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Protective Effect of Green Tea Polyphenol EGCG Against Neuronal Damage and Brain Edema After Unilateral Cerebral Ischemia in Gerbils

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Previous studies have demonstrated that a green tea polyphenol, (−)-epigallocatechine gallate (EGCG), has a potent free radical scavenging and antioxidant effect. Glutamate leads to excitotoxicity and oxidative stress, which are important pathophysiologic responses to cerebral ischemia resulting in brain edema and neuronal damage. We investigated the effect of EGCG on excitotoxic neuronal damage in a culture system and the effect on brain edema formation and lesion after unilateral cerebral ischemia in gerbils. In vitro, excitotoxicity was induced by 24-hr incubation with *N*-methyl-D-aspartate (NMDA; 10 μM), AMPA (10 μM), or kainate (20 μM). EGCG (5 μM) was added to the culture media alone or with excitotoxins. We examined malondialdehyde (MDA) level and neuronal viability to evaluate the effect of EGCG. In vivo, unilateral cerebral ischemia was induced by occlusion of the right common carotid artery for 30, 60, or 90 min and followed by reperfusion of 24 hr. Brain edema, MDA, and infarction were examined to evaluate the protective effect of EGCG. EGCG (25 or 50 mg/kg, intraperitoneally) was administered twice, at 30 min before and immediately after ischemia. EGCG reduced excitotoxin-induced MDA production and neuronal damage in the culture system. In the in vivo study, treatment of gerbils with the lower EGCG dose failed to show neuroprotective effects; however, the higher EGCG dose attenuated the increase in MDA level caused by cerebral ischemia. EGCG also reduced the formation of postischemic brain edema and infarct volume. These results demonstrate EGCG may have future possibilities as a neuroprotective agent against excitotoxicity-related neurologic disorders such as brain ischemia.

Key words: antioxidant; excitotoxicity; stroke; neuroprotection

The excitatory amino acid glutamate plays an important role in pathogenesis of neuronal injury after cerebral ischemia (Rothman and Olney, 1986), and is a principal initiator of the excitotoxic events. Excessive activation of excitatory neurotransmission triggers neuronal death through ionotrophic glutamate receptors (Choi, 1988). Activation of *N*-methyl-D-aspartate (NMDA), AMPA, kainate, and metabotropic glutamate receptors causes excessive intracellular calcium accumulation, which induces production of cytotoxic molecules including nitric oxide (NO) and free radicals (Dykens et al., 1987; Dawson et al., 1991; Coyle and Puttfarcken, 1993; Ciani et al., 1996). This can manifest as either necrosis or apoptosis of the neurons (Glazner et al., 2000; Lu and Mattson, 2001). This pattern is common in brain injury such as that arising from trauma, epileptic seizures, neurodegenerative diseases, and cerebral ischemia (Mattson et al., 1995).

Cerebral ischemia followed by reperfusion results in production of superoxide (Nelson et al., 1992). Oxygen free radicals have been implicated widely in the pathogenesis of brain injury due to ischemia and reperfusion (McCord, 1985; Chan, 1994;). It has been suggested that oxidative stress is maybe involved in ischemic and postischemic brain edema (Abe et al., 1988). In addition to direct neuronal damage of brain ischemia, oxygen free radicals, released under various pathologic conditions, are known as mediators of the blood-brain barrier (BBB) opening. The increase of BBB permeability allows the extravasation of high molecular-weight materials like the protein albumin (Nelson et al., 1992). This phenomenon results in edema formation, which is an important acute clinical complication and a major factor contributing to a poor outcome after an ischemic insult. To reduce brain

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damage and improve the neurologic outcome after ischemia, the efforts to develop new therapeutic strategies like antioxidants are essential.

Green tea is one of the most popular beverages in the world and has biologically important polyphenols. (−)-Epigallocatechin gallate (EGCG) is the most active major polyphenol of green tea and primarily responsible for the green tea effect. It has been proved EGCG shows a potent antioxidant property (Guo et al., 1996; Lee et al., 2003). EGCG possesses two triphenolic groups in its structure, which have been reported to be important for its potent antioxidant activity (Matsuo et al., 1997). Recent in vitro and in vivo studies revealed the protective effects of EGCG and green tea extract on various forms of neuronal damages due to free radicals. Guo et al. (1996) reported that EGCG reduced lipid peroxidation injury in synaptosomes. Lin et al. (1998) demonstrated that green tea extract exerted a protective effect against iron-induced oxidative stress in the rat brain. In particular, EGCG reduced neuronal damage after transient global ischemia (Lee et al., 2000), β-amyloid protein-induced neurotoxicity (Choi et al., 2001), and AMPA-induced calcium influx and neuronal damage (Bae et al., 2002).

We examined whether the potent antioxidant EGCG reduces excitotoxic neuronal damage in culture system and the brain edema and neuronal lesion after unilateral brain ischemia in a gerbil model.

MATERIALS AND METHODS

The experimental protocol and procedure were approved by our institutional animal care and use committee and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Cell Culture and Excitotoxicity

Primary cultures of rat hippocampal neurons were prepared from embryonic (Day 17–18) Sprague-Dawley rats according to Song et al. (1998). Briefly, rats were decapitated and isolated hippocampi were dissected. Cells were seeded onto poly-D-lysin (50 μg/mL)-coated 6-well plates at a final density of 1.5×10^6 cells per each. Cells were maintained in serum- and phenol red-free Neurobasal medium, supplemented with B27 (GibcoBRL, Grand Island, NY) and cultivated in a humidified atmosphere of 95% air and 5% CO_2. One-half of the culture medium volume was replaced by fresh medium 3 days after initial plating, and cells were then maintained with a half change of medium every 2 days. Seven days after plating, one-half of the medium was replaced again, and cultured cells were exposed to excitotoxicity. Excitotoxins such as NMDA (10 μM), AMPA (10 μM), or kainate (20 μM) were applied to the hippocampal cultures alone or concurrently with EGCG (10 μM), MK-801, or 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). After this procedure, hippocampal cultures were incubated for 24 hr, and then cell viability was determined and malondialdehyde (MDA) measured.

Cell Viability Test

To assess directly the number of viable and nonviable neuronal cells after excitotoxin treatments, cells were plated in 6-well plates and treated for 24 hr. At the end of each experiment, cells were trypsinized and pelleted together with cells of the culture medium. After staining for 10 min with 0.2% trypan blue solution, unstained (viable) and stained (nonviable), neuronal cells were counted in a hemocytometer chamber under a microscope. Cell viability was expressed as percentage ratio of viable cells to total number of neuronal cells.

Measurements of MDA Concentration in Culture Model

The amount of MDA, an indirect marker of lipid peroxidation, was measured by the thiobarbituric acid (TBA) assay (Buege and Aust, 1978). The harvested cells were mixed with TBA and placed in a boiling water bath for 15 min. The sample was then centrifuged at $3{,}000 \times g$ for 5 min, after which the absorbance of the supernatant was read at 535 nm in a spectrophotometer (Model DU650, Beckman). The MDA concentration was calculated by using an excitation coefficient of $1.56 \times 105\ M^{-1}cm^{-1}$.

Animals and Treatment

Male Mongolian gerbils (*Meriones ungiculatus*) weighing 60–80 g were used in this study. These animals were housed in laboratory cages and maintained on a 12-hr light-dark cycle, with ad lib access to food and water throughout the study period. Mongolian gerbil is unique in its susceptibility to ipsilateral cerebral ischemia after unilateral common carotid artery occlusion and has been used widely as a model of experimental focal cerebral ischemia (Levin and Payan, 1966). The gerbils were treated with EGCG (25 or 50 m/kg, i.p.; Sigma Chemical Co., St. Louis, MO) twice: at 30 min before and immediately after ischemia. EGCG was dissolved in normal saline. In the ischemic control group, the vehicle (normal saline, i.p.) was also administered twice: at 30 min before and immediately after ischemia.

Surgery for Unilateral Cerebral Ischemia

The gerbils were anesthetized with chloral hydrate (400 mg/kg, i.p.). Chloral hydrate applied intraperitoneally in spontaneously breathing animals is currently one of the most common methods of rodent anesthesia in experimental research. After the injection of chloral hydrate, we started surgery with no reaction of the gerbil to pain stimuli such as tail pressure or neck skin incision. During maintenance of anesthesia, we checked every 10–15 min if there was no spontaneous moving of long nose hairs. In the supine position, a midline ventral incision of 2 cm was made in the neck. The right common carotid artery was exposed, separated carefully from the vagus nerve, and occluded for 30, 60, or 90 min with a microclip. Blood flow during occlusion and reperfusion after removal of the clip was confirmed visually and the incision was closed. The rectal temperature was monitored and maintained at 37 ± 0.5°C with a feedback-controlled heating pad (CMA, Stockholm, Sweden) and an incandescent light placed over the head. In the sham-operated group, the neck incision was made only to expose right common carotid artery without occlusion. The other procedures were identical to those of the other groups.

Histology

The gerbils were sacrificed 24 hr after ischemia. They were anesthetized deeply with diethyl ether and perfused transcardially with cold heparinized phosphate-buffered saline (PBS, pH 7.2) and 10% formalin in PBS. The brains were removed from the skull and fixed in the same fixative for 24 hr. Brains were then embedded in paraffin and representative coronal sections (6-μm thick), which included the dorsal hippocampus, were obtained by a rotary microtome. Tissue sections were stained with hematoxylin and eosin.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End-Labeling (TUNEL) Staining

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining was carried out with a kit (Roche Molecular Biochemicals, Mannheim, Germany). Tissue sections were deparaffinized in xylene and hydrated in a sequence of ethanol washes followed by a final wash in PBS. Nuclei of tissue sections were stripped of proteins by incubation with proteinase K (20 μg/mL in 10 mM Tris/HCl, at 37°C) for 15 min. The slices were then washed and incubated in 0.3% hydrogen peroxide to remove endogenous peroxidases. After equilibration, each section was incubated with 50 μL of TUNEL mixture (5 μL of terminal deoxynucleotidyl transferase and 45 μL of fluorescence-labeled nucleotide) for 60 min at 37°C. The sections were treated with horseradish peroxidase conjugated anti-fluorescence antibodies. We detected the double-strand breaks in genomic DNA using 2,3′-diaminobenzidine tetrahydrochloride (0.5 mg/mL in 50 mM/L Tris-HCl buffer, pH 7.4) as a substrate for the peroxidase.

Evaluation of Brain Edema

All gerbils were decapitated at 24 hr of reperfusion. Brains were removed quickly from the skull, hemispheres were divided, and the cerebellum and brain stem were discarded. Samples were weighed immediately on an electronic analytical balance (model AJ100; Mettler Instrument, Highston, NJ) to measure the wet weight. Samples were then dried in a oven at 100°C for 24 hr to measure the dry weight and water content determined as (wet weight − dry weight)/dry weight (Masada et al., 2001).

Measurement of Infarct Volume

At 24 hr of reperfusion, the brain tissue was removed and sliced into five 2.0-mm thick coronal sections. Brain slices were stained with 2% triphenyltetrazolium (TTC) in saline at 37°C for 30 min and then fixed with 10% formaldehyde (pH 7.2) for 24 hr. The cerebral infarction volume was measured by an image analysis software (SigmaScan Pro, Jandel) followed by correction for brain swelling as described by a previous report (Lin et al., 1993). The complete pale area on each section was considered as the area of cerebral ischemic damage. The investigator who analyzed the brain sections was blinded to the experimental groups.

MDA Measurement in Brain Tissue

At 24 hr of reperfusion, gerbils were anesthetized deeply with diethyl ether and perfused through the heart with cold heparinized PBS (pH 7.2). Immediately after perfusion, the brain was removed and divided into two hemispheres and stored at −75°C until homogenization. Both cerebral hemispheres were homogenized in ice-cold Tris-HCl buffer (20mM; pH 7.4) to produce 10% homogenate. Aliquots (1 mL) of homogenates were isolated and 1 mL of 0.67% TBA reagent was added to the homogenates. This reaction mixture was placed into boiling water for 15 min. The solution was centrifuged at $3{,}000 \times g$ for 5 min at 4°C. The absorbance of the supernatant (MDA) was measured at 535 nm (Waterfall et al., 1995). Results are expressed as the ratio of ischemic side to nonischemic side.

Statistical Analysis

All data of this study are expressed as mean ± SEM. Statistical assessments were carried out using one-way analysis of variance (ANOVA) followed by post-hoc Scheffe's test. Significance refers to results where $P < 0.05$ was obtained.

RESULTS

Effects of EGCG Against In Vitro Excitotoxicity

Figure 1A and 1B demonstrated that 24-hr exposure to excitotoxin NMDA (10 μM), AMPA (10 μM), or kainate (20 μM) resulted in a large depletion of hippocampal cells from the cultures compared to that in vehicle-treated controls ($P < 0.01$, respectively). When cultures were exposed to excitotoxins and EGCG (5 μM), hippocampal cells survived (Fig. 1A,B). An almost equivalent result was obtained using a noncompetitive antagonist of the NMDA receptor, MK-801 (5 μM), or a selective AMPA/kainate antagonist, CNQX (5 μM) (Fig. 1A,B). In the present study conditions, there were no significant differences in the protective effects of EGCG, MK-801, and CNQX against excitotoxicity.

After 24-hr exposure to 10 μM NMDA, 10 μM AMPA, or 20 μM kainate, MDA production was increased significantly ($P < 0.01$, respectively) to about threefold that of vehicle-treated controls and EGCG only treatment (Fig. 1C). EGCG treatment decreased significantly ($P < 0.05$) the excitotoxin-induced increase of MDA concentrations, similar to the effects of MK-801 and CNQX. EGCG and glutamate receptor antagonists also showed similar neuroprotective effects (Fig. 1C).

Effects of EGCG Against Unilateral Cerebral Ischemia In Vivo

Brain edema formation. We tried three different paradigms (30, 60, or 90 min) of unilateral ischemic duration for evaluation of brain edema. Gerbils that underwent 60 or 90 min of unilateral brain ischemia had a significant increase in brain tissue water content in the ipsilateral hemisphere, compared to that in sham-operated animals ($P < 0.05$ and $P < 0.01$, respectively; Fig. 2). Gerbils, which underwent 30 min of unilateral brain ischemia had no significant influence on edema formation compared to sham-operated animals. Animals that received EGCG treatment at the dose of 50 mg/kg had significantly less edema formation in the ipsilateral hemisphere than did vehicle-treated ischemic animals ($P <$

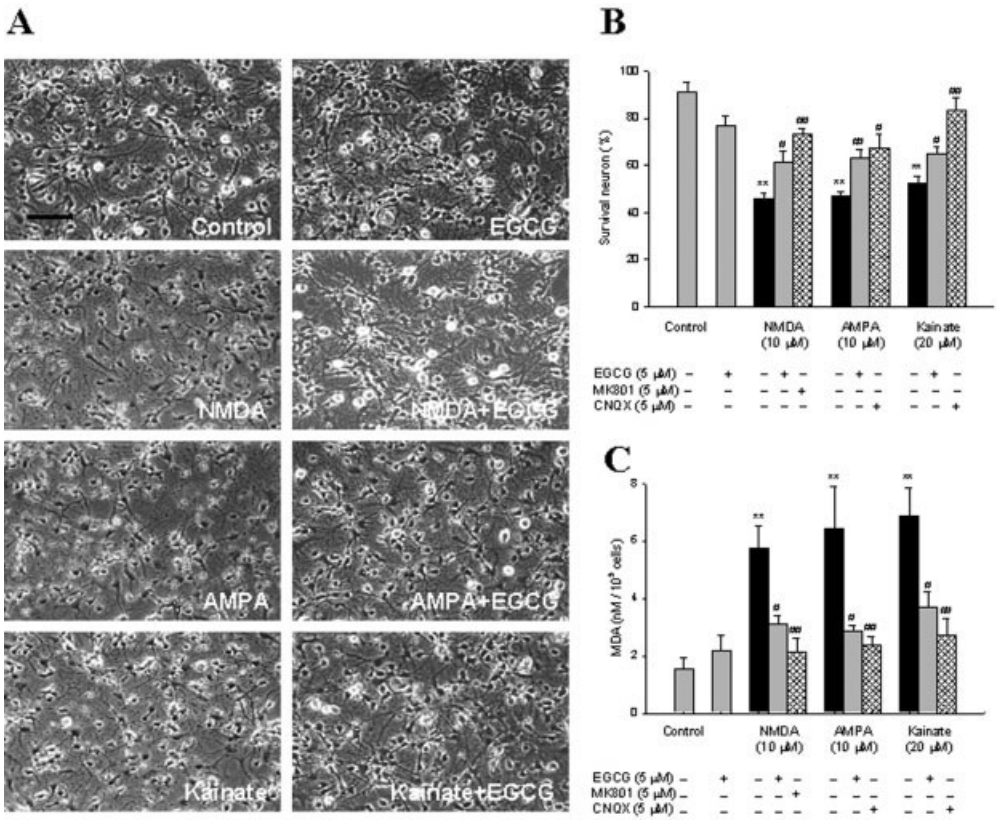


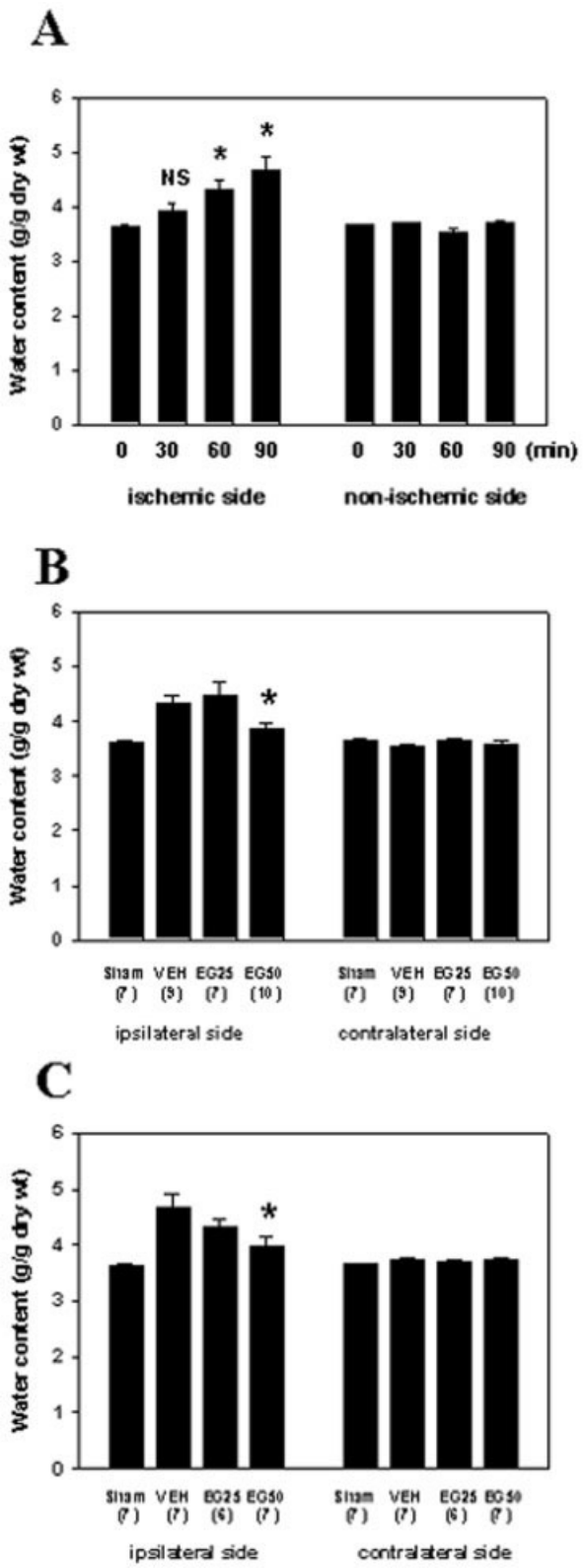
Fig. 1. Effects of excitotoxins on hippocampal neuronal viability and protection by EGCG, MK-801, or CNQX. **A:** Representative photomicrographs of excitotoxin-induced neuronal injury and effects of treatments with EGCG (5 μM), MK-801 (5 μM), or CNQX (5 μM). **B:** Exposure of hippocampal cells to excitotoxins for 24 hr with or without EGCG, MK-801, or CNQX. With treatment of excitotoxins, cultures show remarkable depletion of cells and EGCG blocks excitotoxic neuronal cell injuries after 24 hr exposure to NMDA, AMPA or kainate, respectively. MK-801 or CNQX show similar neuroprotective effect, as compared to EGCG. **C:** MDA production in response to excitotoxins and protection by EGCG in rat hippocampal neurons. MDA concentrations were measured after 24 hr exposure to excitotoxins with or without EGCG, MK-801 or CNQX. Data are shown as means ± SEM of five to seven separate experiments. $\star\star P < 0.01$ compared to controls; $\#P < 0.05$, $\#\#P < 0.01$ compared to corresponding single treatment of NMDA, AMPA, or kainate. Scale bar = 50 μm.

0.05, Fig. 2). A lower EGCG dose (25 mg/kg), however, failed to attenuate the edema formation after unilateral brain ischemia (Fig. 2). In the edema formation experiment, it seemed that 60 min duration of ischemia was optimal in this unilateral brain ischemia model. We focused therefore on the 60-min groups in the rest of our experiments.

Histologic findings of neuronal damage after unilateral brain ischemia. Representative histologic features show 60-min ischemic insult and 24 hr of reperfusion in gerbil brain. Histopathologic changes for all ischemic-lesioned animals were confined to the rostral and caudal parietal cortex, nucleus caudate-putamen, ventrolateral thalamus, and the hippocampus according to Hermann et al. (2001) (Fig. 3). Neuronal changes, including shrunken cell bodies, perineuronal vacuolations, and pyknotic nuclei were prominent. Pan-necrosis, occurred in the ipsilateral side of the brains (Fig. 3). After brain ischemia, the ipsilateral hemisphere of gerbils was swollen with severe edema, and a midline shift of the brain was seen. Cerebral cellular edema was also seen in the ipsilateral side of the histologic sections (Fig. 3). To examine DNA damage after unilateral cerebral ischemia, but not for assessment of EGCG effect, we carried out the TUNEL assay. No TUNEL-positive cells were visible in the non-ischemic contralateral hemispheres (left sides of each of the panels). Dark-brown colored TUNEL-positive cells were prominent in the ipsilateral ischemic hemispheres.

Ratio of MDA concentration (ipsilateral vs. contralateral side). To examine the protective effect of EGCG administration, the MDA concentration ratio of ipsilateral and contralateral hemisphere was determined. After 24 hr of reperfusion subsequent to 60 min of ischemia, the MDA ratio was increased compared to that in sham-operated control animals ($P < 0.01$). EGCG at the dose of 25 mg/kg did not attenuate the MDA ratio. EGCG at the dose of 50 mg/kg, however, attenuated the MDA concentration ratio significantly ($P < 0.01$, Fig. 4).

Ischemia-induced infarction formation. The effect of EGCG on infarct volume after 60 min of unilat-



eral common carotid artery occlusion and 24 hr of reperfusion is shown in Fig. 5A and 5B. EGCG treatment at the dose of 50 mg/kg resulted in a significant 69.7% reduction in total infarct volume compared to that in the vehicle-treated ischemia group ($P < 0.01$). A lower EGCG dose (25 mg/kg), however, failed to reduce the infarct volume (Fig. 5A,B).

DISCUSSION

Effects of EGCG Against In Vitro Excitotoxicity

We found that treatment with the green tea polyphenol EGCG reduced cell death after exposure to the glutamate receptor agonist NMDA, AMPA, or kainate in primary hippocampal cell cultures. Excitatory amino acid-related excitotoxicity may be implicated in both acute and chronic neurodegeneration in a sequence of events in which oxidative stress due to reactive oxygen species also occurs (Meldrum and Garthwaite, 1990; White et al., 2000). Apparently, these reactive oxygen species play a major role in excitotoxic injury to neurons. They result in lipid peroxidation (White et al., 2000). In addition to cell viability, we examined MDA concentration in cultures after exposure to glutamate receptor agonists and found MDA concentrations were increased significantly 24 hr after exposure with these excitotoxins. We also found EGCG reduces excitotoxin-induced MDA production significantly. Consistent with our study, Mattson et al. (1995) reported that exposure of cultured rat hippocampal neurons to glutamate results in accumulation of cellular peroxide, and well-known antioxidants such as vitamin E and propyl gallate can suppress glutamate-induced cell death. The pharmacologic antioxidant properties of EGCG are mediated by free radical-scavenging activity, attenuation of lipid peroxidation, and blockade of neuronal nitric oxide synthase (nNOS) induction (Chan et al., 1998; Lee et al., 2000).

In our previous study, we tested antioxidant effects of EGCG against H_2O_2- or ferrous ion-induced lipid peroxidation in brain homogenate and compared these to well-known potent antioxidants such as trolox, lipoic acid, and melatonin. EGCG showed a greater antioxidant effect than did trolox, lipoic acid, and melatonin (Lee et al., 2003).

Fig. 2. Brain water content 24 hr after unilateral ischemia. Measurements were made from ipsilateral and contralateral hemispheres. **A:** Brain water content 24 hr after unilateral cerebral ischemia of 30, 60, or 90 min. NS, no statistical significance compared to sham-operated group. $\star P < 0.05$ compared to sham-operated group. **B:** Gerbils received unilateral 60 min ischemia injury with or without EGCG administration or sham operation. Sham, sham-operated group ($n = 7$); VEH, ischemia-injured group with vehicle administration ($n = 9$); EGCG25 and EGCG50, ischemia-injured group with EGCG administration (25 or 50 mg/kg, n = 7 and 10, respectively). $\star P < 0.05$ compared to vehicle-treated group. **C:** Gerbils received unilateral 90 min ischemia injury with or without EGCG administration or sham operation. Sham, sham-operated group ($n = 7$); VEH, ischemia-injured group with vehicle administration ($n = 7$); EGCG25 and EGCG50, ischemia-injured group with EGCG administration (25 or 50 mg/kg, $n = 6$ and 7, respectively). $\star P < 0.05$ compared to the vehicle-treated group.

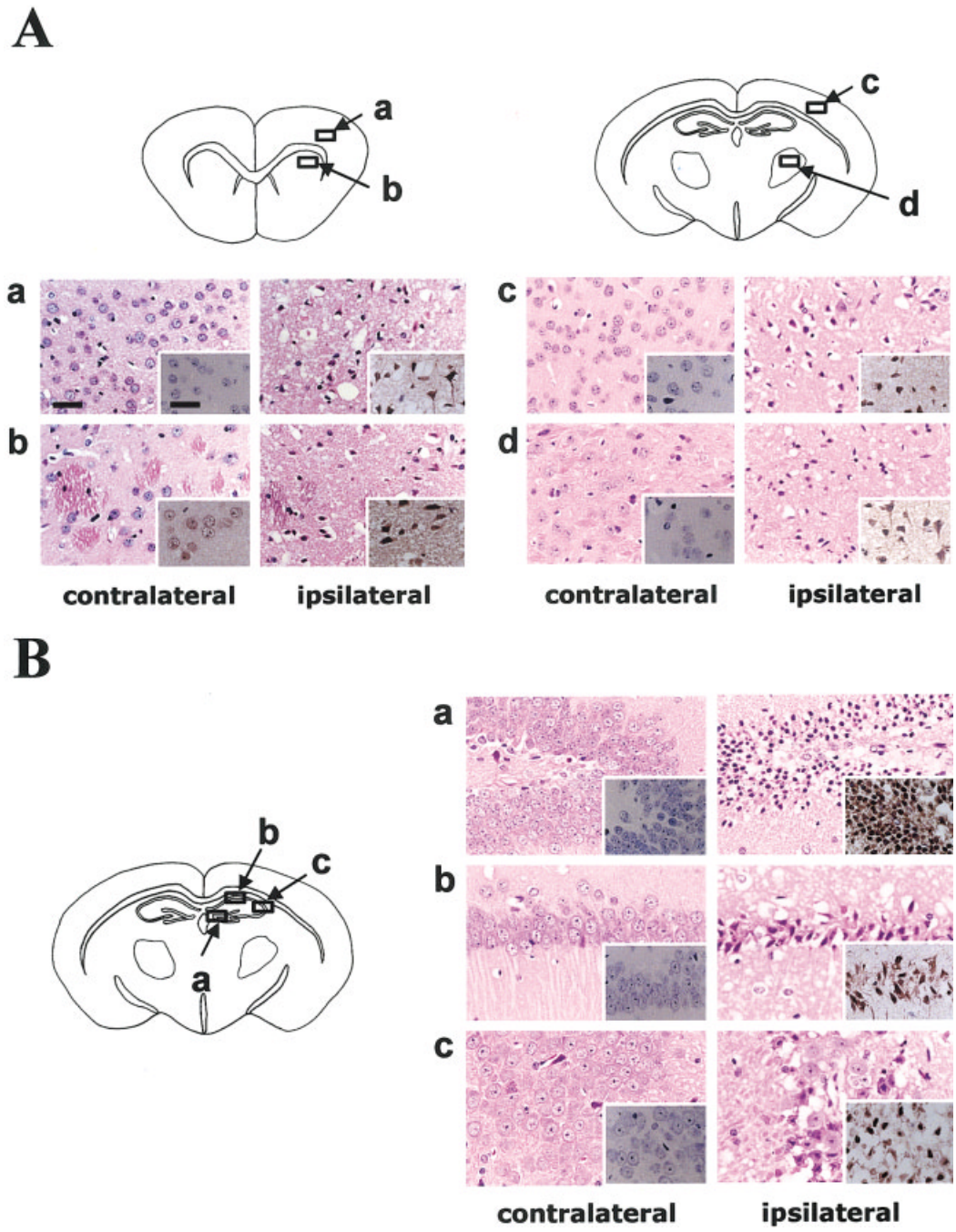


Fig. 3. Representative photomicrographs of gerbil brain sections stained with hematoxylin and eosin or TUNEL (insets). Pathologic features of brain sections at 24 hr after unilateral brain ischemia of 60 min. Schematic line drawings of brain sections and boxes (**a–d** in **A** and **a–c** in **B**) indicate the brain regions with representative histologic features. In A, rostral parietal cortex (a); the nucleus caudate-putamen (b); caudal parietal cortex (c); and the ventrolateral thalamus (d). In B, dentate gyrus (a); CA1 region of pyramidal cell layer (b); and CA3 region of pyramidal cell layer (c). Undamaged contralateral side is shown in the left side of each of the panels in A and B. Ischemic ipsilateral side is shown in the right side of each of the panels in A and B. In ipsilateral side, pyknotic nuclei of damaged neuronal cells are obvious. Neuronal perikarya in cerebral cortex are demonstrating characteristic morphologic features of shrinkage and triangulation of nucleus and cytoplasm and increased eosinophilia. There are no TUNEL-positive cells in nonischemic contralateral sides, left sides of each of the panels. Dark-brown colored TUNEL-positive cells are prominent in each ipsilateral hemisphere. Scale bar = 20 μm. *Reprinted with modifications from NEUROSCIENCE, Vol 102, Issue 4, 779-787, Herman et al., with permission from Elsevier.*

We also reported recently that EGCG attenuates AMPA-induced neuronal damage by inhibiting calcium influx into the neuronal cell (Bae et al., 2002). To assess the protective effect of EGCG against glutamate receptor agonist-induced cell death, we tested MK-801, competitive antagonist of NMDA receptor, and CNQX, competitive antagonist of AMPA/kainate receptors. They showed patterns of protective effects similar to that seen with EGCG.

Although further studies will be needed to find out the exact protective mechanisms of EGCG against excitotoxic neuronal damage, EGCG may be important in treatment of the excitotoxicity-related neurological diseases because of its potent antioxidant effect.

Effects of EGCG Against Unilateral Cerebral Ischemia In Vivo

This study shows the protective effect of EGCG, the green tea polyphenol against lipid peroxidation, brain edema, and brain infarction after unilateral cerebral ischemia. It has been already suggested that oxygen radicals

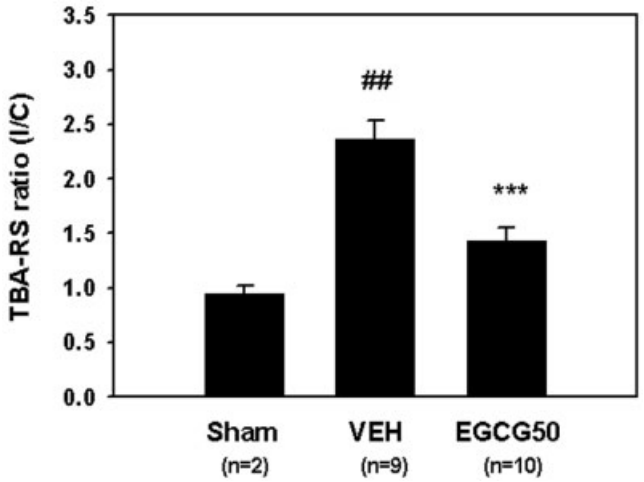


Fig. 4. Relative MDA concentrations from ipsilateral (I) and contralateral (C) hemispheres 24 hr after unilateral ischemia. Gerbils received unilateral 60-min ischemia and 24 hr of reperfusion with or without EGCG administration or sham operation. Sham, sham-operated group ($n = 4$); VEH, ischemia-injured group with vehicle administration ($n = 9$); EGCG25 and EGCG50, ischemia-injured group with EGCG administration (25 or 50 mg/kg, $n = 7$ and 10, respectively). $\#\#P < 0.01$ compared to sham-operated group. $**P < 0.01$ compared to vehicle-injected ischemia group.

play a role in postischemic neuronal damage (Chan et al., 1998; Kawase et al., 1999) and brain edema (Abe et al., 1988). Recently, a variety of studies have examined the neuroprotective properties of antioxidants in brain ischemia (Block et al., 1995; O'Neill et al., 1997; Chan et al., 1998). We examined the protective effect of a potent antioxidant, EGCG, against unilateral brain ischemia. An animal study demonstrated previously that EGCG passes the blood-brain barrier and reaches the brain parenchyma (Suganuma et al., 1998). We administered EGCG intraperitoneally at the dose of 25 or 50 mg/kg. In our experimental model, the lower EGCG dose (25 mg/kg) produced no significant reductions in lipid peroxidation, brain edema, and infarction in comparison to that with vehicle alone. Animals that received EGCG at the dose of 50 mg/kg, however, displayed significant attenuation in the pathologic outcomes after ischemia. In this study, because of the simple surgery to induce unilateral brain ischemia, we used the hemispheric ischemia model of gerbil as a focal brain ischemia. This gerbil model may produce ischemia with variable severity (Yanagihara, 1978), compared to other popular focal brain ischemia models such as middle cerebral artery occlusion model. As mentioned above, 30-min ischemia did not produce significant increase of brain water content. We also tried ischemia of longer duration (60–90 min) than that of previous studies of unilateral ischemia in gerbils (10–30 min) (Kitagawa et al., 1996; Hermann et al., 1998, 2001). Ischemic of 60 or 90 min duration produced significant increase in water content compared to that in sham-operated control animals. We therefore decided to use 60-min ischemia to produce neuronal damage in this study. Based on a significant increase of neuronal damage, brain water content, and infarction size compared to that in the sham-operated animals, we confirmed the reliability of unilateral carotid artery occlusion of 60 min.

To our knowledge, Chiou et al. (1994) reported for the first time EGCG-induced protection against neuronal damage induced by ischemia in retina tissue. Recently, the neuroprotective effect of EGCG against neuronal damage was reported via in vitro and in vivo studies. EGCG treatment attenuated neuronal damage in cultured hippocampal cells after an exposure to neurotoxin, β-amyloid protein (Choi et al., 2001) and dose-dependently reduced hippocampal neuronal damage after transient global isch-

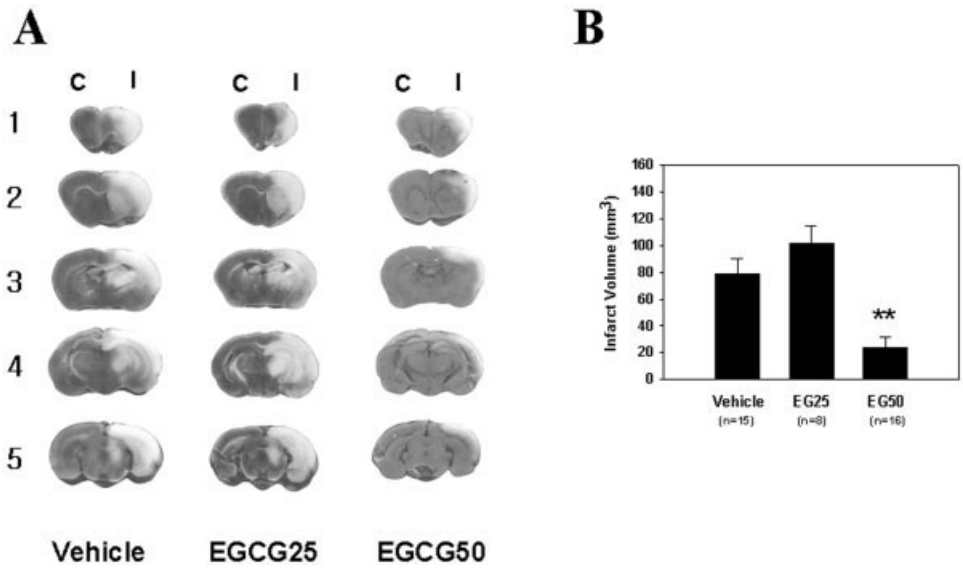


Fig. 5. Effects of administration of EGCG against infarct volume after unilateral brain ischemia. A: Representative TTC stain of gerbil brain from the group treated with vehicle and a representative TTC stain of gerbil brain from the group treated with EGCG (25 or 50 mg/kg). I, ipsilateral side; C, contralateral side. **B:** Effects of EGCG administration (25 or 50 mg/kg) on the TTC-determined infarction volume at 24 hr of reperfusion in gerbils after 60 min of unilateral common carotid artery occlusion. VEH, vehicle-treated gerbils ($n = 15$); EG25, EGCG (25 mg/kg)-treated gerbils ($n = 8$); EG50, EGCG (50 mg/kg)-treated gerbils ($n = 16$). $**P < 0.01$ compared to vehicle-treated gerbils.

emia in a gerbil model (Lee et al., 2000). In addition, EGCG showed neuroprotective effect against dopaminergic neuronal degeneration in a mouse Parkinson's disease model induced by *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (Levites et al., 2001).

Although the mechanisms underlying the neuroprotective effect of EGCG are not fully understood, there are several possible pieces of pharmacologic evidence for neuroprotection through antioxidant effects. First, EGCG can reduce lipid peroxidation in vitro (Guo et al., 1996) and in vivo (Sano et al., 1995). Particularly, Lin et al. (1998) reported that EGCG protects synaptosomes from the damage of lipid peroxidation, which depends on iron-chelating and free radical-scavenging activity toward hydroxyl radicals. Second, EGCG seems to exert its antioxidant effect by inhibiting xanthine oxidase (Aucamp et al., 1997). Inhibition of xanthine oxidase can lower the oxygen free radical production in the body. Because free radicals seem to play a major role in the neuropathology of reperfusion injury after ischemia, the ability of EGCG to reduce xanthine oxidase activity could also be the primary mechanism of action for its neuroprotective effects in this study. Third, EGCG is known to decrease activity and expression of inducible nitric oxide synthase (iNOS) and nNOS (Chan et al., 1997; Lin and Lin, 1997). NO produced by iNOS and nNOS plays an important biological role in pathophysiologic processes including neurotoxicity (Moncada et al., 1992) and ischemia-induced delayed neuronal death (Endoh et al., 1994). Selective iNOS inhibitors attenuate iNOS activity in the ischemic brain and reduce cerebral ischemic damage (Iadecola et al., 1995). In addition, nNOS inhibitors provide significant neuroprotection in cerebral ischemia (O'Neill et al., 1997). It has been reported that EGCG protects against ischemic neuronal damage by deoxidizing peroxynitrate/peroxynitrite (Nagai et al., 2002).

Several articles reported recently the possible mechanisms of neuroprotection of EGCG against brain ischemia. Neuroprotective action of EGCG against oxidative stress-induced cell death includes stimulation of protein kinase C (Levites et al., 2002a, 2003) and modulation of cell survival/cell cycle genes and apoptotic-related genes (Levites et al., 2002a, 2003; Weinreb et al., 2003a,b). Levites et al. (2002b) reported green tea extracts prevented nuclear translocation and activation of cell death promoting nuclear factor (NF)-κB. It is well known that reactive oxygen species act as secondary messengers in NF-κB activation.

In summary, this study demonstrates that EGCG has a protective effect against excitotoxic neuronal damage in a culture system and in unilateral cerebral ischemia of a gerbil model. It seems that the potent antioxidant effects of EGCG contributed to its neuroprotective effect against neuronal damage. This agent deserves consideration for further therapeutic trials in the treatment of excitotoxicity-related neurologic disorders, such as stroke.

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