J Korean Med Sci 2000; 15: 327-36
ISSN 1011-8934

Anti-Oxidative Neuroprotection by Estrogens in Mouse Cortical Cultures

Estrogen replacement therapy in postmenopausal women may reduce the risk of Alzheimer's disease, possibly by ameliorating neuronal degeneration. In the present study, we examined the neuroprotective spectrum of estrogen against excitotoxicity, oxidative stress, and serum-deprivation-induced apoptosis of neurons in mouse cortical cultures. 17β-estradiol as well as 17α-estradiol and estrone attenuated oxidative neuronal death induced by 24 hr exposure to 100 μM $FeCl_2$, excitotoxic neuronal death induced by 24 hr of exposure to 30 μM N-methyl-D-aspartate (NMDA) and serum-deprivation induced neuronal apoptosis. Furthermore, estradiol attenuated neuronal death induced by Aβ25-35. However, all these neuroprotective effects were mediated by the anti-oxidative action of estrogens. When oxidative stress was blocked by an antioxidant trolox, estrogens did not show any additional protection. Addition of a specific estrogen receptor antagonist ICI182,780 did not reverse the protection offered by estrogens. These findings suggest that high concentrations of estrogen protect against various neuronal injuries mainly by its anti-oxidative effects as previously shown by Behl et al. Our results do not support the view that classical estrogen receptors mediate neuroprotection.

Key Words: *Estrogens; Neuroprotective Agents; Antioxidants; Alzheimer Disease*

Yeong-Hee Bae, Jee-Yeon Hwang, Yang-Hee Kim, Jae-Young Koh

National Creative Research Initiative Center for the Study of Central Nervous System Zinc and Department of Neurology, University of Ulsan College of Medicine, Asan Medical Center, Seoul, Korea

Received: 6 December 1999
Accepted: 13 March 2000

Address for correspondence
Jae-Young Koh, M.D.
Department of Neurology, University of Ulsan College of Medicine, Asan Medical Center, 388-1 Poongnap-dong, Songpa-gu, Seoul 138-736, Korea
Tel: +82.2-2224-3440, Fax: +82.2-474-4691
E-mail: jkko@www.amc.seoul.kr

*This work was supported by Creative Research Initiatives of the Korean Ministry of Science and Technology.

INTRODUCTION

Recent epidemiological studies have demonstrated that estrogen replacement in postmenopausal women substantially reduces the risk of Alzheimer's disease (AD) (1-4). Furthermore, estrogen may improve cognitive functions in AD patients (5-6). As to neuroprotective mechanisms of estrogen, investigators have shown that it reduces generation of beta amyloid peptides in neurons (7-8), and also protects neurons against the neurotoxicity of beta amyloid peptides (9-11). Estrogen may also directly attenuate N-methyl-D-aspartate (NMDA) receptor-mediated excitotoxic neuronal death (12-15) and oxidative neuronal death (16-18), the mechanisms related to neurodegeneration in AD (19-21).

Although many investigators agree that estrogen has a remarkable neuroprotective effect, there has been little agreement as to the protective concentration ranges and involvement of estrogen receptors. Behl et al. showed that relatively weak estrogens, 17α-estradiol and estrone have similar neuroprotective action as the physiologically active 17β-estradiol (18).

Furthermore, while 17α-estradiol binds and activates estrogen receptors at nanomolar concentrations, estrogens provided neuroprotection against oxidative injury only at concentrations greater than 10 μM levels (10). Consistently, in cells lacking functional estrogen receptors (HT-22), estrogen still attenuated beta amyloid-induced neuronal death in an extracellular glutathione-dependent manner (22). These results support the idea that neuroprotective action by estrogen is independent of estrogen receptors. However, Gridley's group showed that nanomolar concentrations of estrogen can attenuate beta amyloid-induced neuronal death in SK-N-SH human neuroblastoma cells. (9). Moreover, estrone and 17β-estradiol are weak, but still highly effective agonists at estrogen receptors (9). Thus, it remains still unclear whether estrogen receptors are involved in neuroprotection by estrogen congeners.

Another issue is the mechanistic spectrum of estrogen's neuroprotection. Most studies have focused on the possibility that estrogen acts at the anti-oxidative pathway, attenuating beta amyloid neurotoxicity (9-11, 16-18, 23). However, as discussed, estrogen also blocks NMDA receptor-mediated neurotoxicity, where the contribution of oxidative stress is minimal (12-15).

Furthermore, beta amyloid neurotoxicity in certain cases is not highly sensitive to antioxidants. Rather, in

our cortical cultures, the full-length beta amyloid (Aβ1-42) produces free radical-independent apoptosis (24). These findings suggest that beta amyloid neurotoxicity may occur by multiple toxic mechanisms.

In the present study, the spectrum of estrogen's neuroprotective effects was examined using oxidative stress, excitotoxicity and serum-deprivation-induced apoptosis as neuronal death models in mouse cortical culture. In addition, the possibility of involvement of estrogen receptors was examined by using 17α-estradiol, 17β-estradiol, estrone as well as a specific estrogen receptor antagonist ICI 182,780 (7α-[9-[(4,4,5,5,5-Pentafluropentyl)sulphinyl] noyl]-estra-1,3,5(10)-triene-3,17β-diol).

MATERIALS AND METHODS

Cortical cell cultures

Mixed cortical cell cultures, containing both neuronal and glial elements, were prepared as described previously (25) from fetal Swiss Webster mice (Simonsen Laboratory) at 15 days gestation. Dissociated cortical cells were plated in Primaria (Falcon) 15-mm multiwell vessels (0.4×10^6 cells/well) (26) in Eagle's minimal essential medium (Earle's salts) supplemented with 10% heat-inactivated horse serum, 10% fetal bovine serum, glutamine (2 mM) and glucose (10-20 mM).

Cultures were kept at 37℃ in a humidified CO_2-containing incubator (pH 7.1-7.4). After 5 to 10 days in vitro, non-neuronal cell division was halted by one to three days of exposure to 10^{-5} M cytosine arabinoside, and the cells were shifted into a maintenance medium identical to the plating medium, but lacking fetal serum. Subsequent media replacement was carried out twice per week. Only mature (14-24 days in vitro) cortical cultures were selected for study; all comparisons were made whenever possible on sister cultures derived from a single plating. Cortical glial cell cultures were prepared using the same protocol as above, but using cortices removed from early postnatal (postnatal days 1-3) mice instead of fetal mice since neurons removed from older animals do not survive the plating period (27).

Exposure to toxins

Exposure to toxins was carried out at room temperature via the bathing medium, utilizing defined solutions lacking serum, glutamate or lactate dehydrogenase (MEM). Care was taken to wash out the normal medium from cultures prior to adding toxin exposure solutions. After multiple rinsing with MEM, cultures were exposed to toxins. Cells were divided into control and experimental groups. Toxins were made as 100× stock solutions in distilled water and diluted in MEM to working concentrations before experiments. In some indicated experiments, 100 μM $FeCl_3$, 50 μM NMDA, 10 μM trolox, 1 mM buthionine sulfoximine (BSO), 100 μM H_2O_2, 100 nM staurosporine, 2 μM N,N,N',N'-tetrakis [2-pyridylmethyl]ethylenediamine (TPEN) and 20 μM Aβ25-35 were used. And the concentrations of estrogen congeners were ranged with 1 μM to 100 μM and ICI182,780 were 0.01 μM to 10 μM. Then on the following day, the dishes were returned to the culture incubator for assessment of neuronal death. Younger cultures (DIV 6) were used for serum deprivation as the neuronal death model of apoptosis. And each experiments were repeated at least two to four times.

Assessment of neuronal death

Overall cell injury was estimated in all experiments by examination of cultures with phase-contrast microscopy at ×100 to ×400. This examination was usually performed one day after toxin exposure, at which point the process of cell death was largely complete. In some experiments, this examination was verified by subsequent bright-field examination of Trypan Blue staining (0.4% for 5 min), a dye staining debris and non-viable cells. In most experiments, overall neuronal cell injury was also quantitatively assessed by measuring lactate dehydrogenase (LDH) released by damaged or destroyed cells in the extracellular fluid one day after exposure to toxins. A small amount of LDH was always present in the media of cultures carried through the exposure protocol but without addition of toxin. This background amount, determined on sister cultures within each experiment, was subtracted from values obtained in treated cultures.

The absolute value of the LDH efflux produced by toxin exposure was quite consistent within sister cultures of a single plating, but differed somewhat between platings, largely as a function of neuronal density (which varied despite constant original plating densities, presumably reflecting small variations in cell preparation or serum characteristics). Therefore, each observed LDH value was scaled to a mean value produced by exposure to toxins in other sister cultures, in which near complete neuronal death but little glial damage usually occurs (n=4) (28).

Lipid peroxidation assay

Lipoperoxidation was measured by monitoring thiobarbituric acid reactive substances (TBARS) formation according to Ohkawa et al. (29).

In brief, cortical cells were lysed with 500 μL of 2%

sodium dodecyl sulfate solution. The amount of protein in cell lysates was measured by Bio-Rad DC protein assay. Lysates were added serially with 25 μL of 4% butylated hydroxytoluene in ethanol, 500 μL of 10 % phosphotungstic acid in 0.5 M sulfuric acid and 250 μL of 0.7% thiobarbituric acid (TBA). All mixtures were boiled for 50 min, mixed with 1 mL of n-butanol, and centrifuged. After cooling, the supernatant was collected, and the amounts of TBARS were measured spectrophotometically at 535 nM. The amounts of TBARS were scaled according to the standard curve obtained using a mixture of 1 nM tetrahydroxypropane in 1% sulfuric acid TBARS.

Visualization of reactive oxygen species production in cells

Levels of reactive oxygen species in cells were measured using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCF). In brief, cells were incubated for 5 min in the presence of 4 μg/mL DCF and washed in Eagle's minimal essential medium. Cultures were examined under the fluorescence microscope. DCF is very sensitive to illumination at the excitation wavelength, and the fluorescence signal gradually increases with illumination (30). Hence, care was taken to obtain photographs precisely at 30 sec after the illumination onset.

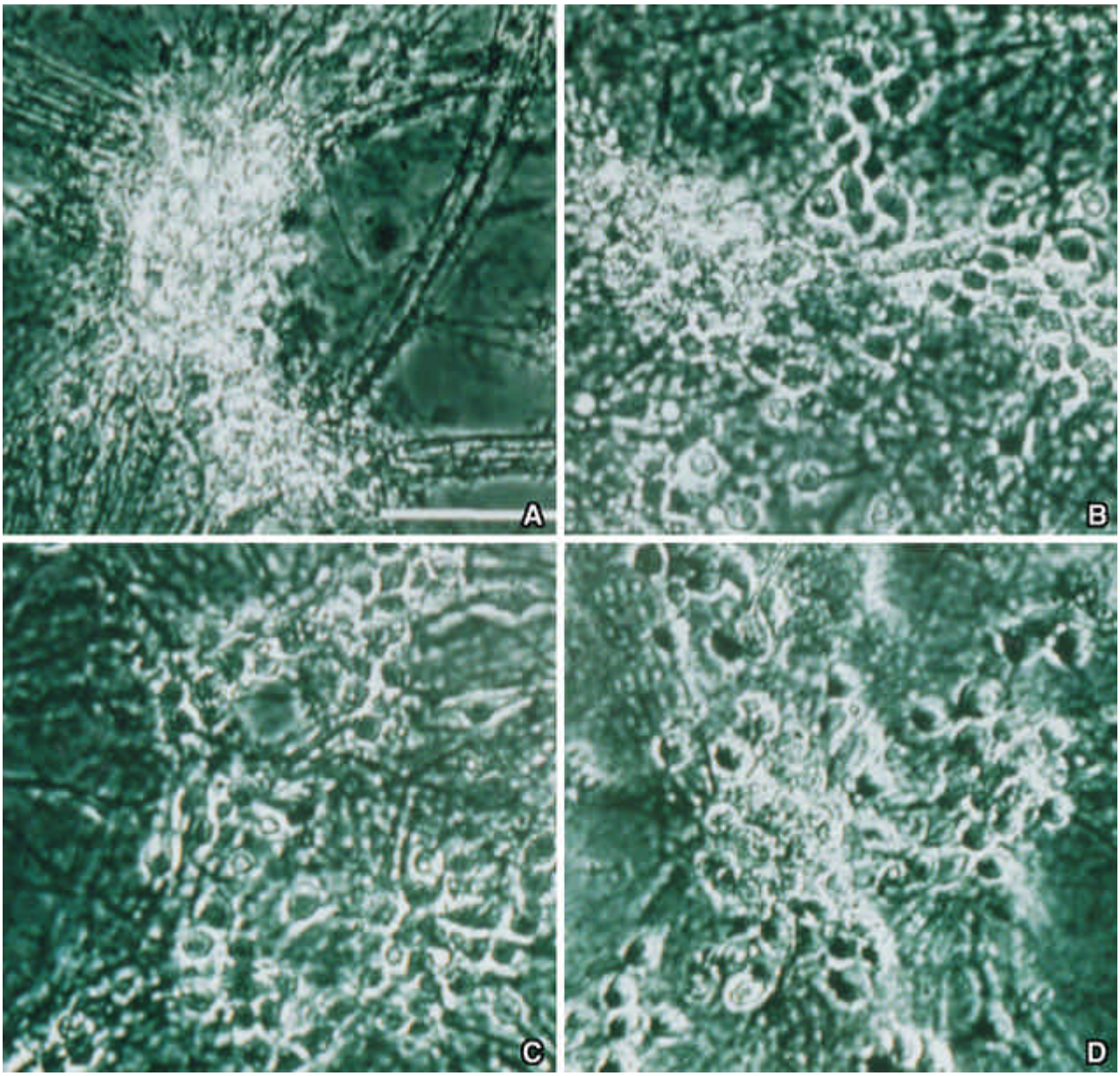


Fig. 1. All three estrogens markedly attenuated iron-induced oxidative neuronal injury. Phase-contrast photomicrographs of cortical cultures after 24 hr exposure to 100 μM $FeCl_3$ alone (A), addition of 30 μM 17β-estradiol (B), 17α-estradiol (C), and estrone (D).

Drugs and chemicals

17β-estradiol, 17α-estradiol, estrone, $FeCl_3$, staurosporine, cyclorheximide (CHX), KCl and Aβ25-35 were purchased from Sigma, brain-derived neurotropic factor (BDNF) from R&D, DCF from Molecular Probes, trolox from Aldrich Chemicals and ICI 182,780 from Tocris.

RESULTS

Previously, we have shown that 24 hr exposure to $FeCl_3$ induces neuronal death mainly via oxidative injury mechanism. As shown in Fig. 1A, exposure to $FeCl_3$ induced widespread neuronal death accompanied by cell body swelling. Addition of 30 μM of 17β-estradiol, 17α-estradiol, or estrone, markedly attenuated $FeCl_3$-induced cell body swelling and neuronal death (Fig. 1 B-D). The protective effect of these estrogens appeared to last for the next 24 hr (data not shown).

Quantitative analysis of neuronal death by measuring LDH efflux, revealed that 10-100 μM concentrations of each estrogen congener were needed to protect against $FeCl_3$-induced cell death (Fig. 2). 17α-estradiol was less potent than the other two. However, at 100 μM, all three almost completely blocked the death induced by $FeCl_3$. At concentrations lower than 3 μM, no protective effect was seen in any case.

Then, we examined the possibility that the estrogens have protective effects against excitotoxicity. Whereas 5 min exposure to 50 μM NMDA induced about 50% of neuronal death 24 hr later, and addition of 30 μM 17β-estradiol, 17α-estradiol or estrone all substantially attenuated NMDA-induced neuronal death (Fig. 3A). However, this protective effect was not likely due to a direct action of estrogens on the NMDA receptor or channels, since NMDA neurotoxicity (50 μM$\times$24 hr) induced in the presence of an antioxidant trolox was not attenuated by the addition of these estrogens (Fig. 3B). Hence, it seems likely that the protective effect of the estrogen was mediated by its antioxidant action, which is involved in NMDA neurotoxicity (12-15).

The involvement of estrogen receptors in estrogen neuroprotection remains controversial. We examined this using a specific estrogen receptor antagonist ICI182,780. As shown above, adding 30 μM 17β-estradiol markedly attenuated $FeCl_3$-induced neuronal death. Indicating that this effect is not mediated by the estrogen receptor, the addition of ICI at concentrations ranging from 10 nM to 10 μM did not reverse the protective effect of 17β-estradiol (Fig. 4).

Then we examined whether estrogen showed neuroprotective effect on serum-deprivation-induced apoptosis. After 24 hr of serum deprivation (DIV 6), addition of 10 μM estrogens significantly attenuated cell death. Again, all three estrogen congeners showed similar effects (Fig. 5). However, this effect was not likely caused by their general anti-apoptosis effect, since serum deprivation induced cell death in the presence of an antioxidant trolox was not attenuated by the addition of these estrogens (Fig. 5D). And neuronal apoptosis induced by staurosporine or a cell-permeant metal chelator TPEN was not attenuated by any of the estrogens (Fig. 6A). Previously, it was shown that all three, SD, staurosporine and TPEN, induced similar patterns of neuronal apoptosis in cortical culture (24, 31-34). Furthermore, serum

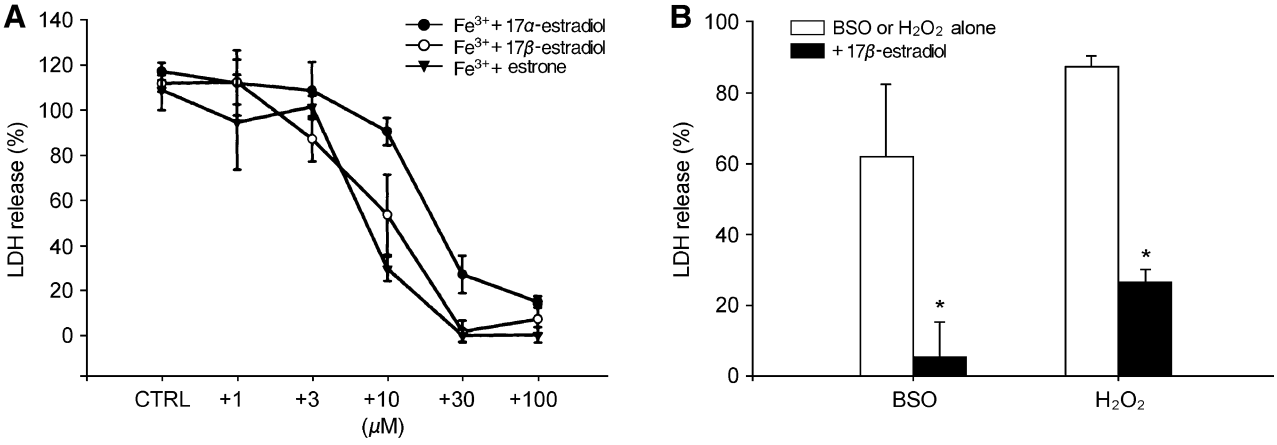


Fig. 2. (A) Concentration-protection relationships of estrogens against iron-induced oxidative neuronal injury with quantitative analysis of neuronal death by measuring LDH efflux. Data are expressed relative to the mean value in mixed cultures exposed 5 mM Glutamate $\pm$S.E.M., n=4 per treatment. Estrogens attenuated iron neurotoxicity at 3-100 μM concentrations in concentration-dependent manners. (B) Estrogen attenuated other oxidative injuries. Bars denoted LDH release in cultures after 24 hr exposure to 1 mM BSO or 100 μM H_2O_2. Data are expressed relative to the mean value in mixed cultures exposed 5 mM Glutamate$\pm$S.E.M., n=4 per treatment. Asterisk denotes difference from control at $p<0.05$ (unpaired Student's t-test, two-tailed).

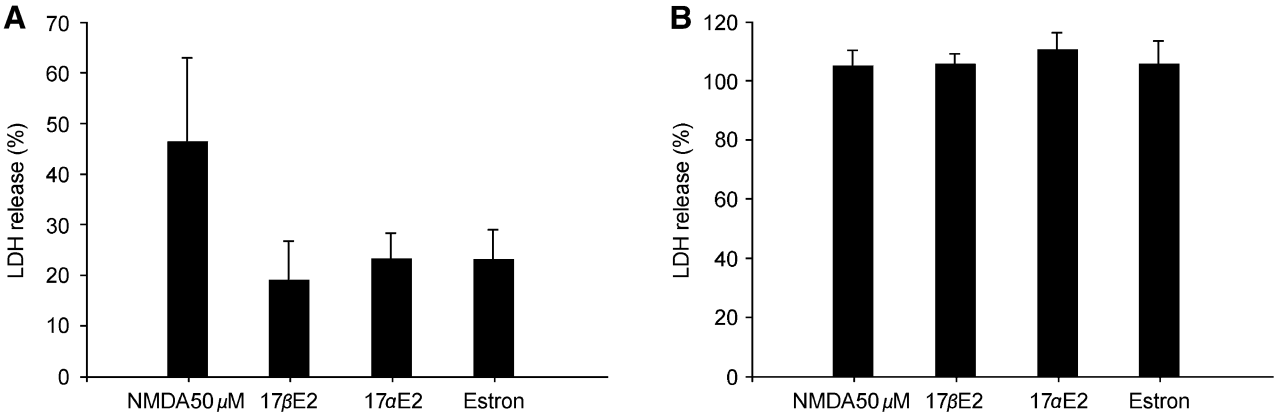


Fig. 3. Estrogens attenuated excitotoxic neuronal injury probably by inhibiting oxidative injury. Bars denoted LDH efflux in cultures 24 hr after 5 min exposure to 100 μM NMDA. Data are expressed relative to the mean value in mixed cultures exposed 5 mM Glutamate±S.E.M., n=4 per treatment. Asterisk denotes difference from control at $p<0.05$ (unpaired Student's t-test, two-tailed). (A) Addition of 30 μM 17β-estradiol, 17α-estradiol, and estrone reduce NMDA-induced neuronal death. (B) In presence of 10 μM trolox, addition of 30 μM 17β-estradiol, 17α-estradiol, and estrone did not attenuated NMDA-induced neuronal death.

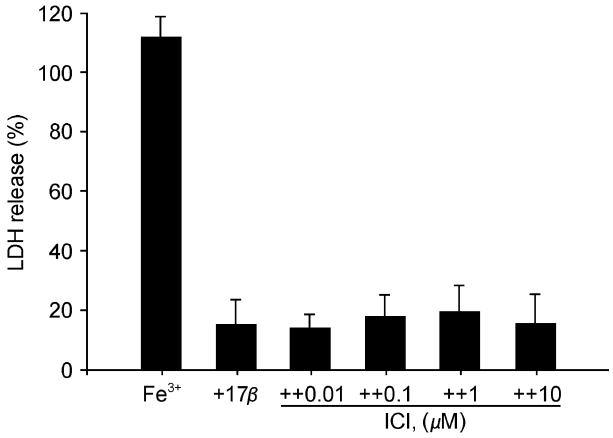


Fig. 4. ICI182,780 does not reverse the neuronal protection by estrogens. LDH efflux in cultures after 24 hr exposure to 100 μM $FeCl_3$ alone, or with addition of 30 μM 17β-estradiol and indicated concentrations of ICI. Data are expressed relative to the mean value in mixed cultures exposed 5 mM Glutamate± S.E.M., n=4 per treatment.

deprivation induced neuronal death in the presence of an antioxidant trolox was not attenuated by the addition of these estrogens (Fig. 6B).

The antioxidative action of estrogens was examined in living cortical neurons using DCF, a radical-sensitive fluorescent dye. Exposure to $FeCl_3$ induced marked increases in intracellular free radical generation in cortical neurons (Fig. 1A). Addition of 30 μM 17β-estradiol completely abrogated the increase (Fig. 3B). The other two estrogens showed similar effect (not shown). Consistently, TBARS assay revealed that Fe-induced increases in lipid peroxidation was markedly attenuated by each three estrogens (30 μM). In fact, these estrogens were as effective as a potent antioxidant trolox (100 μM) (Fig. 7C).

Finally, we examined whether the oxidative neuronal death induced by Aβ25-35 was also attenuated by estrogens. Exposure to 20 μM Aβ25-35 induced mainly neuronal necrosis, which was not attenuated by brain-derived neurotrophic factor (BDNF), cycloheximide or high K^+ medium. All these conditions have been shown to inhibit neuronal apoptosis in various models. Rather, Aβ25-35 induced neuronal death was completely blocked by trolox, indicating that the death is mainly mediated by oxidative injury (Fig. 8). As expected, the addition of 17β-estradiol (10-30 μM) (Fig. 8B) or the other two (not shown) also completely blocked Aβ25-35-induced neuronal death, suggesting the potent anti-oxidative effect of estrogens.

DISCUSSION

The central finding of the present study is that three estrogen congeners, 30 μM of 17β-estradiol, 17α-estradiol and estrone, attenuate diverse forms of neuronal death in cortical culture at μM concentrations. Excitotoxicity, oxidative injury and serum deprivation injury (mainly apoptosis) were all attenuated by adding estrogens. However, the common mechanism of estrogen neuroprotection appears to be independent of estrogen receptors, since adding of a potent yet specific receptor antagonist ICI182,780 did not reverse the protective effect at all. Furthermore, the fact that all the estrogen congeners exhibited similar neuroprotective potency and efficacy despite their obvious differences in efficacy at the estrogen receptor also suggest that estrogen receptors are not involved.

Estrogen replacement may reduce the risk of Alzheimer's disease (1-4). However, the precise mechanism involved here has not been elucidated. At the moment,

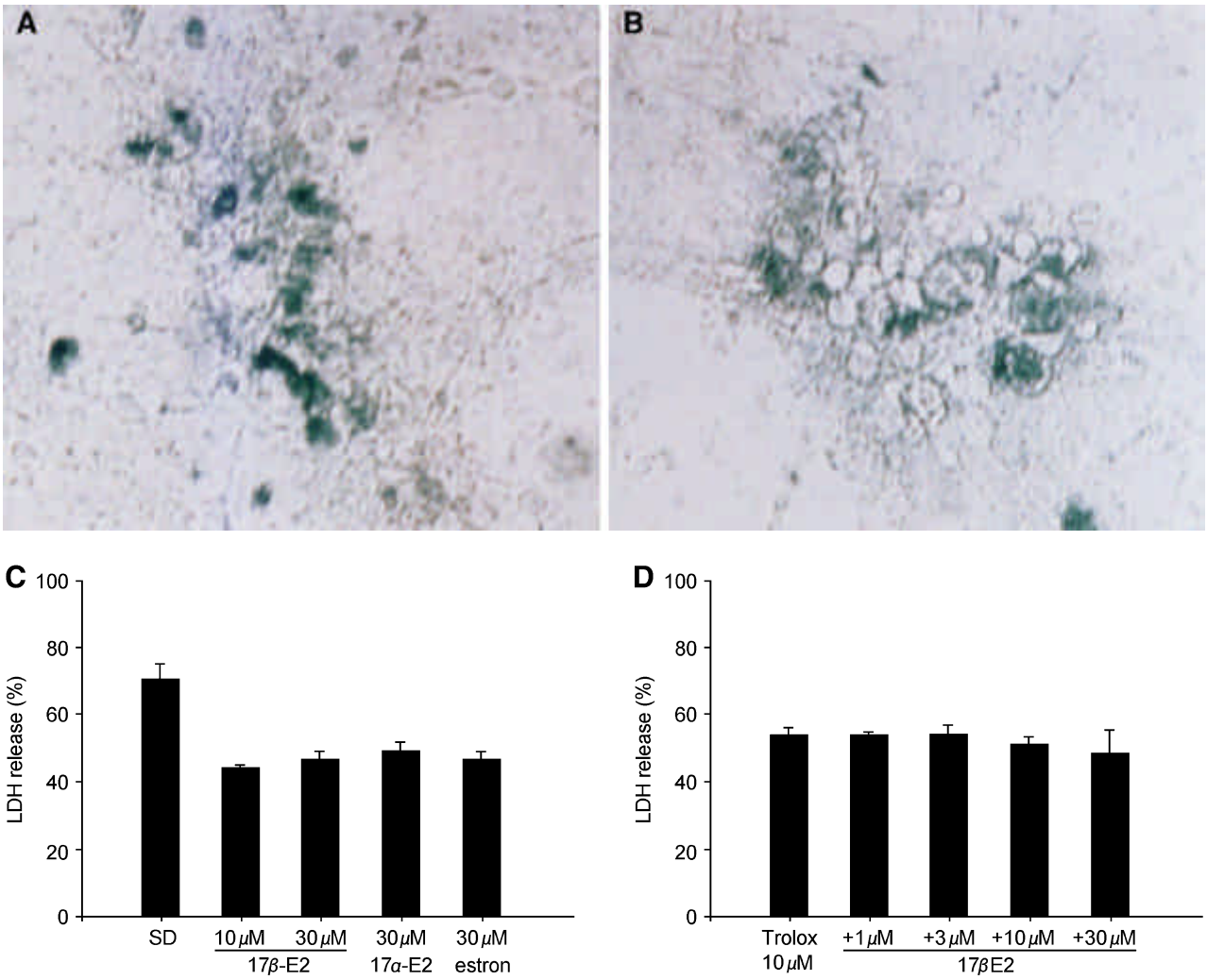


Fig. 5. Attenuation of apoptosis induced by serum deprivation by estrogens. (A, B) Trypan-blue stained cortical cultures (DIV 6) 24 hr after serum deprivation (A) and addition of 10 μM 17β-estradiol (B). (C) LDH efflux in cortical cultures 24 hr after serum deprivation in the absence (indicated as SD) or presence of indicated concentration of estrogens. (D) In presence of 10 μM trolox, addition of indicated concentration of estrogens did not attenuated serum deprivation induced neuronal death. Data are expressed relative to the mean value in mixed cultures exposed 5 mM Glutamate±S.E.M., n=4 per treatment. Asterisk denotes difference from control at $p<0.05$ (unpaired Student's t-test, two-tailed).

many investigators agree that estrogens may be neuroprotective, in part through its anti-oxidative effect (16-18). Consistent with this notion, estrogens exert protective influence over neuronal cells in various cultures. In the present study, we also showed that estrogens attenuated iron-, BSO-, or peroxide-induced oxidative injury at 10-30 μM concentrations, but not at nanomolar concentrations. The lack of stereospecificity suggests that the protective effect of estrogen may come from its chemical structure (e.g. the steroid ring) (18) which may scavenge free radicals, suggesting other steroids such as lazaroid and progesterone also have anti-oxidative effects (12, 35-36). Whether this mechanism is really involved in the effect observed in human AD patients is unknown. However, the high concentrations needed to block oxidative injury makes it less likely that this effect is directly involved. On the other hand, it seems possible that very mild anti-oxidative effect at lower concentrations may still provide significant protection over a long period of time (9, 14, 22).

The non-involvement of estrogen receptors was somewhat expected from several previous studies (9, 22). However, in certain cell systems, estrogen seems to exert protective actions through the activation of the estrogen receptor (14). Hence, it appears possible that estrogens may have multiple mechanisms of neuroprotective action. If estrogen receptors are not required for estrogen neuroprotection, one advantage is that it may be possible to use higher concentrations of estrogen analogues which have no agonistic action at the estrogen receptor. This

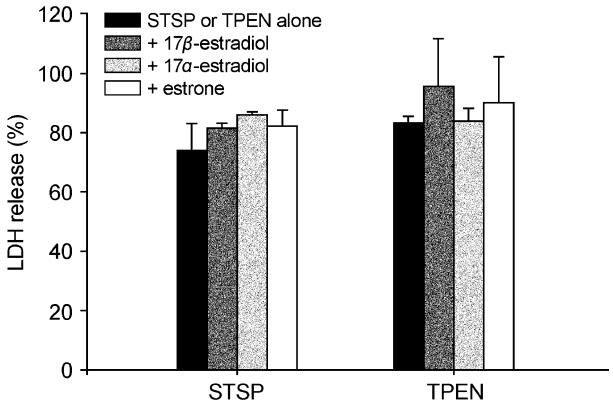


Fig. 6. Other apoptosis induced by staurosporine or TPEN was not prevented by estrogens. LDH release in cortical cultures after 24 hr exposure to 100 nM staurosporine or 2 μM TPEN and addition of 30 μM 17β-estradiol, 17α-estradiol, and estrone. Data are expressed relative to the mean value in mixed cultures exposed 5 mM Glutamate±S.E.M., n=4 per treatment.

will bypass the unwanted side effects of using estrogens, such as menstruation, increased risk of breast and certain gynecological malignancies (37-39). In addition, the high-dose estrogen treatment may be useful in ameliorating acute brain injuries such as ischemia and trauma, where oxidative stress is involved (13).

While apoptosis usually occurs during development to get rid of over-generated cells, recent studies indicate that this mechanism is also operational in pathological conditions. For example, in ischemic neuronal injury, degenerating neurons exhibit features of apoptosis such as internucleosomal DNA fragmentation (40). Furthermore, not only apoptosis-related genes such as caspases and bcl-2 are activated in these conditions, but also their blockade ameliorate the neuronal injury. Also in Alzheimer's disease, apoptosis has been proposed as a mechanism of neuronal loss. Hence, it is interesting that estrogen congeners attenuate serum deprivation-induced neuronal death, which occurs mainly by apoptosis. However, since estrogens fail to attenuate other types of apoptosis, the mechanism may not be a general one. Rather, it seems possible that free radical component of apoptosis (22) may be the site where estrogens act.

The mechanism of neuronal loss in AD is currently unknown. Excitotoxicity, oxidative stress and apoptosis are the major mechanisms thus far proposed. While all three in some ways are related with one another, the specific link to beta amyloid has been established in each mechanism. We showed that soluble beta amyloid, while alone is not neurotoxic, markedly increase excitotoxic injury in cultured cortical neurons (21). The same finding was reported by Mattson et al. (19), who proposed that destabilization of calcium homeostasis underlies this phenomenon. For oxidative stress, there exists more direct evidence. Beta amyloid induces oxidative injury to neurons (Fig. 8) (9-11, 16, 18, 23), and moreover, directly produces free radicals even in test tube conditions. Hence, beta amyloid-produced oxidative injury might be the major mechanism of neuronal loss in AD. Regarding apoptosis, as discussed above, several lines of evidence for it are available. Evidence for DNA damage are found in vulnerable areas of brains in AD (41). In cultures, beta amyloid produces macromolecule synthesis-dependent

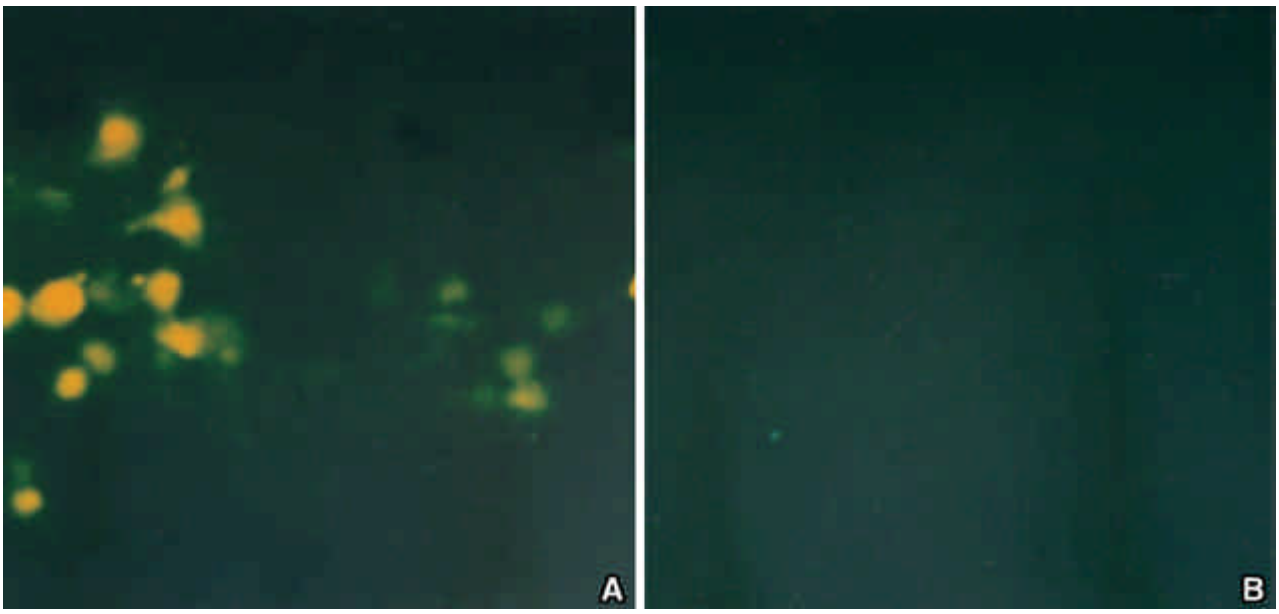
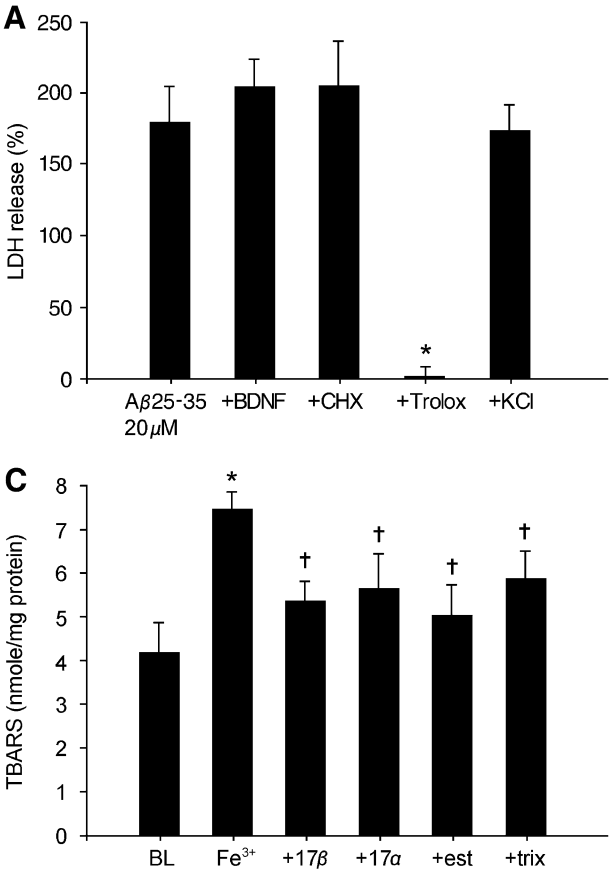


Fig. 7. Estrogens inhibit free radical generation and lipid peroxidation. (A, B) Fluorescent microphotographs of cortical cultures stained with DCF, after 6 hr exposure to 100 μM $FeCl_3$ alone (A) or with addition of 30 μM 17β-estradiol (B).



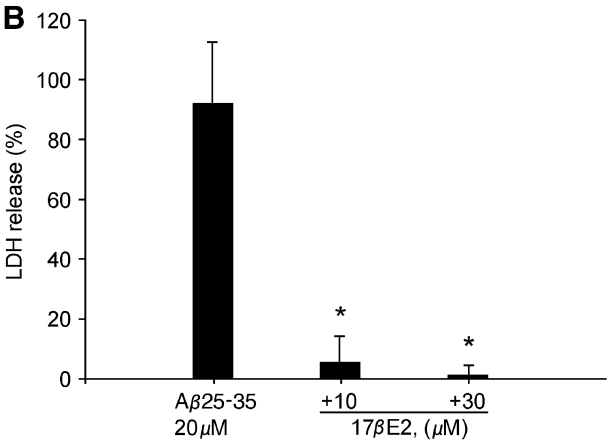


Fig. 8. Estrogen attenuated oxidative neuronal injury by Aβ 25-35. (A) Bars denotes LDH efflux in cultures after 24 hr exposure to 20 μM Aβ25-35 and addition of BDNF, CHX, Trolox or high potassium. Addition of trolox blocked the death, whereas BDNF, insulin-like growth factor (IGF)-1, CHX, or high K^+ did not attenuate it, indicating that the death is mainly mediated by free radicals. (B) LDH efflux in cultures after 24 hr exposure to 20 μM Aβ25-35 alone or with addition of indicated concentrations of 17β-estradiol. Data were expressed relative to the mean value in mixed cultures exposed 5 mM Glutamate± S.E.M., n=4 per treatment. Asterisk denotes difference from control at $p<0.05$ (unpaired Student's t-test, two-tailed). (C) Bars denote lipid peroxidation (% of sham wash controls) in cultures after 24 hr exposure to 100 μM $FeCl_3$ alone or with addition of 30 μM 17β-estradiol, 17α-estradiol, and estrone. (±S.E.M., n=4 per treatment) Sharp denotes difference from cultures after 24 hr exposure to 100 μM $FeCl_3$ alone at $p<0.05$ (unpaired Student's t-test, two-tailed).

apoptosis in some cases (41). Hence, it appears that beta amyloid can induce neuronal death via multiple mechanisms. In this context, it is intriguing that estrogen congeners, at least on surface, attenuate all three types of neuronal injury, excitotoxicity, oxidative injury and apoptosis. This pan-neuroprotective effect of estrogen may be the reason why it may be effective in human AD patients, where multiple degenerative mechanisms may operate simultaneously.

AD is the 4th leading cause of death in the United States and other developed countries. In Korea, we have also seen gradual increases in the incidence of AD pari passu with the increase of aged population. The socioeconomic burden of this devastating, dehumanizing disease is almost incalculable. While several anti-cholinesterases are on the market, their clinical utility is severely limited by side effects, substantial costs, and most importantly, the meager efficacy. Estrogen is a cheap hormone, the only major side effect of which is the potential promotion of estrogen-sensitive malignancies. The present study shows that estrogen is markedly protective against excitotoxicity, oxidative injury and a form of apoptosis in cortical culture at μM concentrations. The fact that estrogen receptor agonism is not required for these neuroprotections, suggests that more potent neuroprotectants completely devoid of estrogen hormone effects could possibly be developed.

REFERENCES

1. Paganini-Hill A, Henderson VW. *Estrogen deficiency and risk of Alzheimer's disease. Am J Epidemiol 1994; 140: 256-61.*
2. Henderson VW, Paganini-Hill A, Emanuel CK, Dunn NM, Buckwalter JG. *Estrogen replacement therapy in older women: comparisons between Alzheimer's disease cases and controls. Arch Neurol 1994; 51: 896-900.*
3. Waring SC, Rocca WA, Petersen RC, O'Brien PC, Tangalos EG, Kokmen E. *Postmenopausal estrogen replacement therapy and risk of AD. Neurology 1999; 52: 965-70.*
4. Tang MX, Jacobs D, Stern Y, Marder K, Schofield P, Gurland B, Andrews H, Mayeux R. *Effect of oestrogen during menopause on risk and age at onset of Alzheimer's disease. Lancet 1996; 348: 429-32.*
5. Birge SJ. *The role of estrogen in the treatment and prevention of dementia. Am J Med 1997; 103(suppl 3A): 19-25.*
6. Fillit H, Weinreb H, Cholst I, Luine V, McEwen B, Amador R, Zabriskie J. *Observations in a preliminary open trial of estradiol therapy for senile dementia-Alzheimer's type. Psycho-*

neuroendocrinology 1986; 11: 337-45.

7. Xu H, Gouras GK, Greenfield JP, Vincent B, Naslund J, Mazzarelli L, Fried G, Jovanovic JN, Seeger M, Relkin NR, Liao F, Checler F, Buxbaum JD, Chait BT, Thinakaran G, Sisodia SS, Wang R, Greengard P, Gandy S. *Estrogen reduces neuronal generation of Alzheimer β-amyloid peptides. Nat Med 1998; 4: 447-51.*
8. Jaffe AB, Toran-Allerand CD, Greengard P, Gandy SE. *Estrogen regulates metabolism of Alzheimer amyloid β precursor protein. J Biol Chem 1994; 269: 13065-8.*
9. Green PS, Gridley KE, Simpkins JW. *Estradiol protects β-amyloid (25-35)-induced toxicity in SK-N-SH human neuroblastoma cells. Neuroscience Lett 1996; 218: 165-8.*
10. Behl C, Widmann M, Trapp T, Holsboer F. *17-beta estradiol protects neurons from oxidative stress-induced cell death in vitro. Biochem Biophys Res Commun 1995; 216: 473-82.*
11. Mook-Jung I, Joo I, Sohn S, Kwon HJ, Huh K, Jung MW. *Estrogen blocks neurotoxic effects of beta-amyloid (1-42) and induces neurite extension on B103 cells. Neuroscience Lett 1997; 235: 101-4.*
12. Goodman Y, Bruce AJ, Cheng BB, Mattson MP. *Estrogens attenuate and corticosterone exacerbates excitotoxicity, oxidative injury, and amyloid b-peptide toxicity in hippocampal neurons. J Neurochem 1996; 66: 1836-44.*
13. Regan RF, Guo Y. *Estrogens attenuate neuronal injury due to hemoglobin, chemical hypoxia, and excitatory amino acids in murine cortical cultures. Brain Res 1997; 764: 133-40.*
14. Singer CA, Rogers KL, Strickland TM, Dorsa AM. *Estrogen protects primary cotical neurons from glutamate toxicity. Neuroscience Lett 1996; 212: 13-6.*
15. Weaver CE Jr, Park-Chung M, Gibbs TT, Farb DH. *17 beta-Estradiol protects against NMDA-induced excitotoxicity by direct inhibition of NMDA receptors. Brain Res 1997; 761: 338-41.*
16. Lacort M, Leal AM, Liza M, Martin C, Martinez R, Ruiz-Larrea B. *Protective effect of estrogens and catecholestrogens against peroxidative membrane damage in vitro. Lipids 1995; 30: 141-6.*
17. Vedder H, Anthes N, Stumm G, Wurz C, Behl C, Krieg JC. *Estrogen hormones reduce lipid peroxidation in cells and tissues of the central nervous system. J Neurochem 1999; 72: 2531-8.*
18. Behl C, Skutella T, Lezoualc'h F, Post A, Widmann M, Newton CJ, Holsboer F. *Neuroprotection against oxidative stress by estrogens: structure-activity relationship. Mol Pharmacol 1997; 51: 535-41.*
19. Mattson MP, Guo ZH, Geiger JD. *Secreted form of amyloid precursor protein enhances basal glucose and glutamate transport and protects against oxidative impairment of glucose and glutamate transport in synaptosomes by a cyclic GMP-mediated mechanism. J Neurochem 1999; 73: 532-7.*
20. Mattson MP, Pedersen WA. *Effects of amyloid precursor protein derivatives and oxidative stress on basal forebrain cholinergic systems in Alzheimer's disease. Int J Dev Neurosci 1998; 16: 737-53.*
21. Koh JY, Yang LL, Cotman CW. *Beta-amyloid protein increases the vulnerability of cultured cortical neurons to excitotoxic damage. Brain Res 1990; 533: 315-20.*
22. Green PS, Gridley KE, Simpkins JW. *Nuclear estrogen receptor-independent neuroprotection by estratriens: a novel interaction with glutathione. Neuroscience 1998; 84: 7-10.*
23. McEwen BS, Alves SE. *Estrogen actions in the central nervous system. Endocr Rev 1999; 20: 279-307.*
24. Ahn YH, Kim YH, Hong SH, Koh JY. *Depletion of intracellular zinc induces protein synthesis-dependent neuronal apoptosis in mouse cortical culture. Exp Neurol 1998; 154: 47-56.*
25. Choi DW, Maulucci-Gedde MA, Kriegstein AR. *Glutamate neurotoxicity in cortical cell culture. J Neurosci 1987; 7: 357-68.*
26. Choi DW, Koh JY, Peters S. *Pharmacology of glutamate neurotoxicity in cortical cell culture: attenuation by NMDA antagonists. J Neurosci 1988; 8: 185-96.*
27. Booher J, Sensenbrenner M. *Growth and cultivation of dissociated neurons from embryonic chick, rat and human in flask cultures. Neurobiology 1972; 2: 97-105.*
28. Koh JY, Choi DW. *Quantitative determination of glutamate mediated cotical neuronal injury in cell culture by lactate dehydrogenase efflux assay. J Neurosci Methods 1987; 20: 83-90.*
29. Ohkawa H, Ohishi N, Yagi K. *Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Ann Biochem 1979; 95: 351-8.*
30. Greenlund LJS, Deckwerth TL, Johnson EM Jr. *Superoxide dismutase delays neuronal apoptosis: a role for reactive oxygen species in programmed neuronal death. Neuron 1995; 14: 303-15.*
31. Galli G, Fratelli M. *Activation of apoptosis by serum deprivation in a teratocarcinoma cell line: inhibition by L-acetylcarnitine. Exp Cell Res 1993; 204: 54-60.*
32. Rawson CL, Loo DT, Duimstra JR, Hedstrom OR, Schmidt EE, Barnes DW. *Death of serum-free mouse embryo cells caused by epidermal growth factor deprivation. J Cell Biol 1991; 113: 671-80.*
33. Martin DP, Schmidt RE, DiStefano PS, Lowry OH, Carter JG, Johnson EM Jr. *Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. J Cell Biol 1988; 106: 829-44.*
34. Koh JY, Wie MB, Gwag BJ, Sensi SL, Canzoniero LM, Demaro J, Csernansky C, Choi DW. *Staurosporine-induced neuronal apoptosis. Exp Neurol 1995; 135: 153-9.*
35. Camins A, Gabriel C, Aguirre L, Sureda FX, Pubill D, Pallas M, Escubedo E, Camarasa J. *U-83836E prevents kainic acid-induced neuronal damage. Arch Pharmacol 1998; 357: 413-8.*
36. Zhou Y, Gopalakrishnan V, Richardson JS. *Actions of neurotoxic beta-amyloid on calcium homeostasis and viability of PC12 cells are blocked by antioxidants but not by calcium channel antagonists. J Neurochem 1996; 67: 1419-25.*

37. Purdie DM, Bain CJ, Siskind V, Russell P, Hacker NF, Ward BG, Quinn MA, Green AC. *Hormone replacement therapy and risk of epithelial ovarian cancer. Br J Cancer 1999; 81: 559-63.*
38. Andren-Sandberg A, Hoem D, Backman PL. *Other risk factors for pancreatic cancer: hormonal aspects. Ann Oncol 1999; 10 (suppl 4): 131-5.*
39. Roach M III. *Current status of androgen suppression and radiotherapy for patients with prostate cancer. J Steroid Biochem Mol Biol 1999; 69: 239-45.*
40. Barinaga M. *Stroke-damaged neurons may commit cellular suicide. Science 1998; 281: 1302-3.*
41. Cotman CW, Su JH. *Mechanisms of neuronal death in Alzheimer's disease. Brain Pathol 1996; 6: 493-506.*