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Research Report

Neuroprotective effects of ginsenoside Rb1 on transient cerebral ischemia in rats

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ABSTRACT

Previous experiments showed that ginsenoside Rb1 (GRb1) reduced infarct and neuronal deficit in rats followed by transient cerebral ischemia. The mechanism of this neuroprotective function is unclear. Here, we tested whether the effect of GRb1 can be achieved through preventing ischemic neuronal death, modulating apoptotic-related genes and affecting glial-derived neurotrophic factor (GDNF) expression in rats subjected to occlusion of the middle cerebral artery. When GRb1(40 mg/kg, i.p.) was administered immediately after reperfusion, the apoptotic cells in the GRb1 group were decreased significantly from 12 to 72 h of reperfusion compared to the ischemia group by TdT-mediated dUTP–biotin nick-end labeling. Immunostaining and Western blotting analysis showed that the expression of GDNF from 3 to 120 h of the GRb1 group was significantly increased compared to the ischemia group, and GDNF expression peaked at 48 h after reperfusion. The enhanced GDNF mRNA in the GRb1 group was not detected by RT-PCR and *in situ* hybridization compared to the ischemia group, but GDNF mRNA at 48 h after reperfusion was strongly increased in both the ischemia and GRb1 group when compared to other time points. The number of bcl-2-positive cells was significantly increased from 12 to 120 h of reperfusion compared to the ischemia group. However, the number of bax-positive cells in the GRb1 group was significantly declined compared to the ischemia group. In the GRb1 group, the number of neuronal apoptosis inhibitory protein-positive cells from 12 to 120 h after reperfusion was evidently higher than that in the ischemia group. Therefore, ginsenoside Rb1 prevents ischemic neuronal death induced by transient cerebral ischemia, and this mechanism of which is related to increase the expression of the antiapoptotic genes and modulate the expression of GDNF.

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Abbreviations: CCA, common carotid artery; ICA, internal cerebral artery; TdT, terminal deoxynucleotidyl transferase; GRb1, ginsenoside Rb1; NAIP, neuronal apoptosis inhibitory protein; DAB, diaminobenzidine; NGF, nerve growth factor; RT-PCR, reverse transcription-polymerase chain reaction; MCA, middle cerebral artery; ECA, external cerebral artery; TUNEL, TdT-mediated dUTP–biotin nick-end labeling; MCAO, the occlusion of middle cerebral artery; GDNF, glial-derived neurotrophic factor; PBS, phosphate-buffered saline; BDNF, brain-derived neurotrophic factor

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Ginseng root (*Panax ginseng* C.A. Meyer) has been a very important component of Chinese prescriptions for thousands of years. Ginseng root consists of two major ingredients: crude ginseng saponin and crude ginseng non-saponin fractions. To date, more than 40 ginsenosides have been isolated from the ginseng root and identified chemically. They can be classified into three major groups according to their chemical structures: protopanaxxdiol, protopanaxatriol and oleanolic acid saponins. Among them, ginsenoside Rb1 (GRb1), ginsenoside Rg1 and ginsenoside Ro are the representative substance (Shibata et al., 1985).

Previous experiments have shown that GRb1 is one of the neuroprotective molecules within the ginseng root. GRb1 was found to exert beneficial effects on memory and learning by facilitating cholinergic function and increasing synaptophysin level in the hippocampus (Inhee et al., 2001). GRb1 increased the neurite outgrowth of chick dorsal root ganglia (Nicole et al., 2001) and cultured cerebral cortex neurons (Kim et al., 1998). Against toxic insults, GRb1 protected the neurons in the spinal cord culture from excitotoxicity induced by glutamate and kainic acid. GRb1 also reduced oxidative stress caused by hydrogen peroxide (Liao et al., 2002), and promoted neurite lengths and neurite number of dopaminergic cells after exposure to MPP^+ and glutamate (Radad et al., 2004a; Radad et al., 2004b). GRb1 could increase the expression of trkA mRNA in the basal forebrain and NGF mRNA in the hippocampus, but there was no significant change of BDNF and NT-3 (Salim et al., 1997). The beneficial effects of ginsenosides were mediated through scavenging the free radicals (Lim et al., 1997), improving energy metabolism, and preserving the structural integrity of the neurons (Jiang and Qian, 1995). GRb1 also affectively blocked the calcium over-influx into the neurons (Liu et al., 2001) and inhibited Na^+ channel activity (Liu et al., 2001). Recent reports confirmed that GRb1 attenuated Tau protein hyperphosphorylation of hippocampal neurons in rats, implying that GRb1 has potential neuroprotective effects on Tau-related neuropathology (Li et al., 2005; Zeng et al., 2005).

The previous study showed that GRb1 administration before the occlusion of middle cerebral artery (MCAO) decreased infarct by 20%, as well as reduced the neurologic deficit and inhibited Ca^{2+} accumulation (Zhang et al., 1996). GRb1 significantly prolonged the response latency of ischemic gerbils and rescued a significant number of ischemic CA1 pyramidal neuron, and this neuroprotective activity of GRb1 was confirmed by the electron microscope counts of synapses in individual strata of the CA1 field of ischemic gerbils pretreated with the GRb1 (Wen et al., 1996). These results indicated that GRb1 is one of the neuroprotective molecules that protect brain from ischemia and reperfusion. However, the underlying mechanisms of GRb1 neuroprotection need to be elucidated further. When rats are subjected to MCAO, the main pathological features are neuronal injuries and apoptosis. GDNF is a strong neurotrophic factor for many kinds of neuron, especially for motor neuron and dopaminergic cell. Therefore, in this study, using the transient ischemia model, we tested whether or not neuroprotective effects of GRb1 act through preventing neuronal apoptosis, mediating apoptotic-related genes and GDNF expression.

1. Results

1.1. *Effects of GRb1 infusion on TUNEL-positive cells in rats subjected to 2 h of MCAO and reperfusion*

In the normal and sham-operated groups, about 0–3 TUNEL-positive cells per slide were present in the parenchyma (Fig. 1A). From 3 to 120 h after MCAO, the TUNEL-positive cells were mainly presented in the preoptic area, the striatum, the frontoparietal cortex, and the CA1 of hippocampus in the ipsilateral hemisphere of the ischemia group (Fig. 1B). When infarct of preoptic area was matured at 48 h of reperfusion, most TUNEL-positive cells appeared in the inner boundary of infarct. At 3 h of reperfusion, the number of TUNEL-positive cells was increased, peaked at 24 h, and then declined onward, but the number of TUNEL-positive cells at 120 h after reperfusion was significantly more than that of the control groups ($P<0.01$). Most of the TUNEL-positive cells are neurons and were characteristic of fragmented nuclear. The TUNEL-positive cells also included endothelial cells of the blood vessels, and the epithelial cells of choroid plexus. Compared to the ischemia group, the TUNEL-positive cells in the GRb1 group were strikingly decreased at 12 h after reperfusion, and continued to decrease at 72 h after reperfusion ($P<0.05$, Fig. 1C). These data are shown in Fig. 2.

1.2. *Effects of GRb1 treatment on NAIP expression of cerebral tissue in rats subjected to MCAO and reperfusion*

In the normal and sham-operated rats, NAIP weak immunostaining was diffusely present in the neurons of parenchyma (Fig. 1D), some choroid plexus cells, and the ependymal cells of ventricle. When rats were subjected to MCAO, NAIP-positive cells showed ischemic changes with scalloped or triangled morphological character, and increased peri-cellular space at 12 h and onward after reperfusion. In addition, most NAIP-positive cells were neurons localized in the ischemic and the peri-ischemic areas. Interestingly, a few astrocytes with normal morphology strongly expressed NAIP in the ventral part of the ischemic striatum (Fig. 1E). In contrast, no NAIP-positive cells could be observed in the contralateral striatum (Fig. 1F). The temporal pattern of NAIP expression is shown in Fig. 2. The number of NAIP-positive cells started to increase at 3 h, peaked at 12 h, then declined up to 120 h after reperfusion. Notably, the number of the NAIP-positive cells at 120 h after reperfusion was less than that of the control groups ($P<0.05$).

In the GRb1 group, the distributive pattern and the feature of NAIP-positive cells were similar to those of the ischemia group, but from 12 to 120 h after reperfusion, the number of the positive cells was significantly more than that of the ischemia group ($P<0.01$). Moreover, the number of NAIP-positive cells was increased at 3 h of reperfusion, peaked at 48 h, then declined up to 120 h. Nevertheless, the number of the NAIP-positive cells at 120 h was still more than that of the control groups ($P<0.01$, Fig. 2).

1.3. *Effects of GRb1 infusion on bcl-2 and bax expression in rat subjected to 2 h of MCAO and reperfusion*

In the normal group, there were a few moderate bax-positive cells in the parenchyma and weak bax immunostaining in the

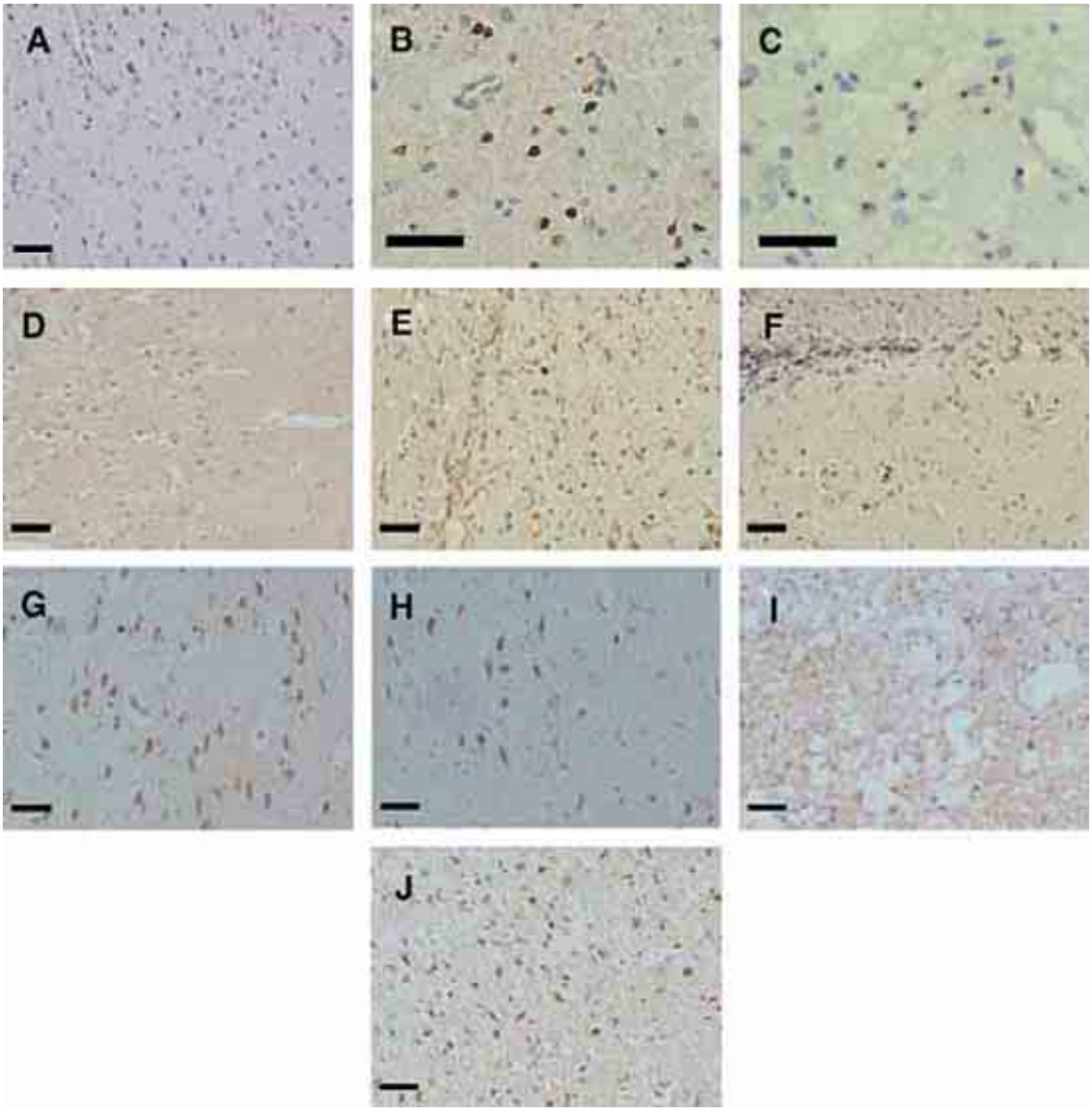

Fig. 1 – Effects of ginsenoside Rb1 on apoptosis and apoptotic-related gene expression in rats subjected to 2 h of middle cerebral artery occlusion and 3 to 120 h of reperfusion (A–C, apoptotic cells by TUNEL; D–F: NAIP-positive cells; G–H, bax-positive cells; I–J: bcl-2-positive cells). (A) No apoptotic cells were observed in the normal rat. (B–C) Apoptotic cells in the frontoparietal cortex at 48 h of reperfusion in the ischemia (B) and in the GRb1 group (C) were observed. (D) Weakly NAIP-positive neurons were present in frontoparietal cortex in the control groups. (E–F) In the GRb1 group, NAIP-positive astrocytes were present in ventral part of the ischemic striatum (E) and in the contralateral striatum (F) at 120 h of reperfusion; (G–H) bax-positive cells were present in striatum at 12 h of reperfusion in the ischemia group (G) and in the GRb1 group (H). (I–J) bcl-2 immunoreactivity was observed in striatum at 120 h of reperfusion in the ischemia group (I) and in the GRb1 group (J) (scale bar = 100 um).

epithelial cells of the choroid plexus. In the sham-operated group, the bax immunostaining was stronger than that of the normal group, but there was no significant difference in the number of bax-positive cells. In the ischemia group, the bax-positive cells presented mainly in the boundary zone of the ischemic core, and there were only very few bax-positive cells in the ischemic core. Moreover, the bax-positive cells were strikingly increased in regions that were not supplied by MCA, such as in the dentate gyrus, the cortical amygdaloid nucleus, the primary olfactory cortex, and the paraventricular thalamic nucleus. Bax-positive cells exhibited normal morphological features in the regions not supplied by MCA, and also exhibited ischemic changes in the ischemic zone. From 3 to 120 h of the reperfusion, the number of bax-positive cells was evidently increased when compared to that in the normal and sham-operated group ($P<0.01$, Fig. 3).

When GRb1 was administered immediately after reperfusion, the only change was the decline of the number of bax-positive cells in each time point when compared to that of the ischemia group ($P<0.05$, Figs. 1G, H and Fig. 3).

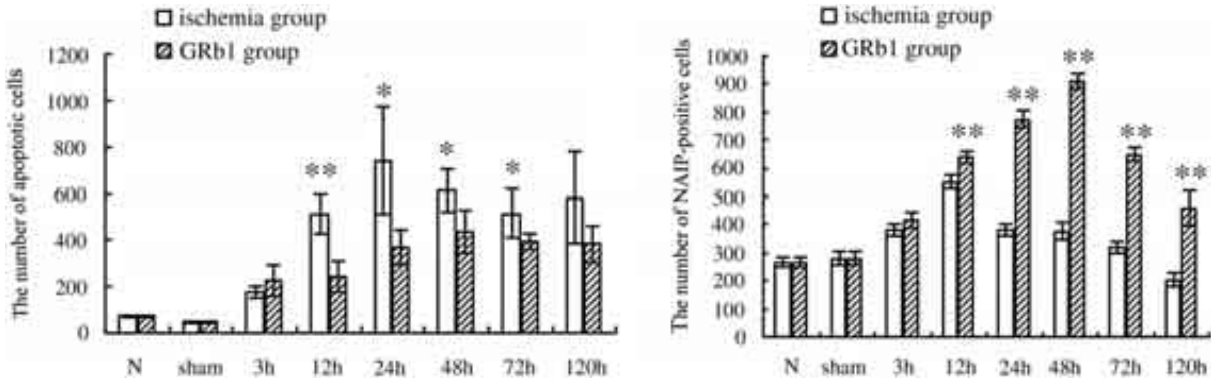

Fig. 2 – Effects of ginsenoside Rb1 on the apoptotic cells and the expression of neuronal apoptosis inhibitory protein (NAIP) in rats subjected to 2 h of middle cerebral artery occlusion and 3 to 120 h of reperfusion. The infusion GRb1 immediately after the onset of reperfusion resulted in a decrease of apoptotic cells but a significant increase in NAIP in comparison with the ischemia group. Note that expression of NAIP in GRb1 group significantly increased compared with the normal and the sham-operated groups. Each value represents mean ± SD (n=4). *P<0.05, **P<0.01 is significantly different from the corresponding time points of the ischemia group.

In the normal and sham-operated group, a very few bcl-2-positive cells were present throughout the parenchyma. Most of the immunoreactivity product existed in the neuroplasm, but a few existed in the neuropil. Bcl-2-positive cells localized primarily at the outer boundary zone of the frontoparietal cortex, the striatum, and the preoptic area when rats were subjected to MCAO and 3- to 120-h reperfusion. Bcl-2-positive cells hardly showed any ischemic change. Surviving neurons and activated glial cells strongly expressed bcl-2 protein. The number of bcl-2-positive cells in the given time was significantly higher in comparison with the control groups (Fig. 3).

In the GRb1 group, the salient change was the significant increase of the number of bcl-2-positive cells at 12 h of reperfusion and onward in comparison with the same time point of the ischemia group (P<0.05, Figs. 1I, J). These data are shown in Fig. 3.

1.4. *Effects of GRb1 treatment on GDNF mRNA expression of cerebral tissue in rats subjected to MCAO and reperfusion in situ hybridization and by RT-PCR*

The low hybridization signals were primarily localized on the axon and the membrane of the neurons in the normal and sham-operated groups (Fig. 4A). From 3 to 120 h of reperfusion, the enhanced GDNF hybridization signals appeared mainly in the fasciculus, which were located in the ischemic regions (Fig. 4B). Strangely, strong hybridization signals were detected in the swollen and fragmented fasciculus in the striatum (Fig. 4C). Weak GDNF mRNA was detected in some neurons located in the ischemic regions, such as in the corpus striatum, the frontalparietal cortex, and the hypothalamus. The hybridization signals were maximal at 48 h after reperfusion (Fig. 5).

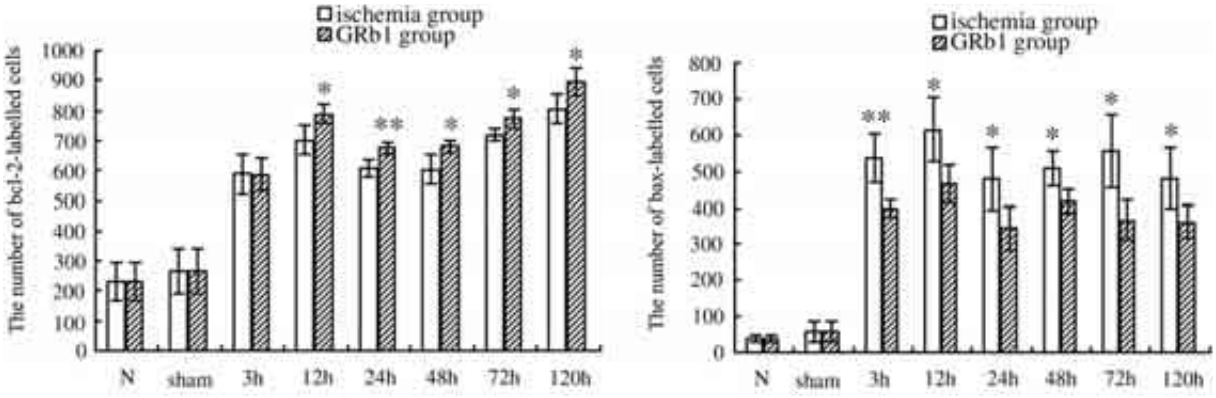

Fig. 3 – Effects of ginsenoside Rb1 on bcl-2 and bax expression in rats subjected to 2 h of middle cerebral artery occlusion and 3 to 120 h of reperfusion. The infusion GRb1 immediately after the onset of reperfusion resulted in a significant increase in the expression of bcl-2 and a significant decrease in the expression of bax in comparison with the ischemia group. Each value represents mean ± SD (n=4). *P<0.05, **P<0.01 is significantly different from the corresponding time points of the ischemia group. Note that the expression of bcl-2 and bax in both the GRb1 treatment and the ischemia group enhanced significantly compared with the normal and the sham-operated group (P<0.01).

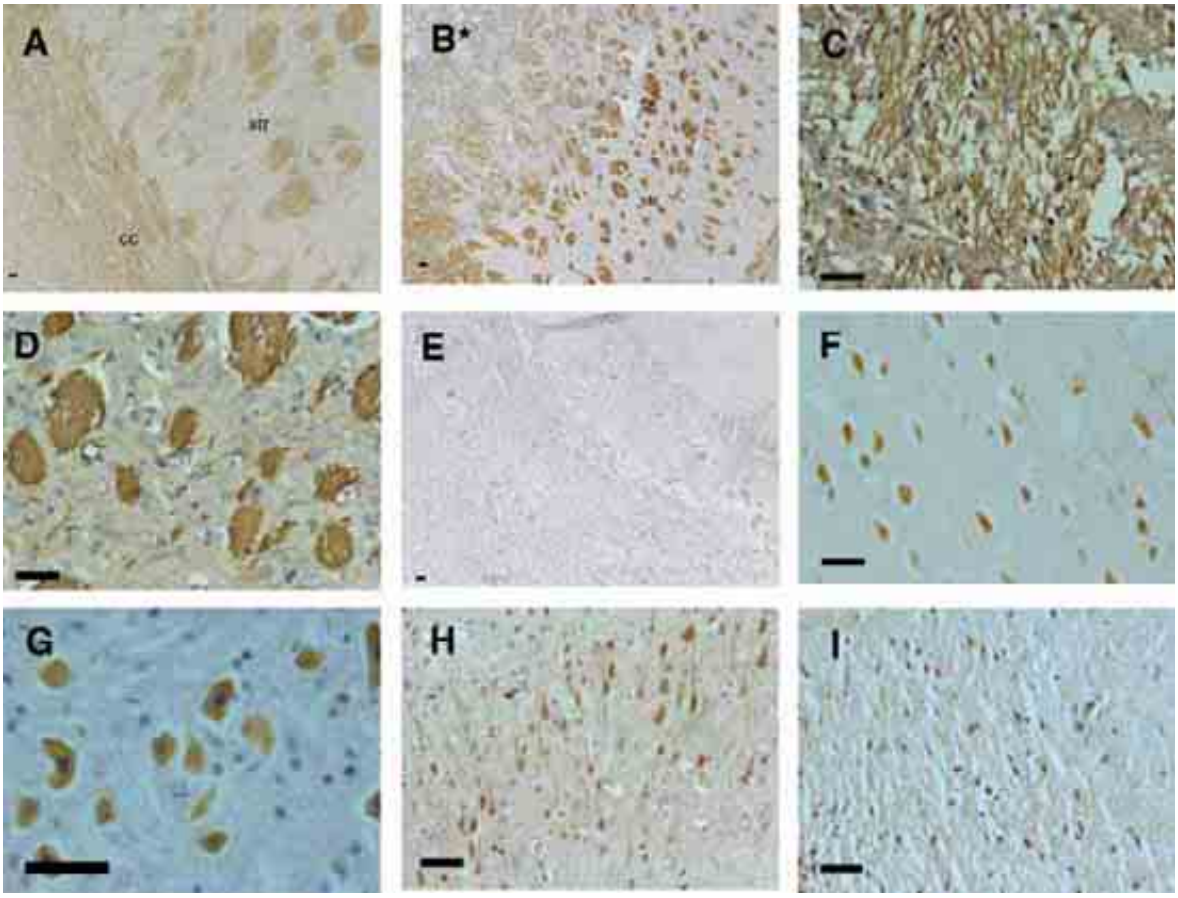

Fig. 4 – Effects of ginsenoside Rb1 on glial-derived neurotrophic factor (GDNF) mRNA (A–E, *in situ* hybridization) and GDNF protein (F–I, immunohistochemistry) expression in rats subjected to 2 h of middle cerebral artery occlusion and 3 to 120 h of reperfusion. (A) GDNF hybridization signals of the hemisphere in normal rat. (B) Enhanced GDNF hybridization signals in the ischemic areas at 24 h after ischemia. (C) In the ischemia group, strong GDNF hybridization signals were present in the swollen and fragmented fasciculus in the striatum at 120 h after reperfusion. (D) In the GRb1 group, strong GDNF hybridization signals were present in fasciculus of striatum at 120 h after reperfusion; (E) no any GDNF hybridization signal was observed when the GDNF cDNA probe was omitted in the hybridization solution at 72 h of reperfusion in ischemia group. (F) GDNF immunostaining was present in neurons in the ischemic cortex at 48 h of reperfusion in the ischemia group. (G) Neurons in thalamus (not supplied by MCA) strongly expressed GDNF at 48 h of reperfusion in the ischemia. (H) In the GRb1group, enhanced GDNF immunostaining appeared in the peri-frontoparietal cortex at 48 h of reperfusion. (I) In the GRb1 treatment, GDNF strong immunoreactivity was presented in the glial cells in the infarcted area of the preoptic area at 48 h after reperfusion. (I) Neurons in thalamus (not supplied by MCA) strongly expressed GDNF at 48 h of reperfusion in the ischemia group. cc: Corpus callosum, str: striatum (scale bar = 100 um).

In the GRb1 group, the distributive pattern of GDNF mRNA was similar to that of the ischemia group (Fig. 4D). The hybridization signals in the corresponding time points of the GRb1 group showed no striking increase compared to those of the ischemia group (Fig. 5). No GDNF hybridization signal was observed when GDNF cDNA probe was omitted (Fig. 4E).

The temporal profile of GDNF mRNA expression by RT-PCR is shown in Fig. 6. Fig. 6 shows that in the ischemia group, the expression of GDNF mRNA was higher at 3 h and 48 h after reperfusion than other subgroups. In the GRb1 group, from 3 to 120 h after reperfusion, the higher expression of GDNF mRNA was observed at 48 h after reperfusion. Compared with the ischemia group, no evident change of GDNF mRNA in GRb1 subgroups was observed except at 3 h after reperfusion.

1.5. *Temporal profile of GDNF protein expression by immunostaining and Western blotting*

Faint GDNF immunoreactivity was present throughout the parenchyma in the normal and the sham-operated groups. The GDNF-positive cells were neurons. At 3 h after reperfusion, the number of moderate GDNF-positive neurons was rapidly increased in the ischemic frontoparietal cortex and the striatum, and a few weak GDNF-positive neurons were present in the preoptic area. At 48 h of reperfusion, the number of positive cell appeared mainly in the ischemic frontoparietal cortex (Fig. 4F), and only a few of the positive cells in the striatum and the preoptic areas appeared at the ipsilateral hemisphere. Moreover, the color of the staining was stronger when compared with other subgroups. A striking feature at

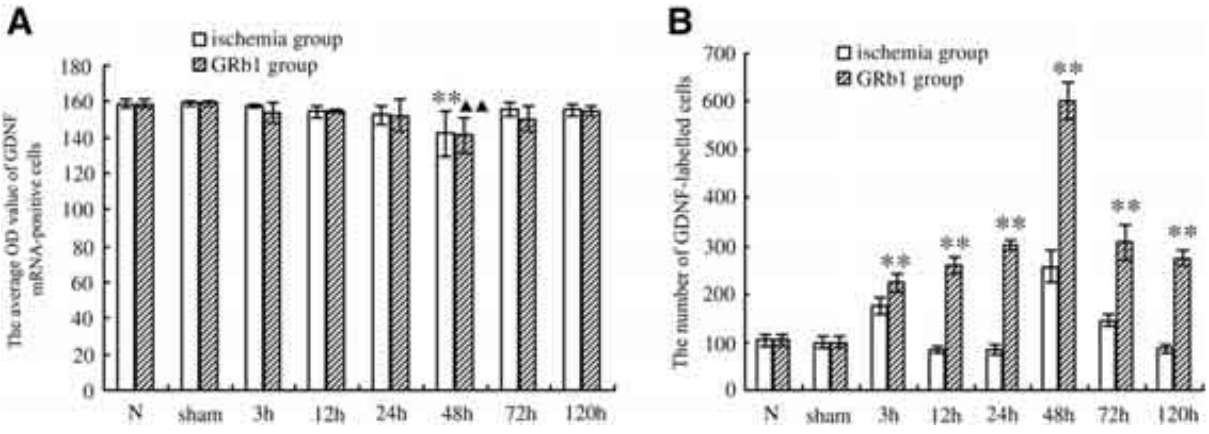

Fig. 5 – Effects of ginsenoside Rb1 on glial-derived neurotrophic factor GDNF mRNA expression (A) and protein (B) in rats subjected to 2 h of middle cerebral artery occlusion and 3 h to 5 days of reperfusion. No statistically significant difference of GDNF mRNA was observed at any time point of reperfusion between the ginsenoside Rb1 group and the ischemia group. But note that GDNF mRNA at 2 days after reperfusion was significantly higher than those of other subgroups within the ischemia group and the ginsenoside Rb1 group(,▲▲ $P<0.01$ vs. other time points of ischemia and GRb1 group, respectively). In contrast, there was a significant increase in the expression of GDNF protein in the subgroups of GRb1 treatment in comparison with that of the ischemia subgroups. Each value represents mean ± SD ($n=4$). ** $P<0.01$ vs. the ischemia group.**

this stage was the presence of GDNF immunostaining in the glial cells. From 3 to 120 h after reperfusion, GDNF staining was localized in some neurons at the outer boundary zone of the ischemic lesion, regions not supplied by MCA (Fig. 4G), and most of the choroid plexus cells in the ventricle. Fig. 5 shows that the number of GDNF-positive cells started to increase at 3 h, declined from 12 to 24 h, then increased again at 48 h, and finally decreased up to 120 h after reperfusion.

In the GRb1 treatment, the distributive pattern was similar to that of the ischemia group (Fig. 4H). An apparent change was an increase in the GDNF-positive glial cells, especially in the ischemic regions (Fig. 4I). This indicated that the activated glial cells expressed GDNF. In contrast, the number of GDNF-positive cells elevated at 3 h after reperfusion, continued to increase until 48 h, then decreased up to 120 h of reperfusion (Fig. 5). Nevertheless, the number of GDNF-positive cells from 3 to 120 h after reperfusion was significantly more than that of control groups ($P<0.01$). Compared with the ischemia group, the number of the GDNF-positive cells in the different time points of the GRb1 group was significantly increased ($P<0.01$, Fig. 5).

As shown in Fig. 7, compared to the normal and sham groups, cerebral ischemia in rats subjected to MCAO and GRb1 treatment also significantly induced the GDNF protein expression by Western blotting analysis. GDNF expression peaked at 48 h after reperfusion in both the ischemia and GRb1 group. Compared with the ischemia group, the GRb1 treatment significantly enhanced the GDNF protein during 3 to 120 h of reperfusion ($P<0.05$, Fig. 7).

2. Discussion

The present study demonstrated that GRb1 infusion after MCAO (at 40 mg/kg, i.p.) significantly decreased TUNEL-positive cells, increased bcl-2, NAIP and GDNF protein expression, and inhibited bax expression. However, no significant change of GDNF mRNA was observed.

2.1. *The GRb1 prevents ischemic neuronal death by modulating expression of NAIP, bcl-2 and bax*

It is well accepted that neurons after cerebral ischemia will suffer necrosis and apoptosis. Our study demonstrated that transient ischemia caused the increase of TUNEL-positive cells with fragmented nuclear. This result was in agreement with our previous study (Yuan et al., 1999). Li et al. (1995) also reported that the number of cells exhibiting DNA fragmentation increased as early as at 0.5 h, peaked at 24–48 h, and persisted for 4 weeks after the onset of reperfusion, and thus suggested that apoptosis contributed to the development of ischemic infarct. In contrast, GRb1 administration after MCAO decreased the number of TUNEL-positive cells ($P<0.05$ vs. the ischemia group). The result suggested that GRb1 prevents apoptotic-like neuronal death caused by cerebral ischemia in rats. Recently, other studies also reported that GRb1 infusion decreased the number of TUNEL-positive cells in the spiral ganglion cells after cochlear ischemia (Fujita et al., 2007). Zhang et al. showed that GRb1 rescued cortical neurons in the ischemic penumbra and reduced the cortical infarct volume by approximately 50%, and the number of TUNEL-positive cells in the hippocampal CA1 field in GRb1-infused ischemic gerbils was fewer than those in vehicle-infused ischemic animals (Zhang et al., 2006). The present results were the consequence of the previous studies in which GRb1 increased the survival of dopaminergic cells (Radad et al., 2004a), and promoted the survival of the cortex neurons in chick and rat embryonic cell culture (Himi et al., 1989).

The underlying mechanisms by which GRb1 reduced ischemic neuron death are still unclear. The pathophysiological mechanisms of ischemic neuronal injury involve the glutamate excitotoxicity and the Ca^{2+} overload, the free

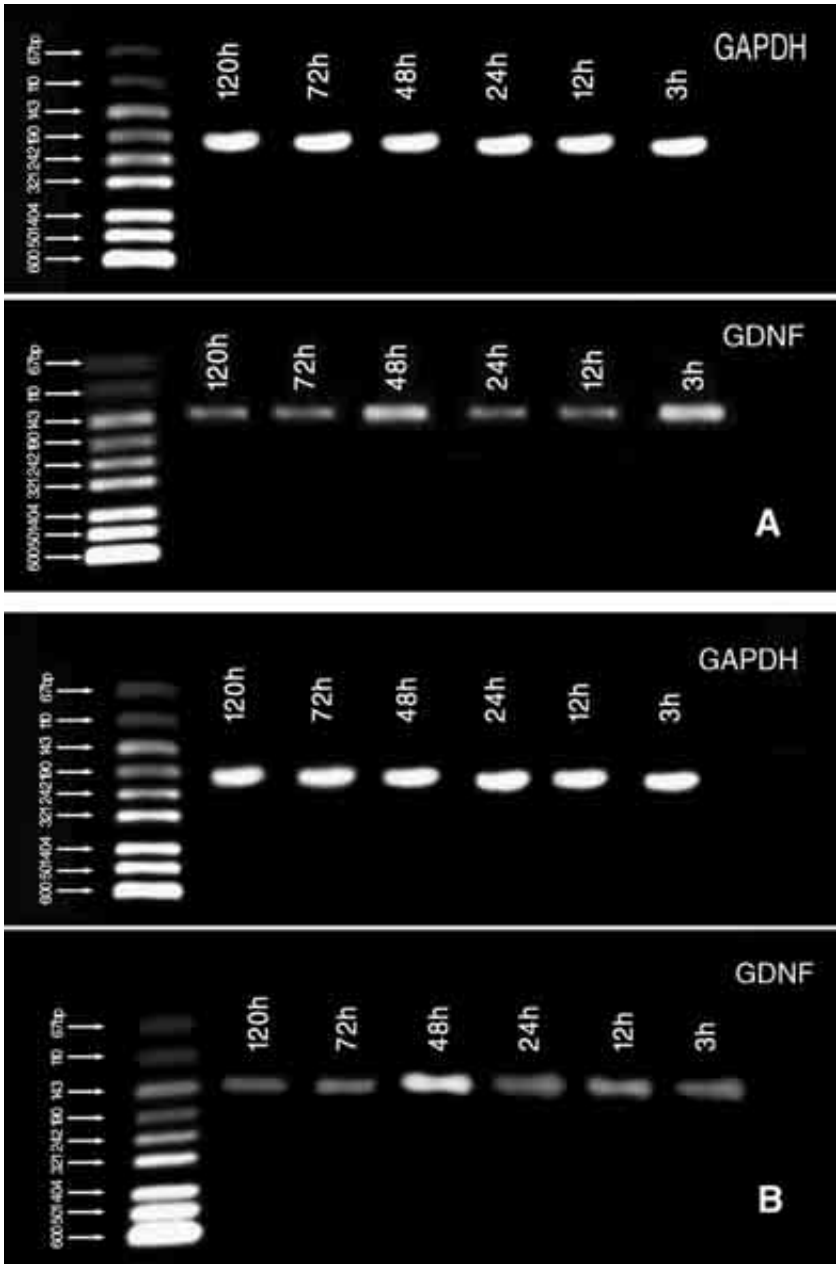

Fig. 6 – RT-PCR products were loaded on the 1.2% agarose gels, and DNA bands were stained with ethidium bromide. A 100-bp DNA ladder was used to identify the size of the products. (A) Ischemia group; (B) ginsenoside Rb1 treatment. GAPDH was the internal control.

radicals, and the changes of genes expression. Up to now, there was no report of whether or not the neuroprotective effect of GRb1 against ischemic cerebral injury in rats involved in the changes of the apoptotic-related genes and the GDNF expression. It is well known that bcl-2 protein prevents apoptosis and bax protein induces apoptosis. The present study showed that the number of bcl-2-positive cells after the infusion of GRb1 was significantly higher at 12 h of reperfusion and onward than that of the ischemia group. In contrast, the number of bax-positive cells strongly declined in the same time points compared to the ischemia group. The results were similar to the study of Diao et al. (2005) who reported that the preconditioning ischemia could induce an increase of bcl-2 and decrease of bax. Diao et al. suggested that the increased bcl-2 and the decreased bax might be one of the mechanisms in antiapoptosis. In addition, Nie et al. (2004) reported that the administration of GRb1 into the primary cultured cerebral cortical neurons by hypoxia dropped the apoptosis rate of neurons, by way of increasing the bcl-2 protein and decreasing Bax protein. Zhang et al. demonstrated that GRb1 prevents neuronal apoptosis through upregulation of the antiapoptotic factor, Bcl-xL, both *in vivo*, and *in vitro*, which is known to suppress activation of procaspase-9 by forming a complex with Apaf-1. This, in turn, prevents the release of cytochrome *c*

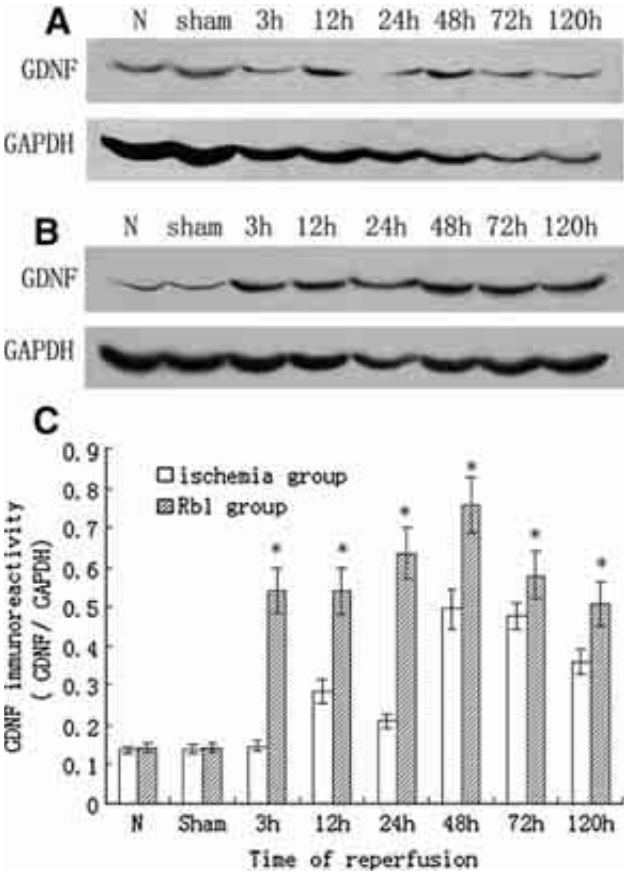

Fig. 7 – Western blot analyzing effects of ginsenoside Rb1 on glial-derived neurotrophic factor GDNF protein in rats subjected to 2 h of middle cerebral artery occlusion and 3 h to 5 days of reperfusion. (A) Ischemia group; (B) ginsenoside Rb1 treatment. GAPDH was the internal control. (C) Statistical analysis according to the results of Western blotting. During 3 to 120 h after reperfusion, GDNF protein in the GRb1 group was significantly increased compared with the ischemia group. Each value represents mean ± SD (n=3). *P<0.05 vs. the ischemia group.

from mitochondria, thereby maintaining cell viability and cell survival (Zhang et al., 2006). Recently, Fujita indicated that postischemic administration of GRb1 preserved the configuration of the spiral ganglion and decreased the number of TUNEL-positive cells with obvious expression of Bcl-xL 1 day after ischemia. They postulated that GRb1 suppressed apoptotic cell death in SGCs by activating the Bcl-xL signaling pathway (Fujita et al., 2007). Therefore, the experiments *in vivo* and *in vitro* suggested that the neuroprotective effects of GRb1 were through the upregulation of bcl-2 and Bcl-xL protein expression and the downregulation of Bax-2 protein expression. GRb1, by way of enhancing the ratio of bcl-2 to bax protein, showed neuroprotective role in rats subjected to MCAO.

Neuronal apoptosis inhibitory protein (NAIP/BIRC1), the inhibitor of apoptosis protein (IAP) family member, suppresses neuronal cell death induced by a variety of insults, including cell death from ischemia and stroke. Reducing NAIP expression via RNA interference techniques resulted in the prevention of L-745,870-mediated protection from oxidative stress. Further, systemic administration of L-745,870 attenuated ischemia-induced damages on the hippocampal CA1 neurons, and upregulated NAIP expression in the rescued hippocampal CA1 neurons in a gerbil model (Okada et al., 2005). The present study first demonstrated that after GRb1 was administrated, the number of NAIP-positive cells at the same time points after ischemia was significantly more than that of the ischemia group. NAIP is an endogenous inhibitor of apoptosis, inactivating caspase-3 and caspase-7 in neuronal tissues. Lesne et al. (2005) demonstrated that NT-3 induced an upregulation of neuronal apoptosis inhibitory protein-1 expression in neurons that promoted the inhibition of amyloid beta-induced neuronal apoptosis. Thompson et al. (2004) reported that systemic administration of kainic acid rapidly elevated NAIP mRNA expression in the hippocampus in mice that lack tumor necrosis factor-alpha receptors, and thus suggested that the induction of the *NAIP* gene contributed to the neuroprotective properties of TNF. Given that NAIP overexpression could reduce neuronal injury by blocking apoptosis, our findings suggest that ischemia may induce NAIP expression to block apoptosis, and the administration of GRb1 may significantly induce the NAIP protein to prevent neuronal apoptosis. Moreover, a few astrocytes in ischemic regions strongly expressed NAIP when rats were subjected to ischemia–reperfusion. This is in agreement with the idea that astrocytes are more endurable than neurons during ischemia injury. The results, from another aspect, further testified that NAIP protein has the potential to inhibit neuron apoptosis.

2.2. *The neuroprotective effects of GRb1 on transient cerebral is to increase the expression of GDNF*

Neurotrophins are a family of the growth factors that attenuate several forms of pathological neuron death. GDNF is a strong neurotrophic factor for motor neuron and other kinds of neuronal cells. Kitagawa et al. (1998) reported that the administration of GDNF may prevent ischemic brain injury after MCAO. Recent study showed that the mechanisms (Nicole et al., 2001) underlying GDNF neuroprotective role in apoptosis involved reducing caspase-3 and nitric oxide synthase activity, increasing heat shock protein, bax-2, and bcl-xl expression, and upregulating superoxide dismutase, glutathione peroxidase and catalase against injuries by the free radical and antioxidative properties. Further, GDNF also reduced the NMDA-induced calcium influx by the mitogen-activated protein kinase pathway. It was reported that GDNF could induce progenitor cell proliferation in adult rats following experimental stroke (Deng and Zhang, 1991; Kobayashi et al., 2006). All these studies showed that GDNF had effectively prevented the development of cerebral injury during cerebral ischemia.

There is a disagreement of the temporal file and the cell location of the GDNF expression in the brain of rat. Our previous study demonstrated that focal cerebral ischemia had induced GDNF protein expression in rat at 3 h and had lasted for 48 h of reperfusion in the peri-ischemic regions and the regions not supplied by MCA (Yuan et al., 2001a,b). We observed that many weak GDNF-positive cells were present

in the normal and the sham-operated groups, and activated microglia strongly upregulated GDNF expression when the rats were subjected to MCAO. Abe and Hayashi (1997) reported that the GDNF mRNA and protein began to be induced in the occluded MCA at 1 h of reperfusion with a peak at 3 h, and almost diminished by 1 day of reperfusion determined by Northern blot analysis and immunohistochemistry. No GDNF was observed in the normal and the sham-operated groups. Abe and Hayashi also reported that the GDNF-positive cells were mainly neurons in the cortex and the striatum, and no glial cell was stained in the brain sections. However, Miyazaki et al. (2001) reported that transient forebrain ischemia-induced reactive astrocytes, as well as the surviving neurons, produced GDNF in 3–7 days after the ischemia in rats. These discrepancies could be due to the different kinds of rat, different ischemic extent, and the different technical sensitivity in different laboratories. In this study, the surviving neurons and activated glial cells strongly expressed GDNF in rats subjected to MCAO, and these results were in agreement with our previous study (Yuan et al., 2001a,b) and Miyazaki et al. (2001).

The present study showed that GRb1 did not significant increase GDNF mRNA by RT-PCR and *in situ* hybridization analysis. Instead, it significantly enhanced the GDNF protein expression by immunohistochemistry and Western blotting. The non-synchronization of mRNA and protein expression could be due to the time that GRb1 was administered, and due to the fact that *GDNF* gene rapidly transcribed in response to the acute cerebral ischemia. In this study, GRb1 was intraperitoneally infused immediately at the onset of reperfusion. It took some time for GRb1 to filter into the cerebral tissue by way of blood cycle and cross the blood–brain barrier. There was a possibility that the *GDNF* gene transfer had been finished within 2 h of ischemia, and GRb1 therefore could not affect the mRNA expression of GDNF, but only influenced the GDNF protein translation. Whereas, the present hypothesis requires to be tested further by quantitative RT-RCR to detect the expression of *GDNF* gene in rats subjected to transient cerebral ischemia.

In conclusion, ginsenoside Rb1 could decrease the neuron apoptosis induced by cerebral ischemia in the rat subject to MCAO, and the neuroprotective mechanisms of ginsenoside Rb1 may be involved in increasing the GDNF, NAIP, bcl-2 protein expression and reducing the bax expression.

3. Experimental procedures

3.1. *Animals*

Adult male or female Wistar rats weighing 250–300 g were used in all experiments (inbred strain, Animal House Center, Sichuan University, Chengdu, Sichuan, PR China). Animals were housed in a colony room under controlled temperature, humidity, and a 12-h light/dark cycle, with food and water available. Animal care and experimental protocols were approved by the Chinese Academy of Sciences, PR China, ensuring that animal numbers and suffering were kept to the minimum. All the experiments were conformed to the international guidelines on the ethical use of animals.

3.2. *Materials*

GRb1 (purity>98%, 110704–200318) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing). Monoclonal anti-mouse bax, bcl-2, polyclonal goat anti-mouse neuronal apoptosis inhibitory protein 1(NAIP), and polyclonal anti-rabbit GDNF were purchased from Santa Cruz Bioctechnology. Antibody binding was detected by using the SP Kit (commercially available Zymed, USA). Total RNA was extracted with the TRIZOL Reagent (Invitrogen, USA). cDNA Synthesis Kit and PCR Kit were from Fermantas (Life Science). Enhanced Sensitive ISH Detection Kit I (Wuhan Boster Biological Technology Co., Ltd. Mk1030, China) was employed to investigate the GDNF mRNA-positive cells in sections.

3.3. *Transient cerebral ischemia model and tissue preparation*

Transient cerebral ischemia was induced by occlusion of middle cerebral artery (MCAO) as previously described (Zea et al., 1989). Briefly, rats were anesthetized with 1% pentobarbital in 0.9%NaCl (30 mg kg^{-1}, i.p.). Under sterile conditions, a ventral neck incision was made and the external carotid artery (ECA), the internal carotid artery (ICA) and the common carotid artery (CCA) were exposed and carefully isolated. A nylon monofilament (18.5–19 mm in length and 0.20 mm in diameter), whose tip was rounded by flame heating, was inserted from the lumen of the ECA to the lumen of ICA to occlude the origin of the right middle cerebral artery (MCA).Two hours after MCAO, the rats were reanesthetized with aether, and reperfusion was performed by withdrawing the intraluminal suture until the tip cleared the ICA lumen and was retained in the stump of the CCA. During surgical procedure of 2-h MCA occlusion, rectal temperature was maintained at 37.0±0.2 °C. Previous results demonstrated that mean arterial blood pressure and the brain temperature were not affected by MCAO or GRb1 infusion (Zhang et al., 2006).

All rats with MCAO exhibited neurological deficits, which were characterized by failure to extend the left forepaw. The rats with neurological deficits were randomly divided into two groups: the ischemia and GRb1 treatment group (GRb1 group). GRb1 (40 mg kg^{-1}) was dissolved in isotonic saline and injected intraperitoneally immediately after the onset of reperfusion; the same volume of saline was injected in the ischemia group. The dose of peripherally administered ginsenoside Rb1 was chosen on the basis of the studies of Zhang et al. (1996, 1990) and Wen et al. (1996). Four rats served as sham-operated controls in which a 15-mm-long nylon monofilament was inserted into the ICA for 2 h. This length of the nylon monofilament was too short to occlude the MCA, and these rats did not exhibit left-side neurological deficits. Four normal rats served as normal control.

Experimental rats were given an overdose of pentobarbital, and sacrificed at 3 h, 12 h, 1, 2, 3, 5 days (n=4–5 per time point). Four rats that served as sham-operated controls were killed at 48 h after withdrawing the intraluminal suture. Rat brains were fixed by transcardial perfusion with saline, followed by perfusion and immersion in the 4% paraformaldehyde in 0.1 mol/l phosphate-buffered saline. A coronal section was

obtained from each rat, and paraffin-embedded coronal slides (5 μm) were cut.

Additional rats (n=2 per time point) were analyzed by RT-PCR for the determination of GDNF mRNA. Experimental rats were sacrificed by decapitation at specified time points following the ischemic paradigm, and their brains were removed for RNA analysis. Cortical regions and striatum of ipsilateral hemisphere for RNA analysis were frozen immediately in liquid nitrogen after removal and they were frozen at −80 °C. Total RNA was extracted from samples without thawing.

3.4. *RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR) analysis*

Total RNA was extracted from the ischemic cerebral tissue by using the TRIZOL Reagent. 2 μg of total RNA was used for cDNA synthesis, using random hexamer primer (0.2 μg/μl) 1 μl and 10 mM dNTP mix (Invitrogen) and M-MLV Reverse Transcriptase (RT, 200 U/reaction; Promega) at 25 °C for 10 min, 42 °C for 60 min. The reaction was stopped by heating at 70 °C for 10 min. Finally, the product was placed on ice. 10 μl of cDNA was used in each subsequent PCR amplification, in an automatic thermocycler, with 2.5 U/reaction of Platinum Taq DNA polymerase and corresponding primers. Reaction conditions were 95 °C for 6 min followed by 33 cycles of 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 1 min. The final extension step was at 72 °C for 7 min. PCR product was analyzed electrophoretically on a 1.2% agarose gel. The ethidium bromide was used to visualize the bands.

The following oligonucleotides were used as PCR primers: GDNF forward primer: 5′-CTGACGAGTGACTCCAATAT-3′, GDNF reverse primer 5′-CCTTTCCCTCTGGAATTCTCT-3′, expected length of GDNF cDNA is 147 bp; and GAPDH was the internal control. GAPDH forward primer: 5′-TGGGTGTGAACCACGAGAA-3′, GAPDH reverse primer: 5′-GGCATGGACTGTGGTCATGA-3′. Expected length of GAPDH cDNA is 187 bp.

3.5. *Preparing for Dig-GDNF-cDNA probe*

A cDNA clone encoding rat GDNF was generated using PCR, and the first-strand cDNA was synthesized from total RNA isolated from the brain tissue of the newborn SD rat. The design of *GDNF* gene-specific primers was based on the published rat GDNF cDNA sequence. The following primers were utilized in the PCR reaction: 5′-TTTGGTACCATGAAGTTATGGGATGTCGT-3′; 5′-TTTAAGCTTTCAGATACATCCACACCGTT-3′. The product of PCR and pAdTrack CMV shuttle vector were cut by *Hin*dI and *Kpn*I, and were ligated by T4 ligase to form the GDNF recombination plasmid. Its authenticity was confirmed by DNA sequence analysis. For use in *in situ* hybridization, cDNA of GDNF was labeled with digoxin–dNTP by PCR: 5 μl 10×PCR buffer, 5 μl 25 mM $MgCl_2$, 2 μl Dig–dNTP Mix, 10 μl GDNF recombination plasmid, 2 μl GDNF primers, 0.6 μl Taq DNA polymerase (3 U/μ1). Reaction conditions were 94 °C for 6 min followed by 33 cycles of 94 °C for 10 s, 50 °C for 20 s, and 72 °C each for 1 min. The final extension step was at 72 °C for 5 min. The resulting PCR product was analyzed electrophoretically and purified, and was diluted with prehybridization solution. The efficiency of cDNA was tested according to commercial procedures.

3.6. *In situ hybridization analysis*

After deparaffinizing, brain sections were treated with 3% H_2O_2 for 10 min at room temperature to eliminate endogenous peroxidase. After washing with distilled water, the sections were treated with fresh pepsin to expose mRNA for 5 min at 37 °C. The sections were prehybridized for 3 h at 40 °C in prehybridization solution (lacking cDNA probe). Hybridization was carried out at 42 °C overnight with 5 μ1 of denatured cDNA probe. The sections were rinsed twice in 2× SSC for 5 min at 37 °C, followed by in the 0.5× SSC and 0.2× SSC wash for 15 min at 37 °C. After being treated with blocking solution, sections were treated with biotinylated secondary antibody against Digoxin for 60 min at 37 °C. After being washed in 0.5 M phosphate-buffered saline (PBS) three times, the sections were treated with SABC (avidin–biotin complex) for 20 min at 37 °C. The resulting sections were visualized by diaminobenzidine (DAB) and were counterstained with Mayer's hematoxylin. Finally, the sections were dehydrated with alcohol, fixed with glycerine gelatin, and were observed under microscope. The procedures of the negative control sections were carried out in similar way but only the cDNA probe was omitted.

3.7. *Immunohistochemistry analysis*

After deparaffinization, sections were placed in boiled citrate buffer (pH 6.0) in a microwave oven (650 to 720 W) to detect the bcl-2 and bax proteins or they were treated with an aqueous solution of 0.1% trypsin and 0.1% $CaCl_2$ pH7.8 to expose the antigen at 37 °C for 45 min for detecting GDNF and NAIP protein. After being blocked in normal 5% horse serum at 37 °C for 30 min, sections were treated with monoclonal primary antibodies against GDNF (dilution 1:100). After sequential incubation with peroxidase conjugated rabbit anti-mouse IgG (dilution 1:100), DAB was then used as a chromogen for light microscopy. Counterstaining of the sections by hematoxylin was also performed. All incubations were performed in a humidified chamber. Negative control sections from each animal was identically stained, except that the primary or the secondary antibody was omitted.

3.8. *In situ detection of DNA fragmentation (TdT-mediated dUTP–biotin nick-end labeling (TUNEL) staining)*

TUNEL was employed to identify the number of cell exhibiting DNA fragmentation by means of light microscope as previously described (Gavrieli et al., 1992). Briefly, after deparaffinizing, the protein in brain sections was digested using the proteinase K and quenching endogenous peroxidase activity with 2% H_2O_2 in PBS. The slides were placed in the equilibration buffer and then in working-strength TdT enzyme, followed by working-strength stop/wash buffer. After 2 drops of anti-digoxigenin-peroxidase were applied to the slides, peroxidase was detected with DAB. Negative controls were performed using the distilled water for TdT enzyme in the preparation of working-strength. Sections were counterstained with hematoxylin.

3.9. *Western blotting analysis for GDNF protein*

Rats in each experimental group were decapitated, and forebrain tissue was immediately obtained. Each specimen was dissected on a bed of ice into hemisphere ipsilateral to the MCAO. The segments were quick frozen in isopentane and stored at −80 °C until homogenization. Then, these segments were thawed on ice, and wet weight in grams was rapidly measured. The tissue pieces were homogenized by adding a 1:5 tissue weight to protein extraction buffer, containing 0.9% NaCI, 7 mM β-mercaptoethanol, 50 mM Tris–HCl (pH 7.5), 2 mM EDTA, and 1% sodium dodecylsulphate (SDS), in a glass homogenizer. The protein concentration of samples was determined using a BCA protein assay kit (Beyotime Institute of Biotechnology, China). Homogenate samples (50 μg) were mixed with an equal volume of a sample buffer and heated at 95 °C for 5 min and resolved by SDS–7.5% polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose membranes. Blots were blocked at room temperature for 1 h in a blocking buffer, Tris-buffered saline-Tween (TBS-T) containing 5% dried milk. Blots were incubated in the primary antibody, mouse anti-rat GDNF (A&D, USA), diluted in the blocking buffer (dilution: 1 μg/ml) for 1 h at room temperature and were washed extensively with TBS-T. They were then incubated in a horseradish peroxidase-conjugated secondary antibody, diluted in the blocking buffer (dilution: 1:1000) for 1 h and washed for an additional hour. The protein band of interest was visualized using an ECL chemiluminescence system (ECL plus; Amersham Biosciences, NJ, USA) and the density of each band was quantified by using an image analysis software.

3.10. *Image analysis and statistical analysis*

The number of immunoreactive cells and apoptotic cells in the coronal section from bregma −0.2 to −1.4 mm was counted in 3 to 4 sections per animal. The number of positive cells (for immunohistochemistry) or optical density of hybridization signals was measured on each section using a computer-based C-CCD camera system (Olympus). The higher the optical density is, the lower the hybridization signals are. The density of positive cells in each section was calculated in the cortex, striatum and the preoptic area of ipsilateral cerebral tissue, respectively.

Data are presented as means ± SD. Differences between mean values for different time points within a group were analyzed using one-way ANOVA, followed by post hoc tests. And independence sample *t*-test was performed to detect the difference between mean values for the same time points between the ischemia group and the GRb1 group. Statistical significance was set at $P<0.05$.

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