CHAPTER IV

RESULTS

1. Effects of Aβ on cultured hippocampal neurons

To examine neurotoxicity of $A\beta_{25-35}$, hippocampal neurons derived from the brains of embryonic day 18 rat fetuses were cultured for 24 hr and then incubated with $A\beta_{25-35}$ at different concentrations and durations. Cell viability was determined using MTT reduction assay as an index of mitochondrial viability and LDH release assay as an indicator of plasma membrane damage. Cultured hippocampal neurons were exposed to $A\beta_{25-35}$ at various concentrations (1-20 μM) for 48 and 72 hr. It was shown that $A\beta_{25-35}$ decreased cell viability in a concentration- and exposure time-dependent manner (Figures 10). $A\beta_{25-35}$ at concentrations of 1, 5, 10, 15 and 20 μM significantly decreased cell metabolic activity by 19.44 $\pm$ 2.37 (P<0.01), 28.90 $\pm$ 2.44, 37.65 $\pm$ 2.69, 52.93 $\pm$ 5.02 and 58.23 $\pm$ 4.36% (P<0.001), respectively, after 48 hr exposure and by 21.11 $\pm$ 2.44 (P<0.01), 46.93 $\pm$ 4.3, 61.44 $\pm$ 3.95, 65.77 $\pm$ 2.49 and 68.50 $\pm$ 2.07% (P<0.001), respectively, after 72 hr exposure when compared with those of the control group. With 72 hr exposure to 5 and 10 μM $A\beta_{25-35}$, the percentages of MTT reduction were significant differences from those of 48 hr exposure to 5 and 10 μM $A\beta_{25-35}$ (P<0.01) (Figure 11).

In addition, low concentrations of $A\beta_{25-35}$ (1 and 5 μM) produced no significant LDH release after 48 hr exposure. However, the higher concentrations of $A\beta_{25-35}$ (10, 15 and 20 μM) significantly increased LDH release approximately by 34.96 $\pm$ 7.62 (P<0.05), 56.95 $\pm$ 7.05 and 68.92 $\pm$ 4.80% (P<0.001), respectively, as compared to control (Figure 12). Following 72 hr exposure, $A\beta_{25-35}$ at the concentrations of 5, 10, 15 and 20 μM markedly increased LDH release by 54.59 $\pm$ 2.57 (P<0.05), 131.77 $\pm$ 14.47, 183.20 $\pm$ 14.14 and 213.47 $\pm$ 16.21% (P<0.001), respectively, as compared to control. With 72 hr exposure to 5, 10, 15 and 20 μM $A\beta_{25-35}$, the percentages of MTT reduction were significant differences from those of 48 hr exposure to 5 (P<0.01), 10, 15 and 20 μM $A\beta_{25-35}$ (P<0.001), respectively.

From preliminary results, it was found that nearly 50% of cells died after exposure to $A\beta_{25-35}$ at the concentration of 5 μM for 72 hr. Based on these results, 72

hr exposure to 5 μM $A\beta_{25-35}$ was used as a challenging neurotoxic insult for cultured hippocampal neurons in further experiments on neuroprotective effects of quercitrin.

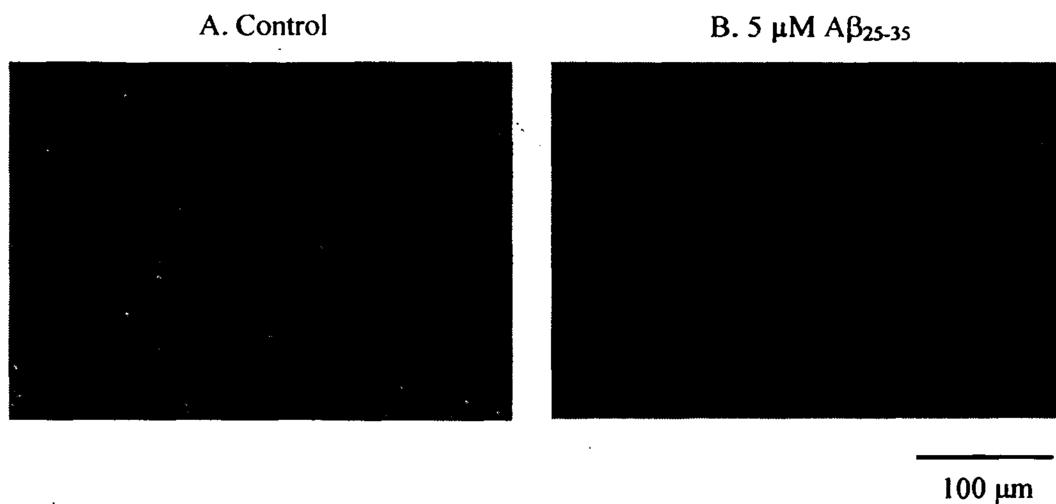

Figure 10 Cytotoxic effect of $A\beta_{25-35}$ on cultured hippocampal neurons. Hippocampal neurons were cultured for 24 hr and then exposed to 5 μM $A\beta_{25-35}$ for 72 hr. After exposure, gross morphological changes were observed by phase-contrast microscopy. Exposure to $A\beta_{25-35}$ (B) apparently induced marked cell injuries and reduction in cell viability as compared to control cultures (A).

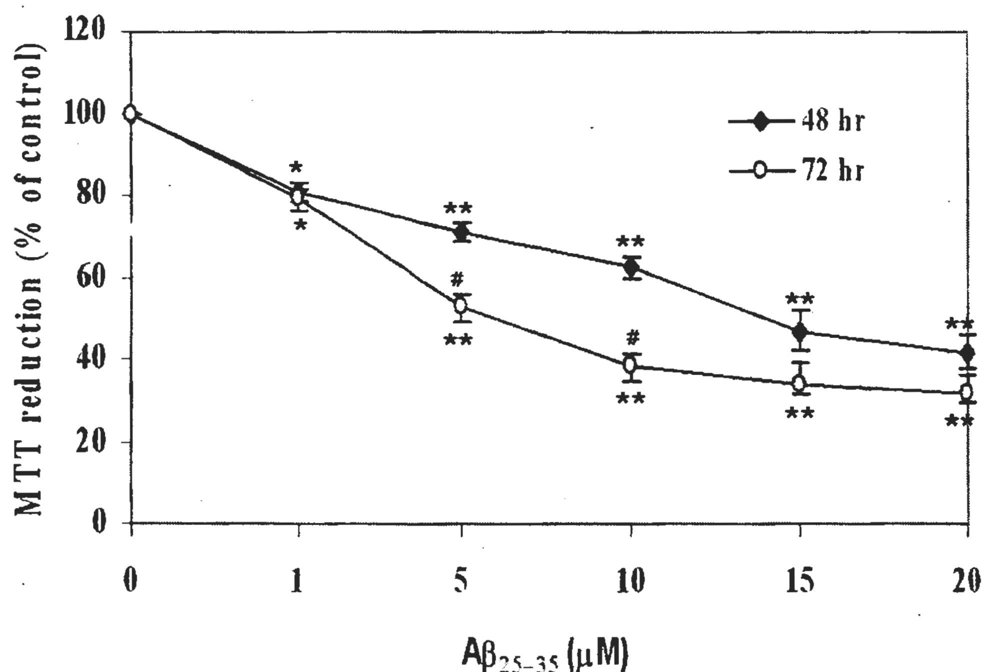

Figure 11 Concentration and time courses of hippocampal neuronal injuries induced by $A\beta_{25\text{-}35}$.

Hippocampal neurons were cultured for 24 hr and then exposed to various concentrations of $A\beta_{25\text{-}35}$ (1-20 μM) for 48 and 72 hr. After exposure, mitochondrial activity was determined by MTT reduction assay.

Data are expressed as mean ± SEM of six samples from at least three independent experiments, each performed in duplicate.

The differences among groups were evaluated with one-way ANOVA and the post-hoc Scheffe's test for multiple comparisons. The difference between two sample groups was analyzed by the independent sample Student *t*-test.

$^{*}P < 0.01$ compared with control cultures, $^{**}P < 0.001$ compared with control cultures.

$^{\#}P < 0.01$ compared between 48 and 72 hr exposure.

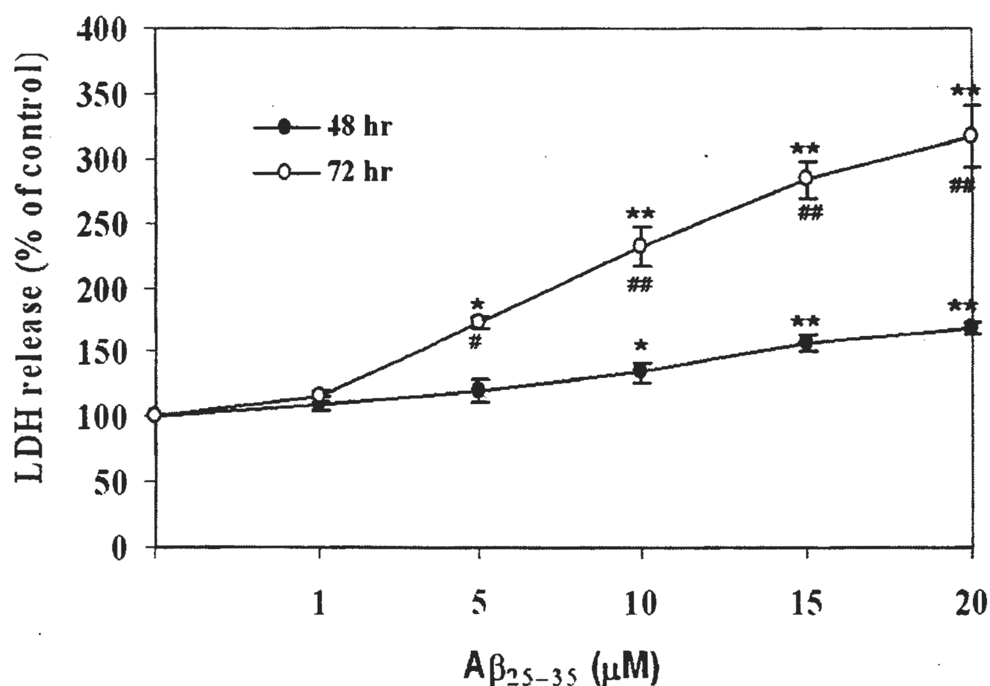

Figure 12 Concentration and time courses of hippocampal neuronal cell death induced by $A\beta_{25-35}$.

Hippocampal neurons were cultured for 24 hr and then exposured to various concentrations of $A\beta_{25-35}$ (1-20 μM) for 48 and 72 hr. After exposure, neuronal cell death was determined by LDH release assay. Data are expressed as mean ± SEM of six samples from at least three independent experiments, each performed in duplicate.

The differences among groups were evaluated with one-way ANOVA and the post-hoc Scheffe's test for multiple comparisons. The difference between two sample groups was analyzed by the independent sample Student *t*-test.

$^{*}P < 0.01$ compared with control cultures, $^{**}P < 0.001$ compared with control cultures.

$^{\#}P<0.01$, $^{\#\#}P<0.001$ compared between 48 and 72 hr exposure.

2. Effects of quercitrin on cultured hippocampal neurons

In the preliminary study, we also studied effects of quercetin on the cell survival of cultured hippocampal neurons. Hippocampal neurons were cultured for 24 hr and were treated with quercetin at 0.001, 0.01, 0.1, 1, 10, 20, 50, and 100 μM for 3 days. Following 3-day exposure, quercetin at concentrations of 0.001 to 1 μM did not affect cell viability, but at higher concentrations of 10, 20, 50, and 100 μM significantly decreased MTT reduction to 47.79 $\pm$ 6.54, 20.60 $\pm$ 0.45, 12.48 $\pm$ 0.45, and 12.50 $\pm$ 0.75%, respectively (Figure 13). Base on preliminary results, we selected quercetin at nontoxic doses (lower than 10 μM) for further study on its neuroprotection. It was apparent that co-exposure with quercetin at concentrations of 0.1, 1, 10, 50, 100, and 1000 nM for 3 days had no protective effect against $A\beta_{25-35}$ -induced neurotoxicity in cultured hippocampal neurons (Figure 14). Therefore, experimental results suggested considerable neurotoxic effects of quercetin to cultured neurons. In this connection, we conceivably selected quercitrin, a glycosidic form of quercetin, for further study on neuroprotection.

To determine an effective concentration range of quercitrin for the study of neuroprotection, we investigated the impact of varying concentrations of quercitrin or 17β-estradiol on hippocampal neuronal survival. Cell viability was verified by MTT reduction analysis. One day after plating, cultured hippocampal neurons were treated with quercitrin or 17β-estradiol at various concentrations for 3 days. Results indicated that after exposure, neither quercitrin nor 17β-estradiol at the concentrations of 0.001, 0.01, 0.1, 1, 10, 20, 50, and 100 μM induced significant changes in cell metabolic activity of cultured neurons (Figure 15). These data provided a clue for using a concentration range of 0.1-100 μM in the further study of neuroprotective effects.

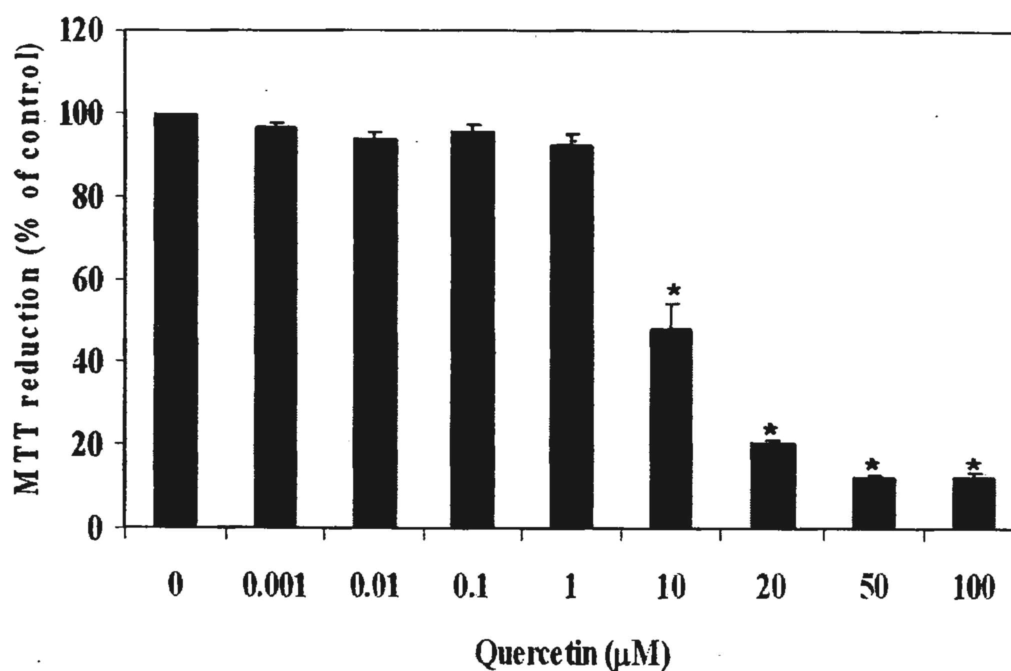

Figure 13 Effect of quercetin on mitochondrial activity of cultured hippocampal neurons.

Hippocampal neurons were cultured for 24 hr and then incubated with different concentrations of quercetin (.001-100 μM) or 72 hr. After incubation, neuronal injury was determined by MTT reduction assay.

Data are expressed as mean ± SEM of six samples from at least three independent experiments, each performed in duplicate. The differences among groups were evaluated with one-way ANOVA and the post-hoc Scheffe's test for multiple comparisons.

*P <0.01 compared with control cultures.

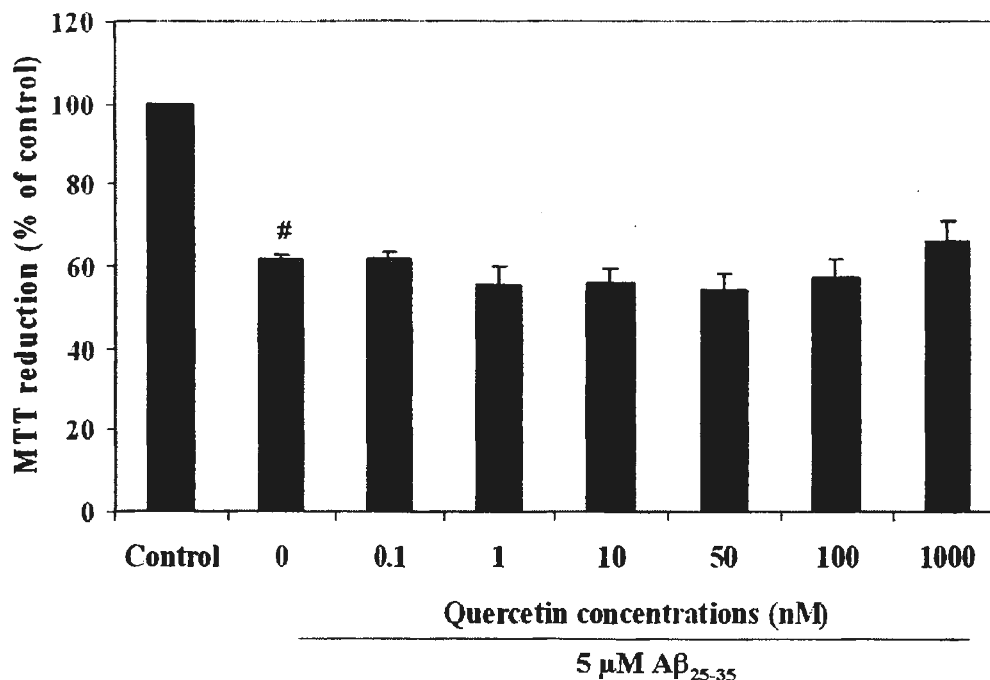

Figure 14 Effect of co-exposure with quercetin on $A\beta_{25-35}$–induced neurotoxicity in cultured hippocampal neurons.

Hippocampal neurons were grown for 24 hr and then treated with different concentrations of quercetin (0.1-100 μM) in combination with 5 μM $A\beta_{25-35}$ for 72 hr. After co-exposure, mitochondrial activity was determined by cellular MTT reduction assay. Data are expressed as mean ± SEM of eight samples from at least four independent experiments, each performed in duplicate. The differences among groups were evaluated with one-way ANOVA and the post-hoc Scheffe's test for multiple comparisons.

$^{\#}P < 0.01$ compared with control cultures.

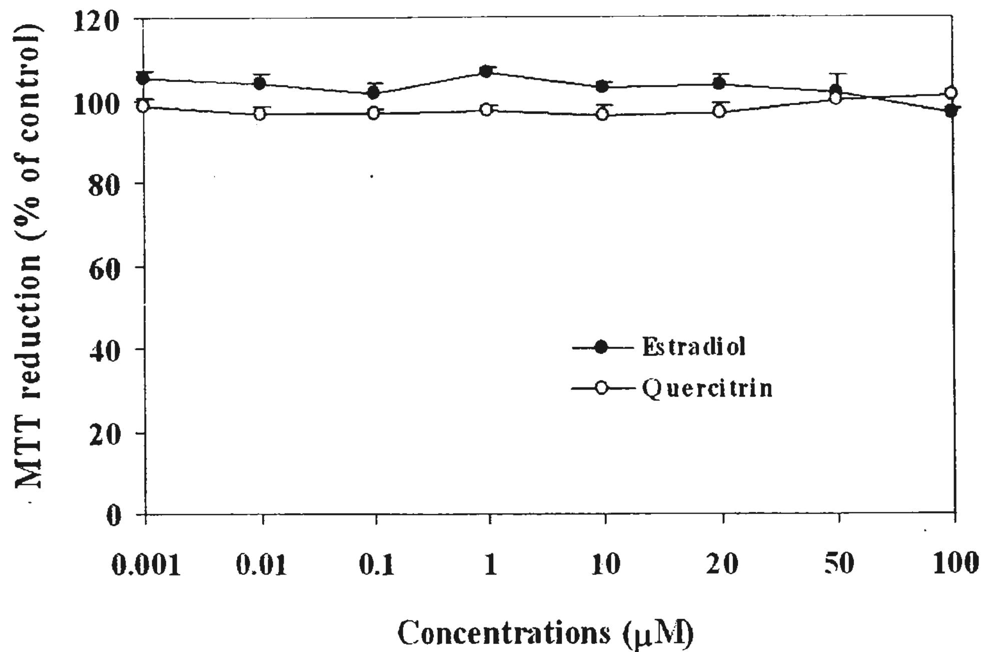

Figure 15 Effect of quercitrin or 17β-estradiol on mitochondrial activity of cultured hippocampal neurons.

Hippocampal neurons were cultured for 24 hr and then incubated with different concentrations of quercitrin or 17β-estradiol (0.001-100 μM for 72 hr. After incubation, neuronal injury was determined by MTT reduction assay. Data are expressed as mean ± SEM of six samples from at least thre independent experiments, each performed in duplicate.

The difference among effects of quercitrin or 17β-estradiol-treated cultures compared to control cultures was evaluated with one-way ANOVA.

No significant different between quercitrin or 17β-estradiol-treated cultures compared to control culture.

3. Effects of co-exposure to quercitrin on Aβ-induced neurotoxicity in hippocampal neuronal cultures

We determine whether quercitrin was able to protect hippocampal neurons against Aβ-induced neurodegeneration. Hippocampal neurons were grown for 24 hr and then co-treated with various concentrations of quercitrin or 17β-estradiol and $A\beta_{25-35}$ for 72 hr. Then, cell survival was analyzed using MTT and LDH assays. After co-incubation of quercitrin and $A\beta_{25-35}$, it was found that $A\beta_{25-35}$ alone at the concentration of 5 μM induced a significant decrease in cell metabolic activity to 44.38$\pm$2.98% as relative to control ($p<0.01$) (Figure 16). Quercitrin at the concentrations of 0.1, 1, and 10 μM had no protective effect against suppression of cell metabolic activity induced by $A\beta_{25-35}$. However, quercitrin at higher concentrations, 50 and 100 μM, significantly increased the percentage of cell metabolic activity from 55.62 $\pm$ 2.98% to 71.43 $\pm$ 2.26% and 55.62 $\pm$ 2.98% to 76.09 $\pm$ 2.81%, respectively, as compared with $A\beta_{25-35}$-treated cultures ($p<0.05$).

In addition, following co-incubation of 17β-estradiol and $A\beta_{25-35}$, it was shown that 5 μM of $A\beta_{25-35}$ significantly decreased cell metabolic activity to 45.85 $\pm$ 3.38% as relative to control ($p<0.01$) (Figure 17). 17β-Estradiol at low concentrations (0.1, 1, and 10 μM) did not show protective effect against suppression of cell metabolic activity induced by $A\beta_{25-35}$, whereas at higher concentrations (50 and 100 μM), it rescued $A\beta_{25-35}$-induced decrease in cell viability from 54.15 $\pm$ 3.38% to 78.27 $\pm$ 5.07% and from 54.15 $\pm$ 3.38% to 81.83 $\pm$ 4.71%, respectively, as compared to $A\beta_{25-35}$-treated cultures ($p<0.05$).

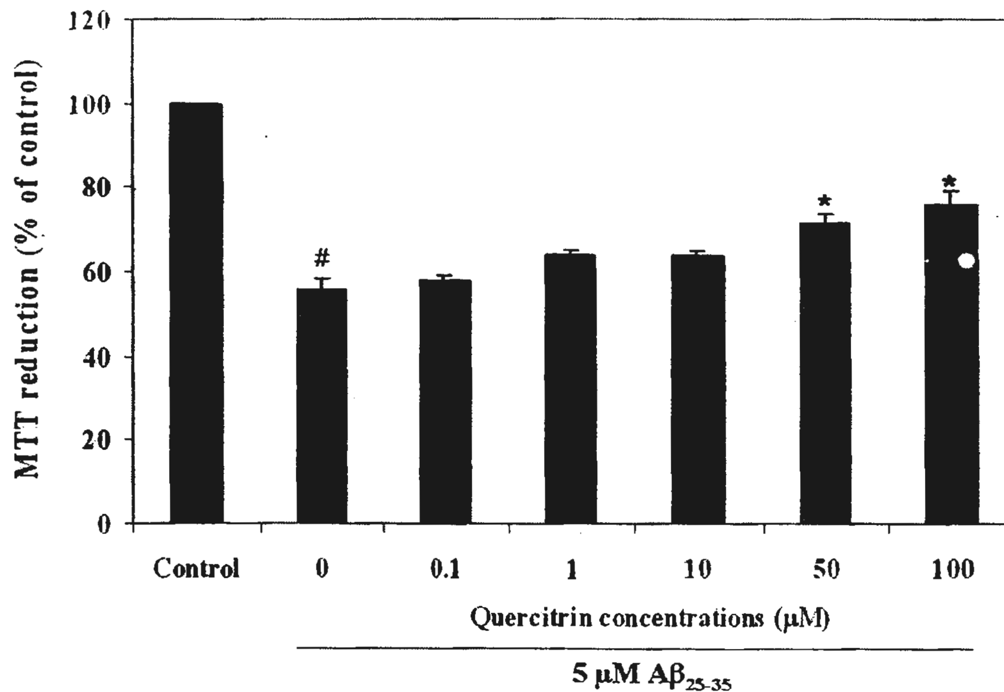

Figure 16 Effect of co-exposure with quercitrin on $A\beta_{25-35}$–induced neurotoxicity in cultured hippocampal neurons.

Hippocampal neurons were grown for 24 hr and then treated with different concentrations of quercitrin (0.1-100 μM) in combination with 5 μM $A\beta_{25-35}$ for 72 hr. After co-exposure, mitochondrial activity was determined by cellular MTT reduction assay. Data are expressed as mean ± SEM of eight samples from at least four independent experiments, each performed in duplicate. The difference among groups was evaluated with one-way ANOVA and the post-hoc Scheffe's test for multiple comparisons.

$^{\#}P < 0.05$ compared with control cultures, $^{*}P < 0.05$ compared with $A\beta_{25-35}$-treated cultures.

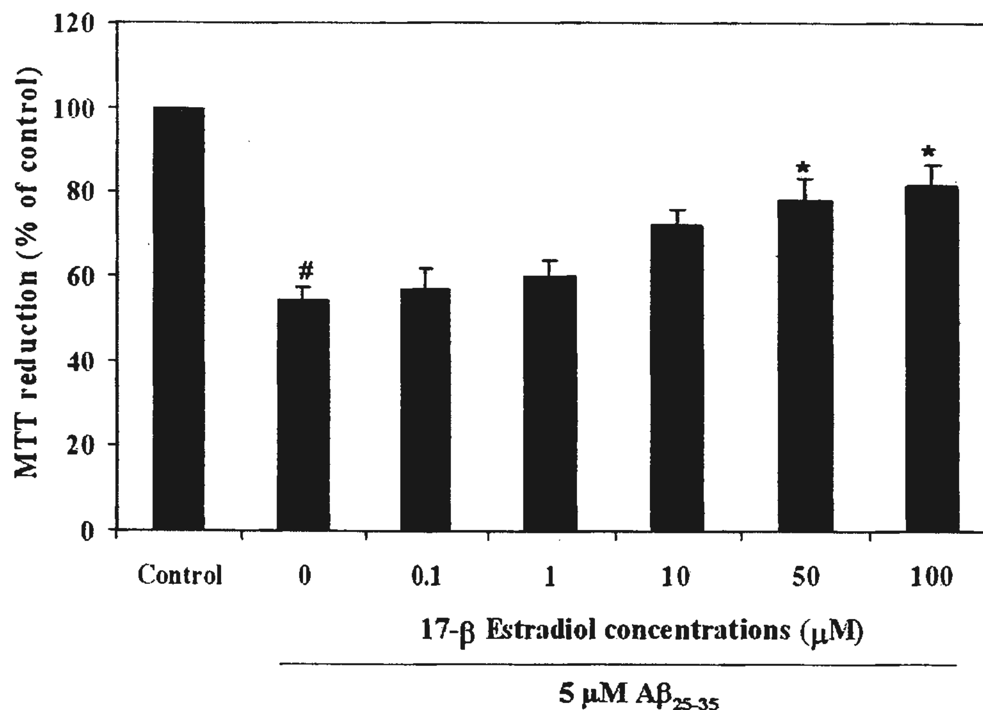

Figure 17 Effect of co-exposure with 17β-estradiol on $A\beta_{25-35}$ -induced neurotoxicity in cultured hippocampal neurons.

Hippocampal neurons were grown for 24 hr and then treated with different concentrations of 17β-estradiol (0.1-100 μM) in combination with 5 μM $A\beta_{25-35}$ for 72 hr. After co-exposure, mitochondrial activity was determined by cellular MTT reduction assay. Data are expressed as mean ± SEM of eight samples from at least four independent experiments, each performed in duplicate. The difference among groups was evaluated with one-way ANOVA and the post-hoc Scheffe's test for multiple comparisons. $^{\#}P < 0.01$ compared with control cultures, $^{*}P < 0.05$ compared with $A\beta_{25-35}$ -treated cultures.

In addition to MTT reduction analysis, LDH release was also evaluated when $A\beta_{25-35}$ was co-incubated with higher concentrations of quercitrin or 17β-estradiol (50 and 100 μM). It was shown that 5 μM of $A\beta_{25-35}$ alone significantly increased LDH release by 68.99 $\pm$ 4.6% when compared to control ($p<0.01$) (Figure 18). Quercitrin at concentrations of 50 and 100 μM significantly reduced the percentage of LDH release from 168.99 $\pm$ 4.60% to 145.02 $\pm$ 2.09% and from 168.99 $\pm$ 4.60% to 140.52 $\pm$ 2.46%, respectively, as compared with $A\beta_{25-35}$-treated cultures ($p<0.05$). 17β-Estradiol at concentrations of 50 and 100 μM significantly reduced the percentage of LDH release from 168.99 $\pm$ 4.60% to 134.60 $\pm$ 2.06% and from 168.99 $\pm$ 4.60% to 127.45 $\pm$ 2.82%, respectively, as compared with $A\beta_{25-35}$-treated cultures ($p<0.01$). However, there were no significant differences in the percentage of LDH release between co-treatment of quercitrin (50 and 100 μM) with $A\beta_{25-35}$ and co-treatment of 17β-Estradiol (50 and 100 μM) with $A\beta_{25-35}$.

These results suggested that higher concentrations of quercitrin or 17β-estradiol (50 and 100 μM) had neuroprotective effect on $A\beta_{25-35}$-induced neurotoxicity in hippocampal neuronal cultures. Therefore, quercitrin and 17β-estradiol at concentrations of 50 and 100 μM were used for subsequent experiments in this study.

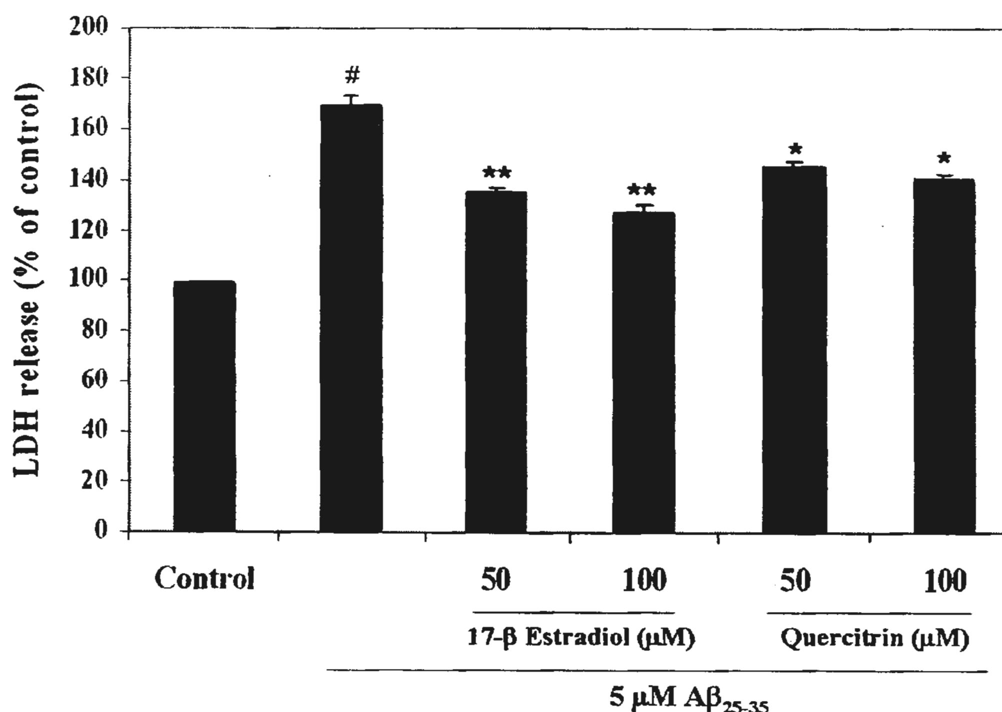

Figure 18 Effect of co-exposure with quercitrin or 17β-estradiol on $A\beta_{25\text{-}35}$-induced neurotoxicity in cultured hippocampal neurons.

Hippocampal neurons were cultured for 24 hr and then treated with different concentrations of quercitrin or 17β-estradiol (50 and 100 μM combination with 5 μM $A\beta_{25\text{-}35}$ for 72 hr. After co-exposure, neuronal cell death was determined by LDH release assay. Data are expressed as mean ± SEM of six samples from at least three independent experiments, each performed in duplicate. The difference among groups was evaluated with one-way ANOVA and the post-hoc Scheffe's test for multiple comparisons. The difference between neuroprotective effects of quercitrin and 17β-estradiol was analyzed by the independent sample Student *t*-test. $^{\#}P < 0.01$ compared with control cultures, $^{*}P < 0.05$ compared with $A\beta_{25\text{-}35}$-treated culture $^{**}P < 0.01$ compared with $A\beta_{25\text{-}35}$-treated cultures.

Oxygen free radical-induced lipid peroxidation has been suggested to play an important role in the $A\beta_{25-35}$-mediated neurotoxicity. Therefore, effects of quercitrin or 17β-estradiol on lipid peroxidation were determined in this study. After co-incubation with quercitrin or 17β-estradiol and $A\beta_{25-35}$ for 3 days, it was found that 5 μM of $A\beta_{25-35}$ significantly increased cellular lipid peroxidation (48.22 $\pm$ 3.31%, $p<0.01$) (Figure 19). Quercitrin and 17β-estradiol significantly prevented $A\beta_{25-35}$-induced increase in cellular lipid peroxidation. Quercitrin at 50 and 100 μM induced a decrease in cellular lipid peroxidation from 148.22 $\pm$ 3.31% to 125.95 $\pm$ 4.44% and from 148.22 $\pm$ 3.31% to 117.17 $\pm$ 5.26%, respectively, as compared with $A\beta_{25-35}$-treated cultures ($p<0.01$). 17β-estradiol at 50 and 100 μM induced a decrease in cellular lipid peroxidation from 148.22 $\pm$ 3.31% to 115.18 $\pm$ 5.15% and from 148.22 $\pm$ 3.31% to 106.63 $\pm$ 4.49%, respectively, as compared with $A\beta_{25-35}$-treated cultures ($p<0.01$). These results suggested that higher concentrations of quercitrin or 17β-estradiol (50 and 100 μM) possessed protective effect against $A\beta_{25-35}$ -induced lipid peroxidation in hippocampal neuronal cultures.

Additionally, It has been suggested that the pathology of neurodegeneration in Alzheimer' disease involves oxidative stress. Therefore, oxidative stress is a contributor to Aβ-induced neurodegeneration and $A\beta_{25-35}$-induced neurotoxicity is in part mediated by ROS. We further investigated the participation of oxidative stress in $A\beta_{25-35}$-induced neurotoxicity and determined whether quercitrin was able to protect hippocampal neurons against Aβ-induced ROS accumulation. The effect of quercitrin or 17β-estradiol on the production of ROS was quantified in this study. After co-incubation with quercitrin or 17β-estradiol and $A\beta_{25-35}$ for different durations of exposure at 6, 12, 18 and 24 hr, it was found that 5 μM of $A\beta_{25-35}$ significantly increased intracellular of ROS at 18 and 24 hr of exposure to 65.71 $\pm$ 13.19 and 51.35 $\pm$ 7.10% ($p<0.05$), respectively, as compared to control cultures (Figure 20).

At 18 hr of incubation time, quercitrin at concentrations of 50 and 100 μM had no significant effects on ROS accumulation in $A\beta_{25-35}$-treated cultures. 17β-estradiol at 50 and 100 μM did not display protective effect against $A\beta_{25-35}$-induced ROS accumulation. These results suggested that quercitrin or 17β-estradiol (50 and

100 μM) had no protective effect against $A\beta_{25-35}$ -induced ROS accumulation in hippocampal neuronal cultures.

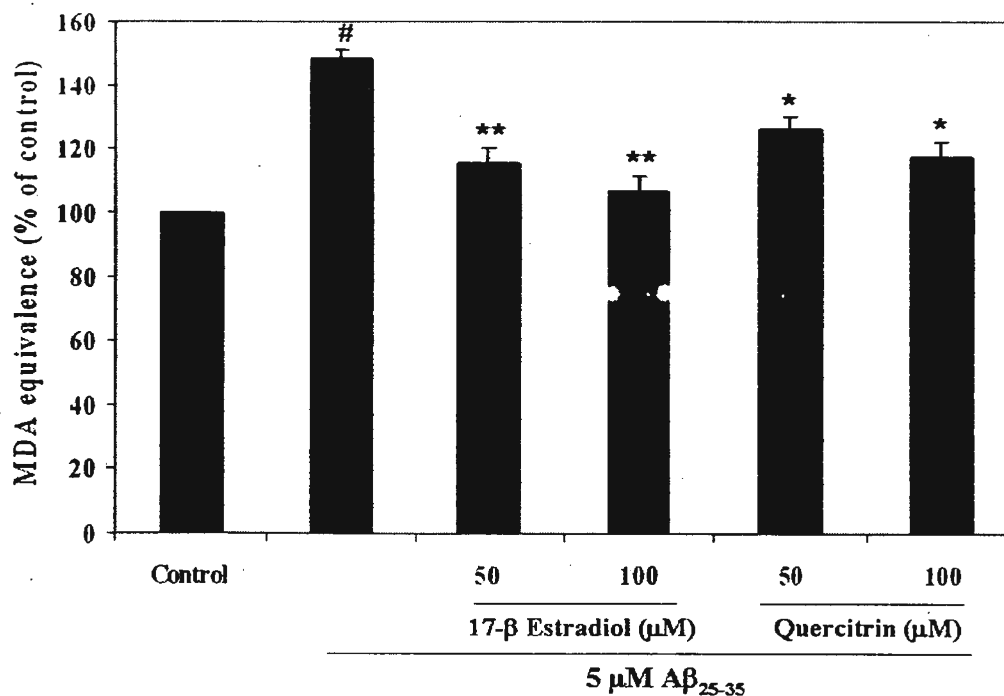

Figure 19 Effect of coexposure with quercitrin or 17β-estradiol on lipid peroxidation level of $A\beta_{25-35}$-exposed hippocampal neuronal cultures. Hippocampal neurons were cultured for 24 hr and then treated with different concentrations of quercitrin or 17β-estradiol (50 and 100 μM) in combination with 5 μM $A\beta_{25-35}$ for 72 hr. After co-incubation, the production of lipid peroxidation was performed by TBARS assay. Data are expressed as mean ± SEM of six samples from at least three independent experiments, each performed in duplicate. The difference among groups was evaluated with one-way ANOVA and the post-hoc Scheffe's test for multiple comparisons.

$^{\#}P < 0.01$ compared with control cultures, $^{*}P < 0.05$ compared with $A\beta_{25-35}$–treated culture, $^{**}P < 0.01$ compared with $A\beta_{25-35}$-treated cultures).

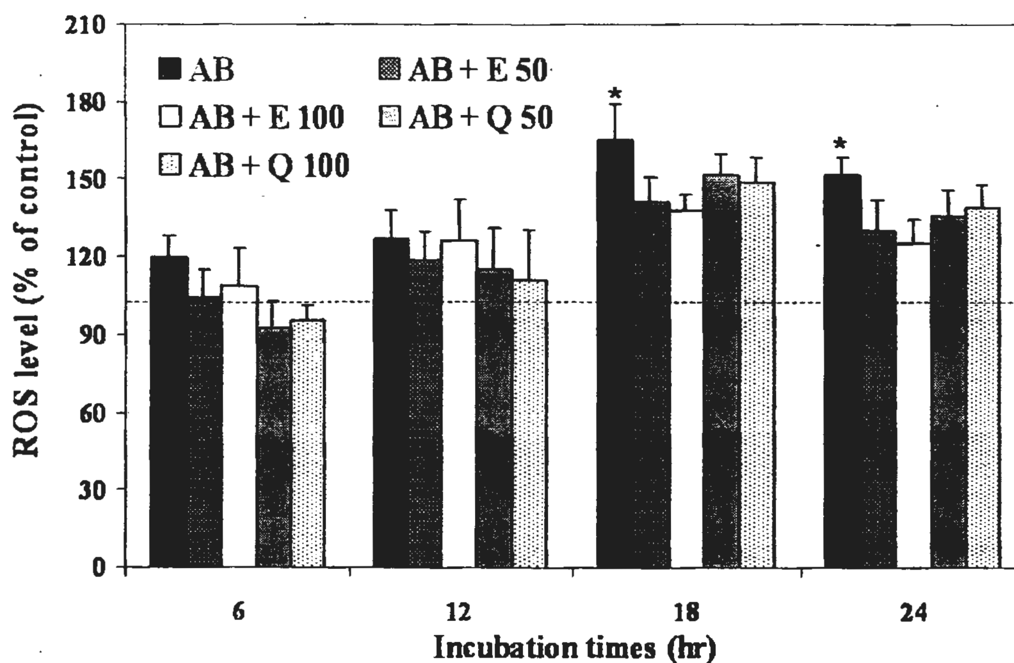

Figure 20 Effect of coexposure with quercitrin or 17β-estradiol on ROS accumulation of $A\beta_{25-35}$-exposed hippocampal neuronal cultures.

Hippocampal neurons were cultured for 24 hr and then treated with different concentrations of quercitrin or 17β-estradiol (50 and 100 μM) in combination with 5 μM $A\beta_{25-35}$ for various time points.

After co-incubation, intracellular ROS level was quantified by a spectrofluorometer using DCFH-DA. Data are expressed as mean ± SEM of six samples from three independent experiments, each performed in duplicate. The difference among groups was evaluated with one-way ANOVA and the post-hoc Scheffe's test for multiple comparisons.

*P <0.01 compared with control cultures.

To determine whether quercitrin altered the antioxidant defense systems of cultured hippocampal neurons when co-exposed with $A\beta_{25-35}$, we carried out assays for various intracellular antioxidant components including GSH, SOD and GPx. It was apparent that exposure of cultured hippocampal neurons to 5 µM $A\beta_{25-35}$ for 3 days caused a marked reduction of celluler GSH content by 65.42 $\pm$ 5.53% as compared to control ($p<0.01$) (Figure 21).

It was found that 3-day co-exposure of 100 µM 17β-estradiol in combination with 5 µM $A\beta_{25-35}$ significant increased cellular GSH content from 34.58 $\pm$ 5.53 to 64.69 $\pm$ 5.42 ($p<0.05$) when compared to $A\beta_{25-35}$-treated cultures whereas both 50 µM and 100 µM of quercitrin failed to prevented a decrease in cellular GSH content caused by $A\beta_{25-35}$. Therefore, it was evident that 17β-estradiol at a concentration of 100 µM possessed a protective effect against β_{25-35}-induced GSH depletion in hippocampal neuronal cultures.

Regarding intracellular antioxidant systems, the SOD activity of hippocampal neurons decreased after exposure to 5 µM $A\beta_{25-35}$ but not statistically significant as compared to control cultures (Figure 22). No significant effects were found in cultured neurons co-exposed with quercitrin or 17β-estradiol and $A\beta_{25-35}$ when compared to neurons treated with $A\beta_{25-35}$ only. In part of the GPx activity, it was shown that 5 µM $A\beta_{25-35}$ alone significantly decreased GPx activity by 31.03 $\pm$ 5.53% when compared to control ($p<0.05$) (Figure 23). Co-exposure of $A\beta_{25-35}$ with quercitrin at concentration of 50 and 100 µM induced a significant increase in GPx activity from 136.35 $\pm$ 4.75% to 167.88 $\pm$ 6.13% and from 136.35 $\pm$ 4.75% to 170.32 $\pm$ 4.61%, respectively ($P<0.05$), when compared to $A\beta_{25-35}$-treated cultures. However, no changes in the level of GPx enzyme in hippocampal neurons were found when neurons co-exposed with $A\beta_{25-35}$ and 17β-estradiol.

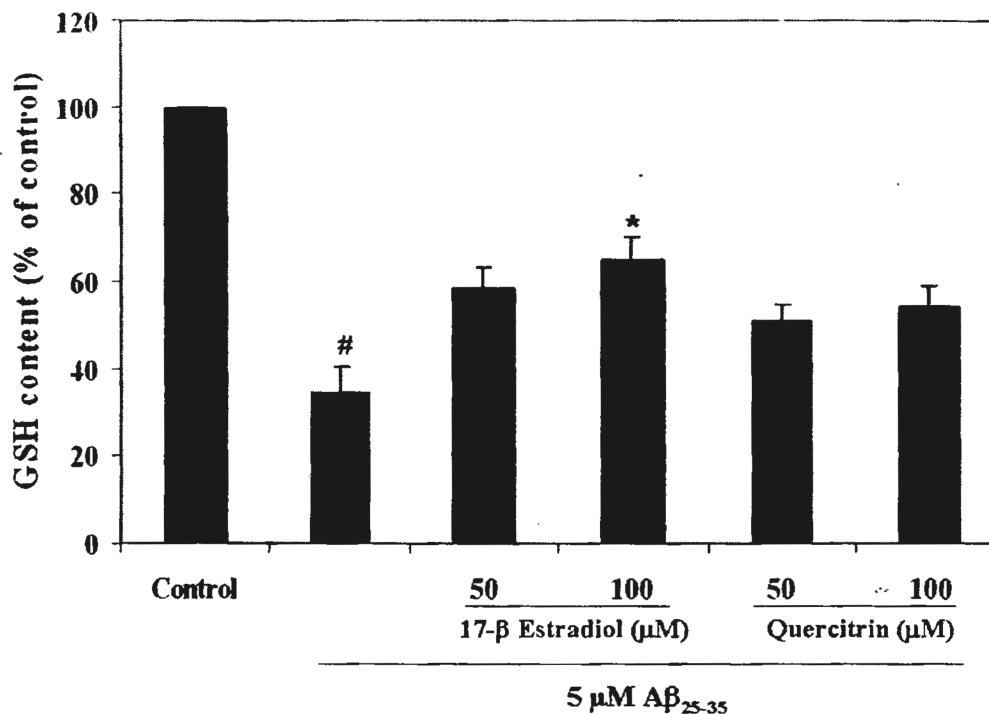

Figure 21 Effect of coexposure with quercitrin or 17β-estradiol on GSH levels of $A\beta_{25\text{-}35}$-exposed hippocampal neuronal cultures.

Hippocampal neurons were cultured for 24 hr and then treated with different concentrations of quercitrin or 17β-estradiol (50 and 100 μM) in combination with 5 μM $A\beta_{25\text{-}35}$ for 72 hr. After co-incubation, intracellular GSH content was measured. Data are expressed as mean ± SEM of six samples from at least three independent experiments, each performed in duplicate. The difference among groups was evaluated with one-way ANOVA and the post-hoc Scheffe's test for multiple comparisons.

$^{\#}P < 0.01$ compared with control cultures, * $P < 0.05$ compared with $A\beta_{25\text{-}35}$-treated cultures.

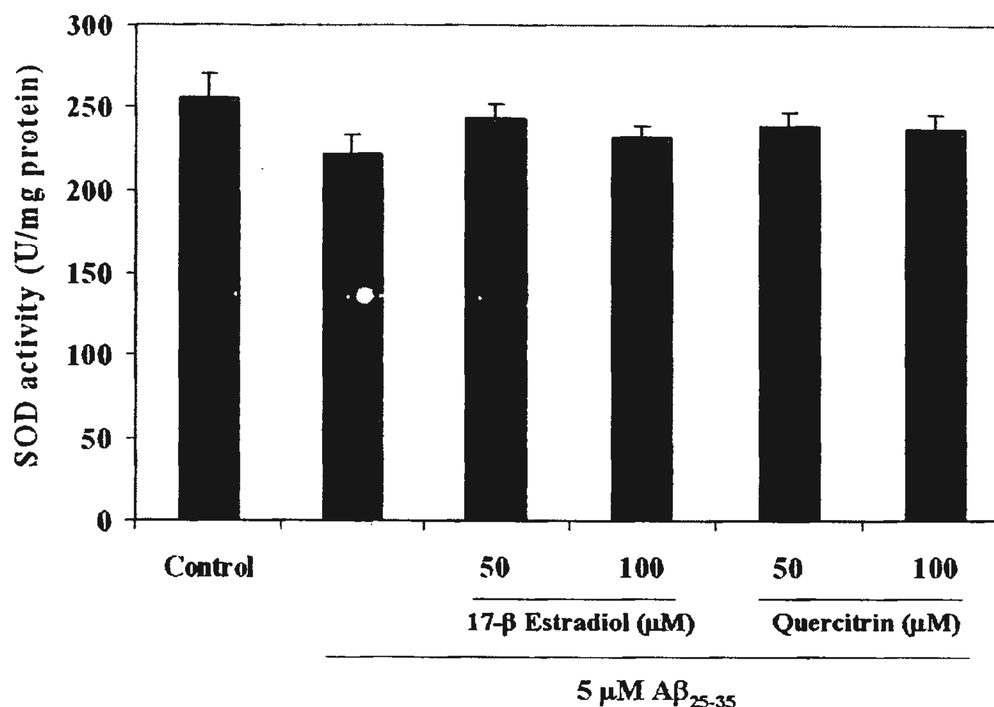

Figure 22 Effect of co-exposure with quercitrin or 17β-estradiol on superoxide dismutase activity of $A\beta_{25\text{-}35}$-exposed hippocampal neuronal cultures. Hippocampal neurons were cultured for 24 hr and then treated with different concentrations of quercitrin or 17β-estradiol (50 and 100 μM) in combination with 5 μM $A\beta_{25\text{-}35}$ for 72 hr. After co-incubation, intracellular SOD activity was detected. Data are expressed as mean ± SEM of six samples from at least three independent experiments, each performed. The difference among groups was evaluated with one-way ANOVA and the post-hoc Scheffe's test for multiple comparisons.

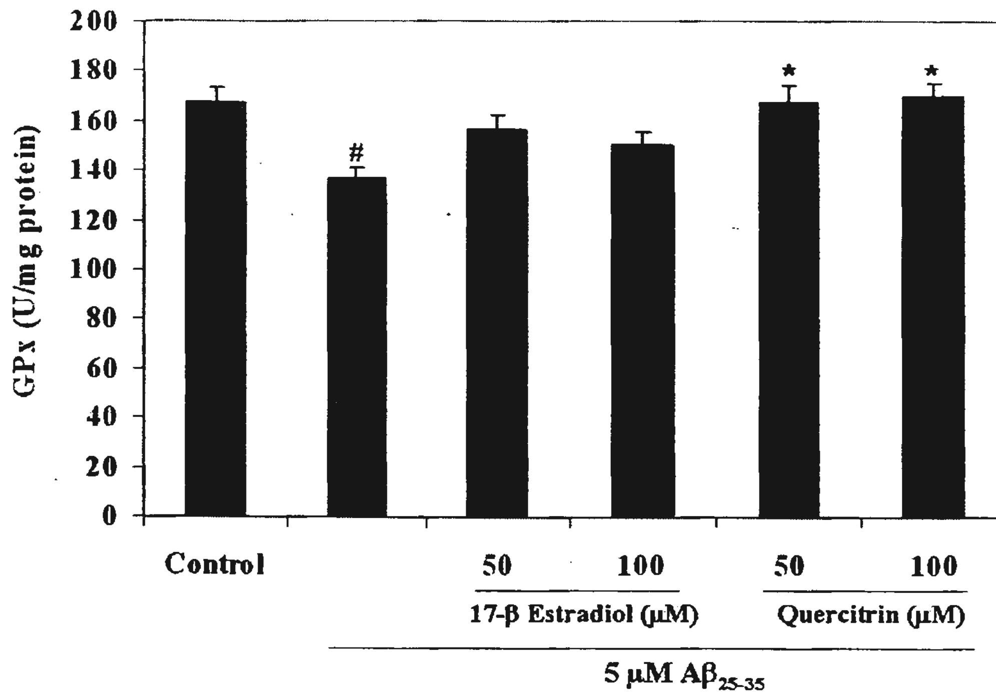

Figure 23 Effect of co-exposure with quercitrin or 17β-estradiol on glutathione peroxidase activity of $A\beta_{25-35}$-exposed hippocampal neuronal cultures. Hippocampal neurons were cultured for 24 hr and then treated with different concentrations of quercitrin or 17β-estradiol (50 and 100 μM) in combination with 5 μM $A\beta_{25-35}$ for 72 hr. After co-incubation, intracellular GPx activity was measured. Data are expressed as mean ± SEM of six samples from at least three independent experiments, each performed in duplicate. The difference among groups was evaluated with one-way ANOVA and the post-hoc Scheffe's test for multiple comparisons. $^{\#}P < 0.05$ compared with control cultures. * $P < 0.05$ compared with $A\beta_{25-35}$-treated cultures.

4. Effects of pre-exposure to quercitrin on Aβ-induced neurotoxicity in hippocampal neuronal cultures

Results from the present study suggested that quercitrin or 17β-estradiol at higher concentrations (50 and 100 μM) had neuroprotective effect against $A\beta_{25-35}$-induced neurotoxicity in hippocampal neuronal cultures under co-exposure condition. Therefore, we further investigated in pre-exposure condition, whether quercitrin or 17β-estradiol at indicated concentrations could exert neuroprotective effect on $A\beta_{25-35}$ -induced neurotoxicity in hippocampal neurons.

Hippocampal neurons were pretreated with quercitrin or 17β-estradiol at concentrations of 50 and 100 μM for 3 days. Then, culture medium was replaced with fresh medium containing $A\beta_{25-35}$ at the concentration of 5 μM for 3 days.

It was showed that 5 μM $A\beta_{25-35}$ alone significantly decreased MTT reduction by 39.51 $\pm$ 3.11% when compared to control cultures ($p<0.01$) (Figure 24). However, pretreatment with quercitrin or 17β-Estradiol at concentration of 50 and 100 μM did not show any changes in mitochondrial activity when compared to $A\beta_{25-35}$-treated cultures. These results indicated that pre-exposure to higher concentrations of quercitrin or 17β-estradiol (50 and 100 μM) did not show neuroprotective effects on $A\beta_{25-35}$ -induced neurotoxicity in hippocampal neuronal cultures.

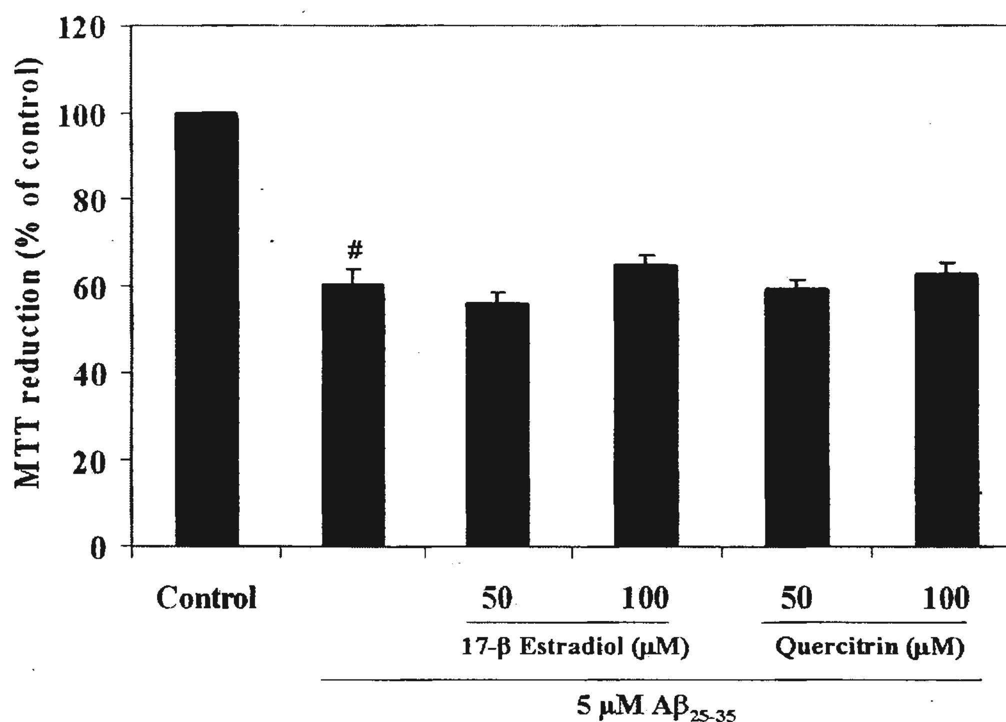

Figure 24 Effect of pre-exposure with quercitrin or 17β-estradiol on $A\beta_{25-35}$-induced neurotoxicity in cultured hippocampal neurons.

Hippocampal neurons were grown for 24 hr and then pretreated with quercitrin or 17β-estradiol (50 and 100 μM) for 72 hr and further incubated with 5 μM $A\beta_{25-35}$ for 72 hr. After incubation period, mitochondrial activity was determined by cellular MTT reduction assay. Data are expressed as mean ± SEM of eight samples from at least four independent experiments, each performed in duplicate. The difference among groups was evaluated with one-way ANOVA and the post-hoc Scheffe's test for multiple comparisons.

$^{\#}P < 0.05$ compared with control cultures.

5. Effects of ER antagonist (ICI 182780) on neuroprotectivity of quercitrin against Aβ-induced neurotoxicity in hippocampal neuronal cultures

We further determined whether protective effects of quercitrin or 17β-estradiol against $A\beta_{25-35}$-induced neurodegeneration were dependent on estrogen receptors. Twenty four hours after plating, cultured hippocampal neurons were preincubated with ICI 182780, a specific estrogen receptor antagonist, or vehicle for 2 hr prior to co-exposure with quercitrin or 17β-estradiol incubation and $A\beta_{25-35}$ for 3 days. Results showed that 5 μM $A\beta_{25-35}$ alone significantly decreased mitochondrial activity as relative to control (40.69 $\pm$ 0.97%, $p<0.01$) (Figure 25). The addition of ICI 182780 at the concentration of 1 μM did not affect cell survival when compared to that of nontreated controls. Quercitrin and 17β-estradiol at concentration of 50 μM significantly antagonized the reduction of mitochondrial activity induced by 5 μM $A\beta_{25-35}$ (from 59.31 $\pm$ 0.97% to 78.09 $\pm$ 3.42% and from 59.31 $\pm$ 0.97% to 80.98 $\pm$ 4.00%, respectively, $p<0.05$) as relative to $A\beta_{25-35}$-treated cultures. Following pretreatment with ICI 182780, co-exposure of quercitrin or 17β-estradiol with $A\beta_{25-35}$ at the concentration of 50 μM did not significantly change the percentage of MTT reduction as compared to that of co-exposure alone. These results confirmed that quercitrin and 17β-estradiol protected hippocampal neurons against Aβ-induced neurotoxicity as previously found. In addition, ER-receptor antagonism might not prevent quercitrin or 17β-estradiol-mediated neuroprotection against $A\beta_{25-35}$.

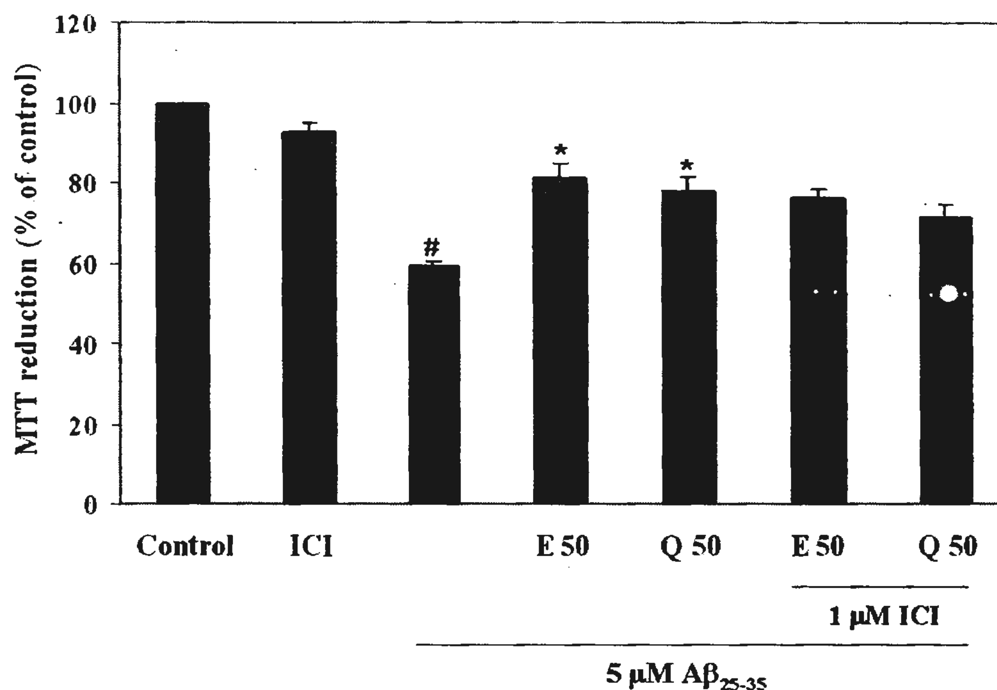

Figure 25 Effect of ER antagonist (ICI 182780) on neuroprotective effect of quercitrin or 17β-estradiol against $A\beta_{25-35}$-induced neurotoxicity in cultured hippocampal neurons.

Hippocampal neurons were cultured for 24 hr and then pretreated with ICI 182780 (ICI) at the concentration of 1 μM. Two hours later, quercitrin (Q) or 17β-estradiol (E) at 50 μM in combination with 5 μM $A\beta_{25-35}$ were added to the culture medium for an additional 72 hr. After co-incubation, mitochondrial activity was measured by cellular MTT reduction assay. Data are expressed as mean ± SEM of six samples from at least three independent experiments, each performed in duplicate. The difference among groups was evaluated with one-way ANOVA and the post-hoc Scheffe's test for multiple comparisons.

$^{\#}P < 0.05$ compared with control cultures, * $P < 0.05$ compared with $A\beta_{25-35}$ -treated cultures.

6. Effects of MEK inhibitor (PD 98059) or PI3K inhibitor (LY 294002) on neuroprotectivity of quercitrin against Aβ-induced neurotoxicity in hippocampal neuronal cultures

We also determined whether protective effects of quercitrin or 17β-estradiol against $A\beta_{25-35}$-induced neurodegeneration were mediated via MAPK or PI3K signaling pathway. Hippocampal neurons were cultured 24 hr and then pretreated with PD 98059 or LY 294002 or vehicle for 2 hr. After pretreatment, cells were co-exposed with quercitrin or 17β-estradiol and $A\beta_{25-35}$ for 3 days. Results showed that 5 μM $A\beta_{25-35}$ alone significantly decreased MTT reduction by 47.10 ± 1.86% and 48.21 ± 2.36%, relative to control (P<0.01) as shown in Figure 26 and Figure 27, respectively. Neither PD 98059 at the concentration of 30 μM nor LY 294002 at the concentration of 40 μM alone had significant effects on cell survival when compared to nontreated controls.

In the experiment with PD 98059, quercitrin or 17β-estradiol at the concentration of 50 μM significantly antagonized the reduction of mitochondrial activity induced by 5 μM $A\beta_{25-35}$ (from 52.90 ± 1.86% to 77.55 ± 3.81% or from 52.90 ± 1.86% to 77.35 ± 4.35%, respectively, p<0.05) as relative to $A\beta_{25-35}$-treated cultures (Figure 26). However, following PD 98059 pretreatment, co-exposure with quercitrin or 17β-estradiol at concentration of 50 μM still significantly inhibited the reduction of mitochondrial activity induced by 5 μM $A\beta_{25-35}$ in a similar degree to that of co-exposures without pretreatment.

A similar result was observed in the LY 294002 experiment, quercitrin or 17β-estradiol at the concentration of 50 μM significantly antagonized the reduction of mitochondrial activity induced by 5 μM $A\beta_{25-35}$ (from 51.79 ± 2.36% to 70.27 ± 2.79% or from 51.79 ± 2.36 to 71.07 ± 3.43, respectively, p<0.05) as relative to $A\beta_{25-35}$-treated cultures (Figure 27). After LY 294002 pretreatment, co-exposure with quercitrin or 17β-estradiol at concentration of 50 μM still significantly antagonized the reduction of mitochondrial activity induced by 5 μM $A\beta_{25-35}$ in a similar degree to that of co-exposures without pretreatment. These results comfirmed that quercitrin and 17β-estradiol protected hippocampal neurons against Aβ-induced neurotoxicity, as previously shown. In addition, MAPK or PI3K inhibition might not prevent quercitrin- or 17β-estradiol-mediated neuroprotection against $A\beta_{25-35}$.

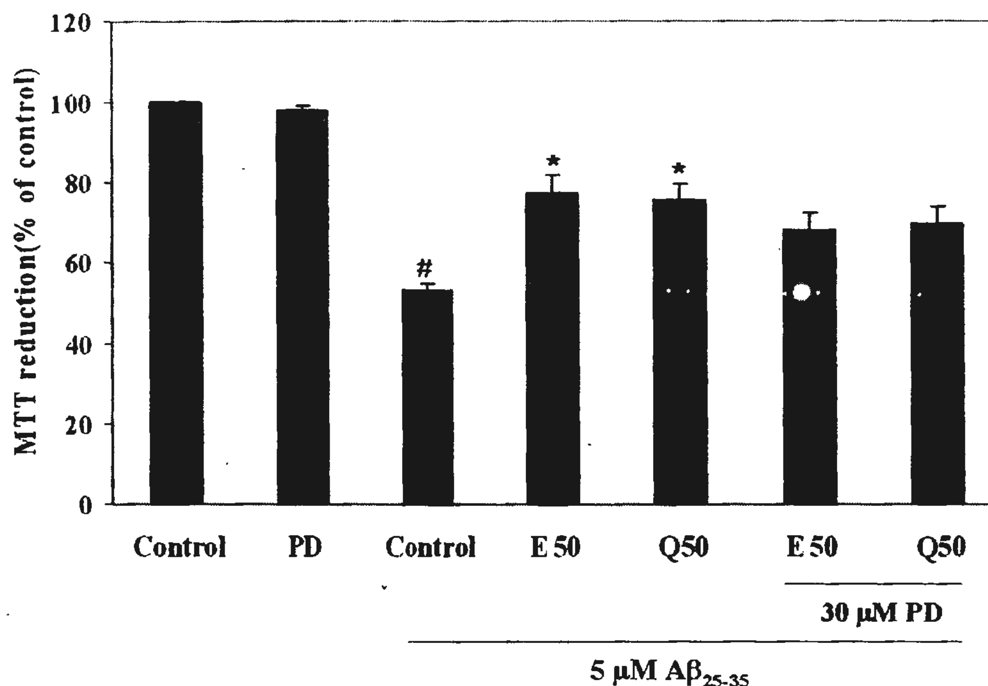

Figure 26 Effect of MEK inhibitor (PD 98059) on neuroprotective effect of quercitrin or 17β-estradiol against $A\beta_{25-35}$-induced neurotoxicity in cultured hippocampal neurons.

Hippocampal neurons were cultured for 24 hr and then pretreated with PD 98059 (PD) at the concentration 30 μM. Two hours later, quercitrin (Q) or 17β-estradiol (E) at 50 μM in combination with 5 μM $A\beta_{25-35}$ were added to the culture medium for an additional 72 hr. After co-incubation, mitochondrial activity was measured by cellular MTT reduction assay. Data are expressed as mean ± SEM of six samples from at least three independent experiments, each performed in duplicate. The difference among groups was evaluated with one-way ANOVA and the post-hoc Scheffe's test for multiple comparisons.

$^{\#}P < 0.05$ compared with control cultures, * $P < 0.05$ compared with $A\beta_{25-35}$ -treated cultures.

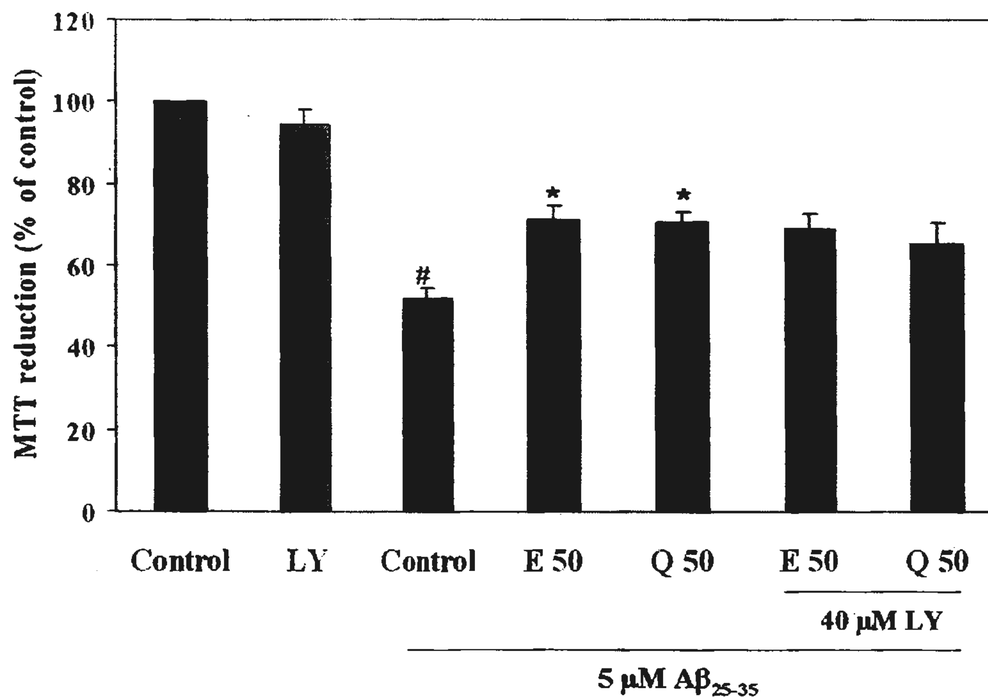

Figure 27 Effect of PI3K inhibitor (LY 294002) on neuroprotective effect of quercitrin or 17β-estradiol against $A\beta_{25-35}$-induced neurotoxicity in cultured hippocampal neurons.

Hippocampal neurons were cultured for 24 hr and then pretreated with LY 294002 (LY) at the concentration 1 μM. Two hours later, quercitrin (Q) or 17β-estradiol (E) at 50 μM in combination with 5 μM $A\beta_{25-35}$ were added to the culture medium for an additional 72 hr. After co-incubation, mitochondrial activity was measured by cellular MTT reduction assay. Data are expressed as mean ± SEM of six samples from at least three independent experiments, each performed in duplicate. The difference among groups was evaluated with one-way ANOVA and the post-hoc Scheffe's test for multiple comparisons.

$^{\#}P$ <0.05 compared with control cultures, * P <0.05 compared with $A\beta_{25-35}$ -treated cultures.

7. **Effects of quercitrin on apoptotic signaling, cell survival and cell death factors in cultured hippocampal neurons**

Caspase activation plays a critical role in the apoptosis of neurons. Caspase-3 is an important biomarker of apoptotic process. To measure caspase-3 activity, the substrate, DEVD-pNA, that composed of the chromophore, p-nitroanilide (pNA) and synthetic tetrapeptide, DEVD (Asp-Glu-Val-Asp) was used. In the presence of caspase-3, the substrate was cleaved and free pNA light absorbance was quantified using spectrophotometer. The effects of quercitrin and 17β-estradiol on the caspase-3 activity induced by $A\beta_{25\text{-}35}$ was shown in Figure 28. Incubation with 5 μM $A\beta_{25\text{-}35}$ for 3 days increased caspase-3 activity to 1.88 ± 0.09 folds as compared to non-treated control ($P<0.001$). Whereas, co-incubation with quercitrin at 50 and 100 μM significantly reduced the increase in caspase-3 activity to 1.47 ± 0.08 and 1.46 ± 0.1 folds, respectively ($P<0.05$). Similar result was observed when co-incubated with 17β-estradiol at 50 and 100 μM, it significantly attenuated the increased caspase-3 activity to 1.45 ± 0.09 and 1.39 ± 0.08 folds, respectively ($P<0.05$).

Bcl-2 (anti-apoptotic protein) and Bax (pro-apoptotic protein) are known to play a key role in cell life and cell death. Bcl-2 and Bax levels were assessed by Western blots to determine whether these key regulators of apoptosis were involved in neuroprotective mechanisms of quercitrin. We therefore investigated the effect of co-exposure with quercitrin or 17β-estradiol on cellular levels of Bcl-2 and Bax of $A\beta_{25\text{-}35}$-exposed hippocampal neuronal cultures.

Hippocampal neurons were cultured for 24 hr and then treated with the combination of quercitrin or 17β-estradiol at concentrations of 50 and 100 μM and $A\beta_{25\text{-}35}$ at the concentration of 5 μM for 3 days. The results indicated that $A\beta_{25\text{-}35}$ itself induced a decrease in Bcl-2 levels (a 26 kDa protein band in Western blotting) as compared to the control (Figure 29). After co-incubation with $A\beta_{25\text{-}35}$, quercitrin or 17β-estradiol at 50 and 100 μM apparently increased Bcl-2 protein levels. In the case of Bax, $A\beta_{25\text{-}35}$ induced an increase in Bax levels as compared to the control (Figure 30). After co-incubation with $A\beta_{25\text{-}35}$, quercitrin or 17β-estradiol at 50 and 100 μM apparently decrease levels of Bax protein. In term of cytochrome c, $A\beta_{25\text{-}35}$ induced an increase in cytochrome c levels (a 12 kDa protein band in Western blotting) as compared to the control (Figure 31). After co-incubation with $A\beta_{25\text{-}35}$, quercitrin or 17β-estradiol at 50 and 100 μM apparently decrease levels of cytochrome c release.

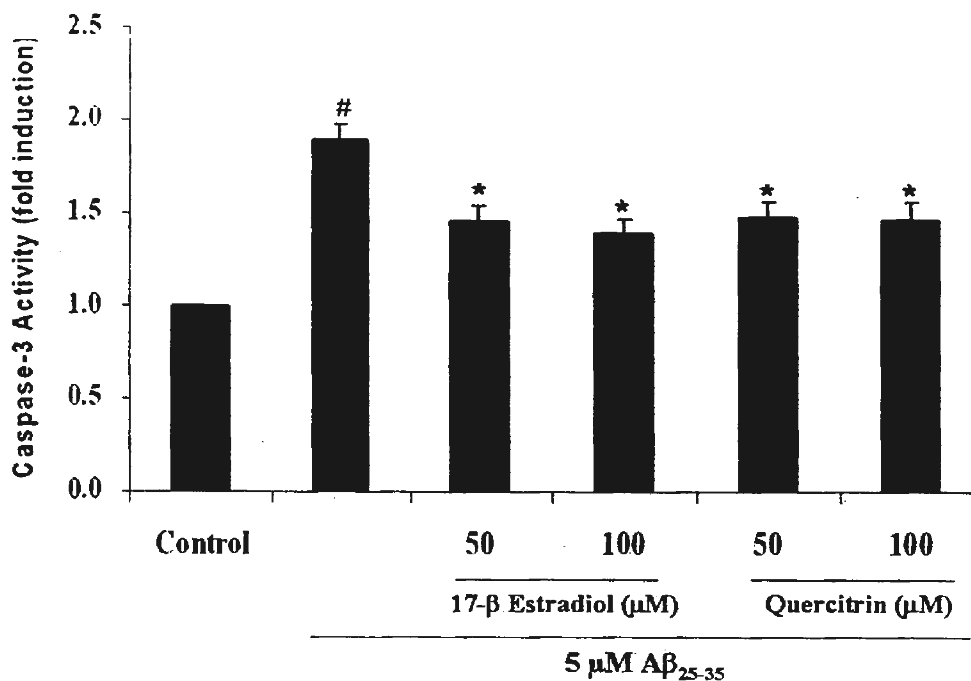

Figure 28 Effect of coexposure with quercitrin or 17β-estradiol on caspase-3 activity of $A\beta_{25\text{-}35}$-exposed hippocampal neuronal cultures.

Hippocampal neurons were cultured for 24 hr and then treated with different concentrations of quercitrin or 17β-estradiol (50 and 100 μM) in combination with 5 μM $A\beta_{25\text{-}35}$ for 72 hr. After co-incubation, caspase-3 activity was determined. Data are expressed as mean ± SEM of six samples from at least three independent experiments, each performed in duplicate. The difference among groups was evaluated with one-way ANOVA and the post-hoc Scheffe's test for multiple comparisons. $^{\#}P < 0.001$ compared with control cultures, * $P < 0.05$ compared with $A\beta_{25\text{-}35}$-treated cultures.

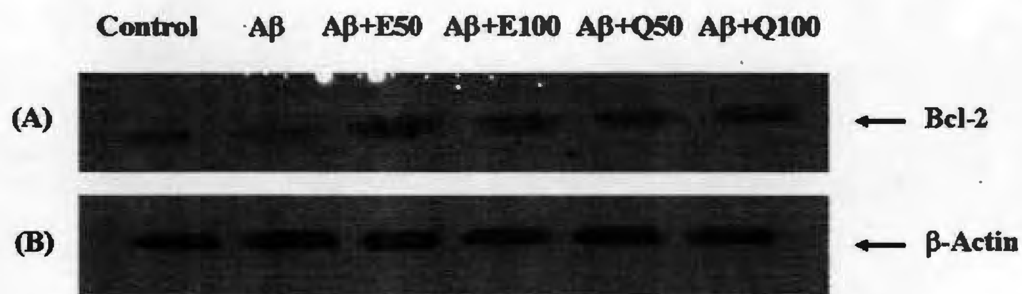

Figure 29 Effect of co-exposure with quercitrin or 17β-estradiol on Bcl-2 protein levels of $A\beta_{25-35}$-exposed hippocampal neuronal cultures.

Hippocampal neurons were cultured for 24 hr and then treated with different concentrations of quercitrin or 17β-estradiol (50 and 100 μM) in combination with 5 μM $A\beta_{25-35}$ for 72 hr. The supernatant extract of cultured neurons (30 μg protein/lane) was separated by 12% SDS-PAGE. Immunoblotting was performed using a specific antibody for Bcl-2.

(A) A representative gel picture showing a 26-kDa band of Bcl-2 protein

(B) A representative gel picture showing a 42-kDa band of β-actin

Representative data from two independent experiments with similar results were shown in this figure.

Figure 30 Effect of co-exposure with quercitrin or 17β-estradiol on Bax protein levels of $A\beta_{25-35}$-exposed hippocampal neuronal cultures.

Hippocampal neurons were cultured for 24 hr and then treated with different concentrations of quercitrin or 17β-estradiol (50 and 100 μM) in combination with 5 μM $A\beta_{25-35}$ for 72 hr. The supernatant extract of cultured neurons (30 μg protein/lane) was separated by 12% SDS-PAGE. Immunoblotting was performed using a specific antibody for Bax.

(A) A representative gel picture showing a 21-kDa band of Bax protein

(B) A representative gel picture showing a 42-kDa band of β-actin

Representative data from two independent experiments with similar results were shown in this figure.

Figure 31 Effect of co-exposure with quercitrin or 17β-estradiol on cytochrome c release of $A\beta_{25-35}$-exposed hippocampal neuronal cultures.

Hippocampal neurons were cultured for 24 hr and then treated with different concentrations of quercitrin or 17β-estradiol (50 and 100 μM) in combination with 5 μM $A\beta_{25-35}$ for 72 hr. The supernatant cytosolic fraction of cultured neurons (30 μg protein/lane) was separated by 12% SDS-PAGE. Immunoblotting was performed using a specific antibody for cytochrome c.

(A) A representative gel picture showing a 26-kDa band of cytochrome c

(B) A representative gel picture showing a 42-kDa band of β-actin

Representative data from two independent experiments with similar results were shown in this figure.