



Eugenol Inhibits Excitotoxins-Induced Delayed Neurotoxicity, Oxidative Injury and Convulsion

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ABSTRACT. In previous our studies, we have reported that eugenol derived from *Eugenia caryophyllata* (Myrtaceace) exhibits acute N-methyl-D-aspartate (NMDA)- and oxygen/glucose deprivation-induced neurotoxicity in primary cortical cultures and protects hippocampal neurons from global ischemia. In this study, we investigated whether the extracts and fractions of *E. caryophyllata* or eugenol shows the neuroprotective effects against delayed neuronal injury evoked by NMDA or α -amino-3-hydroxy-5-methylisoxazole propionate (AMPA), and oxidative damage induced by arachidonic acid-, hydrogen peroxide-, FeCl₂/ascorbic acid-, and buthionine sulfoximine (BSO) in primary cortical cultures. We examined the neurotoxicity of eugenol itself in cultures and inhibitory effect of eugenol on NMDA- or kainate (KA)-induced convulsion in BALB/c mice. Each water, methanol extract and methanol fraction of *E. caryophyllata* was significantly attenuated NMDA-induced delayed neurotoxicity, respectively. Eugenol exhibited a significant inhibitory action against the convulsion evoked by NMDA and KA, and reduced delayed or brief neurotoxicity induced by NMDA, AMPA, and various oxidative injuries. These results suggest that eugenol derived from *E. caryophyllata* may contribute the neuroprotection against delayed-type excitotoxicity and excitotoxins-mediated convulsion through the amelioration of oxidative stress.

Keywords: *Eugenia caryophyllata*, Eugenol, NMDA, Kainate, Free radicals, Murine cortical cultures.

INTRODUCTION

It has been known that a massive release of glutamate, intracellular overload with free calcium, and enhanced production of free radicals are three major process of excitotoxic cell death in cerebral ischemia (Siesjo *et al.*, 1995). During these cascade processes, three glutamate receptor subtypes, such as N-methyl-D-aspartate (NMDA), Kainate (KA) and α -amino-2-hydroxy-5-methyl-4-isoxazole propionate (AMPA) are known to be involve in neuronal death, especially NMDA recep-

tors (Choi, 1988, 1992). Overactivation to NMDA receptor increases calcium-dependent release of arachidonic acid during excitotoxic insults (Dumuis *et al.*, 1988; Lazarewicz *et al.*, 1990; Sanfeliu *et al.*, 1990; Viu *et al.*, 1998). The massive accumulation of arachidonic acid produces downstream in the neurodegenerative cascade process can also induce excitotoxic injury through subsequent formation of reactive oxygen species (ROS) (Dugan *et al.*, 1996; Wie *et al.*, 1999). Hydrogen peroxide (H₂O₂), non-radical oxidant, is produced during normal metabolism. However, excessive production of H₂O₂ in various neurodegenerative diseases can result in neuronal injury (Halliwell, 1989; Kristian and Siesjo, 1998). Exposure to H₂O₂ evoked calcium-dependent neuronal death, and both apoptotic and necrotic cell

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death in cultured neurons (Kaneko *et al.*, 2006; Whittemore *et al.*, 1994). Glutathione (GSH) is the principal endogenous antioxidant in the mammalian central nervous system and the most important regulator of the redox status in living tissues. Dysfunction of GSH metabolism has been associated with many disorders of the CNS, including ischemic/reperfusion injury (Keelan *et al.*, 2001). A depletion in GSH induced by L-buthionine-(S,R)-sulfoximine (BSO), which inhibits glutathione synthesis, could cause potentiate excitotoxic vulnerability, leading to apoptosis or necrosis (Bridges *et al.*, 1991; Merad-Boudia *et al.*, 1998; Kang *et al.*, 2001). Eugenol (4-allyl-2-methoxyphenol), a major component of essential oil obtained from the clove (*E. caryophyllata* Myrtaceae), cinnamon, basil, nutmeg and so on, has widely been used for food flavouring and topical dental analgesic agent (Maralhas *et al.*, 2006). The pharmacological action of eugenol is known to show antiischemic (Won *et al.*, 1998), antinociceptive (Ohkubo and Shibata, 1997), anti-inflammatory (Sharma *et al.*, 1994; Kim *et al.*, 2003), antioxidant (Ogata *et al.*, 2000; Ito *et al.*, 2005). Previously, we have reported that eugenol inhibits the *in vitro* and *in vivo* neuronal injury system induced by NMDA or superoxide radicals (O_2^-) in cultured cortical cells (Wie *et al.*, 1997) and by global ischemia via hypothermic action (Won *et al.*, 1998), respectively. In this study, we evaluated whether what extract or fraction of *E. caryophyllata* exhibit protective effect on delayed NMDA neurotoxicity. In addition, we checked whether eugenol exhibits blockade effect on delayed-type neurotoxicity in culture system and convulsion induced by NMDA and kainate, non-NMDA agonist including various oxidative injury paradigms.

MATERIALS AND METHODS

Preparation of *E. caryophyllata* extracts and fractions

We obtained water, methanol extracts and fractions of *E. caryophyllata* from Institute of Natural Medicine, Hallym University (Chuncheon, South Korea). Briefly, the dried buds of *E. caryophyllata* was purchased from Oriental medicine market (Seoul) and authenticated. The dried sample was extracted with distilled water and 50% methanol by refluxing for 6 h twice to attain 23% and 24% yield, respectively. By adding water and hexane, the methanol extract was fractionated to give hexane fraction and water residue. The aqueous residue was subsequently further fractionated to give ethyl acetate, chloroform and butanol fractions using the same method. The residual aqueous phase was concentrated under reduced pressure to give the water fraction.

Neuronal and Glial cultures

Mixed cortical cell cultures containing both glia and neurons were prepared from ICR mice at gestation day 15~16 as described by Rose *et al.* (1993). Briefly, dissociated neocortical cells ($2.5\text{--}3.0 \times 10^5$ cells/well) were plated onto primaria-coated 24-well plates (Falcon) containing glial bed in a plating medium consisting of Eagle's minimal essential medium (MEM; Earle's salts, supplied glutamine free) supplemented with 20 mM glucose, 2 mM L-glutamine, 5% fetal bovine serum, and 5% horse serum. Cytosine arabinoside (10 μ M) was added 5 days after plating to halt the growth of non-neuronal cells. The cultures were maintained at 37°C in a humidified CO₂ incubator and used for experiments between 14 and 17 days *in vitro* (DIV). Glial cultures were prepared from postnatal (days 1~3) mice, and plated at 0.75 hemispheres/24-well plate in plating medium supplemented with 10% horse serum/10% fetal bovine serum and 10 ng/ml epidermal growth factor. After two weeks *in vitro*, cytosine arabinoside was added to the cultures, which were fed weekly with the same medium with 10% horse serum used for mixed cultures.

Measurement of neurotoxicity

Neuronal injury was quantitatively estimated by measuring lactate dehydrogenase (LDH) released from damaged cells into the bathing medium 20~24 h after NMDA, α -amino-3-hydroxy-5-methylisoxazole propionate (AMPA), arachidonic acid, hydrogen peroxide (30 min exposure) and FeCl₂/ascorbic acid (2 h exposure) treatments, or 48 h after treatment with BSO. Brief (< 2 h) or delayed (24~48 h) exposures to excitotoxins or various compounds causing oxidative damages are usually carried out at room temperature and maintained at 37°C in a humidified CO₂ incubator until LDH measurement. For brief neurotoxicity, cultures are washed with growth medium by aspirating the volume in each well down to 125 μ l and adding 750 μ l of HEPES Control Salt Solution (HCSS) with a repeat pipeter. This step is aspirating the volume down to 125 μ l and pipeting back 250 μ l of HCSS plus desired toxic agonist. To terminate exposure, cultures are washed three times with HCSS (750 μ l/well), then one times with Media Stock (MS, 750 μ l/well) and final time with MS (250 μ l/well) so that the final volume is approximately 375 μ l. Delayed exposures to neurotoxic agonists are carried out with MS solution. The cultures are washed with MS by aspirating the volume in each well to 125 μ l, and adding 750 μ l of MS. This step is repeated second time, and then eugenol was added after aspirating the volume down to 125 μ l and adding 250 μ l of MS with neurotoxins.

For morphological confirmation, neuronal cells were stained with neuron-specific enolase antibody (NSE, BioGenex) and trypan blue. We added 10 μM glycine (final concentration) to all the culture media used in this study.

Anti-convulsion test

To evaluate anti-convulsive action, intracerebroventricular (i.c.v.) injections of NMDA (0.3 μg) or kainate (0.5 μg) were made according to the procedure of Haley and McCormick (1957). Each animal received a single i.c.v. administration of NMDA or kainate using Hamilton syringe in BALB/c mice. The i.c.v. injection volume was 5 μl . The i.c.v. injection sites were verified by injecting a similar volume of 1% methylene blue and observing the distribution the dye in the ventricular space. Following i.c.v. injection, mice were monitored for seizure activity. We considered that onset time of convulsion was determined when mice started to display

clear signs of clonic or clonic-tonic seizure activity. Eugenol was injected 30 min before NMDA or kainate administration at concentration range with 10–300 mg/kg intraperitoneally in a single dose. The same of vehicle (1% Tween 80 in saline) was used as a control.

Statistical analysis

We tested statistical significance with one-way ANOVA using a post-hoc Student-Neuman-Keuls Procedure for multiple comparisons.

RESULTS

Protective effects of water, methanol extract and methanol fraction of *E. caryophyllata* on NMDA-induced neurotoxicity

Mixed cortical cell cultures exposed to 20 μM NMDA for 24 h (delayed neurotoxicity) caused 75–90% neu-

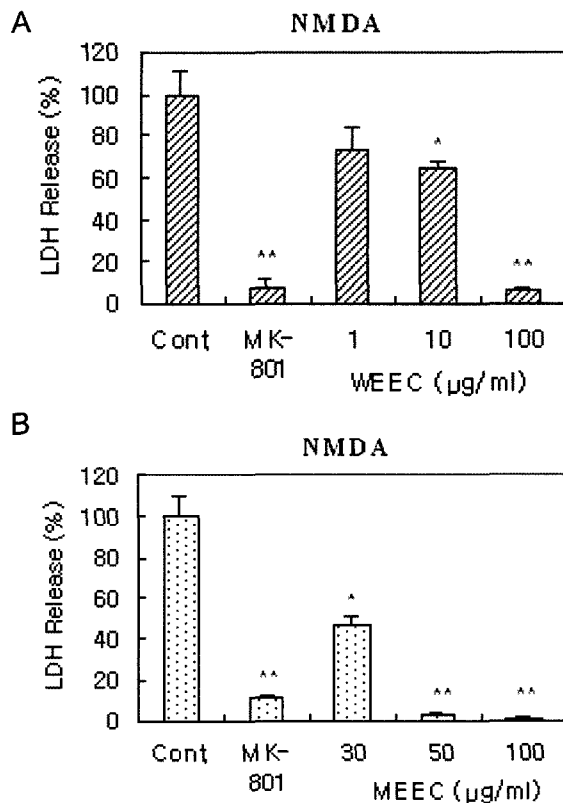


Fig. 1. Water extract (WEEC; A) and methanol extract (MEEC; B) of *E. caryophyllata* attenuate NMDA-induced neurotoxicity. Sister cultures were exposed for 20–24 h to 20 μM NMDA alone (control), or in the presence of MK-801 (10 μM) or WEEC and MEEC, as indicated concentrations ($\mu\text{g/ml}$). Bars show LDH efflux the following day (mean \pm SEM, $n = 4$). The differences were evaluated with a one-way ANOVA and post-hoc Student-Neuman-Keuls test for multiple comparisons (* $p < 0.05$, ** $p < 0.01$ vs. controls).

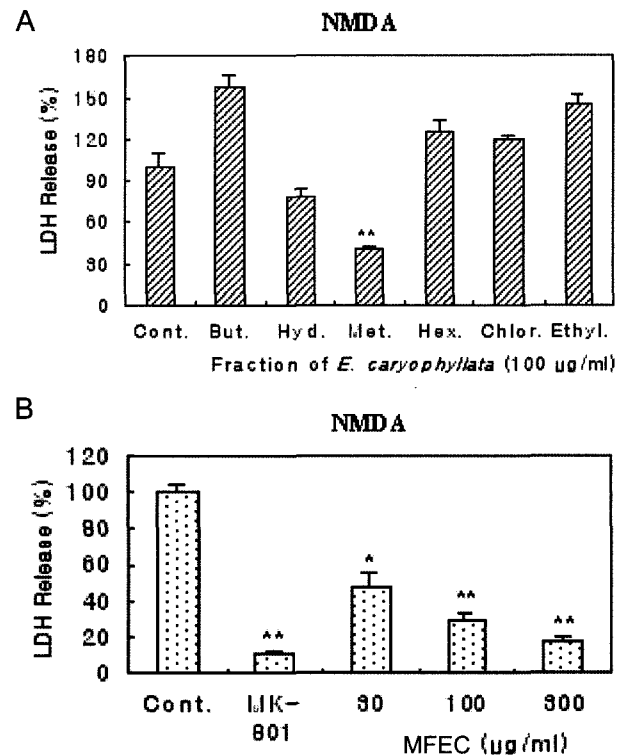


Fig. 2. Methanol fraction of *E. caryophyllata* (MFEC) attenuates NMDA-induced neurotoxicity. (A) Sister cultures were exposed for 20–24 h to 20 μM NMDA alone (control), or in the presence of MK-801 (10 μM) or fractions of butanol, hydrogen, methanol, hexane, chloroform, and ethyl acetate as indicated concentrations ($\mu\text{g/ml}$). (B) Concentration-dependent effect of MFEC. Bars show LDH efflux the following day (mean \pm SEM, $n = 4$). The differences were evaluated with a one-way ANOVA and post-hoc Student-Neuman-Keuls test for multiple comparisons (* $p < 0.05$, ** $p < 0.01$ vs. controls).

ronal damage as measured by elevation of LDH release to the bathing medium after 20–24 h. When 10–100 $\mu\text{g/ml}$ water extract of *E. caryophyllata* (WEEC) and 20 μM NMDA were added simultaneously to the cortical cultures, neuronal injury was significantly inhibited (35–93%) in a dose-dependent manner (Fig. 1A). The methanol extract of *E. caryophyllata* (MEEC) also exhibited similar neuroprotective effect at the range with 30–100 $\mu\text{g/ml}$ (53–99%) compared with those of WEEC (Fig. 1B). Among six fractions of *E. caryophyllata*, the methanol fraction (30–300 $\mu\text{g/ml}$) only exhibited protective action against NMDA-induced delayed neurotoxicity in a concentration-dependent fashion (Fig. 2A, 2B). In these studies, MK-801 (10 μM), non-competitive NMDA receptor antagonist almost blocked the NMDA neurotoxicity.

Eugenol inhibits NMDA- or AMPA-induced delayed excitotoxicity

Exposure of cultured cells to 20 μM NMDA or 20 μM AMPA caused neuronal injury by 80–90%. Concurrent exposure of cultured cells to 50–300 μM eugenol and

20 μM NMDA or 20 μM AMPA attenuated neuronal injury concentration-dependent fashion.

Eugenol exhibited significant neuroprotective effects (44–92% inhibition) on NMDA neurotoxicity between 100 and 300 μM concentration (Fig. 3A). However, it was showed significant neuroprotection (45–51% inhibition) between 200–300 μM concentration in AMPA-induced neurotoxicity (Fig. 3B). The inhibitory action of eugenol against NMDA-induced neurotoxicity showed much better than that of AMPA-induced neurotoxicity at the same concentration. MK-801 (10 μM), non-competitive NMDA receptor antagonist or 50 μM DNQX ((6,7-dinitroquinoxaline-2,3(1H,4H)-dione), AMPA/kainate receptor antagonist blocked the neurotoxicity induced by NMDA or AMPA, completely. To eliminate the participation of NMDA receptor action, 10 μM MK-801 was exposed to both the control and experimental group in AMPA toxicity experiment. For representative morphological confirmation, we stained neurons with trypan blue in NMDA neurotoxicity experiment (Fig. 4).

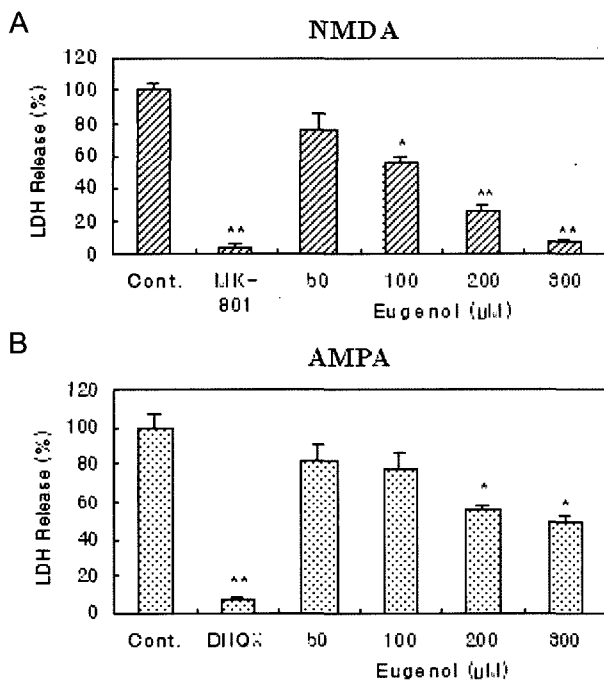


Fig. 3. Eugenol attenuates delayed NMDA- and AMPA-induced neurotoxicity. Sister cultures were exposed for 20–24 h to 20 μM NMDA (A) or 20 μM AMPA (B) alone (control), or in the presence of MK-801 (10 μM) or eugenol as indicated concentrations (μM). Bars show LDH efflux the following day (mean \pm SEM, $n = 4$). The differences were evaluated with a one-way ANOVA and post-hoc Student-Neuman-Keuls test for multiple comparisons (* $p < 0.05$, ** $p < 0.01$ vs. controls).

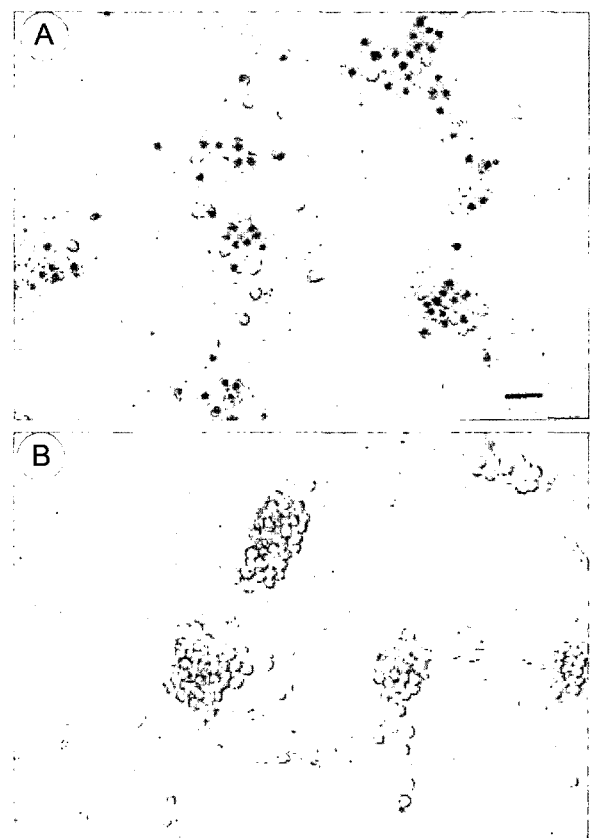


Fig. 4. Morphological evidence of neuroprotection with eugenol in 20 μM NMDA-induced neurotoxicity. Trypan blue staining of sister cultures 20–24 h after NMDA exposure alone (A) or in the presence of 300 μM eugenol (B). Scale bar, 50 μm .

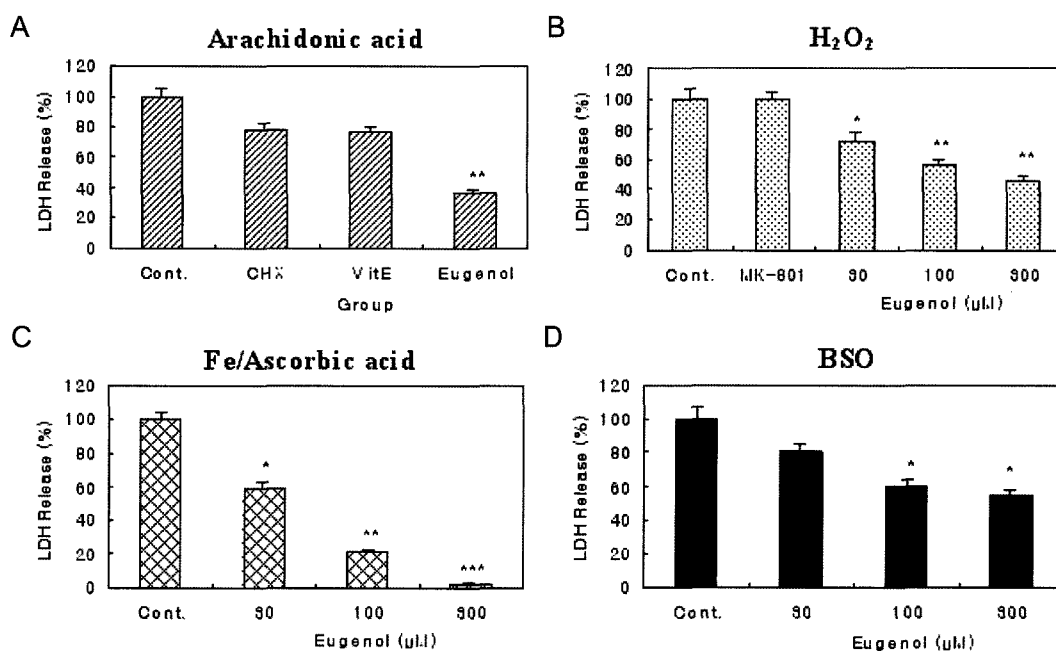


Fig. 5. Eugenol attenuates oxidative injuries. Sister cultures were exposed to each 50 μ M arachidonic acid for 24 h (A), 50 μ M FeCl₂/25 μ M ascorbic acid for 2 h (B), 300 μ M H₂O₂ for 30 min (C), 0.5 mM BSO for 48 h (D) alone (control), or in the presence of eugenol as indicated concentrations (μ M). Bars show LDH efflux the following day (mean \pm SEM, $n = 4$). The differences were evaluated with a one-way ANOVA and post-hoc Student-Neuman-Keuls test for multiple comparisons (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. controls).

Protective effect of eugenol on oxidative injuries induced by various compounds

Exposure of neuronal cultures to 50 μ M arachidonic acid (AA) for 24 h induced 80% neuronal injury. Co-exposure of AA with eugenol (300 μ M) provided significant neuroprotection by 64% (Fig. 5A). Cycloheximide (CHX), new protein synthesis inhibitor and vitamin E (VitE) reduced the AA-induced neurotoxicity by 23% and 24%, respectively although there was statistically not significant (Fig. 5A). Eugenol (30~300 μ M) significantly inhibited hydrogen peroxide (300 μ M for 30 min in HCSS)-induced neuronal injury by 24~58% (Fig. 5B). MK-801 (10 μ M) didn't exhibit a neuroprotective action at high dose of hydrogen peroxide. Eugenol also decreased the neurotoxicity induced by Fe²⁺ (50 μ M)/ascorbate (25 μ M) treatment for 2 h to 41~98% (Fig. 5C). Exposure of neuronal cultures to 1 mM buthionine sulfoximine (BSO), an inhibitor of endogenous glutathione synthesis induced 70~80% neuronal injury. Co-treatment of cultured neurons with 30~300 μ M eugenol for 24 h reduced the BSO-induced neurotoxicity by 20~45% (Fig. 5D). Morphologically, compared with normal neurons (Fig. 6A, 6B), Fe²⁺/ascorbate-induced loss of neuronal cell bodies was apparent 20~24 h after challenge of it by phase contrast (Fig. 6A, 6C, 6E) or immunocytochemistry (Fig. 6B, 6D, 6F) using neuron-specific eno-

lase (NSE) antibody (Fig. 6C, 6D). Many intact neuronal cell bodies were observed in eugenol-treated group (Fig. 6E, 6F). We checked whether the neurotoxicity of eugenol itself exist until 48 h. When the cultures were exposed to 10~300 μ M eugenol, none of eugenol-treated group was found significant neuronal injury (data not shown).

Inhibitory effect of eugenol on NMDA- and kainate-induced convulsion

We tested the effect of eugenol on the onset time of convulsions induced by NMDA (0.3 μ g) injection intracerebroventricularly. When eugenol (10~300 mg/kg) was administered intraperitoneally (*i.p.*) 30 min before NMDA injection, the onset time of convulsion was delayed dose-dependently, and exhibited about two times prolongation at the maximal dose (300 mg/kg) compared to that of control (Fig. 7A). When eugenol (100 mg/kg) was administered intraperitoneally 30 min before kainate injection, the onset time of convulsion was significantly extended by 56% compared to that of control (Fig. 7B).

DISCUSSION

In this study, we found that methanol fraction of *E.*

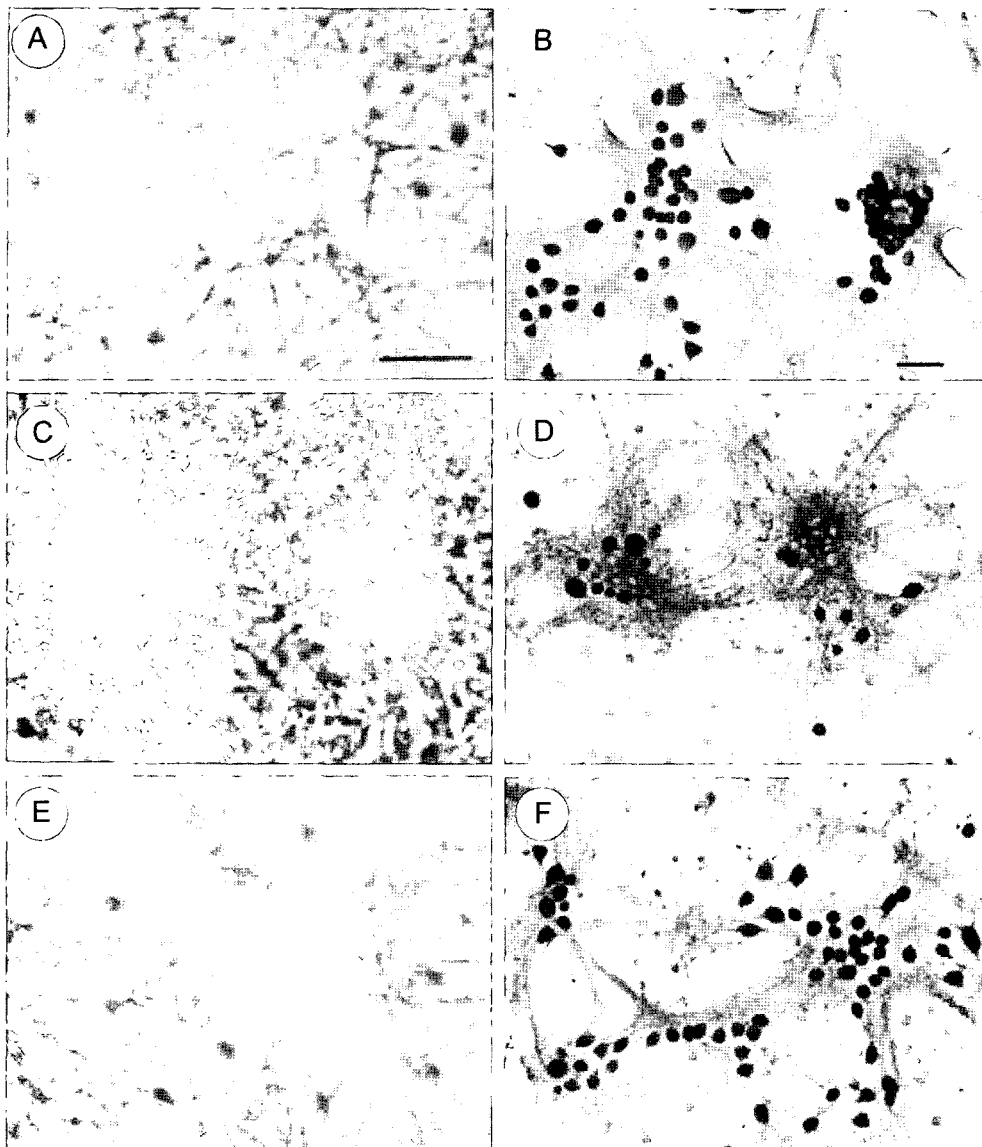


Fig. 6. Morphological evidence of neuroprotection with eugenol in Fe^{2+} /ascorbate-induced neurotoxicity. Phase-contrast photomicrographs of sister cultures 20~24 h after a 2 h exposure to Fe^{2+} alone (C, D) or in the presence of 300 μM eugenol (E, F), or sham wash (A, B). Neurons (B, D, and F) were stained with neuron-specific enolase antibody (BioGenex, USA). Scale bar, 50 μm .

caryophyllata has the most remarkable inhibition on the NMDA neurotoxicity compare to those of other fractions.

We also identified that eugenol, one of the main components derived from clove oil (*E. caryophyllata*), has both neuroprotective action on delayed NMDA and AMPA neurotoxicity. These results suggest that eugenol not only has the modulatory ability on NMDA receptor but also AMPA, non-NMDA receptor in slowly triggered excitotoxicity. However, the modulatory action on NMDA receptor by eugenol shows much larger than that of AMPA receptor. Previously, we reported that eugenol has an inhibitory action on acute NMDA and oxygen-

glucose deprivation (OGD)-induced neurotoxicity by amelioration of free calcium influx via NMDA receptor and scavenging action of superoxide radicals (Wie *et al.*, 1997). Recently, it has been suggested that OGD in primary rat cortical neurons can activate 5-lipoxygenase, the enzyme metabolizing arachidonic acid (AA) to produce proinflammatory leukotrienes, via partly mediating NMDA receptors (Ge *et al.*, 2006). The overstimulation of glutamate receptors during ischemic injury can trigger calcium-mediated release of AA in neurons (Dumuis *et al.*, 1988; Sanfeliu *et al.*, 1990) or astrocyte (Stella *et al.*, 1994).

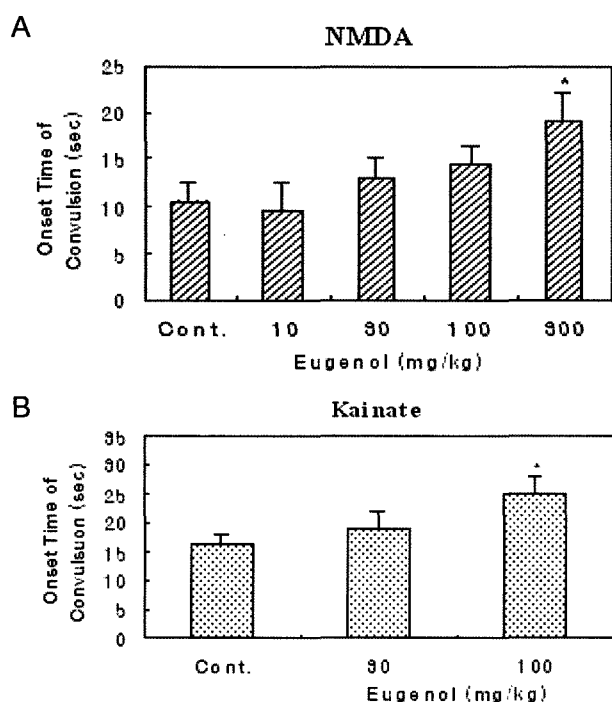


Fig. 7. Eugenol prolongs NMDA- (A) and kainate- (B) induced onset time of convulsion. Eugenol was administered intraperitoneally at the concentrations indicated 30 min before the intracerebroventricular injection of NMDA (0.1 μ g) and kainate (0.5 μ g). The differences were evaluated with a one-way ANOVA and post-hoc Student-Neuman-Keuls test for multiple comparisons (* $p < 0.05$ vs. controls).

The massive accumulation of AA produced downstream in the neurodegenerative cascade process can also induce necrosis or apoptosis through reactive oxygen species (ROS) (Dugan *et al.*, 1996). Overstimulation of NMDA receptors has been known to produce superoxide radicals (Lafon-Cazal *et al.*, 1993) and free radicals generated during NMDA-stimulated metabolism of arachidonic acid (Dumuis *et al.*, 1988; Rothman *et al.*, 1993) may involve in oxidative injury. In these results, eugenol well inhibits AA-induced neurotoxicity in cortical cultures. Judging from these points of view, eugenol may modulate NMDA receptors through inhibiting AA metabolism in downstream level of NMDA-induced neurotoxic cascade process. Neuronal injury evoked by ROS, such as hydrogen peroxide, superoxide and hydroxyl radicals, has been implicated in the pathogenesis of both acute and chronic insults to the CNS (Dugan *et al.*, 1995). Superoxide ($O_2^{\cdot -}$) generation increased after treatment with NMDA, AMPA, and kainate (Bindokas *et al.*, 1996). We observed that eugenol has various antioxidative properties and especially powerful protective effect on hydroxyl radicals ($\cdot OH$) generated by Fe^{2+} -mediated Fenton reaction. Recently, Ogata

et al. (2005) have reported that eugenol exhibits direct scavenging action on hydroxyl radicals without iron chelating action. This result suggests that eugenol may partly contribute to the neuronal survival through direct interaction between hydroxyl radicals produced during excitotoxicity or oxidative stress and eugenol. The other diverse antioxidant abilities of eugenol could contribute the attenuation on NMDA- and kainate-induced convulsion in *in vivo* system. In addition, eugenol inhibits the neuronal damage evoked by BSO-induced glutathione depletion. Brongholi *et al.* (2006) reported that OGD decreased glutathione levels in rat hippocampal slices. Conclusively, these protective actions of eugenol on neurotoxicity and convulsion induced by excitotoxins may partly due to downstream regulation of excitotoxic cascade process through multiple antioxidative actions.

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