

The functional effect of epigallocatechin gallate on ischemic stroke in rats

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We evaluated the efficacy of epigallocatechin gallate (EGCG) for improving function in rats with transient middle cerebral artery occlusion (MCAO). Three procedures underwent for each groups; MCAO and EGCG treatment, MCAO without treatment (MCAO control), and sham operation. Function was evaluated on days 1, 5, 10, and 14 after ischemia, using a modified sticky-tape test and the percentage weight borne on the paretic leg. Infarct volume did not differ between the EGCG-treated and MCAO control groups. Compared with the MCAO control group, the EGCG-treated group showed improved forelimb function and had normal function by day 10 ($P < 0.05$). The improvements persisted until day 14 post-ischemia. In contrast, hind limb function did not differ between the EGCG-treated and MCAO control groups. Thus, EGCG improved certain aspects of function of forelimb until day 14 after MCAO in rats.

Key words: catechin, EGCG, epigallocatechin gallate, functional improvement, middle cerebral artery occlusion

INTRODUCTION

Several million people per year are attacked by stroke and there is still no established treatment for lessening the neurologic damage. A large proportion of medical expenditures is used in the treatment of acute ischemic stroke and many stroke victims are left with residual disability. Several studies have examined various methods of reducing neuronal damage after ischemic stroke. Catechin, extracted from green tea, has been suggested to have a neuroprotective effect (Sutherland et al. 2006, Fraser et al. 2007).

Green tea (*Camellia sinensis*) has been used as a medicine for thousands of years (Sutherland et al. 2006). Several epidemiological and experimental studies have investigated the neuroprotective effect of epigallocatechin gallate (EGCG), the main polyphenol in green tea, on ischemic stroke and have demonstrated several possible mechanisms of action

(Sutherland et al. 2006, Tanabe et al. 2008, Arab et al. 2009). For example, EGCG has been verified to reduce, through an antioxidative effect, the neuronal damage that is caused by iron-induced oxygen free radicals (Choi et al. 2004, Xie et al. 2008). EGCG has been reported to reduce the delayed cell death near the hippocampus and the excitotoxic neuronal damage in ischemic lesions following transient ischemia (Nagai et al. 2002, Zhang et al. 2008). The effect of EGCG on neuronal damage has typically been measured within 2 days after ischemic stroke (Choi et al. 2004), and the effect of EGCG on function following transient focal ischemia has not been investigated. Additionally, many similar treatments have shown positive effects on stroke in animal studies, but equivocal results in clinical investigations (Gladstone et al. 2002). Thus, animal studies that assess the effect of EGCG treatment on function are necessary to bridge the gap between the laboratory and the clinic. A recent study investigated the effect of EGCG on memory and learning after ischemia and demonstrated a functional improvement in a transient middle cerebral artery occluded rat (Haque et al. 2008). This study was performed to verify

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whether EGCG has a positive effect on functional improvement in a transient middle cerebral artery occluded rat.

The aim of the present study was to investigate whether EGCG treatment can improve upper and lower limb functions and reduce neuronal damage within 2 weeks after induction of unilateral brain ischemia in a transient middle cerebral artery occlusion (MCAO) rat model.

METHODS

Subjects

Forty-five male Sprague-Dawley rats weighing 365 ± 25 g (mean \pm SD) were purchased from the Orient Bio Company (Sungnam, Korea) and used in the present study. All experimental procedures were approved by the Animal Experimental Committee of the Catholic University of Korea. Rats were randomly assigned to three groups: stroke with EGCG treatment (MCAO with EGCG, $n=20$), stroke with no treatment (MCAO control, $n=15$), and sham operation (Sham, $n=10$).

Induction of focal cerebral ischemia and epigallocatechin gallate administration

The rats were anesthetized with an intraperitoneal (i.p.) injection of 1% ketamine (30 mg/kg) and xylazine hydrochloride (4 mg/kg), and left middle cerebral artery occlusion was induced using the monofilament thread method, as first described by Longa and coauthors (1989) and revised by Ma and others (2006). After 2 h of occlusion, the two MCAO groups were

reperused. Immediately after reperfusion, EGCG (50 mg/kg, i.p.; Sigma, St. Louis, MO, USA) was administered to the treatment group (Choi et al. 2004). The stroke control and sham control groups received an i.p. injection of an equivalent amount of saline solution. In the sham operation group, the left common carotid artery was exposed to the same level occlusion as that in the two MCAO groups; only adherent soft tissues were removed, arterial ligation and opening were not performed (Lee et al. 2006).

Estimation of cerebral infarction size

Two weeks after reperfusion, all rats in the two MCAO groups were re-anesthetized with 1% ketamine (30 mg/kg, i.p.) and decapitated. The brains were carefully removed and sectioned coronally at 2-mm intervals beginning at the frontal pole. The coronal slices were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) in phosphate-buffered saline, followed by formalin fixation and a saline rinse (Isayama et al. 1991). A calibrated image of the posterior surface of each slice was obtained (NIH Image), and the infarct areas were summed to determine the lesion volumes. The lesion volume is presented as volume percentage of the lesion compared with the contralateral hemisphere to void the impact of edema (Zhao et al. 2005).

Functional evaluation

All tests were carried out the day before ischemia was induced and on days 1, 5, 10, and 14 after ischemia. The tests were performed at predetermined

Table I

The percentage of infarct volume compared with the contralateral hemisphere on day 14 after ischemia for the MCAO with EGCG treatment and MCAO control groups			
Volume	MCAO with EGCG % (mm ³)	MCAO control % (mm ³)	<i>P</i> value
Total volume	6.87 ± 2.03 (24.3 ± 7.7)	8.27 ± 1.38 (29.7 ± 4.6)	0.141
Volume of cortex	3.58 ± 1.02 (12.7 ± 4.2)	4.38 ± 0.82 (15.7 ± 2.7)	0.050
Volume of striatum	3.29 ± 1.32 (11.5 ± 4.5)	3.90 ± 0.78 (14.0 ± 2.8)	0.253

Values are mean \pm SD. *P*-values are for the Mann-Whitney *U* test.

times, to exclude behavioral changes associated with circadian rhythms. A 5-min resting period was allowed between each test. The examiner had no prior knowledge of the experimental conditions. Functional assessments were performed in a random order, to reduce potential bias (Lim et al. 2008).

Assessment of sensorimotor functions in the forelimb

A modified sticky-tape (MST) test was performed to evaluate forelimb function (Sughrue et al. 2006, Komotar et al. 2007). A sleeve was created using a 3.0 × 1.0-cm piece of yellow paper tape (Write-On™ Label Tape; Bel-Art Products, Pequannock, NJ, USA) and was subsequently wrapped around the forepaw so that the tape attached to itself and allowed the digits to protrude slightly from the sleeve. When created correctly, the tape sleeve could not be removed. The typical response is for the rat to vigorously attempt to remove the sleeve by either pulling at the tape with its mouth or brushing the tape with its contralateral paw. The rat was then placed in its cage and observed for 30 s. Two timers were started: the first ran without interruption and the second was turned on only while the animal attempted to remove the tape sleeve. The ratio of the right (affected)/left (unaffected) forelimb performance was recorded. The contralateral and ipsilateral limbs were tested separately. The test was repeated three times per test day, and the best two scores of the day were averaged.

Assessment of sensorimotor functions in the hindlimb

Motor activity was assessed using a hind limb weight-bearing apparatus (Linton Incapacitance Tester; Storling Co., Wood Dale, IL, USA); stationary readings were taken over a 3-s period (Lim et al. 2008). The percentage weight borne (PWB) on the right leg was determined using the following formula:

$$\text{PWB on right leg} = \left[\frac{\text{weight on right leg}}{\text{weight on right leg} + \text{weight on left leg}} \right] \times 100$$

The hind limb weight-bearing apparatus recorded the mean value of three measurements taken for 3 s each. A total of three sets of readings (9 measurements) were taken per rat, and the mean values were calculated and used in the analysis.

Statistical analysis

All data are presented as the mean ± SD. The difference in infarct volume between the two MCAO groups was evaluated using the Mann-Whitney *U* test. Differences between groups with respect to MST performance and PWB on the hind limb were evaluated using the Kruskal-Wallis test, followed by the Mann-Whitney *U* test with the Bonferroni correction. All tests were two-tailed, and *P* values ≤ 0.05 were deemed significant.

RESULTS

Seventeen rats died during the experiment, leaving 9 rats in the MCAO with EGCG group (11 deaths), 10 rats in the MCAO control group (5 deaths), and 9 rats in the sham group (1 death). Of the 17 rats that died, three did not recover from MCAO and died after the anesthesia wore off. The remaining 14 rats expired within 4 days of the operation. Postmortem evaluations revealed extensive infarction of the left hemisphere with signs of cerebral edema in four rats, and evidence of intracranial hemorrhage in eight rats. The remaining two rats did not have postmortem evalua-

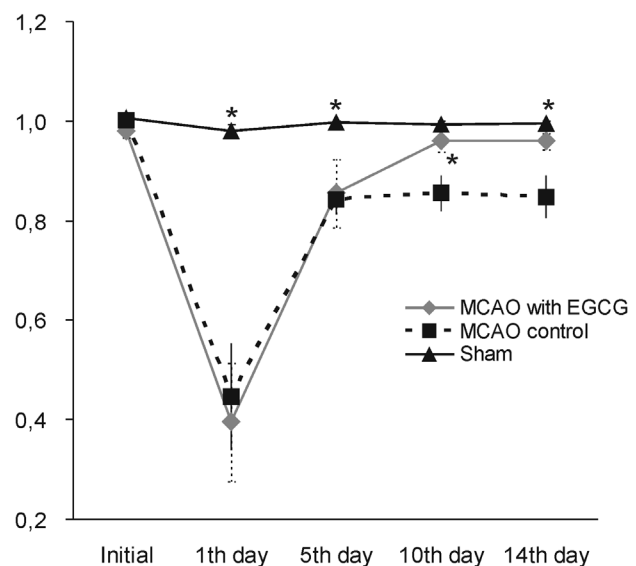


Fig. 1. Modified sticky-tape test (MST) performance expressed as a ratio of right (affected)/left (unaffected) forelimb. The values of all groups are shown mean with SEM (standard error of the mean) over time. The cut-off value for statistical significance was **P* < 0.016. The performance of the MCAO with EGCG group improved on days 10 and 14, and the value was significantly higher than that of the MCAO control group on day 10.

tions because of technical errors. These 17 rats were excluded from the final analysis.

Size of cerebral infarction

The infarct volume of the MCAO control group was slightly larger than that of the MCAO with EGCG group (Table I). There was no difference in the total or striatal infarct volume between groups ($P>0.05$). Although the cortical infarct volume was smaller in the MCAO with EGCG group, the difference was not significant ($P=0.05$).

Comparison of sensorimotor functions in the forelimb

No significant difference in the MST baseline was observed between groups (MCAO with EGCG; 0.98 ± 0.04 ; MCAO control, 1 ± 0.04 ; sham; 1.01 ± 0). After MCAO, the MST values of the MCAO control group were significantly lower than those of the sham group on test days 1–14 ($P<0.01$). The MST values of the MCAO groups were significantly lower than those of the sham group on days 1 and 5 after MCAO (Day 1: MCAO with EGCG, 0.40 ± 0.36 ; MCAO control, 0.45 ± 0.33 ; sham, 0.98 ± 0.04 ; Day 5: MCAO with EGCG, 0.85 ± 0.21 ; MCAO control, 0.84 ± 0.10 ; sham, 1 ± 0.01) However, the performance of the MCAO with EGCG group improved on days 10 and 14, and the value was significantly higher than that of the MCAO control group on day

10 (MCAO with EGCG, 0.96 ± 0.06 ; MCAO control, 0.85 ± 0.11 ; sham, 0.99 ± 0.02 ; $P=0.013$). Although the value of the treatment group was also higher on day 14, the difference was not significant (MCAO with EGCG, 0.96 ± 0.05 ; MCAO control, 0.85 ± 0.13 ; sham, 0.99 ± 0.01 ; $P=0.053$; Fig 1).

Comparison of sensorimotor functions in the hind limb

The PWB baseline did not differ significantly between the groups. The PWB on the paretic hind limb was significantly lower in the two MCAO groups compared with the sham group on test days 1–14 ($P<0.01$). The PWB on the paretic hind limb in the MCAO with EGCG group was not significantly greater than that in the MCAO control group at any point during the study (Table II).

DISCUSSION

Despite the impacts of stroke on several million people worldwide every year, no treatment exists to minimize the neurological damage. People who suffer stroke often experience persistent functional impairment. The present study investigated the effects of EGCG on the recovery of function following ischemic stroke. To show a long-lasting effect of EGCG on function in a MCAO rat model, we used an optimal dose of EGCG based on the findings of several studies (Choi et al. 2004, Rahman et al. 2005). Function was

Table II

The percentage weight borne on the paretic hind limb after MCAO (%)			
Day	MCAO with EGCG	MCAO control	Sham
Preoperative	49.80 ± 1.95	51.10 ± 1.90	50.49 ± 1.80
Postoperative Day 1	$45.02 \pm 2.71^*$	$46.75 \pm 1.57^*$	49.96 ± 0.48
Postoperative Day 5	$46.18 \pm 3.24^*$	$46.00 \pm 2.19^*$	50.99 ± 0.82
Postoperative Day 10	$47.48 \pm 2.02^*$	$46.68 \pm 1.78^*$	50.27 ± 0.97
Postoperative Day 14	$47.15 \pm 2.36^*$	$47.54 \pm 2.00^*$	50.28 ± 1.43

Values are mean percentage \pm SD. (MCAO) Middle cerebral artery occlusion; (EGCG) epigallocatechin gallate; (Sham) sham operation. $*P<0.016$, compared with sham group.

evaluated using the methodology reported in previous studies (Sughrue et al. 2006, Komotar et al. 2007, Lim et al. 2008). The results of the present study revealed that EGCG improved function of the forelimb within 2 weeks post-ischemia. This timeframe is consistent with previous reports of an EGCG effect on neuroprotection, cognitive enhancement, and the inhibition of apoptosis (Xie et al. 2008, Zhang et al. 2008).

Numerous studies have investigated the therapeutic effects of green tea, black tea, and catechin, possibly because tea is natural and regarded as safe for medicinal use (Takami et al. 2008). Several of these studies have reported that the therapeutic effects of EGCG on stroke are the result of its ability to cross the blood brain barrier (Mandel et al. 2006). Studies of EGCG have demonstrated that it is an effective free radical scavenger, antioxidant, xanthine oxidase inhibitor, and nitric oxide synthesis inhibitor (Choi et al. 2004, Burckhardt et al. 2008, Haque et al. 2008, Zhang et al. 2008). Additionally, EGCG may work through a voltage-gated sodium channel signaling pathway (Deng et al. 2008). However, these studies are limited because they do not show a functional effect of EGCG, and are investigated in the hyper-acute period. In contrast of these studies, one report demonstrated EGCG was associated with an increased risk of intracranial hemorrhage with the passage of time (Rahman et al. 2005). Risk of intracranial hemorrhage might induce high mortality of this study. Thus, additional study for the reason of increasing intracranial hemorrhage should be undertaken in near future.

No substance has been clinically proven to improve function after cerebral infarction, perhaps because several animal studies of neuroprotection have examined only the acute period after ischemia (Choi et al. 2004), whereas therapeutic outcomes in the clinical setting are evaluated over time. Moreover, evidence of neuroprotection is often based on changes in infarct volume in animal studies, but infarct volume does not necessarily correlate with functional outcome in patients. Furthermore, many of the therapeutic interventions shown to be effective in animal studies were not effective in the clinical setting (Gladstone et al. 2002). Thus, our study of EGCG derived from green tea may be of practical use, because EGCG can be readily used as a therapeutic intervention for stroke patients and its effects are easily measured in terms of functional outcomes. The present results demonstrate a positive effect on the forelimb function in rats treated

with EGCG. No significant differences were observed between the EGCG-treated and untreated groups during the first 5 days after ischemic stroke. At 10 days post-treatment, the EGCG-treated group showed significant functional improvement, which persisted for 14 days after treatment. We suggest that the lack of a difference in infarct volume between the treated and untreated MCAO groups during the initial 5 days was the result of gliosis occurring over time. A previous study reported that TTC staining for infarct detection was most useful within the first 5 days after MCAO, and the usefulness diminished over time (Clark et al. 1993). However, another study reported that TTC staining revealed neuroprotection at 14 days after MCAO (Zhang et al. 2005). However, infarct volumes of MCAO control group was smaller than those of previous our study (Lim et al. 2008). Thus, gliosis during two weeks would be culprit of reducing infarct volume.

Our study showed that EGCG treatment improved forelimb function, and the neuroprotective effect of the treatment has been demonstrated by several previous studies (Choi et al. 2004, Xie et al. 2008, Zhang et al. 2008, Lorenz et al. 2009); however, we observed no improvement in hind limb function in the EGCG-treated group. It may be that the EGCG-induced changes were too subtle to be detected in a small group or with the test method employed. Forelimb function was improved and had normalized by day 10 after MCAO, indicating that EGCG improved function in the MCAO rat model. Future studies on the effect of EGCG on cognition and learning in MCAO rats are necessary to fully explain these results. Recent studies on changes in the hippocampus of ischemic rats have shown that EGCG improves memory and learning through its actions as an antioxidant or *via* a voltage-gated sodium channel signaling pathway (Burckhardt et al. 2008, Deng et al. 2008, Haque et al. 2008, Xie et al. 2008). We believe that the effects observed in the present study may be mediated through one of these EGCG mechanisms. EGCG or green tea may induce functional improvement of forelimb in rats with ischemic stroke during the acute or subacute period.

CONCLUSION

The present study demonstrated that treatment with EGCG induced functional improvement in forelimb during a 2-week period after stroke in the MCAO rat model.

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