

EGb761 Co-administrated with FK506 Offer no More Neuroprotective Efficacy than EGb761 Alone during Focal Cerebral Ischemia and Reperfusion Monitored by Microdialysis Coupled with Microbore Liquid Chromatography

Ming-Cheng Lin¹

Abstract

Accumulated dopamine (DA) is believed to be detrimental to the brain. EGb761 and FK506 have been reported to possess neuroprotective efficacy. This study attempted to investigate the metabolic pathway of dopamine and to realize whether the synergistic effect would be achieved when EGb761 co-administrated with FK506. Dynamic changes of dopamine and its metabolites including 3, 4-dihydroxyphenylacetate (DOPAC), homovanillic acid (HVA) and 3-methoxytyramine (3-MT) in the striatum were measured. Histological technique of 2, 3, 5-triphenyltetrazolium chloride (TTC) staining was used for assessing the infarction volume. Data indicated that an approximately 80, 90, and 180 fold increases of the dopamine level was seen in the group of EGb761, control, and combination (EGb761 plus FK506) during ischemia. Interestingly, no significant changes in the level of DOPAC, HVA, and 3-MT were observed among these groups. Histological evidence showed that the mean total infarction size was significantly elevated in the combination group when compared with the control group ($P < 0.05$). Importantly, compared with the control group, the infarction volume at the slices of 4mm on the striatum was significantly higher in the combination group ($P < 0.05$). Accordingly, it seems possible that cerebral ischemia may lead to more accumulated dopamine; meanwhile, most of the accumulated dopamine may be rapidly catabolized through a less energy consumed route and eventually to form more alternative metabolites other than DOPAC, 3-MT and HVA. In addition, EGb761 co-administrated with FK506 offers no more neuroprotective efficacy than EGb761 used alone during cerebral ischemia.

Keywords: EGb761, FK506, Microdialysis, Striatum, Cerebral ischemia, Dopamine

¹Department of Medical Laboratory Science and Biotechnology, Central Taiwan University of Sciences and Technology, Taichung 406, Taiwan. Telephone: 886-4-22391647 ext. 7027, Fax: 886-4-22396761. E-mail: mclin@ctust.edu.tw

Introduction

Cerebral ischemia not only results in delayed neurological mortality but also causes a major of death to the older adults [1]. During cerebral ischemia, lower oxygen supplementation and decreased ATP may lead to a massive release and accumulation of dopamine from nerve terminals to extracellular space [1, 2]. Meanwhile, previous research illustrated that massive dopamine may damage the brain [2, 3]. EGb761, a long history of use in traditional Chinese medicine, which may be used individually or particularly in combination, has been assessed for their broad efficacy in the therapy of cerebral ischemia [4, 5]. A number of animal models pointed out that EGb761 may reduce lipid peroxide, phospholipid content and oxygen free radicals [4-6]. Virtually, based upon its excellent antioxidant efficacy, EGb761 has been used clinically for improving peripheral and cerebral vascular diseases in many countries such as Taiwan, France and Germany [7]. FK506, by itself, has been proposed to exert antioxidant property [8-10].

Hence, FK506 has been applied in acting as a neuroprotective medicine for the treatment of cerebral ischemia [11, 12]. The allied technique of microdialysis coupled with microbore liquid chromatography (LC) and electrochemical detection (ED) is useful not only in autosampling but also in providing the dynamic and precise information of the neurochemicals in the brain [13, 14]. The purpose of this present study was to investigate the metabolic route of dopamine in the striatum after cerebral ischemic injury. On the other hand, we aimed to explore whether the synergistic effect would be achieved when EGb761 co-administrated with FK506. In this present study, occlusion of the unilateral right common carotid artery (CCA) and right middle cerebral artery (MCA) was performed for inducing focal cerebral ischemia. Dynamic alterations of the dopamine levels and its metabolites, including DOPAC, HVA and 3-MT were continually auto-sampling through microdialysis technique and measured by means of microbore liquid chromatography (LC) and electrochemical detection (ED). Furthermore, the histological technique of TTC staining was used to evaluate the infarction volume after ischemic insult.

Materials and Methods

Experimentally, twenty-seven male gerbils (65-80 gm) were randomly divided into three groups, including control group, EGb761 group, and combination (EGb761 plus FK506) group. Control group was fed with normal saline; EGb761 group was fed with 0.6ml EGb761 (100 mg/kg/day, ig.) once a day for 7 days and the combination group was fed with EGb761 (100 mg/kg/day, ig.) once a day for 7 days and the FK506 (0.5 mg/kg, i.p) were given 30 min prior to the occlusion. The gerbil was anesthetized with chlorhydrate (400 mg/kg) intraperitoneally and its body temperature was maintained at 37 °C with a heating pad (CMA/150). A midline neck incision was made and the right carotid artery was exposed and separated from the vago-sympathetic trunks. The right carotid artery was loosely encircled with a 4-O suture for later occlusion. The gerbil's head was placed in a stereotaxic frame (David Kopf, CA, USA) with the nose bar positioned 4.0 mm below the horizontal line. Following a midline incision, the skull was partially removed to expose the right middle cerebral artery. The middle cerebral artery was loosely encircled with an 8-O suture for later occlusion.

The micro dialysis probe (4 mm in length, CMA/12, Carnegie Medicin, Stockholm, Sweden) was stereotaxically implanted into the side of the cortex (AP 0 mm, ML +/- 5 mm, DV -5 mm from bregma). A focal cerebral ischemia was induced by occlusion of the right common carotid artery and the right cerebral artery (CCA+MCA) for 60 min followed by an additional 3 h of reperfusion. Dialysis probes were perfused with Ringer's solution (147 mM Na⁺; 2.2 mM Ca²⁺; 4 mM K⁺, pH 7.0) at 2 µl/min using a CMA/100 micro infusion pump. Dialysates were collected every 15 min in a CMA/140 fraction collector. Standard stock solutions of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxytyramine (3-MT), and homovanillic acid (HVA) were prepared at a concentration of 2 ng/ml in 0.1 M perchloric acid and stored at -70°C in the dark and thawed in an ice bath prior to preparation of a standard mixture. Aliquots of dialysates (5µl) were injected onto a micro bore LC system with a dual potentiostat amperometric detector (BAS-4C with MF-1020 electrode, Bioanalytical Systems, West Lafayette, IN) to measure DA and its metabolites. Potentials for the anodic and the cathodic dual series working electrodes were set at +0.75V and +0.05V with respect to a silver/ silver chloride reference electrode, respectively. Separation of these substances was achieved using a microbore column (150 × 1.0 mm I.D.) packed with 5 µm Inertsil-2 C18 particals (GL Sciences, Tokyo, Japan).

The buffer consisted of 9.60 g monochloroacetic acid, 0.16 g sodium 1-octane sulfonate, 10 mg ethylenediaminetetraacetic acid (EDTA), adjusted to pH 3.0 with 1 M sodium hydroxide. The final volume of the mixture was adjusted to 1 L with double-distilled water. The mobile phase was prepared by mixing 50 ml acetonitrile and 950 ml phosphate buffer. The mixture was filtered through a 0.22 μm Nylon filter under reduced pressure and degassed with helium for 20 min. The flow rate was 60 $\mu\text{l}/\text{min}$ maintaining column pressure at ca. 12.4 Mpa. Isocratic separation was achieved within 30 min. Concentrations of biogenic amines and their metabolites in dialysates were calculated by determining the ratio of each peak area relative to the standard mixture. The identity of each peak on the chromatogram was confirmed by its retention time, redox ratio, and a superimposition-alignment technique, which was provided by Beckman (System Gold Data Analysis Software. Version 8.10, Taiwan Beckman, Taipei, Taiwan). All data were analyzed by ANOVA with PLSD. P value less than 0.05 were considered statistically significant. For the determination of the infarction volumes, gerbils were sacrificed for removing the brains after completion of the reperfusion experiments. 2 mm slices of brain tissue were immersed in a 2% solution of 2, 3, 5-triphenyltetrazolium chloride (TTC) stain as described by Bederson [15]. After 20min, slices were placed in 10% buffered formalin in the dark and refrigerated until photographed. Slices were projected and traced. Cutting out and weighing the traced normal and infarct area quantified infarct size.

Results

In the present study, the extracellular dopamine level before, during and after cerebral ischemia in the striatum was showed in figure 1. At the onset of ischemia, dopamine sharply increased 80, 90, and 180 fold over baseline in the group of EGb761, control, and EGb761 plus FK506, respectively. In the combination group, the peak level of dopamine was significantly 2 fold higher than that of the control group ($P < 0.05$). Similarly, a dramatic elevation of dopamine level in the combination group was seen and persisted for 120 minutes ($P < 0.05$), when compared to the control group. Fig. 2 showed the dynamic changes of the DOPAC levels during cerebral ischemia and reperfusion in the striatum. Compared with the control group, a slightly decreased was seen in the group of EGb761 (up to 65.7% of baseline) and combination (up to 68% of baseline), respectively. No significantly change was seen among these three groups. Extracellular concentrations of 3-MT during transient ischemia and reperfusion in the striatum were listed in figure 3.

The 3-MT levels increased up to 800.5%, 764.9%, and 720.1% of the baseline after ischemia and followed by decreased to 120.3%, 154.3%, and 98.2% of the baseline at the end of reperfusion in the groups of control, EGb761, and combination, respectively. For the measurement of the dynamic changes of the HVA levels during transient ischemia and reperfusion in the striatum, the HVA levels decreased to 50.7%, 46.2%, and 41.7% of the baseline in the groups of control, EGb761, and combination, respectively. At the end of reperfusion, the HVA level was returned to 94.1%, 95.8%, and 90.8% of the baseline level on the groups of control, EGb761, and combination, respectively. No significant patterns were seen within every time-point in each group as shown in figure 4. Fig. 5 showed the mean infarction volume in five slices of brain among three groups after completion of the reperfusion experiments. Evidence from TTC staining showed that the mean infarction volumes in combination group were higher at the slices of 2, 4 and 6mm, especially at the slices of 4mm when compared with the control group ($P < 0.05$). Besides, the mean total infarction sizes were 46.3, 69.0, and 124.9mm³ in the group of EGb761, control, and EGb761 plus FK506, respectively. A significantly higher mean total infarction sizes were found in the combination group when compared with the control group (data not shown).

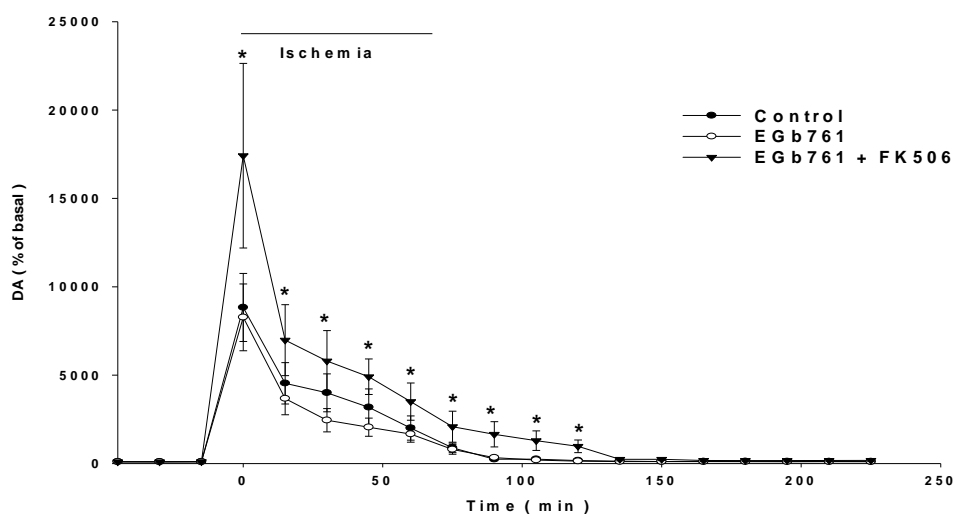


Fig. 1: Time profiles of the effect of EGb761 and EGb761 combined pre-treatment with FK506 on the changes of extracellular dopamine levels in gerbil striatum during 60 min CCA+MCA occlusion and 3 h reperfusion. Data are expressed as Mean \pm SEM (n=9).

$P < 0.05$, ANOVA test followed by LSD test versus control group

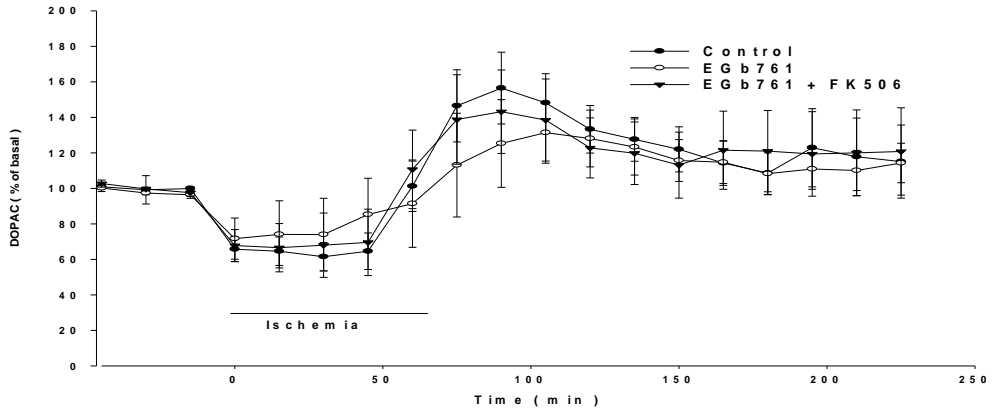


Fig. 2: Time profiles of the effect of EGb761 and EGb761 combined pre-treatment with FK506 on the changes of extracellular DOPAC levels in gerbil striatum during 60 min CCA+MCA occlusion and 3 h reperfusion. Data are expressed as Mean \pm SEM (n=9). $P < 0.05$, ANOVA test followed by LSD test versus control group

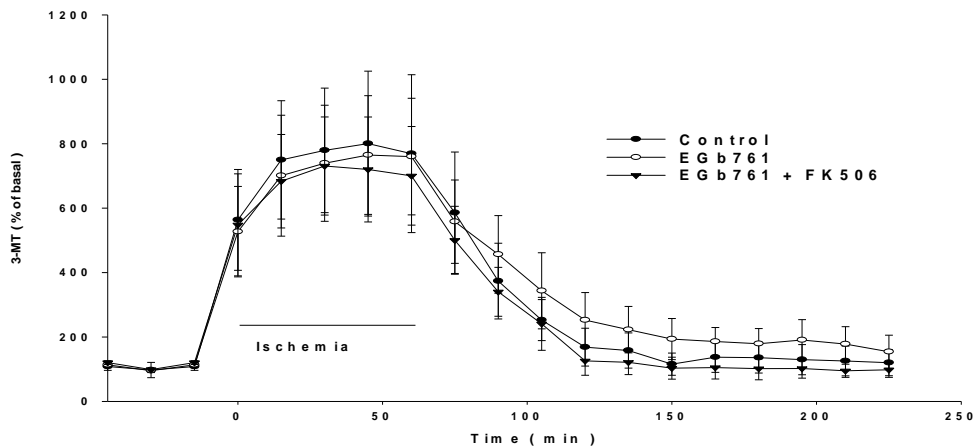


Fig. 3 Time profiles of the effect of EGb761 and EGb761 combined pre-treatment with FK506 on the changes of extracellular 3-MT levels in gerbil striatum during 60 min CCA+MCA occlusion and 3 h reperfusion. Data are expressed as Mean \pm SEM (n=9). $P < 0.05$, ANOVA test followed by LSD test versus control group

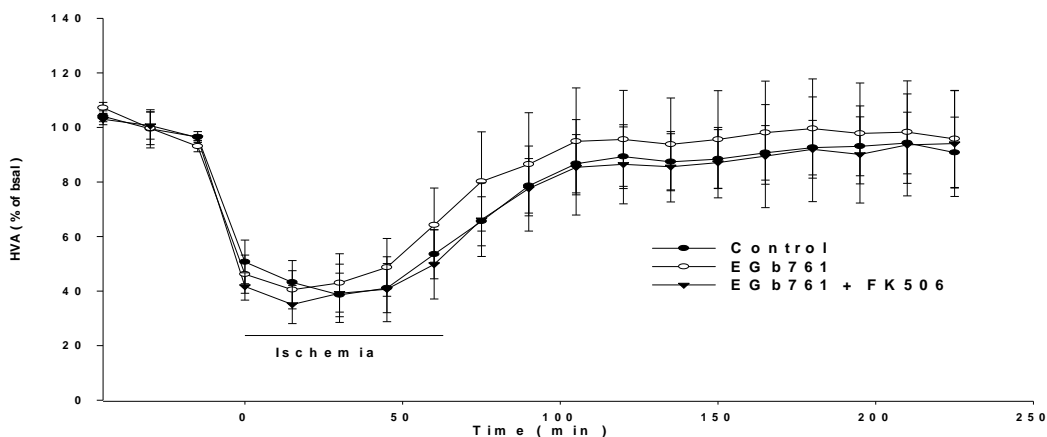


Fig. 4 Time profiles of the effect of EGb761 and EGb761 combined pre-treatment with FK506 on the changes of extracellular HVA levels in gerbil striatum during 60 min CCA+MCA occlusion and 3 h reperfusion. Data are expressed as Mean \pm SEM (n=9). $P < 0.05$, ANOVA test followed by LSD test versus control group

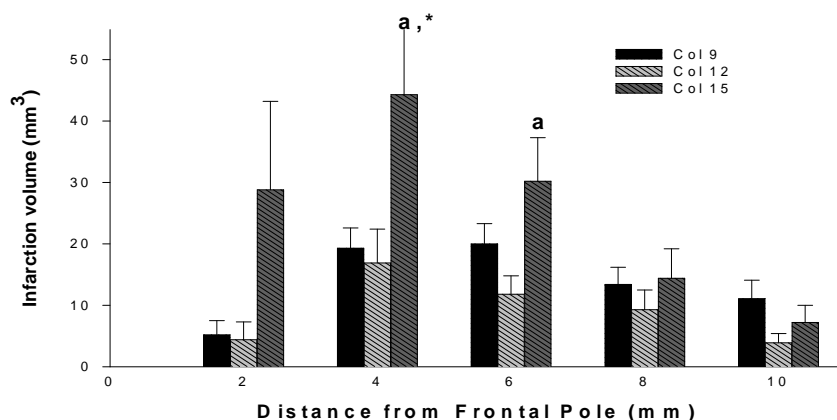


Fig. 5: Illustration of 2-mm thick brain slices stained with TTC stain in gerbil brains treated with control (a), 100mg/kg/day EGb761 ig. (b), and 100 mg/kg/day EGb761 ig. plus 0.5 mg/kg FK506 ip. (c). Data are expressed as mean \pm SEM (n=9). $P < 0.05$, ANOVA test followed by LSD test versus control group

Discussion

Brain represents only about 2% of total body weight and yet accounts for approximately 20% of the total oxygen consumption. Once oxygen supply is reduced to the critical levels, as occurs in severe cerebral ischemia or hypoxia, damage to brain cells occur. In addition, recent paper indicated that the brain has low levels of storage forms of carbohydrates and is highly dependent on oxidative metabolism [16]. As such, it reveals the fact that cerebral ischemia leaving the glycolytic metabolism as a main pathway for ATP production. For the striatum, due to its anatomic structure and thereby, it is believed to be extremely sensitive to energy and oxygen metabolism impairment. Without exception, our present profile showed that the dopamine level was significantly increased to 80, 90, and 180 fold over the baseline levels in the group of EGb761, control, and combination after cerebral ischemia (Fig. 1). In addition to this, comparing the combination group with the control group, a significant elevation of dopamine level was seen during cerebral ischemia and reperfusion period (Fig. 1). Earlier study proposed that the dopamine is a neurotoxic molecule and its accumulation is thought to play a key role in excitotoxic neuronal death [17]. Conversely, dopamine depletion may protect the neurons from ischemia-induced cell death [17]. During cerebral ischemia, the ischemic depolarization of membranes also results from the failure of mitochondria on dopamine reuptake. As a result, the dopamine level was accumulated right. Upon reperfusion, the mitochondria are re-energized and regain oxygen and virtually, the re-uptake rate of dopamine was elevated and eventually, the extracellular dopamine level was declined right and our present profile was in keeping with the previous study [18, 19].

EGb761 and FK506 have been reported to confer direct neuroprotection and to alleviate the ischemic problems in a recent paper [5-9]. On the basis of our experimental data, there are two possible reasons to explain our findings: Firstly, previous research indicated that FK506 tended to decrease the cerebral blood flow not only in the peripheral organs but also in the brain [20, 21]. Therefore, pretreatment Gerbils with FK506 in pre-ischemic stage may decrease the supplementation of oxygen and energy into the brain due to its lower cerebral blood flow and eventually, the outcome is harmful but advantageous to the brain. Conversely, EGb761 has been reported not only in possessing a notable history of safety but also in enhancing the blood flow and the glucose level [22, 23]. Thus, pretreatment Gerbils with EGb761 alone may also prove excellent efficacy for brain in treating ischemia and its related diseases.

Secondly, earlier study pointed out that the neuroprotective effect of FK506 is effective just only on reperfusion period but not on pre-ischemic stage [24-26]. Obviously, based on our dopamine profile and a detrimental effect we achieved here when FK506 was given in pre-ischemic stage. There are two enzymatic pathways that involved in the catabolism of dopamine: one is intraneuronally N-oxidized by monoamine oxidase (MAO) to form DOPAC. The other pathway is extraneuronally O-methylated by COMT to form 3-MT [2, 3]. In the present study, the concentration of DOPAC was declined to under the baseline levels after ischemia. Upon reperfusion, level of DOPAC was dramatically elevated and returned to near the baseline levels among three groups (Fig. 2). During cerebral ischemia, due to the inhibition of monoamine oxidase, level of DOPAC was decreased to under the baseline levels. On reperfusion, the mitochondria function is restoration and thereby, the DOPAC level was elevated in all groups. Previous study illustrated that the DOPAC is mainly metabolized intraneuronally and a major part of extracellular DOPAC is derived from intraneuronal pool of newly synthesized dopamine [13]. Therefore, the elevation of DOPAC may not be accurately reflected in the microdialysate samples.

3-MT is an intermediate metabolite of dopamine. Normally, the 3-MT exists mainly in the extracellular space and its elevation is regarded as an indicator of dopamine release [27, 28]. Presently, an approximately 8 fold increase of the 3-MT level was seen during cerebral ischemia and declined to near the baseline level upon reperfusion among these three groups (Fig. 3). In particular, no significant difference was observed among these three groups. Comparing the dopamine level with the 3-MT level, an approximately 10%, 8% and 5% of the turnover rate for dopamine was converted into the 3-MT during cerebral ischemia period in the group of EGb761, control and combination, respectively. Previous paper pointed out that due to its inherent instability of the catechol molecule and the lowest energy expensed predominant, the accumulated dopamine may readily undergo a non-enzymatic pathway, which an auto-oxidation route, and spontaneously to form reactive quinones, semiquinones and free radicals [29-31]. As stated above, it is also reasonable to suppose our finding that during cerebral ischemia, a large part of the accumulated dopamine may be through an alternative pathway to form others metabolites such as reactive quinones, semiquinones and free radicals and eventually, only a small part of dopamine level was reflected on the concentration of 3-MT through a normal enzymatic catalyzation pathway.

In addition, determination of any chemical component is advanced by means of the availability of an optimum analytical technique. The former liquid chromatography technique has some drawbacks in measuring the concentration of 3-MT due to its sensitivity and resolution power and eventually, the 3-MT level was undetectable except for samples collected during cerebral ischemia and early reperfusion stage [32, 33]. In our present study, the microbore liquid chromatography technique was applied here to overcome the above problems and thereby, very trace amount of the 3-MT level was detectable throughout the experimental process. HVA is the final product of dopamine. Under normal conditions, the HVA is derived from 3-MT through the catalyzation of the enzyme of monoamine oxidase. After cerebral ischemia, due to the enzyme inhibition of monoamine oxidase and consequently, the concentration of HVA are reduced [34, 35]. Presently, during focal cerebral ischemia, the HVA level was decreased to 50.7%, 46.2% and 41.7% of the baseline in the groups of control, EGb761, and combination, respectively. At the end of reperfusion, the HVA level was returned to 94.1%, 95.8%, and 90.8% of the baseline level on the groups of control, EGb761, and combination, respectively. Our result indicated that no significant patterns were seen within every time-point in each group in the present study (Fig. 4).

In order to predict the neuroprotective efficacy in the brain, the TTC staining, a histological technique was used after completion of the reperfusion experiments in the present study [15]. Data from TTC staining showed that the mean infarction volumes in combination group were higher at the slices of 2, 4 and 6mm, especially at the slices of 4mm when compared with the control group (Fig. 5). Besides, the mean total infarction sizes were 46.3, 69.0, and 124.9mm³ in the group of EGb761, control, and EGb761 plus FK506, respectively. A significantly higher mean total infarction sizes were found in the combination group when compared with the control group (data not shown). Remarkably, combined the histological data with the dopamine profile, an effect contrary to our intention we achieved here that EGb761 coadministrated with FK506 in pre-ischemic stage provided no more neuroprotective efficacy than EGb761 used alone. In conclusion, cerebral ischemia may exactly lead to more accumulated dopamine level. Importantly, the accumulated dopamine may be rapidly converted and to form more alternative metabolites other than DOPAC, 3-MT and HVA. In addition, EGb761 co-administrated with FK506 offers no more neuroprotective efficacy than EGb761 used alone during focal cerebral ischemia.

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