Korean J Vet Res(1999) 39(2) : 287~293

Isoeugenol prevents N-methyl-D-aspartate(NMDA)-induced neurotoxicity and convulsion

Myung-bok Wie

Department of Veterinary Medicine, College of Agriculture and Institute of Life Science, Cheju National University, Cheju 690-756, Korea

(Received Jan 24, 1999)

Abstract : Isoeugenol, one of the phenylpropanoid derivatives has been known to inhibit the lipid peroxidation via scavenging effect on hydroxyl or superoxide radical production. We examined whether isoeugenol has a inhibitory effect against N-methyl-D-aspartate(NMDA)-, oxygen/glucose deprivation- and xanthine/xanthine oxidase(X/XO)-induced neurotoxicity or NMDA-induced $^{45}Ca^{+2}$ uptake elevation in primary mouse cortical cultures. We also evaluated whether isoeugenol exhibits inhibitory action on NMDA-induced convulsion in mice. Isoeugenol (30~300μM) attenuated NMDA- and X/XO-induced neurotoxicity by 11~85% and 83~92%, respectively. In the oxygen/glucose deprivation(60 min)-induced neurotoxicity, isoeugenol significantly($p < 0.05$) reduced by 32% at the maximal concentration. However, it failed to ameliorate NMDA-induced $^{45}Ca^{+2}$ uptake elevation. Isoeugenol(0.5g/kg, i.p.) delayed 6.5 times on the onset time of convulsion evoked by NMDA(0.1μg) compared to that of control. These results suggest that the neuroprotective action of isoeugenol may be ascribed to the modulation of massive generation of reactive oxygen species(ROS) occurred during the ischemic or excitotoxic damage, not by directly affecting the NMDA receptor.

Key words : isoeugenol ; N-methyl-D-aspartate ; oxygen/glucose deprivation, xanthine/xanthine oxidase ; $^{45}Ca^{2+}$ uptake.

Introduction

Enhanced production of reactive oxygen species, especially superoxide radicals(O_2-) generated by overactivation of N-methyl-D-aspartate(NMDA) receptor in cerebral ischemic injury is believed to play an important role in the pathogenesis of central nervous system injury[1,2]. Furthermore, other studies have reported that xanthine/xanthine oxidase(X/XO)-induced production of superoxide anions can modulate the activity of NMDA receptor via its redox site[3,4]and trigger L-glutamate release[5]. Although blockade of the NMDA receptor

Address reprint requests to Dr. Myung-bok Wie, Laboratory of Veterinary Pharmacology, Department of Veterinary Mediicne, College of Agriculture, Cheju National University, Cheju 690-756, Republic of Korea.

is necessary to suppress ischemic and excitotoxic neuronal death, its clinical application has been limited due to psychosis in animals and humans[6,7].

Isoeugenol(4-propenyl-2-methoxyphenol), which is derived from plant products, is known to act as an antioxidant by scavenging both hydroxyl radicals produced by ferrous- or ferric ion-induced lipid peroxidation and superoxide anions generated by the X/XO system[8]. Recently, we reported that eugenol(4-allyl-2-methoxyphenol), another phenylpropanoid compound, attenuated the neurotoxicity induced by NMDA treatment or oxygen/glucose deprivation by modulating both superoxide radical production and NMDA receptor itself in neuronal cultures[9].

It also protected CA1 hippocampal neurons in global ischemic injury[10]. Therefore, we examined whether isoeugenol also provides a neuroprotective effect on NMDA application or oxygen/glucose deprivation in neuronal cultures. We also determined the inhibitory effects of isoeugenol on NMDA-induced convulsions in mice.

Materials and Methods

Cortical cells, containing both glia and neurons, were prepared from ICR mice at gestation day 15~16, using previously described methods[11]. Briefly, dissociated neocortical cells(2.5~3.0 hemispheres) 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 20mM glucose, 2mM L-glutamine, 5% fetal bovine serum, and 5% horse serum. Cytosine arabinoside was added 4 to 5 days after plating to halt the growth of non-neuronal cells. The cultures were maintained at 37℃ in a humidified CO_2 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 0.5~0.75 hemispheres/24-well plate, in plating medium supplemented with 10% horse serum : 10% fetal bovine serum, and 10ng/mL epidermal growth factor. After 2 weeks *in vitro*, cytosine arabinoside was added to the cultures, which were fed weekly with the same medium used for mixed cultures. Oxygen and glucose deprivation were brought about by abruptly switching the culture medium for glucose-free, deoxygenated Earle's balanced salt solution(BSS_0 ; dilution $>$ 1 : 1,000) in an anaerobic chamber as previously described[9,12,13]. Oxygen/glucose deprivation was terminated by switching the medium with oxygenated MEM containing 5.5mM glucose and 2mM glutamine, and returning the cultures to the normoxic CO_2 incubator. Neuronal injury was assessed by measuring the lactate dehydrogenase(LDH) released from damaged cells into the bathing medium 1 day after adding the NMDA or the ischemic insult[13,14]. For the $^{45}Ca^{2+}$ uptake studies, cultured cells were washed with HEPES-buffered control salt solution (HCSS), and then incubated with NMDA(300μM)

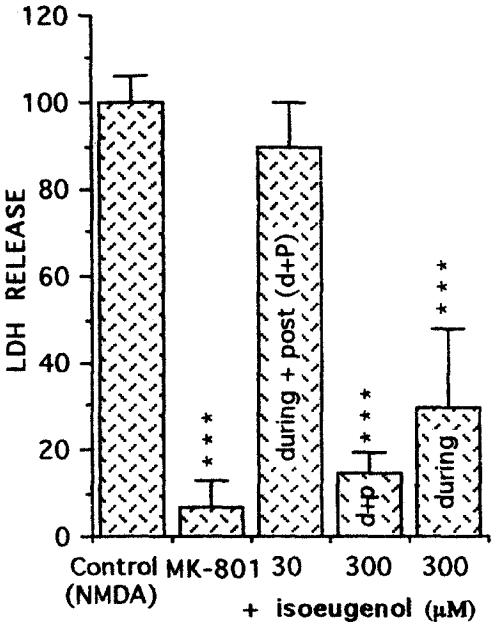

Fig 1. Isoeugenol attenuates NMDA-induced neurotoxicity. Sister cultures were exposed to 300μM NMDA for 5 min either alone(control), or in the presence of MK-801(10μM) or isoeugenol, at the concentrations indicated. The bars show the LDH efflux the following day(mean ± SEM, n = 4 culture well/condition). 'During' denotes the concurrent addition of NMDA and MK-801 or isoeugenol for 5 min. 'During' plus 'post' denotes cotreatment with NMDA and isoeugenol for 5 min, with additional isoeugenol for 20~24h after washout. Asterisks indicate a significant difference(***$p < 0.001$) from the control by a one-way ANOVA using a post-hoc Student-Neuman-Keuls test for multiple comparisons.

in the presence of a maximal concentration(300μM) of isoeugenol(Sigma Chemical, St. Louis, MO, USA) in HCSS containing $^{45}CaCl_2$(Amersham, UK ; final activity, 1.0μCi/mL). After 5 min, the exposure solution was washed out with 4 x HCSS and the cells were lysed by adding warmed 0.2% sodium dodecyl sulfate(SDS) solution. An aliquot of lysed cells was added to a scintillation cocktail solution to count beta emissions as previously described[9,12,15]. We added 10μM glycine (final concentration) to all the media used in this study. We also observed whether isoeugenol was capable of delaying the onset of convulsions induced by the intracerebroventricular injection of NMDA(0.1μg/mL) in ICR mice.

Isoeugenol was injected at concentrations of 10,100, or 500mg/kg intraperitoneally in a single dose. The same volume of vehicle (1% Tween 80 in saline) was used as a control. We tested statistical significance with a one-way ANOVA using a post-hoc Student-Neuman-Keuls Procedure for multiple comparisons.

Results

Exposure of 300μM NMDA for 5 min(acute neurotoxicity)

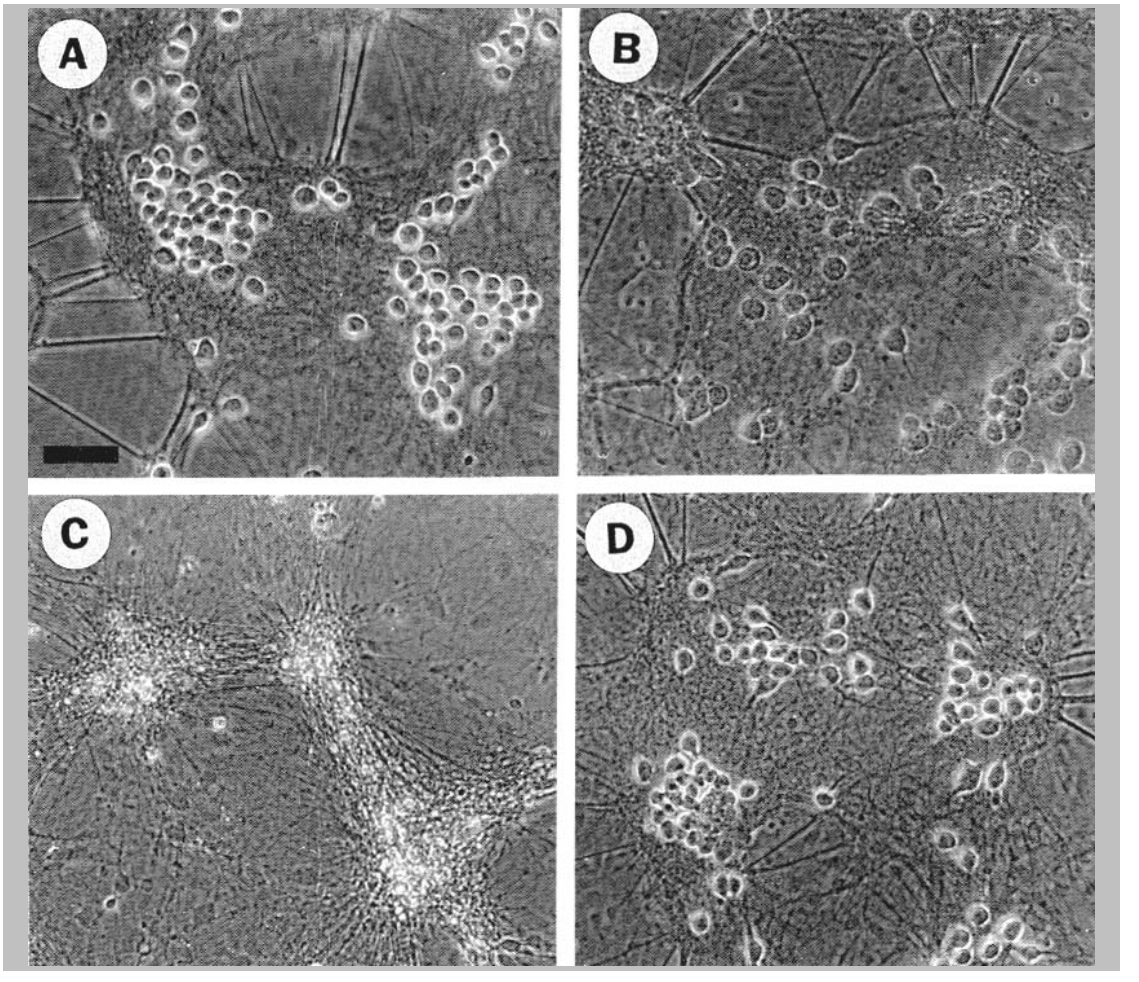

Fig 2. Morphological evidence of neuroprotection by isoeugenol in NMDA-induced neurotoxicity. Photomicrograph of representative fields taken with phase contrast following 1 or 24h after NMDA exposure. Mixed cortical cultures were exposed to 300μM NMDA for 5 min in the absence(B, C) or presence(D) OF 300μM isoeugenol. Sham wash(A), NMDA alone, 1h(B) and 24h(C) after NMDA exposure, cotreatment of isoeugenol and NMDA(D). Scale bar, 50μM.

caused 70~80% increase in LDH release in mixed cortical cell cultures, as determined by measuring the LDH release in the bathing medium at 20~24h after NMDA treatment. When 30 or 300μM isoeugenol was added both during-(simultaneous coapplications of NMDA and isoeugenol for 5 min) and post-(repetitive addition with isoeugenol for 20~24h after cessation of NMDA and isoeugenol coapplication) treatment in acute NMDA neurotoxicity, the neuronal damage was reduced by 11% and 85%, respectively(Fig 1). However, when isoeugenol(300μM) was added only during NMDA exposure(5 min), its neuroprotective effect was reduced by 70%, and 10μM MK-801(dizocilpine), non-competitive NMDA receptor antagonist blocked NMDA-induced neurotoxicity completely(Fig 1). Morphological changes of NMDA (300μM)-induced acute neuronal swelling(Fig 2B, 1h after NMDA exposure) and neuronal cell loss(Fig 2C, 24h after NMDA exposure) were apparently observed as compared to that of sham control(Fig 2A). However, the cotreatment of NMDA and isoeugenol(during+post) almost blocked the NMDA neurotoxicity(Fig 2D, 24h after both NMDA and isoeugenol exposure).

The $^{45}Ca^{2+}$ uptake in mixed cultures exposed to 300μM NMDA for 5 min was elevated about 8-fold(5,800cpm) compared to that of sham control(Fig 3). Simultaneous coapplications of MK-801(10μM) and NMDA totally blocked neuronal $^{45}Ca^{2+}$ uptake elevation induced by NMDA treatment (Fig 3).

At the maximum concentration(300μM) of isoeugenol, $^{45}Ca^{2+}$ uptake slightly ameliorated(by 13%, $p > 0.05$, Fig 3).

When the cultures were exposed to oxygen/glucose deprived BSS_0 for 60 min, neuronal swelling was observed soon(photograph not shown here) after the switch from oxygenated MEM and the neuronal cells were damaged moderately(60~70%) at 20~24h after the cessation of oxygen/glu-

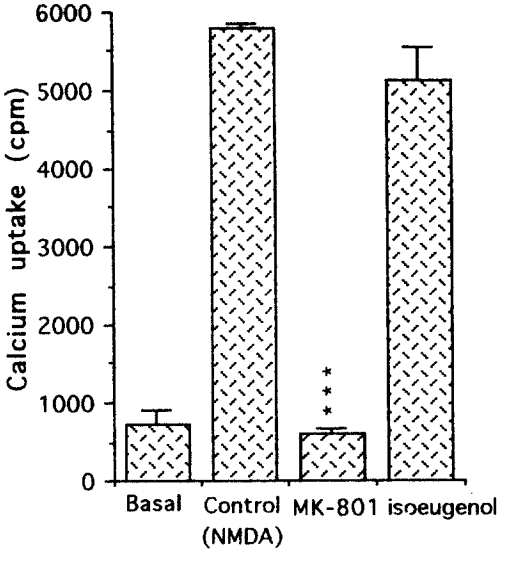

Fig 3. Isoeugenol failed to inhibit NMDA-induced $^{45}Ca^{2+}$ uptake elevation significantly. Sister cultures were exposed to 300μM NMDA for 5 min in the presence of extracellular $^{45}Ca^{2+}$. Immediately following the exposure, the cells were washed and lysed, and intracellular $^{45}Ca^{2+}$ levels measured. The bars represent the mean neuronal $^{45}Ca^{2+}$ uptake after a 5 min NMDA exposure in the presence of MK-801(10μM) or isoeugenol(300μM). Asterisks indicate a significant difference(***$p < 0.001$) from the control.

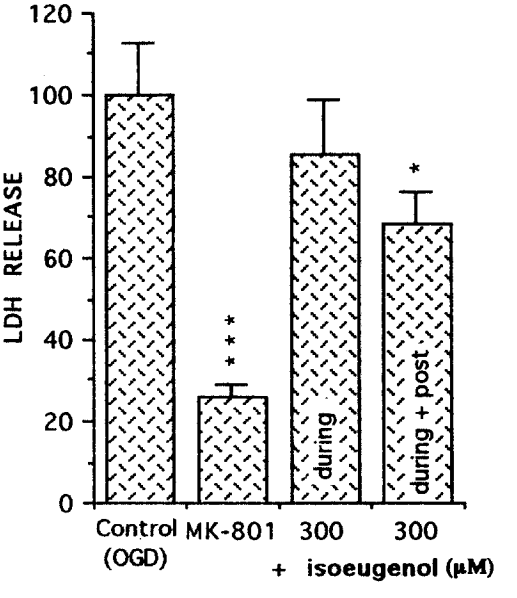

Fig 4. Isoeugenol protests neuronal cells from oxygen/glucose deprivation(OGD)-induced neurotoxicity. Sister cultures were deprived of both oxygen and glucose for 60 min with no drug added(control) or in the presence of MK-801(10μM) or isoeugenol at the concentrations indicated. Early neuronal cell body swelling and later neuronal degeneration following OGD treatment were clearly observed. The bars show the LDH efflux on the following day(mean±SEM, n=4 culture wells/condition). Asterisks indicate a significant difference(*$p < 0.05$, ** *$p < 0.001$) from the control.

cose deprived injury. When MK-801(10μM) was added to the cultures during the oxygen/glucose deprivation time, the neuronal injury was significantly reduced by 75%(Fig 4). With 300μM isoeugenol, the neuronal damage induced by oxygen/glucose deprivation was significantly($p < 0.05$) reduced by 32%(both during and post), but not significantly reduced($p > 0.05$) with during exposure only(15% inhibition) (Fig 4). We also evaluated the protective effect of isoeugenol on X/XO-induced neurotoxicity. Exposure of neuronal cultures to X/XO(0.5mM/10mU/mL) for 10 min in HCSS produced 70~90% neuronal injury. Simultaneous coapplications of X/XO and isoeugenol(30~300μM) markedly attenuated(83~92%) the neurotoxicity, while MK-801 did not show any significant protective effect(Fig 5).

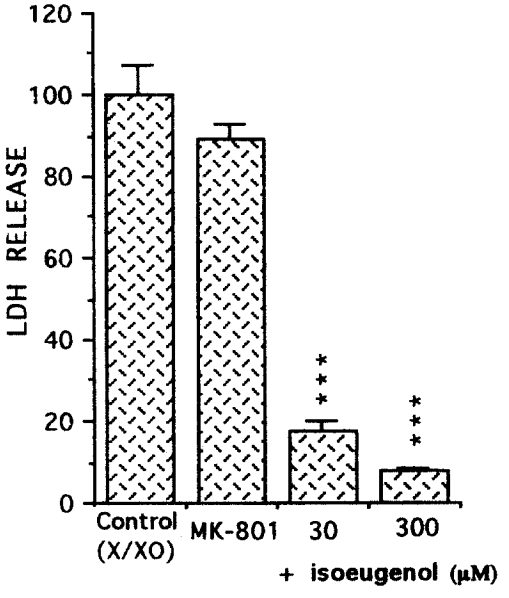

Fig 5. Isoeugenol protects neuronal cells from X/XO(0.5mM/10mM/mL)-induced neurotoxicity. Sister cultures were treated with X/XO for 10 min with no drug added(control) or in the presence of MK-801(10μM) or isoeugenol at the concentrations indicated. The bars show the LDH efflux on the following day(mean±SEM, n = 4 culture wells/condition). Asterisks indicate a significant difference(***$p < 0.001$) from the control.

We tested the effect of isoeugenol on the onset of convulsions induced by NMDA(0.1μg) injection intracerebroventricularly. When isoeugenol(0.5g/kg) was injected intraperitoneally 30 min before NMDA injections, the onset of convulsions was delayed about 6.5 times as long as controls (Fig 6).

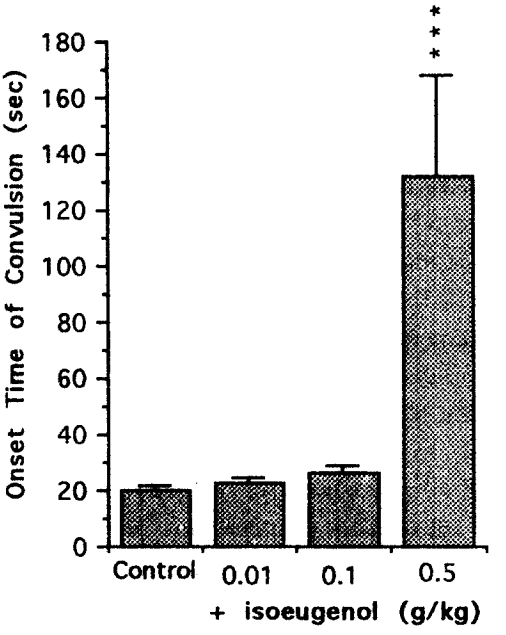

Fig 6. Isoeugenol significantly delays the onset convulsions induced by NMDA. Isoeugenol was administered intraperitoneally at the concentrations indicated 30 min before the intracerebroventricular injection of NMDA(0.1μg). Asterisks indicate a significant difference(***$p < 0.001$) from the cotrol.

Discussion

In this study, we found that isoeugenol has a significant protective action on the neurotoxicity induced by NMDA, oxygen/glucose deprivation and X/XO. However, it did not significantly ameliorate NMDA-induced clacium uptake elevation. In the oxygen/glucose deprivation study, the neuroprotective action of isoeugenol was weak, and it was only seen when isoeugenol was added at the maximum concentration (300μM) with a longer exposure time(during and post) than required to protect neurons from NMDA or X/XO neurotoxicity. These results indicate that the neuroprotective action of isoeugenol, at least against excitotoxic injury, is due to an antioxidant mechanism, rather than to modulating NMDA receptors directly. In our previous studies, we demonstrated that eugenol(4-allyl-2-methoxyphenol) has a neuroprotective

effect on NMDA- and oxygen/glucose deprivation-induced neurotoxicity by modulating both NMDA receptors and antioxidant action in murine neocortical cultures.

Eugenol was twice as potent as isoeugenol in inhibiting NMDA-induced $^{45}Ca^{2+}$ uptake elevation. Eugenol also showed better neuroprotection than isoeugenol in oxygen/glucose deprivation-induced neurotoxicity. These results suggest that the optimal strategy to protect neuronal cells from ishcemic damage may be to modulate both NMDA receptors and antioxidant action, rather than either one alone. Although eugenol blocked excitotoxicity better than isoeugenol, isoeugenol gave much stronger neuroprotection against superoxide radical-mediated neuronal injury[9] and inhibited lipid peroxidation to a greater degree than eugenol in brain homogenates[8]. As far as blocking NMDA neurotoxicity is concerned, isoeugenol seems to have a different pharmacological action and antioxidant effect than trolox, the vitamine E analog[16]. We found that isoeugenol(0.5g/kg) significantly delayed NMDA-mediated convulsions in mice. However, we could not determine exactly how isoeugenol regulates NMDA-mediated neurotoxicity in both *in vitro* and *in vivo* models. We presumed that isoeugenol may exhibit a neuroprotection via the ameliorative effect against metabolic stress and generation of free radicals play in ischemic damage, partly by affecting the redox site on the NMDA receptor. Free radicals produced in traumatic or ischemic insults can accelerate neurodegenerative diseases[17,18]. Because the NMDA receptor blockers exhibit psychotic problems[7] and the impairment of learning and memory[19], our findings would be able to give a help to establish the development strategy of ideal drugs for the therapy of ischemic diseases. Therefore, our results suggest that isoeugenol may potentially be used to prevent NMDA-related ischemic insults and seizures via and antioxidant mechanism.

References

1. Dugan LL, Sensi SL, Canzoniero LM, *et al*. Mitochondrial production of reactive oxygen species in cortical neurons following exposure to N-methyl-D-aspartate. *J Neurosci*, 15:6377-6388, 1995.
2. Lafon-Cazal M, Pietri S, Culcasi M, *et al*. NMDA-dependent superoxide production and neurotoxicity. *Nature*, 364:535-537, 1993.
3. Aizenman E, Hartnett KA, Reynold IJ, Oxygen free radicals regulates NMDA receptor function via a redox modulatory site. *Neuron*, 5:841-846, 1990.
4. Aizenman E. Modulation of N-methyl-D-aspartate receptors by hydroxyl radicals in rat cortical neurons *in vitro*. *Neurosci Lett*, 189:57-59, 1995.
5. Satoh T, Numakawa T, Abiru Y, *et al*. Production of reactive oxygen species and release of L-glutamate during superoxide anion-induced cell death of cerebellar granule neurons. *J Neurochem*, 70:316-324, 1998.
6. Olney JW, Labruyere J, Wang G, *et al*. NMDA antagonist neurotoxicity : mechanism and prevention. *Science*, 254:1515-1518, 1991.
7. Farber NB, Wozniak DF, Price MT, *et al*. Age-specific neurotoxicity in the rat associated with NMDA receptor blockade : potential relevance to schizophrenia? *Biol Psychiatry*, 38:788-796, 1995.
8. Rajakumar DV, Rao MN. Dehydrozingerone and isoeugenol as inhibitions of lipid peroxidation and as free radical scavangers. *Biochem Pharmacol*, 46:2067-2072, 1993.
9. Wie MB, Won MH, Lee KH, *et al*. Eugenol protects neuronal cells from excitotoxic and oxidative injury in primary cortical cultures. *Neurosci Lett*, 225:93-96, 1997.
10. Won MH, Lee JC, Kim YH, *et al*. Postischemic hypothermia induced by eugenol protecs hippocampal neurons from global ischemia in gerbils. *Neurosci Lett*, 254:101-104, 1998.
11. Rose K, Goldberg MP, Choi DW. *In vitro* biological systems. In : Tyson CA and Frazier JM, eds. *Methods in Toxicology*. San Diego : Academic Press, 46-60, 1993.
12. Goldberg MP, Choi DW. Combined oxygen and glucose deprivation in cortical cell culture : calcium-dependent and calcium independent mechanisms of neuronal injury. *J Neurosci*, 13:3510-3524, 1993.
13. Gwag BJ, Lobner D, Koh JY, *et al*. Blockade of glu-

tamate receptors unmasks neuronal apoptosis after oxygen-glucose deprivation *in vitro*. *Neuroscience*, 68: 615-619, 1992.

14. Koh JY, Choi DW. Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. *J Neurosci Methods*, 20:83-90, 1987.
15. Hartley DM, Kurth MC, Bjerkness L, *et al*. Glutamate receptor-induced $^{45}Ca^{2+}$ accumulation in cortical cell culture correlates with subsequent neuronal degeneration. *J Neurosci*, 13:1993-2000, 1993.
16. Chow HS, Lynch JJ, Rose K, *et al*. Trolox attenuates cortical neuronal injury induced by iron, ultraviolet light, glucose deprivation, or AMPA. *Brain Res*, 639: 102-108, 1994.
17. Siesjo BK, Agardh CD, Bengtsson F. Free radical and brain damage. *Cerebrovasc Brain Metab Rev*, 1:165-211, 1989.
18. Piantadosi CA, Zhang J. Mitochondrial generation of reactive oxygen species after brain ischemia in the rat. *Stroke*, 27:327-331, 1996.
19. Wozniak DF, Olney JW, Kettinger L 3d, *et al*. Behavioral effects of MK-801 in the rat. *Psychopharmacol*, 101:47-56, 1990.