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Chebulinic acid attenuates glutamate-induced HT22 cell death by inhibiting oxidative stress, calcium influx and MAPKs phosphorylation

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ABSTRACT

Glutamate-induced excitotoxicity and oxidative stress is a major causative factor in neuronal cell death in acute brain injuries and chronic neurodegenerative diseases. The prevention of oxidative stress is a potential therapeutic strategy. Therefore, in the present study, we aimed to examine a potential therapeutic agent and its protective mechanism against glutamate-mediated cell death. We first found that chebulinic acid isolated from extracts of the fruit of *Terminalia chebula* prevented glutamate-induced HT22 cell death. Chebulinic acid significantly reduced intracellular reactive oxygen species (ROS) production and Ca^{2+} influx induced by glutamate. We further demonstrated that chebulinic acid significantly decreased the phosphorylation of mitogen-activated protein kinases (MAPKs), including ERK1/2, JNK, and p38, as well as inhibiting pro-apoptotic Bax and increasing anti-apoptotic Bcl-2 protein expression. Moreover, we demonstrated that chebulinic acid significantly reduced the apoptosis induced by glutamate in HT22 cells. In conclusion, our results in this study suggest that chebulinic acid is a potent protectant against glutamate-induced neuronal cell death via inhibiting ROS production, Ca^{2+} influx, and phosphorylation of MAPKs, as well as reducing the ratio of Bax to Bcl-2, which contribute to oxidative stress-mediated neuronal cell death.

Glutamate is the main excitatory neurotransmitter in the central nervous system and plays an important role in differentiation, migration, and survival during brain development. However, excessive glutamate release leads to neuronal damage in the brain. There are two different forms of glutamate neurotoxicity, excitotoxicity and oxidative stress.[1,2] Oxidative stress is known as a major causative factor of neuronal cell death in acute brain injuries and chronic neurodegenerative diseases.[3] Previous studies have reported that the existence of excessive concentrations of extracellular glutamate provoked an accumulation of intracellular reactive oxygen species (ROS) through the prevention of cysteine uptake, followed by depletion of intracellular glutathione (GSH) levels.[4] Therefore, the removal of ROS by antioxidants, including N-acetylcysteine (NAC) and flavonoids, significantly reduces glutamate-induced neuronal cell death.[4,5]

Mitogen-activated protein kinases (MAPKs), a family of serine/threonine protein kinases, are implicated in multiple cellular functions in cell survival and cell death.[6] It has been recently reported that an increase in intracellular ROS mediates the activation of the MAPK signaling pathway, resulting in multiple cellular effects, such as inflammation, differentiation, and proliferation, as well as cell death.[7,8] Glutamate-mediated oxidative stress triggers a persistent activation of extracellular signal-regulated kinase (ERK), which contributes to cell death in primary cortical cultures and neuronal cell lines.[9] Additionally, glutamate-induced oxidative stress increases the ratio of Bax to Bcl-2 leading to neuronal death.[10] The Bcl-2 family includes anti-apoptotic (Bcl-2 and Bcl-X_L) and pro-apoptotic members (Bax and Bad).[11] Bcl-2 and Bax are the major members of the Bcl-2 family. Bax promotes apoptosis while Bcl-2 prevents apoptosis by counteracting the activity of Bax. Previous studies have demonstrated that glutamate increased the

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ratio of Bax/Bcl-2 expression during apoptotic neuronal cell death in differentiated neuronal cell lines.[10,12] Therefore, it is a useful strategy when preventing glutamate-mediated neuronal cell death to find a potent neuroprotectant which inhibits the activation of MAPKs, reducing the ratio of Bax to Bcl-2.

It has been reported that the extracts of *Terminalia chebula* (*T. chebula*) had a strong antioxidant effect and showed the neuroprotective effect against ischemic brain injury.[13] This indicates that the bioactive compounds of *T. chebula* may be beneficial to prevent neuronal cell death in neuropathological condition. Chebulinic acid, a well-known hydrolysable ellagitannin, is one of the major bioactive compounds in the extract of *T. chebula*.[14] Previous studies reported that chebulinic acid showed various biological effects such as a strong antioxidant, the suppression of cancer cell growth, and the inhibition of contractile response in cardiovascular muscle.[15–18] Additionally, chebulinic acid isolated from the fruit of *T. chebula* has shown a protective effect against gastric ulcers.[19] Although chebulinic acid has a many biological effects, there is no report about its effect against neuronal cell death so far. Therefore, in the present study, we investigated the protective effect of chebulinic acid against glutamate-induced neurotoxicity and underlying mechanism.

In this study, we first examined the effect of chebulinic acid on glutamate-induced HT22 cell death. To evaluate the protective effect against glutamate-induced cell death, HT22 cells were exposed to 5 mM glutamate in the absence or presence of indicated concentrations of chebulinic acid for 24 h. The results indicated that chebulinic acid (Fig. 1A) showed a strong protective effect against glutamate-induced HT22 cell death at concentrations of 25 and 50 μM (Fig. 1B). Furthermore, we confirmed the characteristics of the morphological changes to cells treated with glutamate and chebulinic acid. The results showed that chebulinic acid almost completely blocked HT22 cell death induced by glutamate (Fig. 1C). As a positive control, the effect of N-acetyl cysteine (NAC) against glutamate-induced HT22 cell death was tested. The results showed that the cell viability was significantly increased by the treatment of NAC compared with glutamate-treated cells (Fig. 1D). To the best of our knowledge, our results in the present study demonstrate for the first time that chebulinic acid prevented glutamate-induced neurotoxicity in HT22 cells.

Oxidative stress is a major event during neuronal cell death in acute and neurodegenerative diseases. Although glutamate is an important neurotransmitter, contributing to physiological brain function, it also introduces oxidative stress, leading to neuronal cell death at higher concentrations.[1,2] Therefore, the prevention of oxidative stress is considered a strategy to prevent neuronal cell death.

Previous studies have reported that chebulinic acid has strong antioxidative activity.[14,20] Thus, we then confirmed the antioxidative effect of chebulinic acid using *in vitro* 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging assay, a technique commonly used to evaluate the antioxidant activity of certain compounds. Consistently, our data also showed that chebulinic acid has a strong DPPH scavenging capacity (Fig. 2A). This suggests that the protective effect of chebulinic acid against glutamate-induced can be associated with its antioxidative property. This prompted us to investigate whether chebulinic acid can prevent the accumulation of intracellular ROS and calcium ions ($[Ca^{2+}]_i$) induced by glutamate

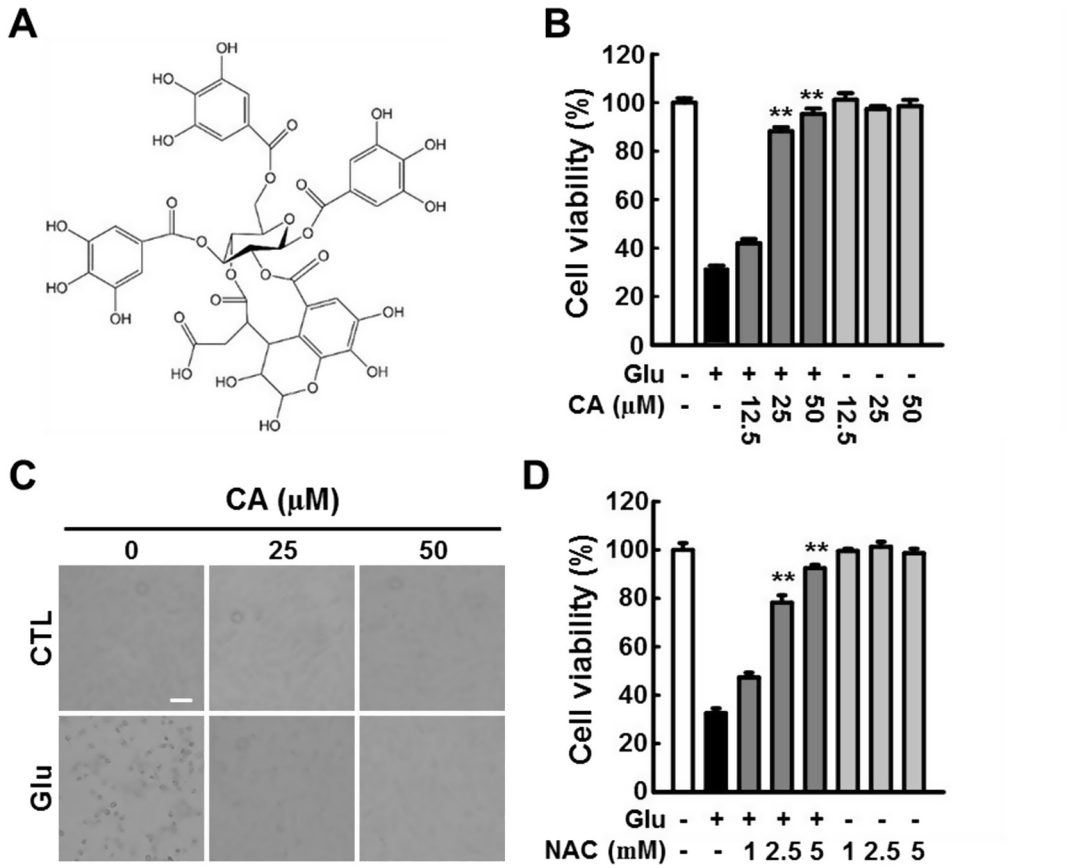

Fig. 1. Chebulinic acid attenuates glutamate-induced cell death in HT22 cells. (A) Chemical structure of chebulinic acid (CA) isolated from *T. chebula*. (B) HT22 cells were treated with 5 mM glutamate and the indicated concentrations of chebulinic acid for 24 h. Cell viability was determined using a CyTox assay. Bars represent the percentage of cell viability (mean ± S.E.M, $^{**}p < .001$ compared with glutamate-treated cells). (C) Phase contrast images were acquired using microscope at 24 h after treatment with glutamate in the presence of chebulinic acid. Scale bar, 50 μm. (D) The indicated concentrations of N-acetylcysteine (NAC) was tested as a positive control for preventing glutamate-induced HT22 cell death (mean ± S.E.M, $^{**}p < .001$ compared with glutamate-treated cells).

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