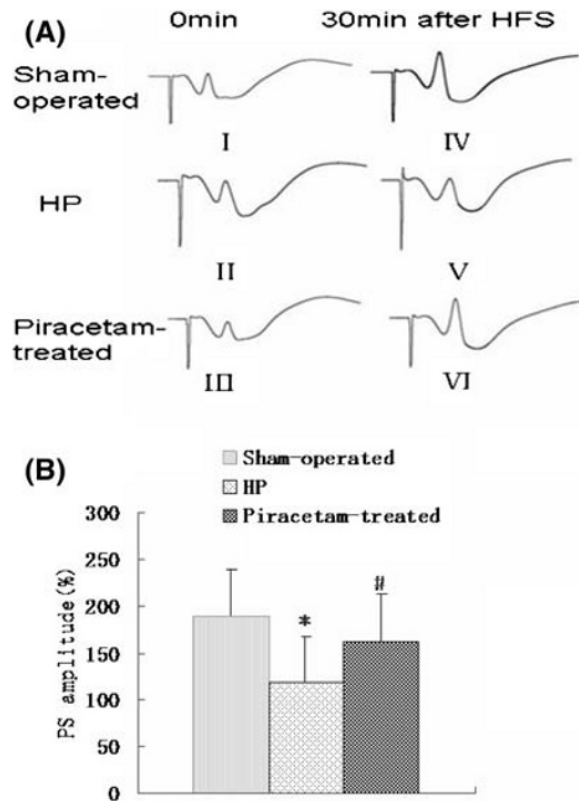


Fig. 7 (A) Changes in LTP after cerebral hypoperfusion in the perforant path-CA3 synapses. LTP was blocked in the HP group. Piracetam ameliorated the inhibition. (B) Changes of the amplitude of the population spike pre-tetanus and post-tetanus (HFS). I and IV: Sham-operated group (treated with only saline, Sham-operated, $n = 7$); II and V: HP group (pretreated with saline 1ml/kg, HP, $n = 7$); III and VI: Piracetam-treated group (oral administration of piracetam 600 mg/kg, once per day for 30 days, Piracetam-treated, $n = 8$); I, II, III before HFS; IV, V, VI after HFS. Significant differences of post-hoc analysis are indicated as * $P < 0.01$ versus sham-operated and # $P < 0.05$ versus HP. Vertical lines indicates SEM ($N = 7-8$)

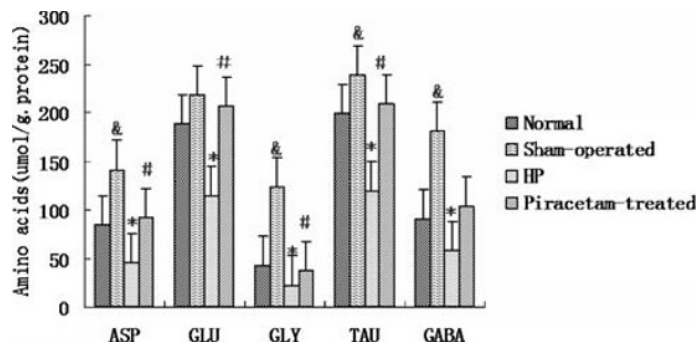


Fig. 8 Effects of piracetam on the content of amino acids. Rats were administrated piracetam (oral administration of piracetam 600 mg/kg, once per day for 30 days, Piracetam-treated, $n = 8$) after surgery, pretreated with saline (1 ml/kg, HP, $n = 7$). The sham-operated group (Sham-operated, $n = 7$) was treated with only saline. Significance with one-way analysis of variance (ANOVA) followed by the Fisher LSD test is indicated as [&] $P < 0.05$ versus Normal group, * $P < 0.05$ versus Sham-operated group and # $P < 0.05$ versus HP group. Vertical lines indicates SEM ($N = 7-8$)

produced by occlusion of both common carotid arteries and vertebral arteries. Our 2VO model was appropriate for the study on chronic survival, because this model can simulate the decrease in cognition and neurodegeneration that occurs in dementia (Ni and Matsumoto 1995).

In our study, we observed that 30 days after the operation, cerebral ischemia induced marked amnesic effects along with neuronal damage, including: (1) spatial learning and memory deficits shown by longer escape latency and shorter time spent in the target quadrant; (2) significant neuronal loss and nuclei condensation in the cortex and hippocampus especially in CA1 region. These results, in accordance with previous reports (Wang et al. 2000), indicate that cerebral ischemia is related to cognitive impairment and neuronal death. Piracetam could ameliorate the cognitive deficits of the vascular dementia model in rats.

It is now considered that the ischemic damage of the brain was related to the enhanced toxicity of the excitatory amino acids, and many researchers have reported that the glutamate level increased in the extracellular fluid during the acute ischemia period and then rebounded to the normal level with the prolongation of the ischemia process (Phillis and O'Regan 2003; Phillis et al. 1996). There are few reports about how the level of amino acids changed during chronic hypoperfusion in rats brain tissue except some reports of increased amino acid levels (Feng et al. 2004; Zhou et al. 2002). In our experiments, we observed that the content of excitatory and inhibitory amino acids was significantly decreased in the hypoperfused group compared to the sham-operated group, after both groups received high frequency stimulation. Piracetam increased the content of all amino acids in this model except GABA. The decreased amino acid content in hypoperfused rats may be explained as follows: affluent excitatory amino acids are released, and they enhance excitotoxicity because of energy failure in the initial stage of ischemia. In the late stage, because of decreased metabolism due to insufficient blood flow, and malfunction of amino acid transport and synthesis, the content of excitatory and inhibitory amino acids decreased below normal levels. The results indicate that piracetam normalizes amino acids metabolism. Such a mechanism correlates to its effects on normalizing ATP metabolism, stimulating phospholipid synthesis and ribosome function, increasing glucose utilization and blood flow by reducing the resistance of cerebral vessels (Bogolepov et al. 1983; Xerri and Zennou-Azogui 2003; Kozubski 1998; Vernon and Sorkin 1991; Platt et al. 1993).

Since the discovery of LTP (Long-Time Potentiation) in the dentate gyrus (Bliss and Lomo 1973), LTP has also been widely investigated as an electrophysiological basis in learning and memory. In the hippocampus, LTP is expressed in trisynaptic circuit, entorhinal cortex to dentate granule cells, granule cells to CA3 pyramidal cells, and CA3 pyramidal cells to CA1 pyramidal cells. However, both anatomical and physiological evidence suggests that the direct perforant path projections to the CA3 and CA1 regions of the hippocampus are substantial. The average pyramidal cells in the CA3 region also receive a direct, monosynaptic input from the entorhinal cortex (EC), via the fibers of the perforant path (PP) (Berzhanskaya et al. 1998; Martinez et al. 2002). Furthermore, previous studies (Do et al. 2002; Yeckel and Berger 1990) and our recent research (Sun et al. 2004; Xu et al. 2004) demonstrated that stimulation of the PP could activate CA3 pyramidal cells and evoke characteristic field potentials. Therefore, CA3 region field potentials recording by stimulating the PP is one good way to study the mechanism of hippocampal information process and to probe piracetam effects. LTP activated by high frequency stimulation in the Perforant path-CA3 pathway was observed in this study. Our results show that the induction rate of LTP of the HP group

is lower than that in sham-operated group. Piracetam could improve that in this model, which indicated that piracetam could facilitate the synaptic transmission in hippocampus. This observation was in agreement with previous results (O'Neill et al. 2004; Nomura and Nishizaki 2000). Based on the results of the content of amino acids, the mechanism of facilitating the electrophysiology in rat brain of piracetam may be explained by the effects of the increase of the content of glutamate, which is the key excitatory neurotransmitter in central nervous system and related to the excitatory synaptic transmission and neuron plasticity (Han 1999).

The present finding also implicated that the neuroprotective effects of piracetam may involve the anti-apoptotic pathway. It is well documented that oxidative stress, mitochondria dysfunction, and Ca^{2+} overload are involved in the basic molecular and biological process leading to apoptosis (Blalock et al. 2003; Rami 2003). In our experiment, HE staining showed that cortical and hippocampal neurons in hypoperfused rats revealed apoptotic-like morphologic changes. Further, immunohistochemical analysis demonstrated that BAX and P53 were up-regulated during chronic cerebral hypoperfusion. Piracetam attenuated apoptotic-like changes and neuron losses and reversed the shift in the expression pattern apoptosis-related proteins (BAX and P53) induced by ischemia in the cortex. The mechanism of the anti-apoptotic effect of piracetam needs further study. Based on our immunohistochemical results, it is possible that this mechanism involves the inhibition of the expression of apoptosis-promoting genes.

Our findings suggest that chronic hypoperfusion decreases the contents of excitatory and inhibitory amino acids in rat brain and up-regulates the apoptosis-related protein: P53 and BAX. Piracetam inhibited the decrease amino acid content induced by chronic hypoperfusion, ameliorated the dysfunction of learning and memory in HP rats, down-regulated P53 and BAX protein, facilitated the synaptic plasticity, and may be helpful in the treatment of vascular dementia.

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References

- Berzhanskaya J, Urban NN, Barrionuevo G (1998) Electrophysiological and pharmacological characterization of the direct perforant path input to hippocampal area CA3. *J Neurophysiol* 79(4):2111–2118
- Bianchi L, Colivicchi MA, Bolan JP (1998) The release of amino acids from rat neostriatum and substantia nigra in vivo: an adult microdialysis probe analysis. *Neuroscience* 87(1):171–180
- Blalock EM, Chen KC, Sharrow K, Herman JP, Porter NM, Foster TC, Landfield PW (2003) Gene microarrays in hippocampal aging: statistical profiling identifies novel processes correlated with cognitive impairment. *J Neurosci* 23:3807–3819
- Bliss TVP, Collingridge GL (1993) A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* 361(6407):31–39
- Bliss TVP, Lomo T (1973) Long-lasting potentiation of synaptic transmission in the dentate area of the perforant path. *J Physiol (Lond)* 232:331–356
- Bogolepov NN, Gusev EI, Burd GS, Buklina SB (1983) Ultrastructural aspects of acute cerebral ischemia during nootropic administration (experimental study). *Zh Nevropatol Psikhiatr Im S S Korsakova* 83(7):984–990
- Boiko AN, Kabanov AA, Es'kina TA, Sheliakina LA, Shchukin AI, Batysheva TT, Artemova I, Vdovichenko TV, Volovets SA, Ganzhula PA (2005) Phezam efficacy in patients with chronic cerebral ischemic disease. *Zh Nevrol Psikhiatr Im S S Korsakova* 105(1):36–41

- Charles D (2004) Vascular factors in dementia: an overview. *J Neurol Sci* 226:19–23
- David WD (2004) Vascular dementia. *Clin Neurosci Res* 3:437–448
- Davis SM, Lees KR, Albers GW, Diener HC, Markabi S, Karlsson G, Norris J (2000) Selfotel in acute ischemic stroke: possible neurotoxic effects of an NMDA antagonist. *Stroke* 31:347–354
- Do V, Martinez CO, Martinez JLL, Derrick BE (2002) Long-term potentiation in direct perforant path projections to the hippocampal CA3 region in vivo. *J Neurophysiol* 87(2):669–678
- Feng YM, Zheng P, Ren XQ (2004) Effects of one traditional Chinese medicine on glutamate and gamma-aminobutyric acid in experimental vascular dementia rats. *TCM Res* 17(2):22–24
- Gabryel B, Adamek M, Pudelko A, Malecki A, Trzeciak HI (2002) Piracetam and vinpocetine exert cytoprotective activity and prevent apoptosis of astrocytes in vitro in hypoxia and reoxygenation. *NeuroToxicol* 23:19–31
- Gao C, Wang JZ, Chen ME (2003) Effects of intravenous administration of marrow stromal cells on long-term potentiation induction of vascular dementia rats. *Chinese J Clin Rehabil* 7(16):2268–2269
- Guang HM, Du GH, (2006) Protections of pinocembrin on brain mitochondria contribute to cognitive improvement in chronic cerebral hypoperfused rats. *Eur J Pharmacol* 542:77–83
- Gunstad J, Brickman AM, Paul RH, Browndyke J, Moser DJ, Ott BR, Gordon N, Haque O, Cohen RA (2005) Progressive morphometric and cognitive changes in vascular dementia. *Arch Clin Neuro-psychol* 20:229–241
- Han JS (1999) *Principals of neuroscience*. Press of Peking medical university Peking
- Karacostas D, Doskas T, Artemis N (1999) Beneficial effect of piracetam monotherapy on post-ischaemic palatal myoclonus. *J Int Med Res* 27(4):201–205
- Klimenko VN, Belenichev IF, Bashkin IN, Shavrin VA, Vizir VA, Guitur MM (1993) Pharmacologic protection of brain in surgery of the brachiocephalic arteries. *Klin Khir* (7–8):14–17
- Kozubski W (1998) Porównanie skuteczności piracetamu i dekstranu 40 tys. u chorych w podeszłym wieku z niedokrwinnym udarem mózgu. *Wiad Lek* 51(7–8):321–325
- Lee B, Choi YK, Kim H, Kim S, Hahm DY, Lee HJ, Shim I (2003) Protective effects of methanol extract of *Acori graminei* rhizome and *Uncariae Ramulus et Uncus* on ischemia-induced neuronal death and cognitive impairments in the rat. *Life Sci* 74:435–450
- Liao Y, Wang R, Tang XC (2004) Centrophenoxine improves chronic cerebral ischemia induced cognitive deficit and neuronal degeneration in rats. *Acta Pharmacol Sin* 25(12):1590–1596
- Martinez CO, Do VH, Martinez JL, Derrick BE (2002) Associative long-term potentiation (LTP) among extrinsic afferents of the hippocampal CA3 region in vivo. *Brain Res* 940(2):86–94
- Mori K, Togashi H, Ichi K, Ueno, Matsumoto M, Yoshioka M (2001) Aminoguanidine prevented the impairment behavior and hippocampal long-term potentiation following transient cerebral ischemia. *Behav Brain Res* 120:159–169
- Ni JW, Matsumoto (1995) Neuronal damage and decrease of central acetylcholine level following permanent occlusion bilateral common carotid arteries in rats. *Brain Res* 673:290–296
- Nomura T, Nishizaki T (2000) Nefiracetam facilitates hippocampal neurotransmission by a mechanism independent of the piracetam and aniracetam action. *Brain Res* 870:157–162
- O'Neill MJ, Bleakman D, Zimmerman DM, Nisenbaum ES (2004) AMPA receptor potentiators for the treatment of CNS disorders. *Curr Drug Targets CNS Neurol Disord* 3(3):181–194
- Paxinos G, Watson C (1986) *The rat brain in stereotaxic coordinates*. Academic Press, New York
- Phillis JW, O'Regan MH (2003) Characterization of modes of release of amino acids in the ischemic/reperfused rat cerebral cortex. *Neurochem Int* 43:461–467
- Phillis JW, Smith-Barbour M, O'Regan MH (1996) Changes in extracellular amino acid neurotransmitters and purines during and following ischemia different durations in the rat cerebral cortex. *Neurochem Int* 29:115–120
- Platt D, Horn J, Summa JD (1993) On the efficacy of piracetam in geriatric patients with acute cerebral ischemia: a clinically controlled double-blind study. *Arch Gerontol Geriatr* 16(2):149–164
- Rami A (2003) Ischemic neuronal death in the rat hippocampus: the calpain–calpastatin–caspase hypothesis. *Neurobiol Dis* 13:75–88
- Solntseva EI, Zhang SS (1991) Relationship between facilitatory effect of piracetam on memory and glutamate receptors. *Acta Pharmacol Sin* 12(2):145–147
- Sun T, Hu HZ, Xu XL, Yu SB, OuYang CH, Qu L, Lv Q, Guo LJ (2004) Effects of glutamic acid and its receptor antagonist on evoked potentials in hippocampus CA3 region in rats. *Chinese Pharmacol Bull* 20(4):414–417
- Tomczykiewicz K, Domzal T (1997) Piracetam treatment in ischemic stroke. *Neurol Neurochir Pol* 31(6):1101–9
- Vernon MW, Sorkin EM (1991) Piracetam. An overview of its pharmacological properties and a review of its therapeutic use in senile cognitive disorders. *Drugs Aging* 1(1):17–35

- Waegemans T, Wilshe CR, Danniau A, Ferris SH, Kurz A, Winblad B (2002) Clinical Efficacy of Piracetam in Cognitive Impairment: A Meta-Analysis. *Dement Geriatr Cogn Disord* 13:217–224
- Wang LM, Han YF, Tang XC (2000) Huperzine A improves cognitive deficits caused by chronic cerebral hypoperfusion in rats. *Eur J Pharmacol* 398:65–72
- White BC, Sullivan JM, DeGracia DJ, O'Neil BJ, Neumar RW, Grossman LI, Rafols JA, Krause JS (2000) Brain ischemia and reperfusion: molecular mechanisms of neuronal injury. *J Neurol Sci* 179:1–33
- Xerri C, Zennou-Azogui Y (2003) Influence of the postlesion environment and chronic piracetam treatment on the organization of the somatotopic map in the rat primary somatosensory cortex after focal cortical injury. *Neuroscience* 118(1):161–177
- Xu XL, Hu HZ, Sun T, Yu SB, Ouyang CH, Qu L, Lv Q, Guo LJ (2004) Effects of GABA on synaptic transmission of hippocampus CA3 neurons after brain ischemia in rats. *Chinese Pharm J* 39(9): 666–669
- Yeckel MF, Berger TW (1990) Feedforward excitation of the hippocampus by afferents from the entorhinal cortex, redefinition of the role of the trisynaptic pathway. *Proc Natl Acad Sci USA* 87((15):5832–5836
- Zhou XQ, Liu WH, Li H (2002) Effects of one traditional Chinese medicine on excitatory amino acids, learning and memory in vascular dementia rats. *J Hunan College TCM* 22(2):4–8