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Ginkgo biloba Leaf Extract (EGb761) Combined with Neuroprotective Agents Reduces the Infarct Volumes of Gerbil Ischemic Brain

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Abstract: Ginkgo biloba exerts many pharmacological actions. It possesses antioxidant properties, the ability of neurotransmitter/receptor modulation and antiplatelet activation factor. This research is designed to investigate the neuroprotective effects of long-term treatment with EGb761 (a standard form of the extract of Ginkgo biloba leaf) in combination with MgSO₄, FK506, or MK-801 on the infarct volume of male gerbils' brain induced by unilateral middle cerebral artery occlusion (MCAO). Thirty-five gerbils fed a standard diet were intragastrically given water or EGb761 (100 mg/kg/day) for one week. Five randomized groups were established: control (n = 7), EGb761 (n = 8), EGb761 + MgSO₄ (n = 7), EGb761 + FK506 (n = 7), and EGb761 + MK-801 (n = 6). The three drug-combination groups were injected with MgSO₄ (90 mg/kg), FK506 (0.5 mg/kg), or MK-801 (1 mg/kg), respectively 30 min before MCAO. Gerbils were anesthetized and craniectomized to expose the right middle cerebral artery (MCA). The right MCA was constricted with an 8-0 suture to produce a permanent ligation for 24 hours. Postmortem infarct volumes were determined by quantitative image analysis of 2,3,5-triphenyltetrazolium chloride (TTC)-stained brain sections. Results showed that the total infarct volumes of the four treated groups either EGb761 alone or in combination with drugs were lower than the control group by 36.1% (EGb761 alone), 40.3% (EGb761 + MgSO₄), 35.3% (EGb761 + FK506), and 56.4% (EGb761 + MK-801), respectively (p < 0.01). The main affected areas of the brain in the four treated groups were significantly focused between 4 and 6 mm from the frontal pole, when compared to the control group.
(p < 0.01). All animals in the five groups had infarctions in both cortex and subcortex. These results indicate that long-term pre-treatment of EGb761 administered either alone or in combination with drugs significantly effective neuroprotection on infarct volume in gerbil ischemic brains.

Keywords: EGb761; Neuroprotection; Infarct Volume; MgSO4; FK506; MK-801; Middle Cerebral Artery Occlusion of Gerbil.

Introduction

Recent evidence has focused attention on the neuroprotective effects of Ginkgo biloba leaf extract (EGb761) in brain ischemia. EGb761 shows a very strong free radical scavenging ability both in vitro and in vivo brain neurons (Oyama et al., 1994; Seif-El-Nasr and El-Fattaii, 1995; Calapai et al., 2000). The antioxidant function of EGb761 has been reported to protect the brain against hypoxic damage (Oberpichler et al., 1988; Lee et al., 2000; Lee et al., 2003b; Chung et al., 2003), and neuron apoptosis (Ni et al., 1996). Our previous study demonstrated that extracellular glucose and pyruvate levels were preserved and lactate levels were attenuated upon pre-treatment of EGb761 (100 mg/kg, i.p.) 30 min prior to cerebral ischemia (Lee et al., 2000). These free radical scavenger properties of EGb761 could be related to the protection of Na,K-ATPase activity, which is responsible for maintaining and restoring the resting membrane potential, as well as the reduction of lipoperoxidation induced by ischemia (Pierre et al., 1999). The mechanisms of therapeutic efficiency of EGb761 in brain ischemia are complex. It is probable that EGb761 may reduce the extent of brain swelling (Attella et al., 1989; Lafitte et al., 1980) and increase cerebral blood flow (Zhang et al., 2000; Krieglstein et al., 1986).

Brain injury following focal cerebral ischemia develops a complex series of pathophysiological events. Focusing on observations of excitotoxicity made in cultured cortical neurons, intense exposure to glutamate can be separated into two categories (Choi, 1992): (1) immediate neuronal cell swelling occurring within minutes of exposure onset; and (2) delayed cell degeneration occurring over a period of hours after exposure. This delayed degeneration depends on the presence of extracellular Ca2+. An increase in calcium translocating into cells is thought to initiate a series of events causing cytoplasmatic and nuclear damage (Choi, 1992; Dirnagl et al., 1999). With energy depletion, presynaptic reuptake of glutamate is impeded, which further increases the accumulation of glutamate in the extracellular space. Thus, with a consequent influx of Na+ and Ca2+ ions through the channels, N-methyl-D-aspartate (NMDA)-type glutamate receptor, which gate channels for highly permeable Ca2+, can rapidly trigger excitotoxicity (Lee et al., 1999). Based on these data and ideas, several NMDA-antagonist drugs were developed and used in human stroke trials to minimize the effects of the ischemic cascade. However, the results from several completed trials have been disappointing. The combination therapies of neuroprotective agents will become the future of acute treatment of ischemic stroke because of their complementary functions for brain protection (Mitka, 2002; Brott et al., 1996).
EGb761 AND NEUROPROTECTION AGAINST FOCAL CEREBRAL ISCHEMIA

Considerable experimental evidence has indicated that Mg\(^{2+}\) plays the role of an endogenous calcium channel blocker and reduces the frequency of opening of the glutamate-induced channels acting by a ‘fast channel block’ mechanism (Nowak et al., 1984). Magnesium sulfate has been shown to be neuroprotective in several experimental models of ischemic brain injury (Lampl et al., 2001; Izumi et al., 1991; Kaya et al., 2001; Miles et al., 2001; Yang et al., 2000; Şirin et al., 1998; Marinov et al., 1996; Chung et al., 2004). This effect may be due to the inhibition by Mg\(^{2+}\) of the mitochondrial permeability transition (Zoratti and Szabó, 1995). Magnesium may also block Ca\(^{2+}\) activity by a calmodulin-inhibiting effect (Sutoo and Akiyama, 2001).

Calcium influx early after ischemia was also prevented by the administration of MK-801, a noncompetitive antagonist of NMDA receptor. Research indicated that MK-801 provided neuroprotection at the hippocampal CA1 region in the gerbil model of global ischemia (Nakamura et al., 1993; O’Neill et al., 1997; Araki et al., 1993; Gill et al., 1988; Gill et al., 1987; Warner et al., 1991; Zablocka et al., 1995). These results have shown that NMDA receptors are involved in the mechanism of ischemia-induced neuronal degeneration. And MK-801 is substantially neuroprotective in the late post-ischemic period (Gill et al., 1988).

The immunosuppressive action of the drug FK 506 involves inhibition of calcineurin in T-lymphocytes by binding the protein FKBP12 in the gerbil brain during the early phase of cerebral ischemia (Nozaki et al., 1998). Calcineurin can be activated by elevation of intracellular calcium levels under ischemic conditions (Liu et al., 1991). Several studies have shown that FK506 is also a powerful neuroprotective agent in rat models of focal cerebral ischemia by preventing the secondary deterioration of mitochondrial function (Siesjo et al., 1999; Nakai et al., 1997). FK506 protects brain tissue mainly against ‘reperfusion injury’ by preventing the calcineurin-mediated dephosphorylation of neuronal nitric oxide synthase and by reducing nitric oxide production (Dawson et al., 1993). Also, FK506 significantly reduces infarct volume in the rat ischemic brain (Butcher et al., 1997; Kuroda and Siesjö, 1996; Drake et al., 1996; Sharkey and Butcher, 1994; Bochelen et al., 1999; Chung et al., 2004).

On the basis of this data, we set out to investigate the neuroprotective effects of combining MgSO\(_4\), FK506 or MK-801 therapy with EGb761 pre-treatment on histopathological changes in permanent MCAO of the gerbil brain. The aim of the study was to examine the long-term neuroprotective effects of EGb761 in combination either with calcium channel blockades MgSO\(_4\) and MK-801, or with calcineurin inhibitor FK506 in the gerbil model using preischemic treatment paradigms. We also applied a unilateral MCA infarction to Mongolian gerbils, because it does not change intracranial pressure as described by Başkaya et al. (1999). This strain of animal is often used for studies of transient global ischemia because Mongolian gerbils have an incomplete circle of Willis, allowing reliable development of ischemia (Yoshimine and Yanagihara, 1983). The MCAO method of inducing cerebral ischemia can be easily applied to gerbils and results in consistent infarction (Chung et al., 2003; Başkaya et al., 1999), allowing the present study of permanent focal ischemia.
Materials and Methods

Preparation of EGb761 and Drugs

The extract of *Ginkgo biloba* was used in the study. EGb761 produced as Cerenin tablets was purchased from Schwabe Karlsruhe Co. in Germany. MgSO₄, FK506 and MK-801 were dissolved in distilled water.

Animals

Thirty-five gerbils fed a standard diet were intragastrically given water or EGb761 (100 mg/kg/day) prior to cerebral ischemia for one week. Five randomized groups were established: control (n = 7), EGb761 (n = 8), EGb761 + MgSO₄ (n = 7), EGb761 + FK506 (n = 7), and EGb761 + MK-801 (n = 6). The drug-combination groups were injected with MgSO₄ (90 mg/kg), FK506 (0.5 mg/kg), or MK-801 (1 mg/kg), respectively, 30 min before MCAO occurred.

Permanent MCAO

The surgical method in the present study was performed in an air-conditioned environment to maintain the body temperature of gerbil. Procedures for permanent MCAO of the gerbils were slightly modified from the experiment by Yamamoto *et al.* (1987). After the gerbil was anesthetized with chloral hydrate (360 mg/kg, ip.) and allowed to breathe, its head was placed in a stereotaxic frame (Stoelting, IL, USA). Following a midline incision, the skull was partially removed to expose the right MCA. An 8-0 suture was tightened around the MCA for occlusion. Focal ischemic lesion time for each group was determined by the initiation of occlusion of the right MCA for 24 hours.

Pathological Study

For the determination of infarct volumes, gerbils were sacrificed 24 hours after the onset of occlusion. Two mm slices were immersed in a 2% TTC stain as described by Bederson *et al.* (1986). After 20 min, slices were placed in 10% buffered formalin in the dark and refrigerated until photographed. Slices were projected and traced by the computer software “Optimas” (Version 6.2, Media Cybernetics, Silver Spring, MD). Infarct volumes were quantified by weighing the traced infarct area to the ipsilateral hemisphere and normalized by the contralateral hemisphere.

Statistical Analysis

All data were analyzed by one-way ANOVA followed by Least Significant Difference test. p < 0.05 vs. the untreated control group was considered to be statistically significant.
Results

Effects of EGb761 in Combination with Drugs on Infarct Volume

A typical TTC stain of the control, EGb761 alone, EGb761 + MgSO$_4$, EGb761 + FK506 and EGb761 + MK-801 groups is shown in Fig. 1. The infarcts (white color) are seen in the upper right cerebral zone of each slice in all the groups. The five slices were taken from top to bottom at 2, 4, 6, 8, and 10 mm distance from the frontal pole. In each of the treated groups, infarct volume in all five slices was smaller than that of the control group.

Pathological Changes

The total infarct volumes of the four treated groups were lower than the control group by 36.1% (EGb761 alone), 40.3% (EGb761 + MgSO$_4$), 35.3% (EGb761 + FK506), and 56.4% (EGb761 + MK-801), respectively (p < 0.01), as shown in Fig. 2. Pre-treatment of EGb761 alone, as well as in combination with MgSO$_4$, FK506 and MK-801 significantly reduced infarct volumes at 4 and 6 mm from the frontal pole, when compared to the control group (p < 0.01), as shown in Fig. 3. However, there was no difference in either total infarct volumes or section volumes of the combination groups, compared to the EGb761 alone group (p > 0.05).

Figure 1. Image of 2 mm thick brain slices stained with TTC after 24 hours MCAO in gerbil brains administered water, EGb761 alone or combined with drugs.
Figure 2. Effect of different treatments on total infarct volumes visualized by TTC stain in the brains of gerbils following the occlusion of the MCA. The five groups are control (n = 7), EGB761 (n = 8), EGB761 + MgSO$_4$ (n = 7), EGB761 + FK506 (n = 7) and EGB761 + MK-801 (n = 6). Data are expressed as mean ± SD. **p < 0.01, ***p < 0.001 vs. the control group as determined by one-way ANOVA followed by least significant difference test.

Figure 3. Treatment effects on mean infarct volumes at each slice investigated by TTC stain in the brains of gerbils following the MCAO. was the control group (n = 7); was the EGB761 group (n = 8); was the EGB761 + FK506 group (n = 7); was the EGB761 + MgSO$_4$ group (n = 7); was the EGB761 + MK-801 group (n = 6). Infarct volume (mm$^3$) was expressed as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 vs. the control group as determined by one-way ANOVA followed by least significant difference test.
Discussion

In the present study, Fig. 1 showed infarct areas in all animals located in both the cortex and the subcortex (striatum included), as described by researchers (Sharkey and Butcher, 1994; Yoshimine and Yanagihara, 1983; Tamura et al., 1981). The infarct size of the gerbil brain in any treated group was less than that of the control group. Our results were relatively less severe but consistent with previous studies that utilized MCA occlusion either by a microclip or an endovascular suture (Başkaya et al., 1999; Yoshimine and Yanagihara, 1983; Yamamoto et al., 1987). Another advantage of our method was the simultaneous occlusion of the extracranial artery which could prevent brain injury via opening the subarachnoid space if not occluded (Tamura et al., 1981).

The present study demonstrates that long-term pre-treatment of EGb761 either alone or in combination with MgSO\textsubscript{4}, FK506 and MK-801 provides significant cerebroprotection to gerbils subjected to permanent MCAO with respect to total infarct volume, compared to the control group. The protective efficacy on infarct volumes was most clearly marked at 4 and 6 mm from the frontal pole, when compared to the control group (p < 0.01) (Fig. 3). According to the results of Yoshimine and Yanagihara (1983), occlusion of the MCA for 24 hours resulted in advanced ischemic infarction in the cerebral cortex, caudate nucleus, and putamen of the gerbil brain. The present investigation demonstrated that these infarct areas localized mostly at 4 and 6 mm from the frontal pole. Thus, it may suggest that long-term pre-treatment of EGb761 either alone or in combination with MgSO\textsubscript{4}, FK506 and MK-801 does provide cerebroprotective effects on gerbils subjected to permanent MCAO for 24 hours.

Severe brain edema evolves 6 hours to 7 days after ischemic insult, with a peak at 24 hours (Slivka et al., 1995; Lin et al., 1993). Brain swelling of ischemic tissue may result in enlargement of the infarcted zone, leading to overestimation of infarct volume, but the measurement of injury volume with TTC staining should be corrected for brain edema in the controlled cortical impact brain injury model (Başkaya et al., 2000). The present study examined the absolute infarct volume with TTC staining in each group at post injury 24 hours, the time point corresponding with the peak brain edema formation. Consequently, our results could have overestimated the injury volume which, we believe, should be corrected for when testing neuroprotective agents.

Our data showed that pre-treatment with EGb761 for a week was able to reduce the total infarct volume by 36.1% after 24 hours following permanent MCAO in gerbil brain, when compared to the control group (p < 0.01) (Fig. 2). The neuroprotective effects of EGb761 may be due to inhibition of post-ischemic nitric oxide formation and post-ischemic brain edema (Calapai et al., 2000). During ischemia, reactive oxygen species are likely to be excessively produced, resulting in a marked suppression of SOD activity in the rat brain (Seif-El-Nasr and El-Fattaii, 1995). Pre-treatment with EGb761 improved the lipid peroxide and phospholipid content of rat brain mitochondria, as well as normalized the SOD activity of rat brain. In addition, cerebroprotective effects of EGb761 on brain metabolism as well as on cerebral circulation also have been reported. EGb761 retarded the breakdown of brain energy metabolism in hypoxia (Oberpichler et al., 1988) and preserved
energy metabolites in the striatum during focal cerebral ischemia (Lin et al., 2004a). Under ischemic conditions, glucose uptake may be inhibited by EGb761, thus preventing the production of large amounts of lactate being produced (Krieglstein et al., 1986).

On the basis of the complicated cerebroprotective effects of EGb761 treatment, in this study, we demonstrated that pre-treatment of EGb761 in combination with MgSO₄ significantly decreased total infarct volume by 40.3% after continuous MCAO for 24 hours (Fig. 2). Our previous experiments proved that during cerebral ischemia, the mean Mg²⁺ level significantly decreased to approximately 41% and 65% of the baseline levels (Lin et al., 2004b; Yang et al., 2004). Significant neuroprotection with only magnesium has been observed in different models of focal cerebral ischemia, with infarct volume reductions between 25 and 61% (Muir, 2001). Our previous experiments also proved that pre-treatment with MgSO₄ significantly reduced total infarct volume by 25.4% after continuous 24 hours MCAO (p < 0.05) (Chung et al., 2004). A supply of extracellular Mg²⁺ may cause a direct dilation of the large cerebral arteries and consequently reduce the vasoconstrictive effect in the early stages after stroke (Altura and Altura, 1992). Furthermore, the effects of magnesium sulfate administration on brain edema have been to attenuate the blood-brain barrier permeability defect and reduced brain water content experienced after brain injury in rats (Esen et al., 2003). Another hypothetical explanation for the reduction of total infarct volume following pre-treatment of EGb761 in combination with MgSO₄ can be based on the defensive effects of extracellular Mg²⁺ on NMDA receptors against the excessive influx of calcium into cells, acting by a ‘fast channel block’ mechanism (Nowak et al., 1984). According to Tsuda et al. (1991), Mg²⁺ administered up to 24 hours after reperfusion prevented ischemic damage of the rat hippocampus in vitro, but was ineffective at 48 hours post-treatment. It may explain why in this study that pre-treatment of a single dose of Mg²⁺ in combination with EGb761 does provide cerebroprotective effects in gerbils during 24 hours MCAO.

Furthermore, neuroprotective effects of MgSO₄ against hypoxia-ischemia are restricted to the pre-treatment intervention by which MgSO₄ is administered before the insult (Sameshima et al., 1999). Considerable experimental evidence indicates that pre-treatment with magnesium in combination with a free-radical scavenger ameliorated hypoxic-ischemic brain damage in the rats (Schmid-Elsaesser et al., 1999; Thordstein et al., 1993). In our study, combination therapy with EGb761 and MgSO₄ also provided a more significant neuroprotection than that of EGb761 alone, compared to the control group. But the combination therapy has not shown its synergistic effect for neuroprotection, compared to the EGb761 alone group (p > 0.05). The 90 mg/kg dose of MgSO₄ used in this study was selected because it has already been shown to be optimal for producing neuroprotective effects in vivo (Marinov et al., 1996; Lee et al., 1999).

In the present study, EGb761 + FK506 reduced cortical ischemic damage of gerbils after continuous 24 hours MCAO by 35.3% (Fig. 2). Similar to our experiment, Haines et al. (2000) reported that combination therapy using orally administered 25 mg/kg/day EGb761 plus 1 or 5 mg/kg/day of FK506 for 10 days synergistically improved postischemic cardiac function. Therefore, in this study, the combination efficacy of EGb761 plus the single low dosage of 0.5 mg/kg FK506 on cerebral ischemia may prevent FK506 treatment from
producing toxic effects (Pizzolato et al., 1998). Both EGb761 alone and in combination with FK506 therapy in our previous study significantly preserved glucose and pyruvate levels during ischemia (Lin et al., 2004a). These results explained the reduction of infarct volumes by EGb761 alone or in conjunction with FK506 therapy in a focal cerebral ischemic model. In this experimental design, continuous MCAO for 24 hours in gerbil brain simulates the common form of human brain ischemia. As shown in Fig. 1, infarction induced by occlusion of the MCA was restricted to the parietal area of the cerebral hemisphere (Yoshimine and Yanagihara, 1983). Our findings demonstrated that long-term pre-treatment of EGb761 in conjunction with single FK506 administration prior to brain ischemia provided a neuroprotective effect. FK506 alone reduced the volume of ischemic brain damage before MCA occlusion by 41% (Chung et al., 2004). FK506 exerted its neuroprotective effects through its binding to high affinity FKBP12, most abundantly located in the caudate-putamen, within the gerbil brain during the early (2 hours) phase of cerebral ischemia (Nozaki et al., 1998). The administration of FK506 immediately after continuous MCAO significantly reduced the extent of brain cytotoxic edema mainly in the cortical region of rat during both the early (3 hours) stage and possibly the later (24 hours) ischemic period (Ebisu et al., 2001). Although the Bochelen et al. (1999) study showed no reduction in the infarct size, when FK506 was administered before or after MCAO, so FK506 may play a multi-function role in cerebral ischemic series. With FK506 given by the intraperitoneal administration route, the brain content of FK506 rose rapidly at 30 min postinjection, the time at which the excitotoxic or ischemia challenge was initiated. It was sustained at a neuroprotective level for 3 days, as described by Butcher et al. (1997). Therefore, in our present experiment, FK506 treatment 30 min prior to permanent MCAO was reasonable. However, the neuroprotective effects of the combination of EGb761 and FK506 were not better than those obtained with the EGb761 alone (p > 0.05) or the FK506 alone (p > 0.05) in our previous study (Chung et al., 2004).

As therapeutic agents, non-competitive NMDA antagonists are lipophilic compounds that readily penetrate the CNS. The two classes of non-competitive NMDA antagonists are divalent cations (e.g. Mg^{2+}), and compounds such as MK801, both of which act on the ion channel. Mg^{2+} probably blocks the channel directly by physically preventing ionic conductance. MK-801 binds to sites within the channel separate from those for Mg^{2+} when the channel is in its open state, preventing the passage of ions (e.g. Na^+, Ca^{2+}, K^+) (Kemp et al., 1987). The NMDA receptor-operated calcium channel is primarily responsible for calcium influx in the early period of ischemia (Nakamura et al., 1993). Gill et al. (1988) showed that single dose of MK-801 of 1 mg/kg or higher was able to protect hippocampal neurons from ischemia-induced neuronal degeneration even when administered 24 hours after the global ischemia. Psychotogenic and autonomic side effects have limited its clinical utility as an antiepileptic agent at higher doses, implying that these effects could be a consequence of NMDA receptor blockade (Wada et al., 1992; Olney et al., 1989). At the dose of 5 mg/kg i.p. used, MK-801 induced serious side effects which brought about the aggravation of the neurological deficit induced by transient global brain ischemia in the rat (Beaughard et al., 1989). The ability of the non-competitive NMDA
antagonists to penetrate the brain following i.p. administration may confer its therapeutic advantages in the treatment of stroke (Kemp et al., 1987).

Previous studies reported that no effects of MK-801 at a dose of less than 2 mg/kg (administered intraperitoneally) on brain temperature were observed (Nakamura et al., 2001; Hoffman and Boast, 1995). In that study, no attempt was made to maintain body temperature of gerbils during MCAO, MK-801 did not lower the brain temperature at a dose of 1 mg/kg. In the present study, the combination therapy of EGb761 + MK-801 did reduce ischemic brain damage for body temperature free-regulating gerbils in the air-conditioned room. Under these conditions, we found that pre-treatment of EGb761 + MK-801 reduced total infarct volume in gerbil brains by 56.4%. However, the reduction of infarct volume with EGb761 + MK-801 pre-treatment was only slightly more than that with EGb761 alone (p > 0.05) (Fig. 2), but similar to the reductive volume with MK-801 alone: 55.5% in our previous study. Our result, which revealed that brain damage in the cortical region was reduced by pre-treatment with 1 mg/kg MK-801, was in accordance with that of Warner et al. (1991). Based on these findings, it is likely that 1 mg/kg MK-801 pre-treatment attenuated brain edema formation and blood brain barrier permeability both at the core and the periphery of the ischemia in rat brains (Görgülü et al., 2000; Yang et al., 1994). Besides, in our preliminary study, the efficacy of intraperitoneal injections of 0.1, 0.5, and 2.5 mg/kg MK-801 on cortical blood flow demonstrated respective increases of 3.8%, 26.7%, and 61.1% of baseline levels (Lee et al., 2003a). Therefore, the efficacy of the combination therapy of EGb761 + MK-801 on cerebral ischemia could be significant.

Brain damage following cerebral ischemia develops in a series of complex changes. We conclude that our findings indicate great effects of pharmacological treatment with EGb761 alone or in combination with MgSO₄, FK506 or MK-801 in continuous MCAO, based on TTC staining. The minimum effective neuroprotective dose was previously reported to be comparable to the immunosuppressant dose in humans, suggesting that EGb761 + FK506 may have clinical potential for the treatment of stroke. An additive protective effect of different therapeutic strategies on brain damage may be suggested in clinical fields. The present results suggest that there are protective gains to be made using a mixture of agents that are active at different levels of the destructive process taking place after permanent MCAO.

Neuroprotectants are widely used today in the treatment of stroke to minimize the effects of the ischemic cascade. Treatments attempts focus on the reduce of excessive influx of calcium into cells or the prevent of the toxicity of glutamate and other excitatory amino acids as well as on the devastating production of toxic free radical species or of nonradical species that cause inflammation. It would be equally important to enhance production of ATP by raising cerebral blood flow. Based on these principles, in the present study, our results demonstrated that the synergistic effects of EGb761 with drug combinations did have neuroprotection on infarct volumes of gerbil brains for 24 hours MCAO. The reasoning behind long-term pre-treatment of EGb761 is mainly based on improving survival rates (Le Poncin-Lafitte et al., 1982), its preservation of glucose and pyruvate under anaerobic conditions (Lee et al., 2000; Lee et al., 2003b), and its anti-PAF
(platelet-activating factor) as well as anti-free radical effects. In the study, the efficacy of 100 mg/kg EGb761 pre-treatment combined with only one dosage of MgSO₄, FK506 and MK-801 on reduction of infarct volumes in gerbil brains by TTC staining was demonstrated at lower dosages; i.p. injections of 90, 0.5 and 1 mg/kg, although there are limitations of concerning side effects. Furthermore, EGb761 + MK-801 pre-treatment for focal cerebral ischemia should be investigated at 100 mg/kg EGb761 combined with a single dose of 0.5 mg/kg MK-801 in the future. This new trial should be expected to display its potential for clinical usage in preventing brain damage following cerebral ischemia.

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References


Tsuda, T., K. Kogure, K. Nishioka and T. Watanabe. Mg$^{2+}$ administered up to twenty-four hours following reperfusion prevents ischemic damage of the CA1 neurons in the rat hippocampus. *Neuroscience* 44: 335–341, 1991.


