2002. Vol. 3. No. 1. 51-59
Korean Journal of Oriental Medicine

Original Article

# *Bombycis corpus* Extract (BCE) Protects Hippocampal Neurons against Excitatory Amino Acid-induced Neurotoxicity

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Abstract

The present study was undertaken to investigate the effects of BCE on a cultured cell system, NMDA-induced cytotoxicity. In a previous study, it was shown that A β25-35 produces intracellular free radicals and induces the peroxidation of cellular lipids of rat astrocytes. The experiments reported here show that the oriental medical BCE has a powerful ameliorating effect on excitatory amino acid-induced neurotoxicity. This conclusion is strengthened by this effect being demonstrated *in vitro* for NMDA. Under these conditions, BCE had a major attenuating effect on the neurotoxic action of NMDA. The mechanisms by which BCE produces these neuroprotective effects are not yet evident.

**Objective:** To investigate the effect of BCE, which could be a protective agent for free radical generating compounds, and show that A β25-35 is not only a potent lipid peroxide inducer, but also causes change in antioxidative enzymes on cultured rat astrocytes, lipid peroxidation and antioxidative enzyme activities in A β25-35-treated conditions induced by NMDA.

**Methods:** To investigate the effect of BCE on NMDA-induced excitotoxicity in primary hippocampal cultures, mature cultures (older than 10 days in vitro) were incubated with BCE (10 μg/ml) under standard culture conditions for 6-8 h. After that, the cultures were exposed to NMDA for 1 h. After exposure to the toxin, they were then washed with the same medium (without NMDA) and incubated for an additional survival period of 16 h at the end of which they were fixed in either 10% formalin or 4% paraformaldehyde. They were then washed in PBS (pH 7.4) and stored at 4℃ until staining.

**Results:** The neuroprotective effects of BCE on NMDA-induced toxicity *in vitro* is that a number of astrocytes remain, although NMDA clearly reduced the number of βT-III-positive cells. The dose-dependent effect of BCE on neuroprotection is that the lowest dose (1μg/ml) exerted significant protection against the toxic actions of 1 mM NMDA, which is the maximal value for effect of NMDA-induced cell cytotocixity in cultured cells. Pretreatment and posttreatment of neuroprotective effects of BCE on NMDA-induced toxicity in vitro include that co-administration of BCE also protected neurons, although the effect was less marked than in pretreatment. Post administration of BCE also increased neuronal survival compared with cultures without steroids, but not to the same extent as pre-treatment. Neuroprotective effect of BCE on AMPA or kainate-induced toxicity in vitro is that cell counts were restored from 22.1% to 52.1% after AMPA, and increased from 28.8% to 57.3% after kainic acid.

**Conclusions:** BCE had a powerful ameliorating effect on excitatory amino acid-induced neurotoxicity in human neuronal cells, and effectively attenuated the neurotoxic action of NMDA in cultured human neuronal cells *in vitro*. In addition, BCE protected the cultured neurons against both AMPA and kainic acid, and interferes with some process downstream of the initial action of NMDA. Thus, BCE may offer a new and preventive potential treatment for those at risk for age-related neurodegenerative disorders. The present findings point the way toward new preventive treatments for those either at risk for age-related neurodegenerative disorders or additional therapeutic approaches for those suffering acute brain damage.

***Korean Journal of Oriental Medicine 2002;3(1):51-59***

**Key Words:** *Bombycis corpus,* Hippocampal neurons, neurotoxicity, glutamate agonist, cerebral ischemia.

## Introduction

*Bombycis corpus* (BC) or *Bombyx Batryticatus*, a batryticated silkworm and white-stiff silkworm,

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is a drug made from the dried larva of the silkworms, Mobyz mori L., dead and stiffened due to the infection of Beauveria (Bals.) Vuill.(family Moniliaceae). In traditional oriental medical application in Korea, the *Bombycis corpus* water extract (BCE) is used to subdue endogenous wind and convulsions, in the treatment of headache, vertigo, tic and skin prurigo, and to resolve nodulation for the treatment of scrofula, tonsillitis parotitis and purpura[1].

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by a progressive cognitive decline resulting from selective neuronal dysfunction, synaptic loss, and neuronal cell death[2]. It has been suggested that glial cells in AD may play a part in the neurodegenerative cascade that leads to AD. Neurodegenerative disorders usually involve the activation of astrocytes and gliosis (microglia). Glial activation involves morphological changes, as well as changes in expression of a large number of proteins[3]. In AD, activated astrocytes surround the neuritic shell of the amyloid plaque, and activated microglia are near the center of the neuritic shell adjacent to the amyloid core[4]. There are a number of stimuli that cause glial activation. One of the primary inducers of glial activation causes neuronal dysfunction or injury. Although the role of glial activation in AD is uncertain, it is known that reactive glia are associated with amyloid plaques[5]. In addition, some cytokines and inflammatory mediators produced by activated glia have the potential to initiate or exacerbate the progression of neuropathology[5-8].

In a previous study, it was shown that A β25-35 produces intracellular free radicals and induces the peroxidation of cellular lipids of rat astrocytes[9]. We investigated the effect of BCE on cultured rat astrocytes, lipid peroxidation and antioxidative enzyme activities in A β25-35-treated conditions. The protective effect of BCE on cytotoxicity induced by A β25-35 strongly indicated that BCE could be a protective agent for free radical generating compounds, and that A β25-35 is not only a potent lipid peroxide inducer, but also causes change in antioxidative enzymes. From these results, it was concluded that BCE has a protective effect on A β-induced cytotoxicity in cultured astrocyte cells through the inhibition of lipid peroxidation and protection of antioxidative enzymes[10].

Recently, our results showed that BCE has a direct effect on the brain, acting as an allosteric modulator of gamma-aminobutyric acid type A receptors (data not shown), interacting with voltage-gated $Ca_2^+$ channels in CA1 hippocampal neurons, reducing aggression, and improving memory in mice. The functional and clinical significance of age-related or stress-induced declines in neural function is not understood. Both age and stress are associated with neuronal vulnerability to degeneration[11].

We have found that BCE can prevent or reduce the neurotoxic actions of the glutamate agonist *N*-methyl-D-aspartic acid (NMDA) in the hippocampus *in vitro*, as well as that of two other glutamate receptor agonists, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainic acid *in vitro*. The release of glutamate has been implicated in the neural damage after cerebral ischemia and other neural insults[12].

## Materials and Methods

### Extract preparation from *Bombycis corpus*

*Bombycis corpus* (300 g) was obtained from the Oriental Medical Hospital, Dongguk University College of Oriental Medicine, and extracted with

boiling water for 3 hr. Then, the extract was evaporated under reduced pressure by 75%, 85%, 95% ethanol solution. The last extracts were diluted by 0.9% NaCl and filtered. The extract solution was stored at 4°C.

### Preparation of rat hippocampal cultures.

Hippocampi were dissected out of E18 Sprague-Dawley rat fetuses and transferred into DMEM (GIBCO BRL, Bethesda, MD, USA). The tissue was triturated gently by using a glass pipette with a narrow fire-polished tip. After a cell count with a trypan blue vital stain to assess the success of the dissection and triturating procedures, the cell suspensions were cultured at 105 cells per well on 13-mm glass coverslips coated with poly-L-lysine (1 mg/ml; Sigma) and merosin (10 ㎕/ml; Chemicon). Both poly-L-lysine and merosin (used just before the experiment) were used as adhesive agents, the former giving a charge to the glass coverslip and the latter acting as a biological adhesion molecule. They were used together to enhance adhesion of cells onto the coverslips for the purposes of this type of culture technique. The wells were then flooded with culture medium, a total volume of 500 ㎕ of DMEM supplemented with B27 (1:50) growth medium (GIBCO BRL), streptomycin (25 ㎍/ml)/penicillin G sodium (10,000 ㎍/ml)/amphotericin B (0.85%) (Sigma), and 5% fetal calf serum (GIBCO BRL). The coverslips were inverted within 24 h to maximize neuronal survival[15]. On day 5 (plating day is day 0), the cultures in the medium were changed, and the concentration of fetal calf serum was reduced to 1%. On day 10, the medium was replaced with DMEM supplemented with N-2 growth medium (GIBCO BRL) but without FCS. N-2 (insulin 500 ㎍/liter/human transferrin, 10,000 ㎍/liter/progesterone, 0.63 mg/liter/putrescine, 1611 ㎍/liter/selenite, 0.52 mg/liter) was used because it is limited in the range of anti-oxidants and hormones that it contains[13].

### Protective effect of BCE on NMDA-induced excitotoxicity in primary hippocampal cultures

To investigate the effect of BCE on NMDA-induced excitotoxicity in primary hippocampal cultures, mature cultures (older than 10 days *in vitro*) were incubated with BCE (10 ㎍/ml) under standard culture conditions for 6-8 h. After that, the cultures were exposed to NMDA for 1 h. After exposure to the toxin, they were then washed with the same medium (without NMDA) and incubated for an additional survival period of 16 h at the end of which they were fixed in either 10% formalin or 4% paraformaldehyde. They were then washed in PBS (pH 7.4) and stored at 4°C until staining.

Depending upon the experimental group, BCE (10-4 - 10-7 g/ml) was added (at 2% volume in conditioned medium) to or omitted from flasks. After 16 ~ 18 hr, cells were washed twice with warm phosphate-buffered saline (PBS) and serum-free medium added to the flask.

### Determination of cell viability and toxicity assay

Cells were cultured in polyethylenimine-coated 96 well culture plates at a density of 1 x 104 cells per well for lactate dehydrogenase (LDH) assay. LDH activities in the medium were measured by a CytoTox 96 nonradioactive cytotoxicity assay kit Promega) according to the manufacturer's instructions. The results were expressed as percentages of peak LDH release on complete cell lysis (control)[10].

For all findings, each condition represents five separate wells per experiment and is repeated in

two or five independent experiments.

### Immunocytochemical Staining for Glial Fibrillary Acidic Protein (GFAP) and βTubulin III

The cultures were double-stained immunocytochemically by using an mAb to βtubulin(III) (βT-III) (Sigma) and a polyclonal antibody to GFAP (aGFAP) (Dako). GFAP is a widely accepted marker of activated astrocytes. It should be noted that all astrocytes in culture are activated. βT-III is a neuron-specific cytoskeletal protein.

The cultures were incubated with 5% normal goat serum in PBS and 0.2% Triton X-100 (TX-PBS) for 1 h at room temperature. They were then incubated with the primary antibodies [GFAP (1:1000) and βT-III(1:500)] in PBS and 0.2% Triton X-100 overnight at 4 ℃. After that, they were washed three times with PBS and then incubated for 1 h with anti-mouse biotin and anti-rabbit fluorescein secondary antibodies (1:500 in PBS) (Boehringer Mannheim) at room temperature. They were then washed again three times with PBS and incubated for another hour at room temperature with streptavidin sulforhodamine secondary antibody (1:500 in PBS) (Boehringer Mannheim) to enable total cell count. They finally were washed and mounted (inverted) onto clean glass microscope slides with PBS/glycerol mountant (1:1). To prevent evaporation, the edges of the coverslips were sealed to the glass slide with clear nail varnish. After the varnish dried, the slides were cleaned, and stained cells were counted on a fluorescent microscope.

### Examination of cellular morphology

Cell morphology was examined under a Nikon TMS (H-III) inverted microscope equipped with a Nikon FDX-35 photo.

### Cell Counts and Data Analysis

Stained cells were counted in six random 625-μm2 fields across the coverslips by using a minimum of four coverslips per treatment. The identity of coverslips was not known during this procedure. All experiments were repeated across cultures from four different rats for BCE.

Multivariate comparisons were made by using ANOVAs and intergroup comparisons by Scheffe tests. Two group comparisons were analyzed with post hoc t tests.

## Effect of BCE

### 1. Experiment 1

To investigate the effect of BCE on NMDA-induced neurotoxicity, the cultures were treated either with BCE (10 μg/ml) or vehicle 6-8 h before exposure to incremental doses of NMDA (0.1, 1, 10, and 50 mM). In subsequent *in vitro* experiments, a dose of 1 mM NMDA was chosen. Culture medium samples were collected at the end of the experiment (50 mM NMDA only), and lactate dehydrogenase was measured spectrofluorometrically (LDL kit; Sigma diagnostics).

### 2. Experiment 2

The above procedures were repeated except that incremental amounts of BCE (0, 1, 2, 5, and 10 μg/ml) were added before 1 mM NMDA.

### 3. Experiment 3

The significance of the time of application of the BCE was investigated by adding BCE (5.0 μg/ml) to the cultures 6 h before administration of 1 mM NMDA, simultaneously with the NMDA, or 1 h later (after the NMDA was washed off).

Table 1. ILDH Release in the Conditioned Medium of Cultured Cells at 48 h after Treatment with NMDA and BCE.

| Concentration (mM) | LDH (% of maximal release) NMDA | NMDA+BCE |
|---|---|---|
| Control | 2.5±0.14 | 1.6±0.12 |
| 1.0 | 46.6±3.65 | 17.7±2.1** |

** significantly different, P<0.001.

## Results

### 1. Effect of NMDA-induced cell cytotocixity in cultured cells

The toxicities of NMDA were assessed by LDH assay. The NMDA increased LDH release by 46.6% of the maximal value at 1.0 mM concentration (Table 1). For effects of BCE treatment, cells were treated with NMDA for 2 h, thereafter adding BCE at the indicated concentration; LDH activities in the conditioned medium of cultured cells were assayed at 48 h after treatment with BCE. LDH releases from the cells were severely decreased, indicating that BCE treatment reduced the cell injury and protected the cells against NMDA-induced cytotoxicity (Table 1).

The results are expressed as a percentage of the maximal LDH release that was obtained through a complete cell lysis. To assess the effects of the BCE treatment, cells were treated with NMDA for 2 h and further treated with 2 x 10-6 g/ml BCE extract. LDH activities in the culture medium of cultured cells were assayed at 48 h after treatment with indicated concentrations of BCE. Data are expressed as mean ± SD values obtained from five culture wells per experiment, determined in three to five independent experiments.

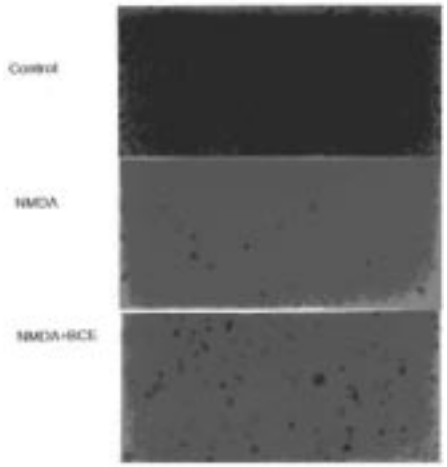


Fig. 1. The effect of BCE on the survival of cells in hippocampal primary cultures exposed to NMDA.

### 2. Neuroprotective effects of BCE on NMDA-induced toxicity *in vitro*

Hippocampal neurons are particularly sensitive to the toxic effects of glutamate analogues 10). Thus, possible neuroprotective effects of BCE on glutamate-mediated toxicity on both neurons and glia *in vitro* were examined for the first time. Cells harvested from the hippocampi of E18 embryos were cultured for 10 days and grown in the presence or absence of BCE (10 μg/ml) for 6-8 days. Individual cells were then treated with incremental doses of NMDA (0.1, 1, 10, and 50 mM for 1 h), fixed, and stained 16 h later histochemically with the Hoechst dye (no. 33342), a nuclear stain (for total cell counts), and immunocytochemically for β-III (to display putative neurons) and GFAP (for astrocytes). Control cultures contained many large β-III-positive cells with neuronal morphology and a smaller population of GFAP-positive astrocytes (Fig. 1).

A) Photomicrographs showing the protective

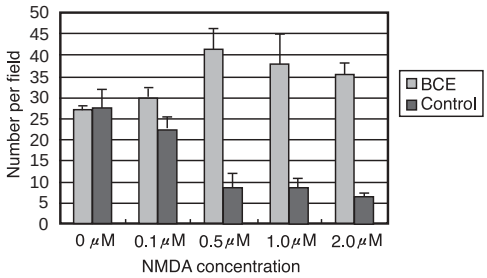


Fig. 1. B) βtubulin-stained cells

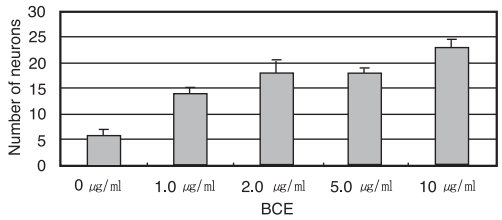


Fig. 2. The effects of incremental doses of BCE (0, 1, 2, 5 and 10 μg/ml) on the numbers of neurons in cultures exposed to 1 mM NMDA.
The means (± SEM) of 4-5 experiments are shown. * P<0.05 compared with baseline (no BCE).

effects of BCE on NMDA-induced toxicity. (Bar = 10 μm).

NMDA clearly reduced the number of phase-bright cells, although a number of astrocytes remain.

B) βtubulin-stained cells

In the absence of BCE, there was a decline in the number of βT-III-positive cells (log-transformed data ANOVA: main effect NMDA, P<0.01); this was prevented by the application of 10 μg/ml BCE (main effect BCE, P<0.01). There was a significant interaction between these factors (P<0.05). Values are mean ± SEM. Each variable is the mean of 4-6 wells.

C) GFAP-stained cells.

There were no significant effects of either NMDA (P>0.01) or BCE (P>0.01) on the survival

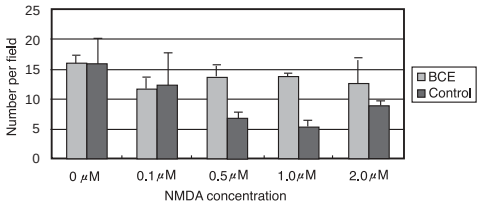


Fig. 1. C) GFAP-stained cells.

of GFAP-stained cells in culture. P<0.01 compared with baseline (no BCE).

### 3. Dose-dependent effect of BCE on neuro-protection

The dose-response features of the neuro-protective effect of BCE were then investigated. Cultures were exposed to incremental amounts of BCE (0, 1, 2, 5, and 10 μg/ml). The lowest dose (1 μg/ml) exerted significant protection against the toxic actions of 1 mM NMDA (n=4; P<0.01) (Fig. 2).

### 4. Pretreatment and posttreatment of neuroprotective effects of BCE on NMDA-induced toxicity in vitro.

As it was not clear whether BCE pretreatment was essential for neuroprotection, in the next experiment we added BCE (10 μg/ml) 6 h before, with, or 1 h after 1 mM NMDA, showing that co-administration of BCE also protected neurons, although the effect was less marked than in pretreatment (P<0.01). Post administration of BCE also increased neuronal survival compared with cultures without steroids, but not to the same extent as pre-treatment (P<0.01) (Fig. 3).

BCE was added 6 h before NMDA (Pre), co-administered with NMDA (Co), or applied 60 minutes afterward (Post). *, P < 0.01 (Scheffe test) compared with controls (no BCE added).

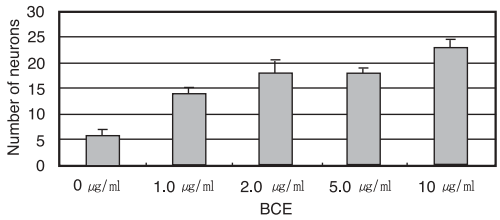


Fig. 3. Effect of changing the time of application of BCE relative to that of NMDA (1 mM) on neuronal survival.

Table 2. Effect of BCE (10 ㎍/ml) on Neurotoxicity induced by either AMPA or Kainic Acid.

| | Neurons per field (mean ± SEM) |
|---|---|
| Control (no toxin + vehicle) | 25.4 ± 3.2 |
| AMPA 25 ㎛ + vehicle | 3.9 ± 0.6 |
| AMPA + BCE (10 ㎍/ml) | 12.4 ± 1.1* |
| Kainic acid 1mM + vehicle | 6.3 ± 0.4 |
| Kainic acid + BCE (10 ㎍/ml) | 16.3 ± 2.1* |

### 5. Neuroprotective Effects of BCE on AMPA or Kainate-Induced Toxicity in Vitro

We examined the ability of BCE to protect neurons in vitro against two other glutamate receptor agonists acting on non-NMDA receptors. BCE (10 ㎍/ml), added 6 h before either AMPA (25 ㎛) or kainic acid (1 mM), also partially protected cultures against the toxic actions of both of these agonists (Table 2). In the absence of BCE, AMPA reduced neuronal cell counts to 22.1% of control values, but these values were restored to 52.1% in the presence of BCE. Similarly, cell counts that were 28.8% of control values after kainic acid increased to 57.3% after BCE was added.

The effect of adding BCE (10 ㎍/ml)6 h before either AMPA (25 ㎛) or kainic acid (1 mM) for 60 min on the mean number of neurons per field (four experiments for each treatment; 4-6 wells per experiment). *P<0.01 (t-test; toxin + vehicle vs. toxin + BCE)

## Discussion

The present study was undertaken to investigate the effects of BCE on a cultured cell system, NMDA-induced cytotoxicity. Cell killing was significantly enhanced by addition of increasing concentrations of NMDA. Pretreatment of BC attenuated this cell killing.

The experiments reported here show that the oriental medical BCE has a powerful ameliorating effect on excitatory amino acid-induced neurotoxicity. This conclusion is strengthened by this effect being demonstrated in vitro for NMDA. Under these conditions, BCE had a majora-ttenuating effect on the neurotoxic action of NMDA. We used a standard concentration of NMDA in vitro (1 mM) that was slightly higher than that of some previous studies because the experiments were performed on inverted cultures[14]. We believe that BCE may play a predominant role in the combined neuroprotective effects in the brain. The protective actions of BCE stand in contrast to those of glucocortoids, which induce hippocampal pyramidal neuron degeneration [11].

The mechanisms by which BCE produces these neuroprotective effects are not yet evident. Although protection of the large pyramidal neurons of the hippocampus was a prominent feature, other cell types (for example, glia) also may have been affected or might be acting as mediators of this effect. The neuroprotective effects of BCE may be related to its known action as a γ-aminobutyric acid type A antagonist[15], although whether γ-aminobutyric acid contributes to NMDA-induced toxicity under the conditions

used in these experiments remains doubtful. There seems no indication that BCE is a glutamate (NMDA) receptor-blocking agent. Whether BCE might alter the number or affinity of NMDA-type glutamate receptors awaits further study. However, our finding that BCE also protects cultured neurons against either AMPA or kainic acid raises questions about the identity of the glutamate receptor involved in these effects. These results suggest that BCE has a generally protective action against glutamate neurotoxicity.

It was also found that BCE was able to protect cultured neurons against NMDA when BCE was added either at the same time as NMDA or even 1 h later. This suggests that BCE interfered with some process downstream of the initial action of NMDA, although what this might be remains to be determined. The present findings point the way toward new preventive treatments for those either at risk for age-related neurodegenerative disorders or additional therapeutic approaches for those suffering acute brain damage.

## Conclusion

The present study was done to investigate the effects of BCE on a cultured cell system, NMDA-induced cytotoxicity. The results are as follows:

1. BCE had a powerful ameliorating effect on excitatory amino acid-induced neurotoxicity in human neuronal cells.
2. BCE effectively attenuated the neurotoxic action of NMDA in cultured human neuronal cells *in vitro*.
3. BCE also protected the cultured neurons against either AMPA or kainic acid
4. BCE interferes with some process downstream of the initial action of NMDA
5. BCE may offer a new and preventive potential treatment for those at risk for age-related neurodegenerative disorders

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