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Journal of Chromatography A, 985 (2003) 387–394
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Ginkgo biloba extract preserves pyruvate and enhances ascorbate in the cortex of gerbils during focal cerebral ischemia A microdialysis–liquid chromatography study

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Abstract

The aim of this study was to evaluate dynamic changes in energy-related metabolites in the cortex of gerbils subjected to focal cerebral ischemia after pretreatment with *Ginkgo biloba* extract. Focal cerebral ischemia was induced by occlusion of the right common carotid artery and the right middle cerebral artery for 60 min in anesthetized gerbils. A microdialysis probe was inserted into the cortex to monitor extracellular lactate, pyruvate and ascorbate during ischemia and reperfusion. The present study demonstrated a dynamic decrease in pyruvate (25% of baseline) and increases in lactate (160% of baseline) and ascorbate (300% of baseline) and a 5-fold increase in the lactate:pyruvate (L:P) ratio during cerebral ischemia in the control group. However, pyruvate levels were preserved and ascorbate levels were enhanced with a chronic pretreatment of *Ginkgo biloba* extract for 8 days (i.p., 100 mg kg^{-1} day^{-1}). Preservation of pyruvate and enhancement of ascorbate observed in this study may be associated with the neuroprotective effects of *Ginkgo biloba* extract.

Keywords: *Ginkgo biloba*; Microdialysis; Pyruvate; Ascorbate; Lactate

1. Introduction

Ginkgo biloba (Ginkgoaceae) is an ancient Chinese tree which has been cultivated and held revered for its health-promoting properties. The extract of dried leaves of *Ginkgo biloba* can play a role in reducing brain damage [1–3]. A standardized extract (EGb 761) of dried leaves of *Ginkgo biloba*, containing 24% ginkgo flavonol glycosides and 6% terpene lactones such as ginkgolides and bilobalide, was developed by Willmar Schwabe [2]. It has commonly been used in Europe, particularly in France and Germany, to treat peripheral vascular and neurological disorders [4]. Its broad spectrum of pharmacological activities allows it to mitigate the numerous pathological hemodynamic, hemorheological, and metabolic insults during cerebral, retinal, cardiac or peripheral ischemia [1–3,5]. Moreover,

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0021-9673/02/$ – see front matter
PII: S0021-9673(02)01911-8

EGb 761 directly protects against necrosis and apoptosis of neurons and improves neural plasticity [6].

Cerebral ischemia results in low oxygen and glucose supply and causes decreased ATP formation [7–9]. Various ATP-driven membrane-bound pumps or reuptake processes that usually maintain the homeostasis of important metabolites or ions become retarded. Moderate to severe neuronal damage may occur following ischemic events. A large amount of lactate can be produced by nerve tissues, including nerve and glial cells, in acute cerebral ischemia. Under anaerobic conditions, pyruvate is reduced to lactate by lactate dehydrogenase. Lactate levels in the brain have been advocated for estimating the severity of stroke and for prognostication of the outcome [10,11]. In addition, the L:P ratio is an important index of outcome and prognosis in experimental animals and clinical patients. Changes in lactate, pyruvate and the L:P ratio may serve as important biochemical markers of cerebral ischemia in experimental animals and clinical studies [12,13].

Microdialysis was introduced 2 decades ago and has been widely used for the sampling of neurochemical substances from the extracellular fluid of the brain [14–16]. Today it is one of the most widely used techniques for in vivo sampling in the brain. Continuous intracerebral microdialysis techniques demonstrate the dynamic chemistry of the brain. It is crucial to learn how the ischemic processes are reflected in localized compartments. In general, ischemic stress induces metabolic derrangement, increasing the lactate and L:P ratio, and decreasing pyruvate, resulting in severe pathological changes. Extracellular lactate and pyruvate levels may indicate the ischemic insult to neuronal tissues. Ascorbate is a biochemical index of early ischemia and can be a useful tool for the evaluation of initial ischemic damage [17]. In addition, ascorbic acid release may also be regulated by glutamate transports which are altered during cerebral ischemia [17,18]. Employing intracerebral microdialysis techniques, dynamic changes in energy related analytes during cerebral ischemia and protection effects of a chronic pretreatment with *Ginkgo biloba* extract were investigated in the present study.

2. Materials and methods

2.1. Sample preparation and assay

Standard stock solutions of pyruvate and lactate were prepared at concentrations of 10 and 100 m*M*, respectively, in 4 m*M* sulfonic acid and stored at 4 °C. The standard mixtures were prepared from a portion of these stock solutions after appropriate dilution with 4 m*M* sulfonic acid. Ascorbate was prepared fresh daily at a concentration of 100 μ*M*. In vitro recovery was also performed in a standard mixture containing pyruvate, lactate, and ascorbate to determine the recoveries of all analytes and the dead volume of the microdialysis system [18]. Microdialysis probes with recoveries of all analytes between 20 and 30% were used to ensure the analytical quality. The precision of assays was tested using standard mixtures and pooled dialysate samples. The intra- and inter-assay precision was assessed ($n=8$) and expressed as relative standard deviations (RSDs) as shown in Table 1. The RSD values for determination of pyruvate, lactate, and ascorbate were $<5\%$ in the standard mixture and pooled dialsate samples. The inter-assay variability assessed with a mixture containing pyruvate, lactate, and ascorbate over 6 consecutive days was $<5\%$. All reagents were of analytical quality unless otherwise stated.

Twelve male gerbils (65–75 g) were randomly divided into control and EGb 761 groups, and were given daily injections (i.p.) of saline or EGb 761 (100 mg kg^{-1} day^{-1}, cerrenin Amp., Schwabe Karlsruhe, Germany), respectively, for 8 days. The

Table 1
Analytical precision (RSD) of intra-assay ($n=8$, at 1-h intervals) and inter-assay ($n=6$, in 6 consecutive working days) stabilities of standard mixtures and pooled dialysates in the LC–UV system

	RSD (%)		
	Pyruvate	Lactate	Ascorbate
Intra-assay			
Standard mixture	2.5	3.1	4.5
Pooled dialysates	2.3	3.3	3.8
Inter-assay			
Standard mixture	3.6	4.2	4.9

last injection was made 1 h prior to ligation. The animals were anesthetized with intraperitoneally administered chloral hydrate (360 mg/kg) and their body temperature was maintained at 37 °C with a heating pad (CMA/150). The right common carotid artery (CCA), exposed through a ventral midline incision in the neck, was carefully separated from the vago-sympathetic trunks and loosely encircled with 8-0 sutures for later occlusion. The gerbil's head was mounted on a stereotaxic apparatus (Stoelting, IL, USA) with the nose bar positioned 3.3 mm below the horizontal line. Following a midline incision, the skull was craniectomized and exposing the right middle cerebral artery (MCA). A 8-0 suture was positioned around the MCA for later ligation. A microdialysis probe (4 mm in length, CMA/12, Carnegie Medicin, Stockholm, Sweden) was stereotaxically implanted into the cortex (AP 0 mm, ML 5 mm, DV −4.0 mm from bregma).

The probe (0.8 mm in diameter) of a laser Doppler blood flow monitor (MBF 3D, Moor Instruments, Axminster, UK) was positioned on the cortex with its tip close to the MCA. The flow signal was averaged with a 5-s time constant, and the signal was recorded continuously on an *x–y* recorder. A focal ischemic lesion was made by occlusion of the right CCA and the right MCA for 60 min, followed by a 3-h reperfusion period.

Dialysis probes were perfused with Ringer's solution (147 m*M* Na^+; 2.2 m*M* Ca^{2+}; 4 m*M* K^+; pH adjusted to 7.0) at 2 μl/min using a CMA/100 microinfusion pump. Dialysates were collected every 15 min in a CMA/140 fraction collector (Carnegie Medicin, Stockholm, Sweden). Dialysates (5 μl) were directly injected onto a LC system with a UV detector set at 214 nm (BAS UV-116, Bioanalytical Systems, West Lafeyette, IN, USA) for the measurement of pyruvate, lactate, and ascorbate [19]. Separation of these substances was achieved using a conventional column (100×4.6 mm I.D.) packed with 10 μm Polypore H (Brownlee Labs., IL, USA).

The mobile phase consisted of 4 m*M* sulfonic acid in double-distilled water (112 μl concentrated sulfonic acid in 1000 ml distilled water). The mixture was filtered through a 0.22-μm nylon filter under reduced pressure. The flow-rate was 0.55 ml/min maintaining column pressure at ~5.2 MPa. The concentrations of pyruvate, lactate, and ascorbate in the dialysates were calculated by determining each peak area ratio relative to the standard mixture. The identity of each peak in the chromatograms was confirmed by their retention times, and a superimposed technique provided by CHEMSTATION (Hewlett-Packard 3365 Series II Chemstation, Taipei, Taiwan).

3. Results

Cerebral blood flow dropped to 60% of basal when the CCA was occluded, and then to less than 5% of basal after the additional occlusion of the MCA as shown in Fig. 1. Cerebral blood flow reached minimum levels within 5 min of occlusion (CCA+MCA) and maintained at this level throughout the occlusion period. The cerebral blood flow returned to its basal level (100%) 5 min after reperfusion and was maintained there throughout the reminder of the experimental period (3 h). The occlusion of the right CCA and MCA prevents cerebral blood flow to the anterior portion of the right side of the brain. The gerbil brain lacks the connection between the carotid and vertebrobasilar blood vessels, which makes an incomplete circle of Willis. Thus, a simple CCA and MCA ligation will prevent blood flow to the anterior portion of the brain. This surgical technique has made the CCA+MCA occlusion an excellent animal model for studying focal cerebral ischemia.

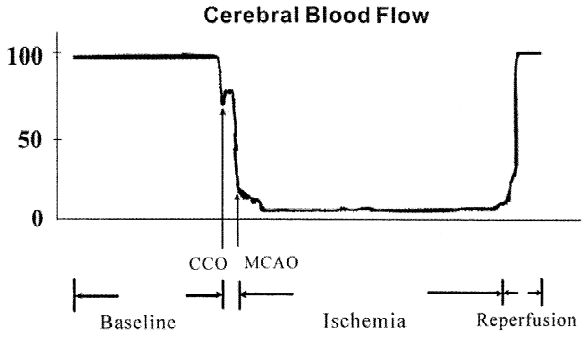

Fig. 1. Cerebral blood flow was recorded by a laser doppler during the microdialysis sampling experiment. Arrows indicate baseline, ischemia and reperfusion periods.

The precision and stability of the LC assays in the determination of pyruvate, lactate and ascorbate were acceptable (<5%) using standard mixtures and pooled microdialysates. The detection limits (signal-to-noise ratio = 5) for pyruvate, lactate, and ascorbate in the present assay were 0.2, 2.0 and 0.5 μ*M*, respectively. Occasionally, each peak was also verified by spiking with authentic standards to see if the addition increased the peak height without changing the retention time and peak shape. The measurement of pyruvate, lactate, and ascorbate in such small volumes and low detection limits has great analytical potential in microdialysis applications.

In general, stable basal levels of pyruvate, lactate and ascorbate were obtained 2 h after implantation of microdialysis probes in anesthetized gerbils. Basal concentrations of pyruvate, lactate and ascorbate in dialysates were 10.3±2.8, 500.7±105.0 and 4.7±2.5 μ*M*, respectively, in the control group. The basal concentrations of pyruvate, lactate and ascorbate were 10.5±4.6, 348.9±106.2 and 4.4±1.4 μ*M*, respectively, in the EGb 761 group. There were no significant differences between the two groups.

During focal ischemia, the right CCA and MCA were occluded for 60 min. Pyruvate, which is an intermediate of both anaerobic and aerobic glucose metabolism, significantly decreased to 26% of basal. It gradually returned to about 62% of basal within 30 min of reperfusion. It remained at this low level throughout the experiment in the control group. The pyruvate profile of the EGb 761 group was similar to that of the control group during the baseline and ligation periods (25% of basal). However, it returned to baseline (124% of basal) within 1 h after reperfusion and remained at this level throughout the rest of the experiment (Fig. 2).

Lactate levels increased to 158% of basal in the first 15-min interval and then decreased to baseline at the end of the occlusion period in the control group. An increase in lactate level to 152% of basal was observed at 30 min and remained elevated after reperfusion in the control group. Lactate levels increased to 156% of basal in the first 15-min interval and then decreased to baseline at the end of occlusion in the EGb 761 group. Lactate increased to 171% of basal at 45 min and was maintained at this high level after reperfusion in the EGb 761 group. There was no significant difference in lactate profiles between the two groups.

The basal L:P ratios were 39 and 53 for the control and EGb 761 groups, respectively. The L:P ratio increased dramatically upon cerebral ischemia to a peak of about 200 and decreased right after reperfusion in both groups. Then, the ratio gradually decreased to about 100 within 3 h of reperfusion in both groups. These data were in agreement with those of other investigators [23]. There was no difference in the L:P ratio during the ligation period. A slight attenuation of the L:P ratio (87% of basal) was observed at the beginning of the reperfusion period in the EGb 761 group. However, L:P ratios were about the same at the end of the reperfusion period.

Ascorbate demonstrated a biphasic pattern of increase in the early ischemia and later reperfusion periods. An increase (to 177% of basal) in the first phase was demonstrated in the microenvironment during the stress of focal cerebral ischemia. Thereafter, ascorbate levels increased drastically, to a peak of 309% of baseline, within 90 min of the start of reperfusion, then gradually decreased to 235% of basal at the end of reperfusion in the control group. There was no difference between the two groups during the ischemic period. However, the increase in ascorbate levels was even higher at 45 min reperfusion to a peak of 752% followed by gradual decreases to 131% at the end of reperfusion in the EGb 761 group.

4. Discussion

Numerous well-controlled clinical studies conducted in Europe and in the USA have revealed that EGb 761 is an effective therapy for a wide variety of disturbances of cerebral functions ranging from cerebral impairment of ischemic vascular origin (i.e. multi-infarct dementia), and early cognitive decline to mild-to-moderate cases of the more severe types of senile dementia (including Alzheimer's disease) and disturbances of mixed origins (i.e. psycho-organic origin) [1–3]. Some clinical studies have shown that EGb 761 improves the capacity of geriatric patients to cope with the stressful demands

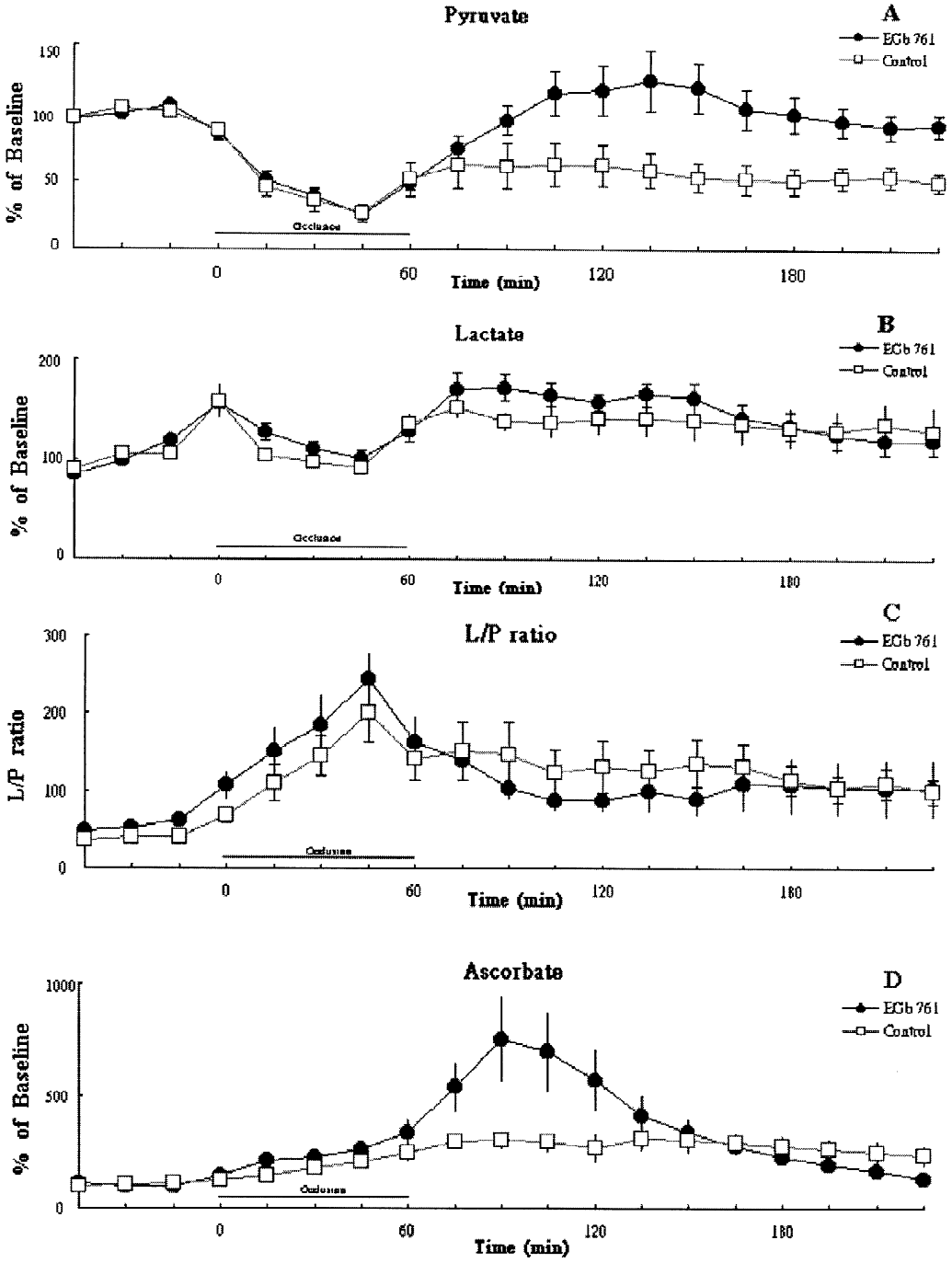

Fig. 2. Time profiles obtained for extracellular levels of (A) pyruvate, (B) lactate, (C) L:P ratio, (D) ascorbate in gerbil cortex of control (blank square) and EGb 761 treated (dark circle) groups during a 60-min focal cerebral ischemia followed by 3 h reperfusion.

of daily life [4]. There is substantial experimental evidence supporting the view that EGb 761 has neuroprotective properties under conditions such as hypoxia/ischemia, seizure activity and peripheral nerve damage [4]. EGb 761 consists of various ginkgo-flavonol glycosides and terpene lactones suggesting that it may exert multiple effects on the cascade of cerebral ischemic events [2]. However, research on the neurochemical effects of EGb 761 is still in its early stages.

Within the last decade, there has been increasing interest in examining biochemical parameters to determine if EGb 761 can play a role in reducing brain damage. At the molecular and cellular levels, some evidence obtained from animal models indicates that EGb 761 can maintain ATP content by protection of mitochondrial respiration and preservation of oxidative phosphorylations [21]. Chronic administration of EGb 761 as a potent treatment can reduce neurotoxin-induced cerebral edema [21]. Preservation of extracellular pyruvate by intracerebral injection of naloxone in anesthetized MCAO rat models has also been observed [19]. In addition, EGb 761 improves cerebral blood flow and increases glucose utilization [6]. Karcher et al. also found that pretreatment with EGb 761 increases brain glucose concentrations and enables rats to survive cerebral hypoxia for a longer period of time [23]. Taken together, this evidence suggests that EGb 761 may reserve glucose or preserve pyruvate within neuronal tissues for emergency energy needs during cerebral ischemia.

Another major metabolic change during cerebral ischemia is lactacidosis. In general, in acute cerebral ischemia there is a decline in high-energy phosphates, an increase in lactate, a decline in pH and a decline in the time courses of these changes. These changes have been well documented in a number of animal models including rats and gerbils [18–20,22]. The lactate profile in this study was in agreement with those of previous reports [18–20]. However, much remains to be learned about the precise relationship between metabolic states and the critical thresholds at which the environmental status of the brain tissue becomes impaired and results in brain dysfunction. Biphasic lactate data clearly demonstrate dynamic changes in gerbil cortex tissues during ischemia and reperfusion. The present study of anaerobic conditions yielded a time profile of dynamic chemical changes in the gerbil cortex while subjected to focal cerebral ischemia. However, there was no significant difference in lactate profiles between the control and EGb 761 group. It is not known whether the change in lactate is an epiphenomenon or if it is involved in neuronal death either directly or as a secondary aggravating factor.

A markedly increased L:P ratio was observed during the ligation period indicating the severity of cerebral ischemia. The basal L:P ratios ranged from 39 to 53 prior to ligation. These ratios increased significantly to 200–243 at the end of the ligation period for both groups, and gradually decreased after reperfusion. In general, increased lactate levels and decreased pyruvate production by tissues were demonstrated during cerebral ischemia, simply because of decreased regional cerebral blood flow and blood supply. The higher utilization rate of glucose also produced relatively higher concentrations of lactate. Increased brain glucose concentrations might indicate a reservoir of lactate source during cerebral ischemia with pretreatment of EGb 761. The preservation of pyruvate was noted at the early reperfusion period. The L:P ratio was indeed attenuated in a parallel manner, but was not significantly different, in the EGb 761 group. However, there was no significant difference in L:P ratio between the two groups.

It has been hypothesized that the brain produces reactive oxygen species during ischemia and at a rate sufficient to escape endogenous antioxidant defenses [21]. Therefore, many antioxidants, such as vitamin E, vitamin C and α-tocopherol, have been proposed to be beneficial for cerebral ischemia in animals and clinical trials [21]. It has also been proposed that EGb 761 interacts as a free radical scavenger and an inhibitor of lipid peroxidation with all or nearly all reactive oxygen species. Many reports have indicated that EGb 761 has free radical-scavenging and antilipoperoxidative properties [21,24]. In the present study, ascorbate levels increased during ligation and reperfusion periods to scavenge generated reactive oxygen species in the control group. The decrease in ascorbate in the first phase was mostly due to the reperfusion of blood flow causing a dilution of local ascorbate level. Further increases in ascorbate levels (309 and 752%) were detected during reperfusion in

both groups. However, increases in ascorbate (up to 752%) were observed at the beginning of the reperfusion period in the EGb 761 group. These enhanced ascorbate levels may be interpreted as an indicator of neuron-beneficial effects. In addition, Crespi reported that ascorbate is a biochemical index of early ischemia and could be a useful tool for the evaluation of initial ischemic damage [17].

Improvement in cognitive functions has been demonstrated, particularly in memory, attention, alertness, vigilance, arousal and mental fluidity following treatment with EGb 761 [4]. EGb 761 is worthy of further investigation as potential neuroprotective agent. Clinically, EGb 761 has proven to have favorable effects on intellectual deficiency, equilibrium disturbances and peripheral artery occlusions, and is a drug with a clear cut indication for these diseases [1–3]. The present study demonstrated that the preservation of pyruvate and/or the enhancement of ascorbate by EGb 761 may contribute to the observed neurological benefits. In addition, the present study yielded useful information on the range and kinetics of chemical changes following acute focal cerebral ischemia with EGb 761 pretreatment. A dynamic microdialysis–LC assay by which the energy states of the brain can be monitored will be valuable in a number of acute experimental animal models or clinical situations. EGb 761 has been reported to help in reducing neurological deficits caused by cerebral ischemia in rat models and clinical trials.

5. Conclusion

This study measured dynamic changes in the extracellular concentrations of pyruvate, lactate and ascorbate, and in the lactate:pyruvate ratio in the cortex of gerbils subjected to CCA and MCA ligation for 60 min. Depletion of pyruvate and increase in lactate were observed and the lactate:pyruvate ratio was raised 5-fold during cerebral ischemia in the control group. Preventive treatment with EGb 761 provided beneficial effects when chronically injected for 8 days. The results of this study also supported earlier findings of beneficial effects of EGb 761 at biochemical levels. To the best of our knowledge, this is the first study to apply microdialysis technique for investigating EGb 761 protection by energy related chemicals during experimental cerebral ischemia. Clear beneficial effects of EGb 761 on the preservation of pyruvate levels and the enhancement of ascorbate levels were demonstrated. A complete explanation of EGb 761's beneficial effects is not yet possible, but antioxidant, free radical scavenging and anti-ischemic actions and its effects on cerebrovascular and neurotransmitter mechanisms seem to be involved. The variable elapsed time between ischemic injury and treatment with EGb 761, the dosages of the drug and the difficulty in the exact quantitation of the injury sustained are questions still to be addressed in the future.

Acknowledgements

This study was supported by grants from Taichung Veterans General Hospital (TCVGH-897201B and 897311D) and the National Science Council (NSC-89-2113M-075A-002), Taiwan, Republic of China.

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