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Influence of Terpene Lactones Treatment on Brain Lipid Peroxidation and Antioxidant Defense Systems of Rats after Focal Ischaemia

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The influence of terpene lactones on the lipid peroxide product, malondialdehyde, glutathione and catalase, glutathione peroxidase, superoxide dismutase activities in rat brain as well as lactate dehydrogenase and creatine kinase activities in rat blood serum after occlusion of middle cerebral artery following reperfusion was investigated. Experimental model of the reversible middle cerebral artery occlusion without craniectomy of rat focal cerebral ischaemia-reperfusion was used. Compared to sham-operated animals, rats subjected ischaemia followed reperfusion exhibited severe neurologic deficits, ischaemia followed reperfusion increased blood serum lactate dehydrogenase and creatine kinase activities as well as brain glutathione peroxidase activity and decreased superoxide dismutase activity as well as glutathione and malondialdehyde content. Administration of terpene lactones (4,8,16 mg/kg, i.p.x2) at 15 min and 6 h after induction of just ischaemia could improve the movement function and normalize the lactate dehydrogenase and creatine kinase activities of the rat serum. Terpene lactones were also able to increase catalase and superoxide dismutase activity, ameliorate the abnormal increment of glutathione peroxidase activity but did not change glutathione content. Significant decrement of malondialdehyde content in cortex and striatum was also observed when terpene lactones were administrated. These results suggested that terpene lactones could affect the process of oxidative damage. This perhaps was related to the beneficial role in the protection against ischaemia-reperfusion injury.

The hypothesis that free radical processes participate in the post-ischaemic neuronal damage has been popular for several years[1-4]. Under normal conditions, the production and elimination of reactive oxygen species are in a balance. During ischaemia, the free radical defense is damaged and reactive oxygen species are overwhelming, therefore peroxidation of membrane lipids occurs and neurons are injured. However, definitive proof of the occurrence is still controversial. Several laboratories have provided evidence that lipid peroxidation occurred *in vivo* either during or after brain ischaemia and hyperfusion[5-8], but other data have failed to support this hypothesis[9,10]. The free radical defense includes enzymatic and non-enzymatic systems.

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Terpene lactones (TL) are terpenoid constituents of *Gingko biloba* extract (EGb), which includes ginkgolides A, B, C, E, J and bilobalide. We have previously obtained the results of TL's beneficial effect on rat focal cerebral ischaemia model[11]. There are phenol hydroxy radicals in some constituents of TL, and also some of these constituents have antagonistic effects on platelet activating factor (PAF). It was reported that the interaction of PAF with platelets, leukocytes, endothelial cells and/or neuronal cells potentiated the accumulation of reactive oxygen species in the brain during post-ischaemic reoxygenation[12]. We conjured that the pro-

tective effect of TL on rat ischaemia might be related to its interaction with the free radical systems. Therefore, in the present study, we tried to investigate the influence of TL on endogenous enzymes including catalase (CAT), glutathione peroxides (GSH-Px) and superoxide dismutase (SOD) activities as well as endogenous non-enzymatic antioxidant, reduced glutathione (GSH) content, and also tried to examine the change of malondialdehyde (MDA) in rat brain homogenates using the reversible middle cerebral artery occlusion (MCAO) without craniectomy of rat focal cerebral ischaemia-reperfusion.

MATERIALS AND METHODS

Total terpene lactones, purity>95% (Ginkgolides A 42.7%, B 33.2%, C 9.5%, bilobalide 9.5%), provided by professor Song You (Department of Biotechnology, Shenyang Pharmaceutical University), was dissolved in a mixture of dimethyl sulfoxide (DMSO), PEG 400 and saline (10:30:60). Fresh drug solutions were prepared just before use[13]. The International Guiding Principles for Animal Research were strictly adhered to throughout the experiment.

Male Wistar rats weighing 280-320 g (Animal Breeding Center of China Medical University, Shenyang, China) were housed under constant enviromental conditions and were allowed free access to pelleted food and water *ad libidum*. During the studies, every effort was made to minimize animal suffering and to reduce the number of animal used. MCAO was performed following the procedure of Longa *et al.*[14] with minor modifications. Rats were anaesthetized by an i.p. injection of 360 mg/kg chloral hydrate. The frequency of spontaneous breathing and heart rate were monitored throughout the experiment. The left external carotid artery (ECA) was separated and after the branches of ECA were ligated, a 5-cm length of 4-0 monofilament nylon suture was inserted into ECA lumen, then gently advanced from the ECA to the internal carotid artery (ICA). After about 19-21 mm of suture (from the origin of ICA) was inserted into ICA, resistance was felt, indicating that the tip of the suture had passed the MCA origin and reached the proximal segment of the anterior cerebral artery. The nylon suture was pulled back after a 2 h MCAO to restore the blood flow to the MCA territory. The enviromental temperature was maintained at 30° throughout the experiment in order to prevent cerebral hypothermia[15]. The motor behavior of the rats was observed and scored according to Zhang *et al.*[16] and Longa *et al.*[14] at 6 h and 24 h after ischaemia by an observer who had no knowledge of which procedure had been performed. Animals were classified into five groups, each group consisted of 8-10 rats. The first group served as sham-operated control, which was performed without inserting the suture, the second group for induction of 2 h ischaemia followed by 24 h reperfusion. The first and the second group were given the mixture of DMSO, PEG 400 and saline without TL. TL (4, 8,16 mg/ kg, i.p.x2) was given to the other three groups of rats 15 min and 6 h after induction of just ischaemia.

Biochemical determinations:

Rat blood was collected by excising their eyes in ice-cold containers without anticoagulant and then rats were decapitated and their skulls were isolated on ice. The cortex, hippocampus and striatum of left MCA territory were homogenized respectively with ice-cold saline as 10% w/v homogenates using glass homogenizers. The homogenate was centrifuged at 4000 g for 10 min and the supernatant was used for assay of catalase, glutathione peroxidase, superoxide dismutase activity as well as reduced glutathione and MDA content according to the following methods. The blood was centrifuged and the serum was gathered for assay of LDH and CK by using commercial kit purchased from Jian-Cheng (Nanjing, China).

CAT was estimated at 405 nm by monitoring the light yellow combining compound of H_2O_2 and added ammomium molybdate described by Cheng *et al.*[17] One unit of catalase has been expressed as the amount of 1 μmol H_2O_2 decomposed per sec per mg of protein. GSH-P_x and GSH was determined by DTNB colouring method[18]. One activity unit of GSH-P_x was expressed as μ mol of GSH decomposed per min per mg of protein.

SOD was assayed according to the method of Martin *et al.*[19] The change in absorbance was monitored at 550 nm. The activity of enzyme was expressed as units/mg protein, where 1 unit of enzyme activity was defined as the amount of enzyme inhibiting the rate of reaction by 50%. MDA was estimated by the thiobarbituric acid reactive substances (TBARS) method, as described by Ohkawa *et al.*[20] and was expressed in terms of MDA formed per mg of protein. Protein content of brain tissues was determined by the method of Lowry *et al.*[21] using bovine serum albumin as standard.

Statistical analyses:

All data were presented as means±SEM. Results of drug treatment group and control group were compared with those of ischaemia-reperfusion group and were analysed for significant statistical difference by using student's t-test. The value of P less than 5% ($P<0.05$) was considered as significant.

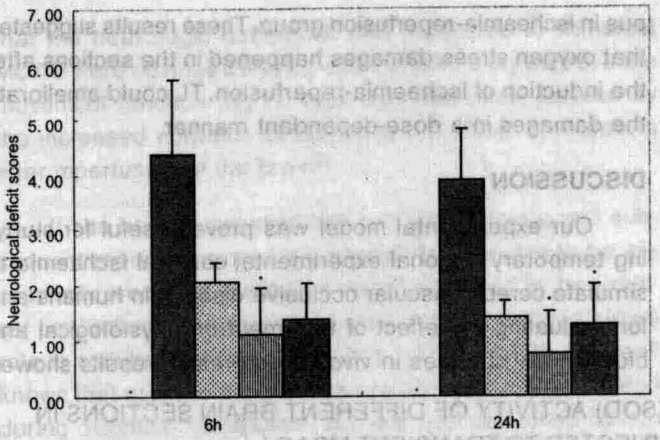

Fig.1: Effect of TL on neurologic deficits of rats subjected to trancient MCAO.

TL 4,8 and 16 mg/kg was given i.p. for 2 times, 15 min and 6 h after induction of just ischaemia. The motor behaviour of rats was scored at 6 h and 24 h after ischaemia followed reperfusion. Values were means±SEM of 6-9 experiments. Statistical significance was determined by students t-test. *represents significant difference when compared with ischaemia-reperfusion group at p<0.05. □ -Sham-operated, ▨ -Ischaemia-reperfusion, ▩ -TL(4 mg/kg), ▥ -TL(8 mg/kg), ▤ -TL(16 mg/kg).

RESULTS

All the rats with MCAO exhibited neurologic deficits while the ischaemia-reperfusion rats suffered more at 6 h and 24 h after MCAO. TL 4,8 and 16 mg/kg improved the movement function of the rats with MCAO (fig.1). In the serum of the rats submitted to ischaemia followed by reperfusion, LDH and CK activity increased significantly. Terpene lactones could ameliorate them (Table 1).

In the cortex homogenates of the rats submitted to ischaemia followed by reperfusion, there was no significant change in CAT activity. Treatment of rats with terpene lactones (TL16 mg/kg) produced a significant increment in CAT activity compared to the ischaemia-reperfusion control (Table 2). Results in Table 2 showed that significant increment in cortex homogenates of left MCA territory of GSH-Px activity in ischaemia-reperfusion rats comparing to sham-operated group rats. TL was able to improve the abnormal phenomena.

There was a significant reduction in SOD activity of the rat cortex and striatum after induction of ischaemia followed by 24 h reperfusion but no change was seen in hippocampus (Table 3). TL was able to increase the SOD activ-

TABLE 1: EFFECT OF TL ON LDH AND CK ACTIVITIES (U/L) OF RATS'SERUM SUBJECTED TO TRANSIENT MCAO.

Group	LDH (U/L)	CK (U/L)
Sham-operation	627±142*	1303±134*
Ischaemia-reperfusion	1161±163	4247±1055
Ischaemia-reperfusion +		
TL 4 mg/kg	916±111	2757±830
TL 8 mg/kg	804±247	2329±665
TL 16 mg/kg	428±89*	2763±643

TL 4,8 and 16 mg/kg was given i.p. for 2 times, 15 min and 6 h after induction of just ischaemia. Blood was collected by excising rats'eyes at 24 h after ischaemia followed reperfusion and LDH and CK activities in serum were detected according to the kits'guide. Values were means±SEM of 5-6 experiments. Statistical significance was determined by students t-test. *represents significant difference when compared with ischaemia-reperfusion group at p<0.05.

TABLE 2: EFFECT OF TL ON CATALASE (CAT) ACTIVITY AND GLUTATHIONE PEROXIDASE (GSH-PX) ACTIVITY OF CORTEX OF LEFT MCA TERRITORY OF RATS SUBJECTED TO TRANSIENT MCAO.

Group	CAT [U/(mg protein)]	GSH-Px (Activity Units)
Sham-operation	1.54±0.83	3.35±0.75*
Ischaemia-reperfusion	0.17±0.38	6.65±0.13
Ischaemia-reperfusion +		
TL 4 mg/kg	0.14±0.56	5.96±0.47*
TL 8 mg/kg	0.38±0.29	4.75±0.62*
TL 16 mg/kg	3.34±1.22*	2.32±0.92*

TL 4,8 and 16 mg/kg was given i.p. for 2 times, 15 min and 6 h after induction of just ischaemia followed reperfusion. At 24 h after ischaemia, brain cortex homogenate was collected for assaying CAT and GSH-Px. Values were means±SEM of 8-10 experiments. Statistical significance was determined by students't-test. *represents significant difference when compared with ischaemia-reperfusion group at p<0.05.

ity when administered 15 min and 6 h after the induction of ischaemia. The effects of TL 4, 8 and 16 mg/kg on SOD activity in ipsilateral striatum were dose-dependent.

Cortex homogenates GSH content was decreased by 34% significantly after reperfusion of the ischaemia rat brain (Table 4). Treatment of rats with TL at 15 min and 6 h after induction of ischaemia did not change the GSH content comparing to the ischaemia-reperfusion control group. Table 4 showed that MDA content was significant increased in rat cortex and striatum but no change was found in hippocampus in ischeamia-reperfusion group. These results suggested that oxygen stress damages happened in the sections after the induction of ischaemia-reperfusion. TL could ameliorate the damages in a dose-dependant manner.

DISCUSSION

Our experimental model was proved useful for studying temporary regional experimental cerebral ischaemia to simulate cerebrovascular occlusive disease in humans and for evaluating the effect of treatment on physiological and biochemical changes in *vivo*[14]. The present results showed

TABLE 3: EFFECT OF TL ON SUPEROXIDE DISMUTASE (SOD) ACTIVITY OF DIFFERENT BRAIN SECTIONS IN LEFT MCA TERRITORY OF RATS SUBJECTED TO TRANSIENT MCAO.

Group	SOD [Units /(mg pro)]		
	Cortex	Hippocampus	Striatum
Sham-operation	213.5±8.9*	182.3±10.0	261.3±8.4*
Ischaemia-reperfusion	182.9±10.1	168.9±7.68	194.3±6.29
Ischaemia-reperfusion +			
TL 4 mg/kg	185.6±10.2	218.4±12.7	234.4±24.0
TL 8 mg/kg	189.6±13.8	215.5±10.8	225.7±12.9*
TL 16 mg/kg	211.2±18.0	207.5±12.4	216.4±6.53*

TL 4,8 and 16 mg/kg was given i.p. for 2 times, 15 min and 6 h after induction of just ischaemia followed reperfusion. At 24 h after ischaemia, brain cortex, hippocampus and striatum homogenates were collected for assaying SOD. Values were means±SEM of 5-6 experiments. Statistical significance was determined by students't-test. *represents significant difference when compared with ischaemia-reperfusion group at $p<0.05$.

TABLE 4: EFFECT OF TL ON GLUTATHIONE (GSH) AND MDA CONTENT OF LEFT MCA BRAIN SECTIONS OF RATS SUBJECTED TO TRANSIENT MCAO.

Group	GSH [mg/(g pro)]	MDA [n mol /(mg pro)]		
		Cortex	Hippocampus	Striatum
Sham-operation	93.9±9.51*	7.77±1.72*	9.06±2.38	3.17±1.01*
Ischa-reperfusion	61.7±8.29	10.3±1.56	8.69±2.64	9.12±1.25
Ischa-reperfusion+				
TL 4 mg/kg	92.0±13.5	9.12±1.58	10.7±1.95	8.81±2.43
TL 8 mg/kg	78.4±20.0	7.26±2.25*	9.59±2.30	7.17±2.22
TL 16 mg/kg	58.6±9.31	6.55±1.50*	10.9±1.24	6.74±1.74*

TL 4,8 and 16 mg/kg was given i.p. for 2 times, 15 min and 6 h after induction of just ischaemia followed reperfusion. At 24 h after ischaemia, brain sections homogenate was collected for assaying GSH (in cortex) and MDA content. Values were means±SEM of 5-6 experiments. Statistical significance was determined by students't-test. *represents significant difference when compared with ischaemia-reperfusion group at $p<0.05$.

that the neurologic deficits of rats subjected to trancient MCAO were obvious while TL could significantly improve the movement function (fig.1). This was also demonstrated by the increased activities of LDH and CK (Table 1) in serum after reperfusion of the brain[22].

It has been suggested that during ischaemia and subsequent reperfusion reactive oxygen species are to be excessively produced. Oxidative damage caused by free radical production has been postulated at least partly to play a role in cellular damage in cerebral ischaemia[23,24]. It is well known that reactive oxygen species are normally generated during oxidative metabolism but are well controlled by enzymatic and non-enzymatic systems. An increase of lipid peroxidation may be resulted from either an overproduction of reactive oxygen species or from a less of efficacy of the scavenging systems. Studies have attempted to measure the evolution of endogenous antioxidant content, a progressive decline of GSH level was found from 10 min to 24 h after an ischeamic period[25,26]. Our data also showed that there was a marked suppression of GSH content in the cortex of left MCAO territory of rat brain after ischaemia followed by reperfusion but TL could not change GSH content (Table 4).

In the present investigation, GSH-Px activity was found to be significantly increased in the cerebral cortex at 24 h of recirculation after 2 h of MCAO, while TL could normalize them when adminstered 15 min and 6 h after induction of ischaemia (Table 2). Just like the aging process, ischaemic damage was also a progressive tissue damage that could be induced by reactive oxygen species[27]. Moreover, the ischaemic conditions are certainly much more severe and free radical generation is much greater in complete than incomplete ischaemia model[1]. Bromont's study[23] showed that increases were observed in thiobarbituric acid-reactive substances levels in parietotemporal cortex, hippocampus and striatum brain regions not during the ischaemic period or after 1 h of recirculation but between 8 and 72 h of recirculation. Therefore, the increment in our experiments of brain GSH-Px activity of ischaemia-reperfusion rats *vs* sham-operated group rats could represent a defensive response of brain to an augmented lipid peroxidation in the earlier phases of changes of lipid peroxidation. In addition, the mechanism of protein synthesis in brain may be programmed to elevate the level of this protective enzyme in response to an increase of reactive oxygen species[28]. Moreover, we found that there were decrement of SOD activity and increment of MDA content in cortex and striatum but no changes in hippocampus (Table 3 and 4). This suggested that in this model the oxidative damages were mainly happened in cortex and striatum. The results were different from Bromont's. The discrepancy might originate from differences in the severity of ischeamia. Our study was performed on a model of reversible regional cerebral ischaemia in rats without craniectomy based on advancing an intraluminal suture from the internal carotid artery (ICA) to occlude the origin of the MCA, whereas Bromont performed the study with the four-vessel occlusion model, which produced more severe forebrain ischaemia. Moreover, we observed that TL (16 mg/kg) could significantly increase the CAT activity in cortex (Table 2), which was obviously helpful to eliminate hydrogen peroxide and furthermore to control the formation of hydroxyl radicals derived from hydrogen peroxide. Hydrogen peroxide was known to be a factor which contributed to brain injury and brain oedema following cerebral ischaemia[29,30], and also in the presence of Fe^{3+} and superoxide anion, hydrogen peroxide was partly converted to hydroxy radicals which were highly reactive with the membrane constituents.

The doses required in this study were different from that in others[13]. There were numerous explanations for the difference, which included the amount of tissue involved in the two insults, the different end points determined in the two studies and the compound itself. It was important to recognize that the mechanism of injury and the efficacy of treatments degree, duration, and other aspects of the experimental model. TL was a mixture that included ginkgolides A, B, C, E, J and bilobalide. All the monomers were contributed to the affection of TL and therefore in our study the doses required were much lower. This was also the advantage of Chinese medicine. Comparing to the monomers, TL was much more easily prepared, and it was an effective position of traditional medicine with bright prospects.

In conclusion, our results suggested that as a kind of terpenoid constituents of *Ginkgo biloba* extract, TL could protect rats against damage resulting from transient cerebral ischaemia by regulating the efficiency of scavenging systems.

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