A Thesis

For The Degree of Master of Veterinary Science

Quercetin attenuates Oxygen-Glucose Deprivation-induced Neurotoxicity in Primary Cortical Cell Cultures

Department of Veterinary Medicine

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2001. 12

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Veterinary Science.

2001. 10

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Abstract

Quercetin attenuates Oxygen-Glucose Deprivation-induced Neurotoxicity in Primary Cortical Cell Cultures

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Quercetin, a naturally occurring plant flavonoid, has been known to be very effective scavengers of free radical on ischemic injury. In this study, the possible role of quercetin in protecting against oxygen-glucose deprivation (OGD)-, excitotoxin-, and free radical-induced or apoptotic neuronal injury in mouse cortical cell cultures was investigated. The inhibitory action of quercetin on the N-methyl-D-aspartate (NMDA)-induced expression of cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) was also examined. Pre- and co-treatment with quercetin inhibited the OGD-, NMDA-, and kainate-induced neurotoxicity. Quercetin also ameliorated free radical-mediated neuronal injury mediated

by arachidonic acid, buthionine sulfoximine, sodium nitroprusside, $ZnCl_2$ and $FeCl_2$. Quercetin reduced the expression of COX-2 in neurons and of 5-LOX in both glia and neurons. However, it did not attenuate staurosporine- and MK-801-induced apoptosis; in fact, in the presence of staurosporine or MK-801, quercetin promoted apoptosis. This effect was blocked by cycloheximide and nerve growth factor. These results suggest that quercetin provides protection against ischemic neural injury, mainly by antioxidant and anti-inflammatory actions.

Keywords: quercetin, oxygen-glucose deprivation, cyclooxygenase, lipoxygenase, arachidonic acid, cortical cultures, NMDA, free radical

Ⅰ. Introduction

The abrupt elevation of reactive oxygen species during brain inflammation plays a key role in various neurodegenerative processes, such as stroke, trauma, and Parkinson's and Alzheimer's diseases (Floyd, 1999; Ishige *et al.*, 2001). During the ischemic insult cascade, anti-inflammatory drugs diminish the effects of cerebral infarction by inhibiting cyclooxygenase-2 (COX-2) expression and prostaglandin E_2 production (PGE_2) (Yrianheikki *et al.*, 1999). Glutamate stimulates the production of arachidonate, and arachidonate also stimulates the release of glutamate (Lipton, 1999). The activation of N-methyl-D-aspartate (NMDA) receptors increases arachidonic acid release (Viu *et al.*, 1998) and COX-2 expression in cultured brain cells (Hewett *et al.*, 2000). The activation of enzymes of arachidonic acid metabolism, especially in 12-lipoxygenase (12-LOX), correlates with neuronal death caused by deprivation of endogenous glutathione (GSH) (Li *et al.*, 1997).

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Quercetin, a natural flavonoid, is well known as a constituent of *Ginkgo biloba* and *Hypericum perforatum* (St. John's wort) and occurs in other fruits and vegetables (Oyama *et al.*, 1994; Butterweck *et al.*, 2000). In recent studies, quercetin decreased neuronal injury in cultured hippocampal cells by inhibiting production of nitric oxide (Bastianetto *et al.*, 2000) and metal ion-induced peroxidation of lipids (Ramanathan and Das, 1992). Quercetin inhibits the release of PGE_2, and the expression of COX-2 and inducible nitric oxide synthase (Raso *et al.*, 2001), although quercetin may have some unfavorable effects under ischemic conditions, such as

inhibiting ischemic tolerance (Nakata *et al*., 1993) and preventing antiapoptotic action (De *et al.,* 2000). Moreover, it has been reported that quercetin reduces expression of both COX and LOX (Kim *et al.,* 1998). Previously, we reported that phenidone, an inhibitor of COX and LOX, exhibits antioxidant and antiapoptotic actions in cultured cortical cells (Wie *et al*., 1999). In this study, we examined whether quercetin protects neuronal cells in primary mouse cortical cultures against neurotoxicity induced by oxygen-glucose deprivation (OGD) or excitotoxins (NMDA or kainate) and against staurosporine- or MK-801-induced apoptosis. We evaluated the inhibitory action of quercetin on various free radical injuries and the NMDA-induced expression of COX-2 and 5-LOX.

Ⅱ. Materials and Methods

1. Neuronal and glial cultures

Mixed cortical cell cultures, containing both glia and neurons, were prepared from ICR mice at 15~16 days gestation, as previously described (Rose *et al.*, 1993). Briefly, dissociated neocortical cells (2.0~2.5 hemispheres) were plated onto primaria-coated 24-well plates (Falcon) containing a glial bed in plating medium consisting of Eagle's minimal essential medium (MEM; Earle's salts, supplied glutamine free) supplemented with 20 mM glucose, 2 mM *L*-glutamate, 5% fetal bovine serum, and 5% horse serum. Cytosine arabinoside (10 μM) was added 5 days after plating to halt the growth of non-neuronal cells. Cultures were maintained at 37℃ in a humidified CO_2 incubator and used for experiments between days 12 and 14 *in vitro*. Glial cultures were prepared from postnatal (1~3 days) 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 10 ng/㎖ epidermal growth factor. After 2 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. We added 10 μM glycine (final concentration) to all the media used in this study. We pre-incubated cultures with quercetin for 20~24 h before initiating neurotoxicity and added it again after the cessation of neurotoxicity, before the release of lactate dehydrogenase (LDH) was measured.

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2. Oxygen-glucose deprivation

Simultaneous oxygen and glucose deprivation was brought about by abruptly switching the culture medium to glucose-free, deoxygenated Earle's balanced salt solution (BSS_0: dilution > 1:1000) in an anaerobic chamber as previously described (Goldberg and Choi, 1993). OGD was terminated by switching the culture medium to oxygenated MEM containing 5.5 mM glucose and 2 mM glutamine, and returning the cultures to a normoxic CO_2 incubator.

3. Immunocytochemistry

Mixed cortical cultures containing both neurons and astrocytes were fixed for overnight at 4℃ in a freshly prepared solution of 4% paraformaldehyde in phosphate-buffered saline, pH 7.4 (PBS), washed with PBS three times, and permeabilized with 0.25% Triton X-100 in PBS for 10 min at room temperature. After washing with PBS, cultures were incubated in 10% normal goat serum (NGS) in PBS for 30 min at room temperature. After blocking, cultures were labeled with goat anti-COX-2 antisera (500 ng/㎖; Santa Cruze, California, CA, USA) and rabbit anti-5-LOX antisera (1:1000; Cayman, Ann Arbor, MI, USA) overnight at 4°C. After three washes, appropriate biotinylated secondary antibodies (anti-goat IgG or anti-rabbit IgG) were diluted 1:200 and added to the cultures for 1 hr at room temperature. The ABC Elite Kit (Vector, Burlingame, CA) for COX or the Histoplus kit (Zymed, South San Francisco, CA, USA) for LOX was added sequentially. The reaction

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product was visualized using 3,3-diaminibenzidine tetrahydrochloride (Vector, Burlingame, CA). All antibodies were diluted in PBS containing 2% NGS. Images were photographed with an Olympus IX-70 microscope and were processed using Adobe Photoshop software.

4. Measurement of neuronal cell injury

In most cases, neuronal cell death was quantitatively assessed by the measurement of lactate dehydrogenase (LDH) released into the cellular bathing medium 20 to 24 h after experimentation. LDH activity was quantified by the rate of oxidation of NADH, which was followed spectrophotometrically at 340 nm (Koh and Choi, 1987). Neuronal damage was assessed by damaged cells 1 day after the administration of excitotoxins or ischemic insult. For studies of apoptosis, LDH levels were measured 2 days after exposing the cells to apoptosis-inducing agents. Morphological confirmation was obtained by immunostaining cells with trypan blue or neuron-specific enolase antibody (Dako, Denmark).

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5. Statistical analysis

Data are expressed as the mean±standard error of mean (S.E.M.) and analyzed for statistical significance by one-way ANOVA using a post-hoc Student-Neuman-Keuls test for multiple comparisons using the Instat (v.2.04) software package from GraphPad (San Diego, CA).

Ⅲ. Results

1. Protective effects of quercetin on excitotoxin- and OGD-induced neurotoxicity

1) Long-term Neurotoxicity

Exposure of mixed cortical cell cultures to 20 μM NMDA for 20～24 h caused LDH release to increase by approximately 70～80%. When 100 μM quercetin was present both before and during NMDA exposure, neuronal damage was reduced by 22% ($p<0.01$) (Fig. 1). Quercetin (100 μM) also inhibited kainate-induced neurotoxicity by 61% ($p<0.01$) (Fig. 1). The NMDA antagonist MK-801 (10 μM) and the non-NMDA receptor antagonist CNQX (50 μM) effectively blocked NMDA- and KA-induced neurotoxicity (Fig. 1). In addition, After the exposure of 20 μM NMDA, cell culture supernatants were collected at 6, 12 or 24 h, and the time course of the LDH activity from cortical cultures was assessed. Results showed that NMDA-induced neurotoxicity resulted in increased LDH release in cortical cell cultures (Fig. 2). 100 μM quercetin inhibited the long-term NMDA-induced neurotoxicity by 53～54% at 6 and 12 h ($p<0.001$). However, LDH level showed a significant increase after 12 h (Fig. 2).

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2) Acute neurotoxicity

When cultures were subjected to OGD by switching from oxygenated MEM to BSS_0 for 50 min, neuronal swelling was observed soon after the switch. At approximately 20～24 h after OGD ceased, approximately 60～70% of the neuronal cells were damaged. When 10 μM MK-801 or 100 μM quercetin was added to the cultures during the OGD period, neuronal injury was reduced by 84% and 36%, respectively ($p<0.01$) (Fig. 1). Mixed cortical cell cultures exposed to 300 μM NMDA for 5 min showed moderate neuronal injury as evaluate by elevated LDH release into the bathing medium after 20～24 h. Pre- and co-treatment of cultures neurons with 1～100 μM quercetin for 20～24 h did not have a neuroprotective effect on NMDA-induced neurotoxicity in the same cultures. However, 100 μM quercetin reduced the acute NMDA-induced neurotoxicity by 25～31% at 6～12 h.

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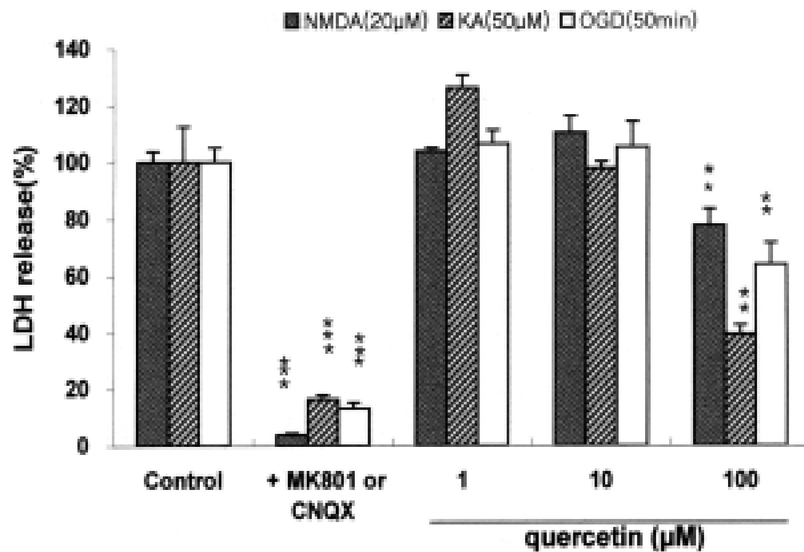

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Fig. 1. Pre- and co-treatment with quercetin (100 μM) attenuates N-methyl-D-aspartate (NMDA)-, kainate (KA)- and oxygen-glucose deprivation (OGD)-induced neurotoxicity. Bars represent LDH release (mean ± SEM, n=4) in sister cultures after 20-24 h of exposure to 20 μM NMDA or 50 μM KA, or after 50 min of OGD (controls), or with the addition of 10 μM MK-801 or 50 μM CNQX, or with the addition of quercetin at the indicated concentrations. The differences were evaluated with a one-way ANOVA and the post-hoc Student-Neuman-Keuls test for multiple comparisons (**; p<0.01, ***; p<0.001 vs. controls).

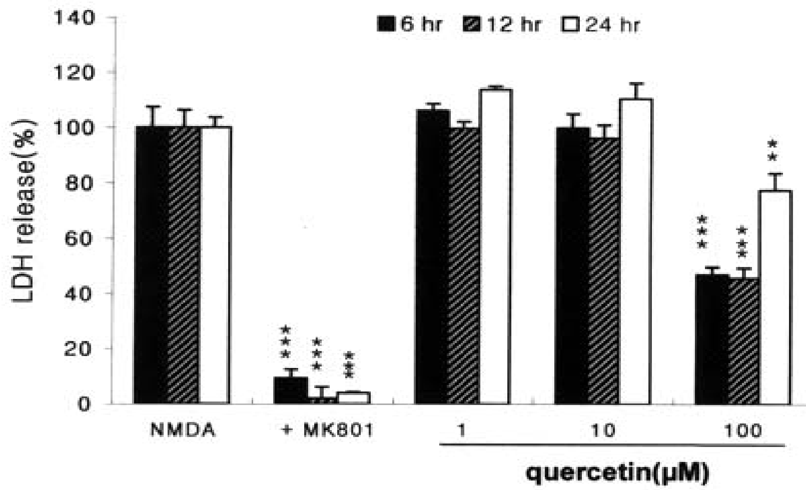

Fig. 2. Dose and time-course effect of quercetin on NMDA-induced neurotoxicity. Bars represent LDH release (mean ± SEM, n=4) after 6, 12 and 24 h of exposure to 20μM NMDA, or with the addition of 10μM MK-801 or with the addition of quercetin at the indicated concentrations. The differences were evaluated with a one-way ANOVA and the post-hoc Student-Neuman-Keuls test for multiple comparisons (**; $p<0.01$, ***; $p<0.001$ vs. controls).

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2. Quercetin ameliorates some free radical-mediated injuries

Exposure of neuronal cultures to 150 μM arachidonic acid (AA) for 20~24 h induced almost complete neuronal death. The LOX inhibitor esculetin (30 μM) reduced this AA-induced neuronal injury by 81%. Interestingly, the effect of quercetin on these cultures was inversely correlated with concentration, so that 1 μM quercetin reduced AA-induced neurotoxicity by 91%, whereas 100 μM quercetin reduced toxicity by 25% (Table 1). In contrast, only the highest concentration of quercetin (100 μM) inhibited the neurotoxicity induced by 40 μM $FeCl_2$ or 1 mM buthionine sulfoximine (BSO), an inhibitor of endogenous glutathione synthesis. In these cases, neurotoxicity was reduced by 92% and 34%, respectively (Table 1). The neurotoxicity evoked by sodium nitroprusside (SNP), which directly produces nitric oxide, was completely blocked (by 97~93%) by 10~100 μM quercetin. We observed that 100 μM L-N^G-nitroarginine methyl ester (L-NAME), a competitive inhibitor of nitric oxide synthase, did not display any protective effect against SNP neurotoxicity. All tested concentrations of quercetin (1~100 μM) significantly inhibited the neurotoxicity induced by 30 μM $ZnCl_2$. Similarly, an iron chelator deferoxamine (200 μM) effectively blocked $ZnCl_2$-induced neurotoxicity. We confirmed this neuroprotection by Immunocytochemistry using a neuron-specific enolase antibody (Fig. 3).

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Table 1. Antioxidant effects of quercetin on free radical-induced neuronal injury[a]

	LDH Released				
	AA(150μM)	BSO(1mM)	$FeCl_2$(40μM)	SNP(50μM)	$ZnCl_2$(30μM)
Control	100.0±7.7	100.0±15.7	100.0±5.0	100.0±7.0	100.0±8.6
1 μM Quercetin	8.7±7.9***	106.5±15.9	120.0±5.4	101.6±13.7	41.6±19.5**
10 μM Quercetin	30.2±7.6***	97.9±5.8	109.0±9.9	2.7±3.4***	6.3±0.9***
100 μM Quercetin	75.3±4.1*	66.3±15.9**	13.3±1.4***	6.8±4.6***	32.9±5.7**

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[a] Cortical cultures were exposed to 150 μM arachidonic acid (AA), 1 mM buthionine sulfoximine (BSO), 40 μM $FeCl_2$, 50 μM sodium nitroprusside (SNP), or 30 μM $ZnCl_2$ in the presence or absence (control) of 1～100 μM quercetin. For the AA and BSO samples, positive controls contained 30 μM esculetin and 100 μM α-tocopherol, respectively. For the $FeCl_2$ and $ZnCl_2$ samples, positive controls contained 200 μM deferoxamine. LDH levels in the medium were measured 24 h (5 h in SNP study) after exposure and are given as the mean ± SEM. (n=4). The differences were evaluated with a one-way ANOVA and the post-hoc Student-Neuman-Keuls test for multiple comparisons (*; $p < 0.05$, **; $p < 0.01$ ***; $p < 0.001$ vs. controls).

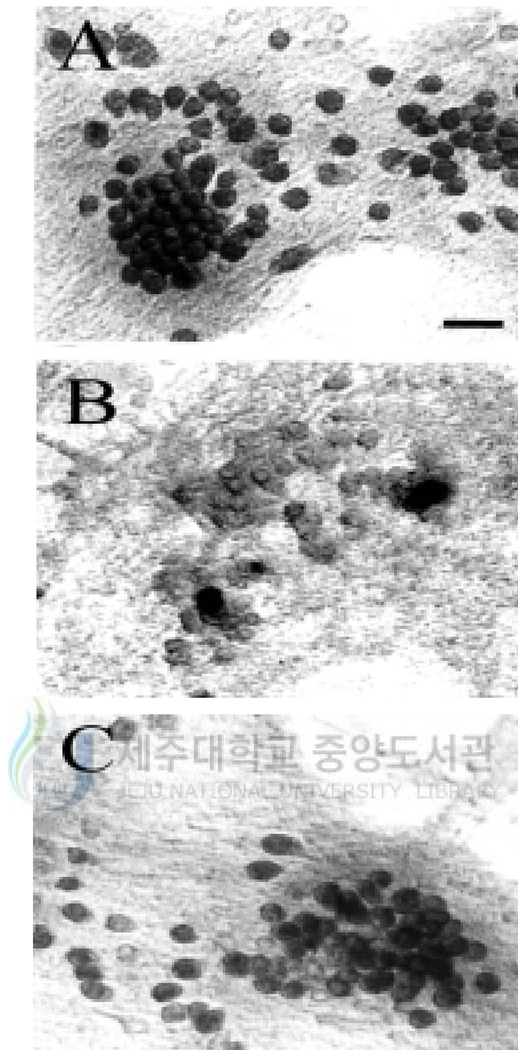

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Fig. 3. Morphological evidence of neuroprotection with quercetin in $ZnCl_2$-induced neurotoxicity. Phase-contrast photomicrographs of sister cultures 20~24 h after exposure to 30 μM $ZnCl_2$ alone (B) or in the presence of 10 μM quercetin (C) or 200 μM deferoxamine (A). Neurons were stained with specific mouse antibody to neuron-specific enolase (Dako, Denmark). Scale bar, 50 ㎛.

3. Quercetin-induced neuronal injury is blocked by cycloheximide (CHX)-and nerve growth factor (NGF)

Apoptotic injuries caused by the NMDA receptor antagonists MK-801 (100 µM), dextromethorphan (100 µM), the protein kinase inhibitor staurosporine (200 nM), or 300 µM cytosine arabinoside (Ara-C) were not inhibited by simultaneous exposure to quercetin (1～100 µM) (Table 2). In fact, quercetin was found to increase neuronal injury in a dose-dependent manner, so that quercetin at 1, 10, 100, and 200 µM caused 23, 33, 87, and 100% neurotoxicity, respectively, at 48 h after exposure (Fig. 4A). Morphological analysis confirmed that shrinkage of neuronal cell bodies occurred as a consequence of quercetin exposure. CHX (0.5 µM), a protein synthesis inhibitor, and NGF (100 ng/㎖) reduced quercetin neurotoxicity by 89～78% and 78～42% at 17-48 h, respectively (Fig. 4B).

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Table 2. Quercetin fails to block apoptotic injury[a]

	LDH Released			
	Ara-C (300μM)	MK-801 (100μM)	DM (100μM)	STSP (200nM)
Control	100.0±15.4	100.0±11.5	100.0±5.3	100.0±5.3
CHX(0.5μM)	25.5±1.6**	21.4±8.2**	42.5±7.3***	21.7±5.9***
1μM Quercetin	107.8±2.9	113.5±14.3	101.1±8.1	90.2±3.6
10μM Quercetin	110.5±15.3	210.5±16.2**	112.7±5.9	169.9±8.3***
100μM Quercetin	207.5±0.8*	157.0±20.4	124.7±10.5	113.7±9.3

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[a] Mixed cortical cell cultures were exposed to 100 μM MK-801, 100 μM dextromethrphan (DM), 200 nM staurosporine (STSP), or 300 μM cytosine arabinoside (Ara-C) in the presence or absence (control) of 1～100 μM quercetin. Positive controls contained 0.5 μM cycloheximide (CHX). LDH levels in the medium were measured 48 h after exposure and are given as the mean ± SEM. (n=4). The differences were evaluated with a one-way ANOVA and the post-hoc Student-Neuman-Keuls test for multiple comparisons (*; $p < 0.05$, **; $p < 0.01$ ***; $p < 0.001$ vs. controls).

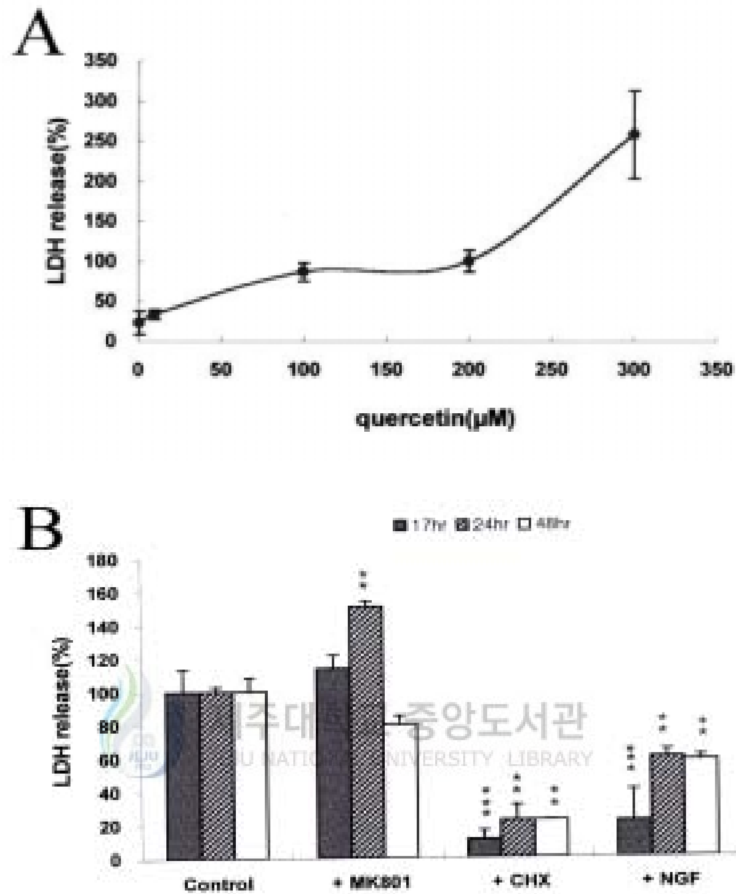
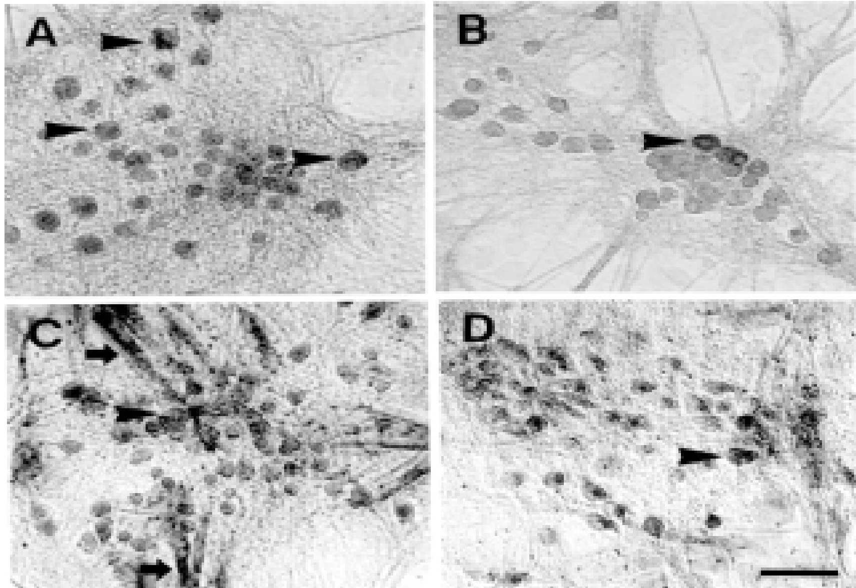


Fig. 4. Quercetin was found to increase neuronal injury in a dose-dependent manner, at 48 h after exposure (A). The neurotoxicity induced by quercetin is attenuated by cycloheximide (CHX) and nerve growth factor (NGF) (B). Bars represent LDH release (mean ± SEM, n=4) in sister cultures after 17-48 h of exposure to 200 μM quercetin with or without (control) the addition of 10 μM MK-801, 0.5 μM CHX, or 100 ng/㎖ NGF. The differences were evaluated with a one-way ANOVA and the post-hoc Student-Neuman-Keuls test for multiple comparisons (**; $p<0.01$, ***; $p<0.001$ vs. controls).

4. Quercetin inhibits the NMDA-induced expression of neural COX-2 and neural and glial 5-LOX

Brief exposure (5 min) of mixed cortical cultures to NMDA (100 μM) caused to increase in COX-2 expression in neurons (Fig. 5A) and in 5-LOX expression in both neurons and glia (Fig. 5C) examined at 1 h after NMDA addition. Quercetin inhibited this effect of NMDA on the expression of COX-2 (Fig. 5B) and 5-LOX (Fig. 5D).



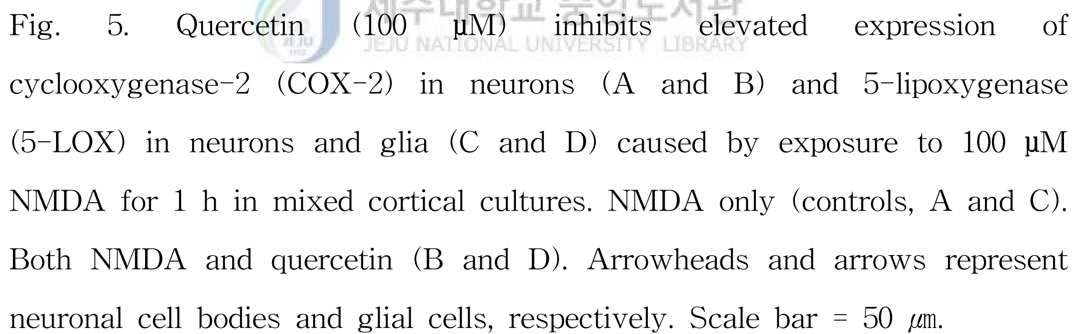

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Fig. 5. Quercetin (100 μM) inhibits elevated expression of cyclooxygenase-2 (COX-2) in neurons (A and B) and 5-lipoxygenase (5-LOX) in neurons and glia (C and D) caused by exposure to 100 μM NMDA for 1 h in mixed cortical cultures. NMDA only (controls, A and C). Both NMDA and quercetin (B and D). Arrowheads and arrows represent neuronal cell bodies and glial cells, respectively. Scale bar = 50 ㎛.

Ⅳ. Discussion

In this study, the antiexcitotoxic, antioxidant (Wie *et al.*, 2000) and anti-inflammatory properties of quercetin were shown to protect cortical cells from OGD-induced neurotoxicity. This protective effect of quercetin is similar to that reported previously for phenidone, an inhibitor of both COX and LOX (Wie *et al.*, 1999). However, quercetin failed to ameliorate the neurotoxicity caused by the apoptosis- inducing agents staurosporine (Koh *et al.*, 1995), MK-801, and dextrometorphan (Hwang *et al.*, 1999).

Previous studies have demonstrated a dual effect of quercetin on apoptosis. On the one hand, quercetin inhibits hydrogen peroxide-induced apoptosis by interventing in the activator protein 1 (AP-1)-mediated apoptotic pathway (Ishikawa *et al.*, 2000). On the other hand, it induces apoptosis in a monoblastoid cell line by inhibiting expression of heat shock proteins (Rong *et al.*, 2000). In our study, apoptosis-like neurotoxicity occurred in a dose-dependent manner 48 h after quercetin exposure, and CHX and NGF attenuated this effect. This result implies that quercetin may make a weak overall contribution to neuroprotective action during OGD- and excitotoxins-induced neuronal injury. In fact, we observed that the early protective effect of quercetin on NMDA-induced neurotoxicity diminished over time.

Although the OGD and excitotoxicity paradigms predict an unfavorable effect of quercetin in neurotoxicity, quercetin exhibited powerful antioxidant effects in our study. Reactive oxygen species and lipid peroxidation are generated in the early phase of Zn^{2+}-induced neurotoxicity (Kim *et al.*,

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1999), and combined Zn^{2+} accumuation and increased free radical generation have been observed in brains after transient global ischemia (Koh *et al.*, 1996). To this point, chelation of Zn^{2+} by quercetin may be a beneficial factor in ameliorating neuronal injury subsequent to cerebral ischemia, since secondary neuro-inflammation contributes significantly to neuronal damage after cerebral ischemia (Yrjanheikki *et al.*, 1999; Hewett *et al.*, 2000;, Kondoh *et al.*, 1999). In this study, quercetin was demonstrated to inhibit the elevation of COX-2 and 5-LOX levels normally evoked by NMDA in the inflammatory process. Interestingly, we observed that the elevation of 5-LOX expression caused by NMDA was much more apparent in glia than in neurons. Although NMDA treatment of glia did not cause morphological damage, the high level expression of 5-LOX in glia may contribute to neuronal injury via the accumulation of toxic arachidonic acid metabolites.

V. Conclusion

These results suggest that quercetin, a COX-2 inhibitor and 5-LOX inhibitor, contributes to neuronal survival by counteracting excitotoxicity, the production of free radicals, and OGD-induced inflammation. However, quercetin fails to block apoptotic injury. Taken together, these results indicate that quercetin protects against OGD-induced neurotoxicity through its cooperative antioxidant and anti-inflammatory actions, but not through an antiapoptotic action.

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초 록

Quercetin은 초대 피질세포배양에서 산소-포도당 결핍 유발 신경독성을 감소시킴

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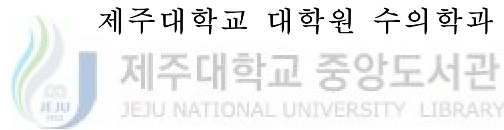

식물성 천연 플라보노이드인 quercetin은 허혈성 손상 시 발생하는 다양한 free radical을 매우 효과적으로 제거하는 것으로 보고되어지고 있다. 이 연구는 quercetin이 생쥐 피질세포배양에서 산소-포도당 결핍 (OGD), 흥분성 신경독성과 free radical 관련 및 아팝토시스성 신경손상에 대한 방어효과가 있는지를 검토하였다. 또한 quercetin이 NMDA 급성 신경독성 시 유발되어 발현되는 cyclooxygenase-2 (COX-2)와 5-lipoxygenase (5-LOX)에 대한 세포변화를 면역염색하여 관찰하였다. 전처리 및 동시 처리한 quercetin은 OGD, NMDA와 kainate로 야기되는 신경독성을 억제하였을 뿐만 아니라, arachidonic acid, buthionine sulfoximine, sodium nitroprusside, $ZnCl_2$ 및 $FeCl_2$로 매개되어 일어나는 다양한 free radical 관련 신경손상을 감소시켰다. 특히 quercetin은 NMDA (100 μM, 5 분) 급성 신경독성 시, 신경세포 내 COX-2 와 신경교 및 신경세포 내 증가된 5-LOX의 발현을 감소시켰다. 그러

나, quercetin은 staurosporine 나 MK-801로 유발되는 아팝토시스성 손상에 대해서는 신경방어효과를 나타내지 않았다. 다시 말해서, staurosporine이나 MK-801의 존재 시 quercetin은 아팝토시스를 진행시킨다고 생각되어진다. 이러한 quercetin으로 인한 신경손상은 cycloheximide와 nerve growth growth factor로 인하여 유의성 있게 ($p<0.01$) 억제되었다. 이상의 결과들로 보아 quercetin은 생쥐 신경세포배양을 이용한 허혈성 손상에 대하여 항산화와 항염증의 주된 활성으로 신경보호효과가 있음을 알 수 있었다.

주요어: quercetin, 산소-포도당 결핍, cyclooxygenase, lipoxygenase, arachidonic acid, 피질세포배양, NMDA, free radicals