

## Betaine Attenuates Glutamate-Induced Neurotoxicity in Primary Cultured Brain Cells

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Effects of betaine on glutamate-induced neurotoxicity were examined on primary cultures of chicken embryonic brain cells and on rat cortical cultures. Betaine was found to attenuate glutamate-induced neurotoxicity both morphologically and biochemically. A 30 min exposure of chicken embryonic brain cells cultured for 12 days to 500  $\mu$ M glutamate produced widespread acute neuronal swelling and neuritic fragmentation. A 2-h pretreatment of cultured chicken embryonic brain cells with 1 mM betaine prior to a 30 min exposure to 500  $\mu$ M glutamate significantly raised the survival rate of neurons in the culture. When chicken embryonic brain cells were pretreated for 2 h with 1 mM betaine followed by exposure to 100  $\mu$ M glutamate for 42 h, lactate dehydrogenase levels within the cells remained at 63% of untreated control values while glutamate-treated controls fell to 0% lactate dehydrogenase. Betaine also exerted attenuating effects on N-methyl-D-aspartate-, kainate- and quisqualate-induced neurotoxicity in a similar manner to that observed with glutamate. Similar neuroprotective effects of betaine were obtained with rat cortical cultures.

**Key words:** Betaine, cultured chicken embryonic brain cells, Glutamate-induced neurotoxicity, Lactate dehydrogenase, Rat cortical cells

### INTRODUCTION

Glutamate neurotoxicity has been implicated in the slow neuronal loss of such neurodegenerative diseases, as Huntington's disease and Alzheimer's disease (Maragos *et al.*, 1987; Young *et al.*, 1988; Gedeas *et al.*, 1986). In addition, when the brain is subjected to hypoxia, hypoglycemia or prolonged seizures, glutamate is known to contribute to the ensuing nerve degeneration (Simon *et al.*, 1984; Coutinho-Netto *et al.*, 1981; Rothman and Olney, 1986; Dingledine *et al.*, 1990; Löscher, 1984). A considerable body of evidence also suggests that the glutamate and related excitatory amino acids such as quisqualate, kainate and N-methyl-D-aspartate (NMDA) cause a specific pattern of neurodegeneration in the brain of experimental animals and in primary cultures of neurons (Monaghan *et al.*, 1989; Koh and Choi, 1988; Rosser, 1982).

In the course of searching for potential natural products against glutamate-induced neurotoxicity in our laboratory, it was found that methanol extract of *Lycii Fructus* exerted attenuating activities on the glutamate-

induced neurotoxicity by microscopic observation. Thus, prior to isolation of the active components from *Lycii Fructus*, betaine, a major component of the crude drug, was chosen for the testing of neuroprotective activity against glutamate-induced neurotoxicity.

In the present study, effects of betaine on glutamate-induced neurotoxicity in primary cultured chicken embryonic brain and rat cortical cells will be described. Our results suggest that betaine significantly attenuates the morphological and biochemical damage caused by glutamate.

### MATERIALS AND METHODS

#### Animals

Chicken embryos were purchased from Hung-il Hatchery (Sungham, Kyunggi-do, Korea). Sprague-Dawley rats were supplied from the Experimental Animal Breeding Center of Seoul National University (Seoul, Korea).

#### Reagents

Dulbecco's modified Eagle's medium (DMEM), Hank's balanced salt solution (HBSS) and trypsin were purchased from Gibco Lab. (Grand Island, NY). Penicil-

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lin-streptomycin, amphotericin B, betaine, L-glutamic acid (glutamate), NMDA, kainate and quisqualate were products of Sigma Chemical Co. (St. Louis, MO). Horse and fetal bovine sera (FBS) and collagen were obtained from Hyclone Lab. (Logan, UT) and Calbiochem Co. (La Jolla, CA), respectively.

### Chicken Brain Cell Cultures

Primary cultures of chicken brain cells were prepared from cerebral hemisphere of 10-day-old chicken embryos as described by Sensenbrenner *et al.* (1972). Dissociated cells were plated at a density of  $0.8 \times 10^6$  cells/dish ( $35 \times 10$  mm). Cultures were grown to confluency in a medium containing 87.5% DMEM, 2.5% chicken embryo extract, 10% horse serum, 10,000 IU/100 ml penicillin and 10,000  $\mu\text{g}/100$  ml streptomycin at  $37^\circ\text{C}$  in a humidified atmosphere of 95% air/5%  $\text{CO}_2$ . Chicken embryo extract was prepared from 10-day-old chicken embryos according to the method of Markelonis *et al.* (1980).

### Cortical Cell Cultures

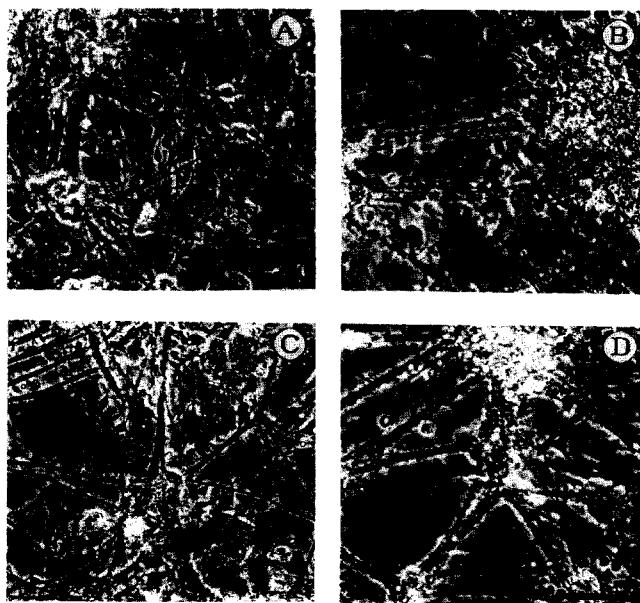
Mixed cortical cell cultures, containing both neuronal and glial element, were prepared as described by Choi (1987) from fetal rats at 17-19 day gestation. Briefly, the cortices were dissected under a dissecting microscope and the meninges were carefully removed. Following incubation with 0.25% trypsin, the tissue was triturated, diluted with DMEM supplemented with 10% FBS. Dissociated cortical cells were plated in  $35 \times 10$  mm vessels (Falcon) at a density of  $1.0 \times 10^6$  cells/dish in DMEM supplemented with 10% FBS. Cultures were kept at  $37^\circ\text{C}$  in a humidified incubator in an atmosphere of 95% air/5%  $\text{CO}_2$ . After 4 days *in vitro*, dividing non-neuronal cells were suppressed by exposure to  $5 \times 10^{-5}$  M 5-fluorodeoxyuridine.

### Estimation of Survival Rate of Neurons

The neurons were counted using a phase-contrast inverted microscope at a magnification of  $200\times$ . The numbers of total neuronal cell bodies and swollen neuronal cell bodies regarded as dead cells were counted 30 min after exposure of the cultures to glutamate or NMDA.

### Assays for Lactate Dehydrogenase

Quantitative assessment of neuronal injury was accomplished by measuring the concentration of the cytosolic enzyme, lactate dehydrogenase (LDH) (Choi and Koh, 1987). Cultures were first washed with DMEM without sodium pyruvate to remove any cellular debris. Then, the remaining cells were lysed in 0.5 ml of 0.5% triton X-100 in 0.1 M phosphate buffer, pH 7.4. LDH values were then measured colorimetri-



**Fig. 1.** Primary cultures of chicken embryonic brain cells. A. Chicken embryonic brain cells cultured for 12 days. B. The effect of 42-h-treatment of  $100 \mu\text{M}$  glutamate on 12-day-cultured chicken embryonic brain cells. C. The effect of 2-h-pretreatment of 1 mM betaine on 42-h-exposure on 12-day-cultured chicken embryonic brain cells to  $100 \mu\text{M}$  glutamate. D. The effect of concurrent treatment of 1 mM betaine with  $100 \mu\text{M}$  glutamate for 42 h on 12-day-cultured chicken embryonic brain cells. bar= $100 \mu\text{m}$

cally using Sigma Diagnostic Kit # 500 at a wavelength of 450 nm. The relative ratio of LDH was determined by dividing the LDH value in the experimental culture by LDH value in control culture.

### Measurement of Intracellular Calcium Concentration

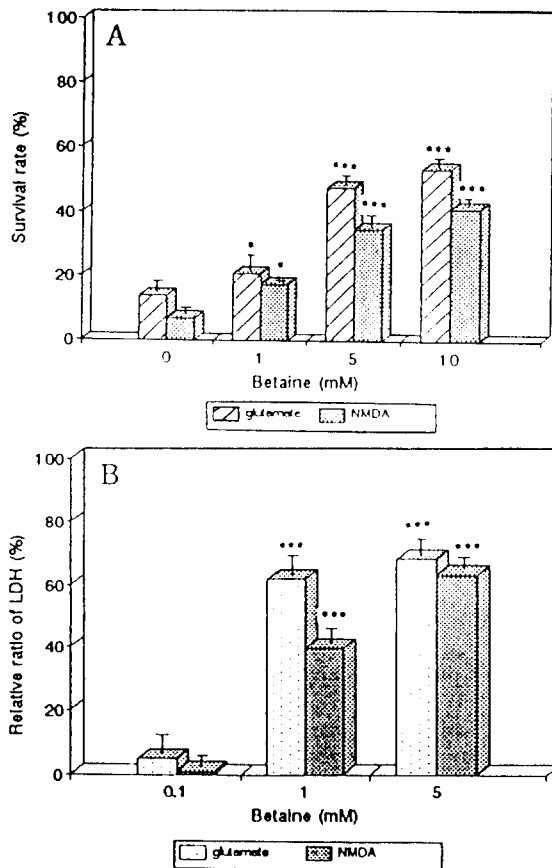
Cultures were washed with DMEM without sodium pyruvate. Then, the remaining cells were lysed in 2 N HCl. Intracellular calcium concentration was measured using Plasma Atomic Emission Spectrometry.

### Statistical Analysis

Levels of statistical significance ( $p < 0.05$ ) were assessed using the "ANOVA" test between means for unpaired data. All results are expressed as mean  $\pm$  standard deviations.

## RESULTS AND DISCUSSION

It was found that glutamate-induced neurotoxicity developed only in mature cultures after 12 days of incubation (Garthwaite and Garthwaite, 1986). Exposure of chicken embryonic brain cells to  $500 \mu\text{M}$  glu-

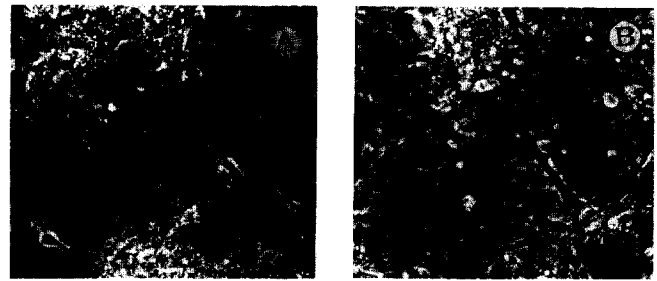


**Fig. 2.** The effect of betaine in chicken embryonic brain cells exposed to glutamate or NMDA. A. The effect of betaine on the survival rate. B. The effect of betaine on LDH values. (\* $p < 0.05$ , \*\*\* $p < 0.001$ )

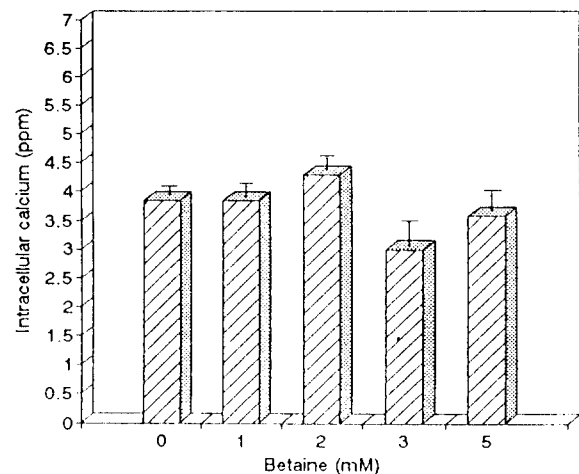
tamate for 30 min caused acute toxicity; immediate swelling of neuronal cell bodies and fragmentation of neurites were observed under phase-contrast microscope as reported in rat cortical or hippocampal neuronal cells (Sensenbrenner *et al.*, 1972; Choi and Koh, 1987).

Pretreatment of cultured chicken brain cells with 1 mM betaine for 2 h prior to glutamate insult seemed to block glutamate-induced neurotoxicity (Fig. 1A, 1B and 1C). The usual neurotoxicity induced by glutamate could not be observed in the presence of 1 mM betaine even if the concentration of glutamate was raised to 10 mM. If betaine was added with glutamate concurrently, it was observed that betaine still prolonged the life span of neuronal cells after glutamate insult (Fig. 1D).

In parallel with these morphological observations, quantitative assessment of the protective effects of betaine against glutamate-induced neurotoxicity was accomplished by estimating the survival rate of neuronal cells among chicken embryonic brain cells and by measuring the levels of cytoplasmic LDH. The survival



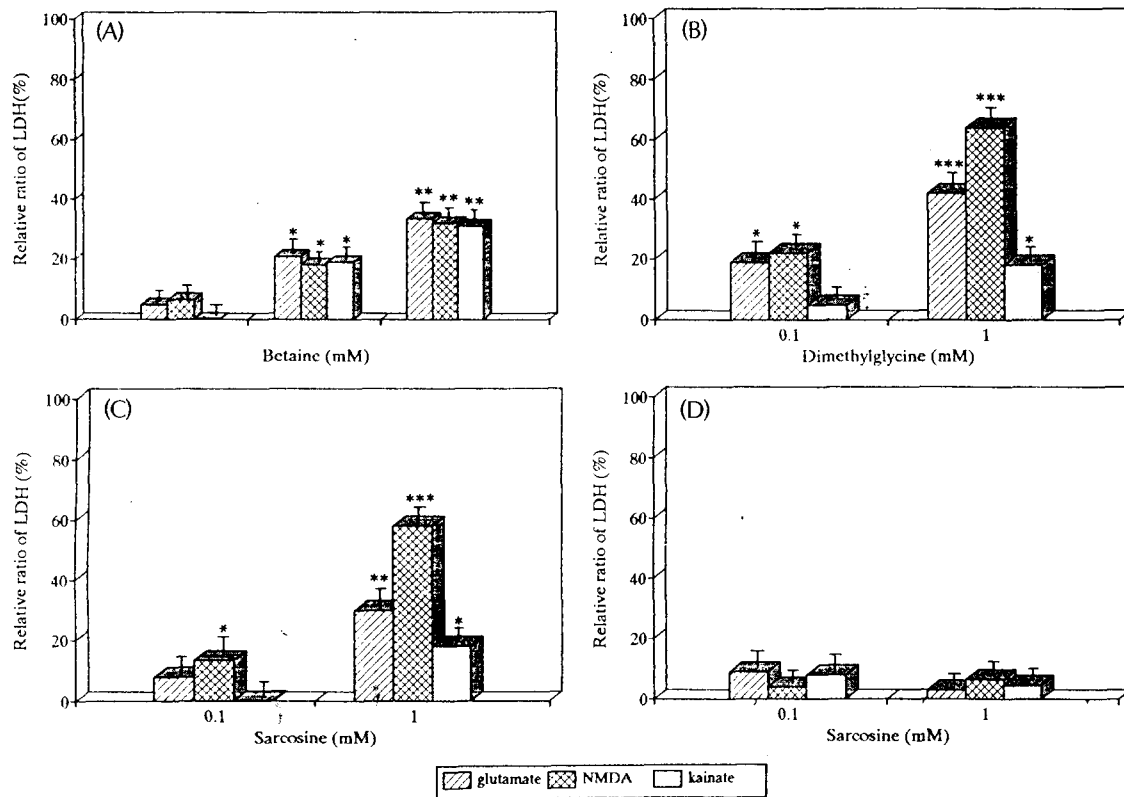
**Fig. 3.** Primary cultures of chicken embryonic brain cells. A. The effect of 500 μM NMDA on 12-day-cultured chicken embryonic brain cells. B. The effect of 2-h-pretreatment of 1 mM betaine on 24-h-exposure of 12-day-cultured chicken embryonic brain cells to 500 μM NMDA. bar=100 μm



**Fig. 4.** The effect of betaine on the intracellular calcium concentration of rat cortical cells exposed to 100 μM glutamate for 24 h.

rate of neuronal cells was determined by considering swollen neuronal cells as dead cells 30 min after treatment of the brain cells with 500 μM glutamate. Pretreatment of chicken embryonic brain cells with betaine for 2 h prior to glutamate insult markedly preserved the cells from glutamate-induced neurotoxicity as demonstrated by both the survival rate of neuronal cells (Fig. 2A) and the levels of cytoplasmic LDH (Fig. 2B). When chicken embryonic brain cells were pretreated for 2 h with 1 mM betaine followed by exposure to 100 μM glutamate for 42 h, cytoplasmic LDH levels within the cells remained at 63% of untreated control values while glutamate-treated controls fell to 0% LDH.

To clarify whether the protective effect of betaine against glutamate-induced neurotoxicity resulted from an antagonistic action on glutamate receptors, three different subtypes of glutamate receptor agonists, NMDA, quisqualate and kainate were employed. Swe-



**Fig. 5.** Effects of betaine, dimethylglycine, sarcosine and glycine on LDH values of rat cortical cells exposed to 100  $\mu$ M glutamate, NMDA or kainate for 24 h.

(A) The effect of betaine, (B) The effect of dimethylglycine, (C) The effect of sarcosine, (D) The effect of glycine (\* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001)

lling of neuronal cell bodies and fragmentation of neurites developed acutely by exposure to NMDA (Fig. 3A). However, pretreatment of brain cells with 1 mM betaine for 2 h prior to exposure to 500  $\mu$ M NMDA blocked this acute swelling and neuronal injury (Fig. 3B). The survival rate of neuronal cells and the levels of cytoplasmic LDH were significantly increased in a dose-dependent manner (Fig. 2B). Similar protective effects of betaine on quisqualate- and kainate-induced neurotoxicities were also observed. From the above results, it may be assumed that the protective effects of betaine may be due to a non-specific antagonistic action on glutamate receptors or through mechanism totally unrelated to these receptors.

To determine whether the protective effect of betaine on glutamate-induced neurotoxicity is species-specific, rat cortical cells in lieu of chicken brain cells were employed. Since betaine had similar protective effects on glutamate-induced neurotoxicity in rat cortical cells, the effect did not seem to be species-specific.

It has been generally accepted that exposure of neuronal cells to glutamate provokes the abrupt uptake of calcium ions into neuronal cells (Monagan *et al.*, 1989; MacDermott *et al.*, 1986). In order to study

whether betaine exerts its protective effect by acting on calcium channels, intracellular calcium concentrations of rat cortical cells were measured (Fig. 4). Betaine had no effect on intracellular calcium concentrations either in normal or glutamate-treated cortical cells. From these results and those mentioned above, it can be postulated that the protective effect of betaine on glutamate-induced neurotoxicity is not linked causally to any of the glutamate receptor subtypes nor to calcium channels.

Since betaine is a derivative of glycine, the protective effects of betaine and some other glycine derivatives on glutamate-induced neurotoxicity were studied. The effects of betaine, dimethylglycine, sarcosine and glycine on glutamate-induced neurotoxicity were assessed by determining the levels of cytoplasmic LDH in rat cortical cells. Cultures were treated with various concentrations of betaine, dimethylglycine, sarcosine and glycine, respectively, for 5 min prior to exposure for 24 h to 100  $\mu$ M glutamate (Fig. 5). Glycine showed no effect on toxicity. This may be due to the fact that the culture medium already contains glycine as a component and thus acts as an agonist against glutamate-induced neurotoxicity (Uckele *et al.*, 1989). The protective effects of betaine, dimethylglycine and sar-

cosine on glutamate-induced neurotoxicity seem to be exerted in a different manner (Fig. 5A, 5B, 5C and 5D). While the effect of betaine was not specific on glutamate receptor subtypes, dimethylglycine and sarcosine showed more specificity for NMDA type receptors than for kainate receptors. Dimethylglycine and sarcosine may compete with glycine at the modulator site of glycine in NMDA-type receptors thus blocking the agonistic activity of glycine on glutamate-induced neurotoxicity.

It may be suggested that betaine might exert its protective effect by simply stabilizing cell membranes thus protecting cells from bursting. The agent is used as an osmotic protectant for microorganisms (Smith *et al.*, 1990; Marthi and Lighthart, 1990). Another possible hypothesis is that betaine may attenuate glutamate-induced neurotoxicity by acting as an antioxidant as postulated for the case of cyanobacterium (Mamedov *et al.*, 1991) since antioxidants attenuate glutamate-induced neurotoxicity (Miyamoto *et al.*, 1989).

#### ACKNOWLEDGEMENT

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