The effect of salmon calcitonin against glutamate-induced cytotoxicity in the C6 cell line and the roles the inflammatory and nitric oxide pathways play

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Research Article

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Abstract

Recent evidence has shown that salmon calcitonin (sCT) has positive effects on the nervous system. However, its effect and mechanisms on glutamate-induced cytotoxicity are still unclear. The current experiment was designed to examine the effect of sCT on glutamate-induced cytotoxicity in C6 cells, involving the inflammatory and nitric oxide stress pathways. The study used the C6 glioma cell line. Four cell groups were prepared to evaluate the effect of sCT on glutamate-induced cytotoxicity. The control group was without any treatment. Cells in the glutamate group were treated with 10 mM glutamate for 24 h. Cells in the sCT group were treated with various concentrations (3, 6, 12, 25, and 50 µg/mL) of sCT for 24 hours. Cells in the sCT + glutamate group were pre-treated with various concentrations of sCT for 1 hour and then exposed to glutamate for 24 hours. The cell viability was evaluated with an XTT assay. Nuclear factor kappa b (NF-kB), tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), neuronal nitric oxide synthase (nNOS), nitric oxide (NO), cyclic guanosine monophosphate (cGMP), caspase-3, and caspase-9 levels in the cells were measured by ELISA kits. Apoptosis was detected by flow cytometry method. sCT at all concentrations significantly improved the cell viability in C6 cells after glutamate-induced cytotoxicity ($p < 0.001$). Moreover, sCT significantly reduced the levels of NF-kB ($p < 0.001$), TNF-α, and IL-6 levels ($p < 0.001$). sCT also decreased nNOS, NO, and cGMP levels ($P < 0.001$). Furthermore, it decreased the apoptosis rate and increased the live-cell rate in the flow cytometry ($P < 0.001$). In conclusion, sCT has protective effects on glutamate-induced cytotoxicity in C6 glial cells by inhibiting inflammatory and nitric oxide pathways. sCT could be a useful supportive agent for people with neurodegenerative symptoms.

1. Introduction

Glutamate, an excitatory neurotransmitter, is most commonly found in the central nervous system. Although it is found in many areas in the central nervous system, the most common areas are the cerebral cortex and hippocampus (Danbolt 2001). It acts as a primary neurotransmitter in some specific regions in the cerebellum such as granule cells. It has two groups of receptors: ionotropic and metabotropic. The ionotropic receptor family consists of three groups: N-methyl-D-aspartate (NMDA) receptors, alpha amino-3-hydroxy-5-methyl-4 isoxazolepropionic acid (AMPA) receptors, and kainate receptors (Mayer 2005). Glutamate, which plays an important role in synaptic plasticity, learning and memory in physiological conditions, is involved in the pathogenesis of some diseases such as Alzheimer's disease, ischemic or hemorrhagic strokes, autism, and amyotrophic lateral sclerosis (Lau and Tymianski 2010). The basic mechanism in the pathogenesis of these diseases is excitotoxicity caused by excessive glutamate stimulation, which leads to excessive calcium ion influx into the cell (Matute et al. 2006). Calcium entrance to the cell triggers mitochondrial dysfunction, and increases intracellular nitric oxide levels, which induces apoptosis mechanisms in the cell (Arundine and Tymianski 2003).

Calcitonin is a single-chain peptide hormone containing 32 amino acids with a molecular weight of approximately 3500 Da. There are different types of calcitonin in the varied species such as human calcitonin and salmon calcitonin (sCT). Calcitonin is secreted by C cells, which are found in the thyroid

gland in mammals, and in the ultimo branchial structure in fish, amphibians, reptiles and birds (Ostrovskaya et al. 2017). The main role of calcitonin is the regulation of mineral metabolism and to help eliminate the increase in calcium levels called 'calcium stress'. It also controls the movements of magnesium and phosphate ions as part of its function of maintaining the ionic balance (Masi and Brandi 2007). There are four types of calcitonin used in the clinic, derived human, pig, eel, and sCT. Calcitonin from fish (sCT and eel calcitonin) have approximately 30–40 times greater biological activity than mammalian calcitonin (human calcitonin and pig calcitonin) (Braga 1994). sCT, due to its more stable structure and high bioactivity, has been applied as one of many treatments in recent years, especially in diseases that cause bone destruction (Chesnut et al. 2008). Moreover, some studies have reported that different types of calcitonin have positive effects on the central nervous system. Furthermore, evidence has been suggested that sCT is closely related to the glutamatergic system, such as the NMDA and AMPA receptors, and glutamate releasing (Kilinc et al. 2018; Taskıran et al. 2020). However, its effect on glutamate-induced cytotoxicity and underlying mechanisms are still unclear. In the present study, the effect of sCT against glutamate-induced cytotoxicity in C6 glial cells involving in the inflammatory and nitric oxide pathways were examined.

2. Materials And Method

2.1. Cell Culture

The C6 Glioma (CRL107) cell line was used in this study because of having appropriate glutamate-induced cytotoxicity (Kritis et al. 2015). C6 Glioma cell lines were obtained from the American Type Culture Collection. The cells were cultured in DMEM (Thermo Fisher Scientific, Altrincham, UK) containing 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich Co., St Louis, MO, USA), and 1% L-glutamine (Sigma-Aldrich Co., St Louis, MO, USA) and 1% penicillin/streptomycin (Sigma-Aldrich Co., St Louis, MO, USA). The cells were maintained at 37° C within a 5% CO2 humidified atmosphere.

2.2. Drug Administration

sCT (Biological Industries, Kibbutz Beit-Haemek, Israel) and glutamate (Sigma-Aldrich Co., St Louis, MO, USA) were dissolved in DMEM, and stock solutions were prepared before treatment.

2.3. Glutamate-Induced Cytotoxicity

Four-cell groups were prepared to evaluate the effect of sCT on glutamate-induced cytotoxicity. The control group was without any treatment. Cells in the glutamate group were treated with 10 mM glutamate for 24 hours. Cells in the sCT group were treated with various concentrations (3, 6, 12, 25, and 50 µg/mL) of sCT for 24 hours. Cells in the sCT + glutamate group were pre-treated with various concentrations (3, 6, 12, 25, and 50 µg/mL) of sCT for 1 hour and then exposed to 10 mM glutamate for 24 hours.

2.4. Cell Viability Assay

Cell viability was assessed using the XTT assay (Roche Diagnostic, MA, USA). C6 Glioma cells were seeded in 96-well plates at a density of 1×104 cells per well in 100-µL DMEM and grown overnight before sCT. The procedure of glutamate-induced cytotoxicity was performed as mentioned above. The following day, after 24 hours of incubation, the medium was removed, and the wells were washed twice with phosphate-buffered saline (PBS). In the last step, 100 µL DMEM without phenol red and a mixture of 50 µL XTT labeling solution were added to all the wells, and then the plates were maintained at 37º C for 4 hours. The plates were shaken, and the absorbance was detected using an ELISA microplate reader (Thermo Fisher Scientific, Altrincham, UK) at 450 nm. All the experiments were performed three times, and the cell viability was measured as a viable cell amount percent compared to the control, as untreated cells.

2.5. Preparation of Cell Homogenates

The cells for each group were collected by sterile tubes. They were centrifuged at 2000 rpm for approximately 10 minutes and the supernatants were removed. The component of cells in under the tubes were suspended by using PBS (pH: 7.4) to dilute cell suspension to the cell concentration of approximately 1 million/ml. The cells were damaged through repeated freeze-thaw cycles to let out the internal components. They were centrifuged at 4000 rpm for 10 minutes at a temperature of 4º C. Then, the supernatants were collected for biochemical analysis. The Bradford protein assay kit (Merck Millipore, Darmstadt, Germany) was used for determination of total protein levels in the samples.

2.6. Measurement of nuclear factor kappa B (NF-kB), tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), nitric oxide neuronal nitric oxide synthase (nNOS), nitric oxide (NO), cyclic guanosine monophosphate (cGMP), caspase-3, and caspase-9 levels in the cells

The levels of NF-kB, TNF-α, IL-6, nNOS, NO, and cGMP in the supernatants of the cells for each group were measured using ELISA commercial kits (BT Lab, Shanghai, China). The operation protocols were according to the manufacturer's instructions. In brief, the standard and tissue samples were added into the plate and incubated for 60 minutes at 37° C. After the washing step, the staining solutions were added and incubated for 15 minutes at 37° C. The stop solution was added and read at 450 nm in the ELISA microplate reader (Thermo Fisher Scientific, Altrincham, UK). Standard curves were plotted to determine the value of samples. The coefficients of variation within and between plates were less than 10%.

2.7. Annexin V Binding Assay

Apoptosis was evaluated using a Muse Annexin V/Dead Cell (Merck Millipore, Darmstadt, Germany) assay. In summary, the cells were seeded into six-well plates and were allowed to attach overnight before treatment. The cells were then treated with sCT (3 and 50 µg/mL), glutamate (10 mM), or their combinations and incubated for 24 hours. After incubation, the annexin V assay was performed according to the report in our previous study (Taskiran et al. 2020).

2.8. Statistical Analysis

The results were expressed as a mean ± standard error of the mean (SEM). The data analyses were performed with SPSS Version 23.0 for Windows. The data were evaluated using a one-way analysis of variance (ANOVA) and the posthoc Tukey test was chosen to identify the differences between the experimental groups. A value of $p < 0.05$ was accepted as statistically significant.

3. Results

3.1. Effect of sCT on cell survival after glutamate-induced cytotoxicity in C6 cells

The protective effects of sCT against glutamate-induced cytotoxicity in C6 cells were evaluated using a XTT cell proliferation assay. In this study, increasing doses of sCT (3–50 μg/mL) on cell survival were performed in both control and glutamate-treated C6 cells. Initially, the cells were pretreated with increasing doses (3–50 μg/mL) of sCT for 1 hour and then incubated with or without 30 mM glutamate for the next 24 hours. As shown in Fig. 1, pre-incubating the C6 cells with glutamate for 24 hours significantly decreased cell survival compared with the untreated control cells ($p < 0.001$; Fig. 1). However, all doses of sCT increased cell survival in C6 cells as compared with glutamate-treated cells ($p < 0.001$; Fig. 1). Furthermore, sCT, in all doses, did not change C6 survival compared with the untreated control cells ($p > 0.05$; Fig. 1).

3.2. Effect of sCT on NF-kB, TNF-α, and IL-6 after glutamate-induced cytotoxicity in C6 cells

The effects of sCT on NF-kB, TNF-α, and IL-6 were measured via ELISA kits after glutamate-induced cytotoxicity in the C6 cells. The cells were pretreated with two doses of sCT (3 and 50 μg/mL) for 1 hour and then incubated or not incubated with 10 mM glutamate for the next 24 hours. Pre-incubating the C6 cells with glutamate during 24 hours significantly increased NF-kB, TNF-α, and IL-6 levels as compared with the untreated control cells ($p < 0.001$; Fig. 2). However, the sCT (3 and 50 μg/mL) significantly decreased NF-kB, TNF-α, and IL-6 levels in C6 cells compared with the glutamate-treated cells ($p < 0.001$; Fig. 2).

3.3. Effect of sCT on nNOS, NO, and cGMP after glutamate-induced cytotoxicity in C6 cells

The ELISA measurements were performed to assess the effects of sCT on nNOS, NO, and cGMP after glutamate-induced cytotoxicity in the C6 cells. The cells were pretreated with two doses of sCT (3 and 50 μg/mL) for 1 hour and then incubated or not incubated with 10 mM glutamate for the next 24 hours. Pre-incubating the C6 cells with glutamate for 24 hours significantly raised nNOS, NO, and cGMP levels compared with the untreated control cells ($p < 0.001$; Fig. 3). However, sCT (3 and 50 μg/mL) significantly reduced nNOS, NO, and cGMP levels in C6 cells compared with the glutamate-treated cells ($p < 0.001$; Fig. 3).

3.4. Effect of sCT on caspase-3 and caspase-9 after glutamate-induced cytotoxicity in C6 cells

The effects of sCT on caspase-3 and caspase-9 were measured by ELISA kits after glutamate-induced cytotoxicity in C6 cells. The cells were pretreated with two doses of sCT (3 and 50 μg/mL) for 1 hour and then incubated or not incubated with 10 mM glutamate for the next 24 hours. Pre-incubating the C6 cells with glutamate for 24 hours significantly raised caspase-3 and caspase-9 levels compared with the untreated control cells ($p < 0.001$; Fig. 4). However, sCT (3 and 50 μg/mL) significantly reduced caspase-3 and caspase-9 levels in C6 cells compared with the glutamate-treated cells ($p < 0.001$; Fig. 4).

3.5. Effect of sCT on apoptosis after glutamate-induced cytotoxicity in C6 cells

The anti-apoptotic effects of sCT were evaluated by flow cytometry after glutamate-induced cytotoxicity in the C6 cells. As shown in Fig. 5, it is exhibited that 10 mM glutamate remarkably increased the proportion of apoptotic cells for 24 hours compared with the untreated control cells ($p < 0.001$; Fig. 5). Moreover, an sCT pretreatment at doses of 3 and 50 μg/mL significantly reduced the apoptotic percentage of C6 cells after glutamate-induced cytotoxicity in the cells ($p < 0.001$; Fig. 5). However, pretreatment of sCT (3 and 50 μg/mL) alone did not demonstrate a significant apoptotic effect in the C6 cells ($P > 0.05$; Fig. 5).

4. Discussion

The present study is the first time that the effects of sCT against glutamate-induced cytotoxicity in C6 cells have been evaluated. sCT pretreatment increased C6 cell survival and reduced cell death after glutamate-induced cytotoxicity in the cells. Furthermore, pretreatment with sCT decreased inflammatory pathway proteins, NF-kB, TNF-α, and IL-6, levels in the C6 cells. Moreover, sCT suppressed the nNOS, NO, and cGMP levels in the C6 cells, and sCT inhibited apoptosis related factors, caspase-3 and caspase-9, levels after glutamate-induced cytotoxicity in the C6 cells.

Several in vivo and in vitro studies have demonstrated that sCT has positive effects on the nervous system. It has been reported that sCT stimulates neuritis elongation in the nerve cells of mollusks (Grimm-Jørgensen 1987). Moreover, it has been claimed that sCT changes the neuronal excitability in different brain regions of rats and decreased intracellular calcium by modulating synaptosomes (Twery and Moss 1985). It has also been found that sCT reduces intracellular calcium levels in response to high potassium stimulation (Welch and Olson 1991). Calcium plays a vital role in glutamate-induced cytotoxicity (Pastukhov and Borisova 2018). The current study found that sCT raised the cell viability and inhibited apoptosis after glutamate-induced cytotoxicity in C6 cells. Therefore, sCT could reduce glutamate-induced cytotoxicity by blocking NMDA and reducing calcium flow into C6 cells.

Inflammation is closely related to the neurodegenerative process in the nervous system (Takeuchi 2013). The activation of NF-kB, the main modulator of inflammation in the organism, leads to releasing of proinflammatory cytokines, such as TNF-α and IL-6, and other chemokines (Bonizzi and Karin 2004; Ulivi et al. 2008). It has been shown that glutamate-induced cytotoxicity increases proinflammatory cytokines, which are associated with nervous system disorders (Chaparro-Huerta et al. 2005). The previous findings have found that sCT has an antiinflammatory effect by inhibiting proinflammatory cytokines production in the brain and blood tissues (Zhang et al. 2017; Taskıran et al. 2020). Furthermore, it has been claimed that other types of calcitonin, such as human calcitonin, decreases IL-1 stimulated chondrocytes activation by suppressing the p50-NF-κB pathway (Bai et al. 2019). In this study, sCT decreased NF-kB, TNF-α, and IL-6 levels after glutamate-induced cytotoxicity in the C6 cells, which is consist with previous studies.

NO is an essential neuromodulators of neurotransmitters in the central nervous system. It is synthesized from the oxidation of the L-arginine amino acid via three types of nitric oxide synthases, which are endothelial NOS (eNOS), inducible NOS (iNOS), and neuronal NOS (nNOS) (Bahremand et al. 2010). nNOS is commonly expressed in neurons (Zhou and Zhu 2009), and it has been reported that C6 cells express nNOS (Feinstein et al. 1994; Raso et al. 2002). The activation of nNOS leads to an increase in NO in the neurons, which initiates soluble guanylate cyclase (sGC) / guanosine monophosphate (cGMP). cGMP, a secondary messenger, gives rise to glutamate releasing in the neurons and causes excitation (Kiss 2001). Moreover, nNOS/NO/cGMP is also involved in glutamate-induced cytotoxicity (Kritis et al. 2015). A previous study has demonstrated that calcitonin protects chondrocytes from lipopolysaccharide-induced apoptosis by suppressing NO production (Zhang et al. 2017). In contrast to this study, it has been claimed that elcatonin, a synthetic eel calcitonin, vasodilates retinal blood vessels in rats by increasing NO levels in endothelial cells (Mori et al. 2015). In this study, it has been found that the rise in nNOS/NO/cGMP levels in the cells after glutamate-induced cytotoxicity. Nevertheless, sCT decreased nNOS/NO/cGMP after glutamate-induced cytotoxicity in the C6 cells.

According to our findings, sCT has a protective effect in glial cell survival after glutamate-induced cytotoxicity in C6 cells. Since glial cells are critical for neurodegenerative diseases, sCT could be a supportive therapeutic agent to the treatment of neurodegeneration related diseases. However, this needs to be proven by further studies.

5. Conclusion

The findings of this study showed that sCT reduced cell death after glutamate-induced cytotoxicity in C6 cells. These effects could be associated with inhibition of inflammation (NF-kB/ TNF-α/ IL-6) and nitric oxide (nNOS/NO/cGMP) pathways. Therefore, sCT could be a useful supportive therapeutic agent for glutamate-related neurodegenerative diseases. However, further investigation is required to answer the questions raised about the probable mechanisms involved.

Declarations

Acknowledgement

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Authors' Contributions

AST designed the study, interpreted the data, and had a major contribution in writing and revising the manuscript. ME performed the experiment, drafted the manuscript and analyzed data. All authors read and approved the final manuscript.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Compliance with Ethical Standards

Funding

None

Conflict of Interest

The authors declare that they have no conflict of interest

Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Figures

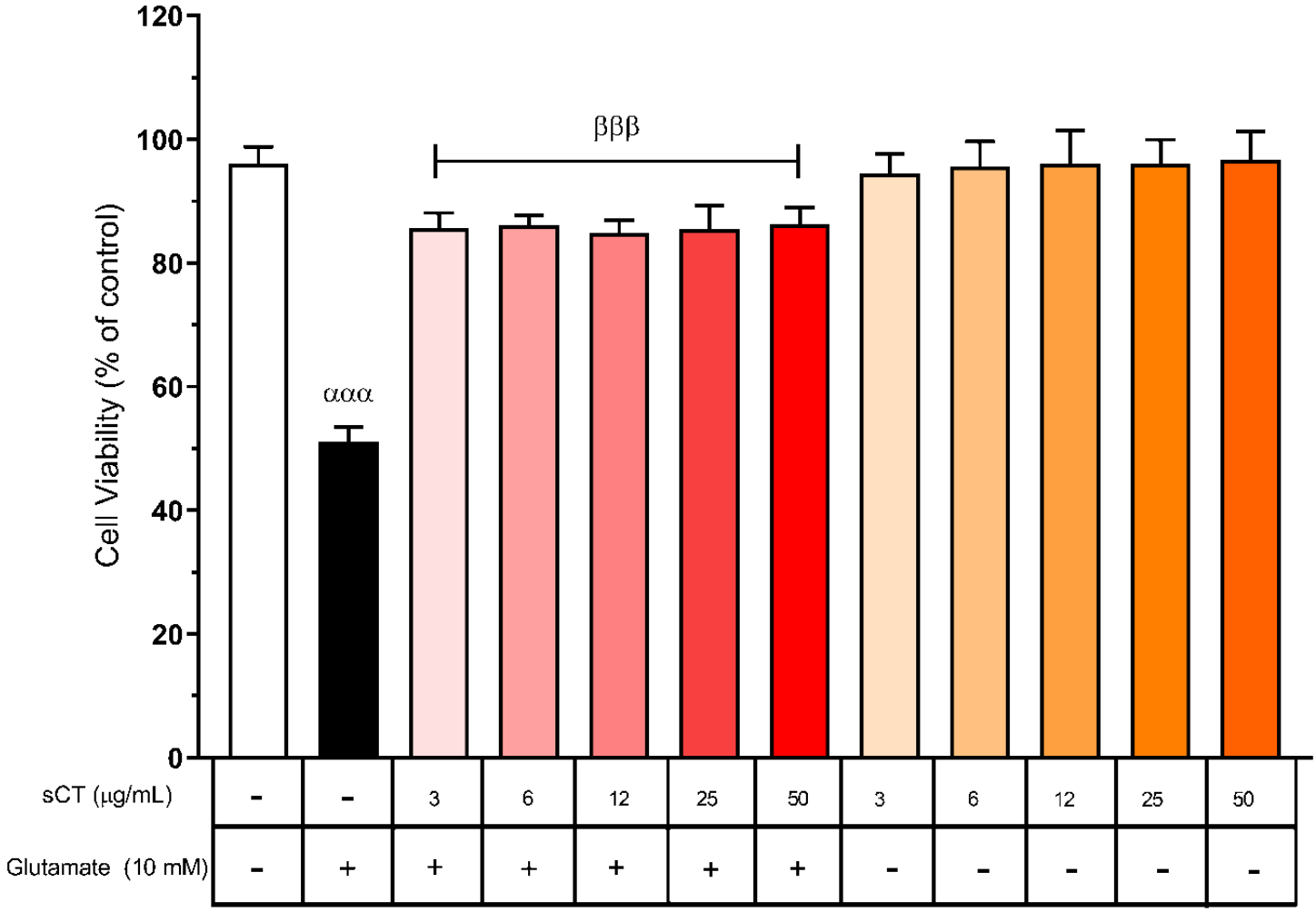

Figure 1

Effect of sCT on cell survival after glutamate-induced cytotoxicity in C6 cells. The data are expressed as mean ± SEM. ααα$p < 0.001 as compared to the untreated control group; βββ$p < 0.001 compared to glutamate-treated group.

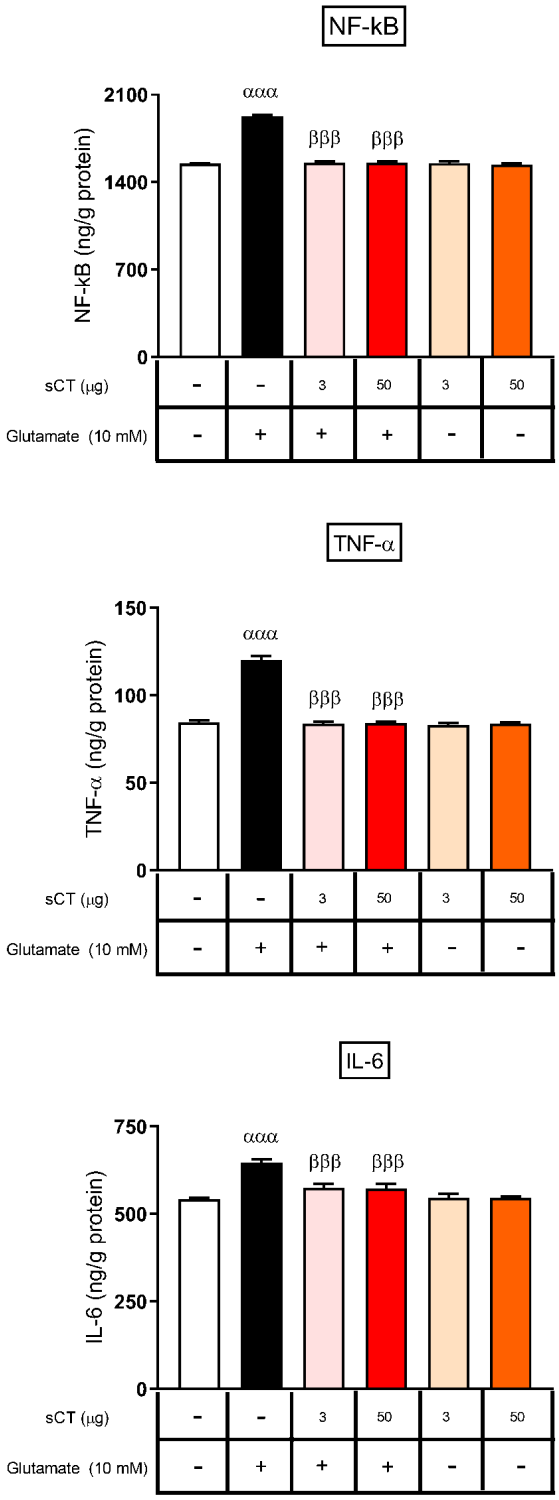

Figure 2

Effect of sCT on NF-kB, TNF-α, and IL-6 levels after glutamate-induced cytotoxicity in C6 cells. The data are expressed as mean ± standard error mean. αααp < 0.001 as compared to the untreated control group; βββp < 0.001 compared to the glutamate-treated group.

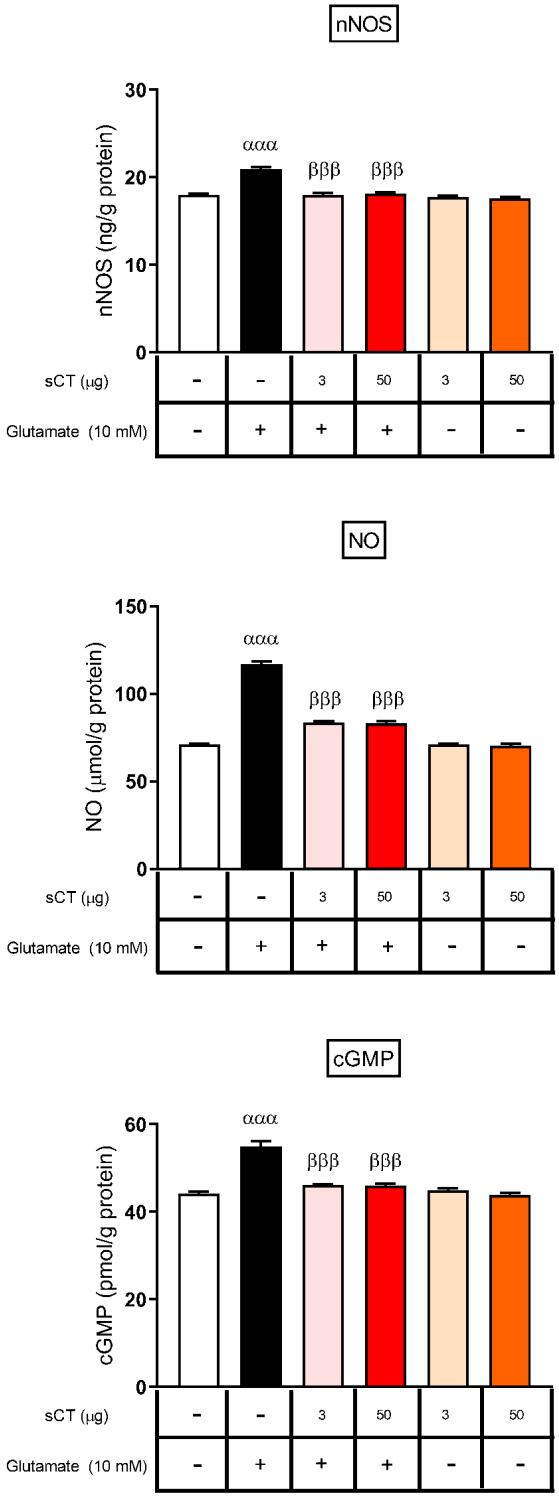

Figure 3

Effect of sCT on nNOS, NO, and cGMP levels after glutamate-induced cytotoxicity in C6 cells. The data are expressed as mean ± SEM. αααp < 0.001 as compared to the untreated control group; βββp < 0.001 compared to glutamate-treated group.

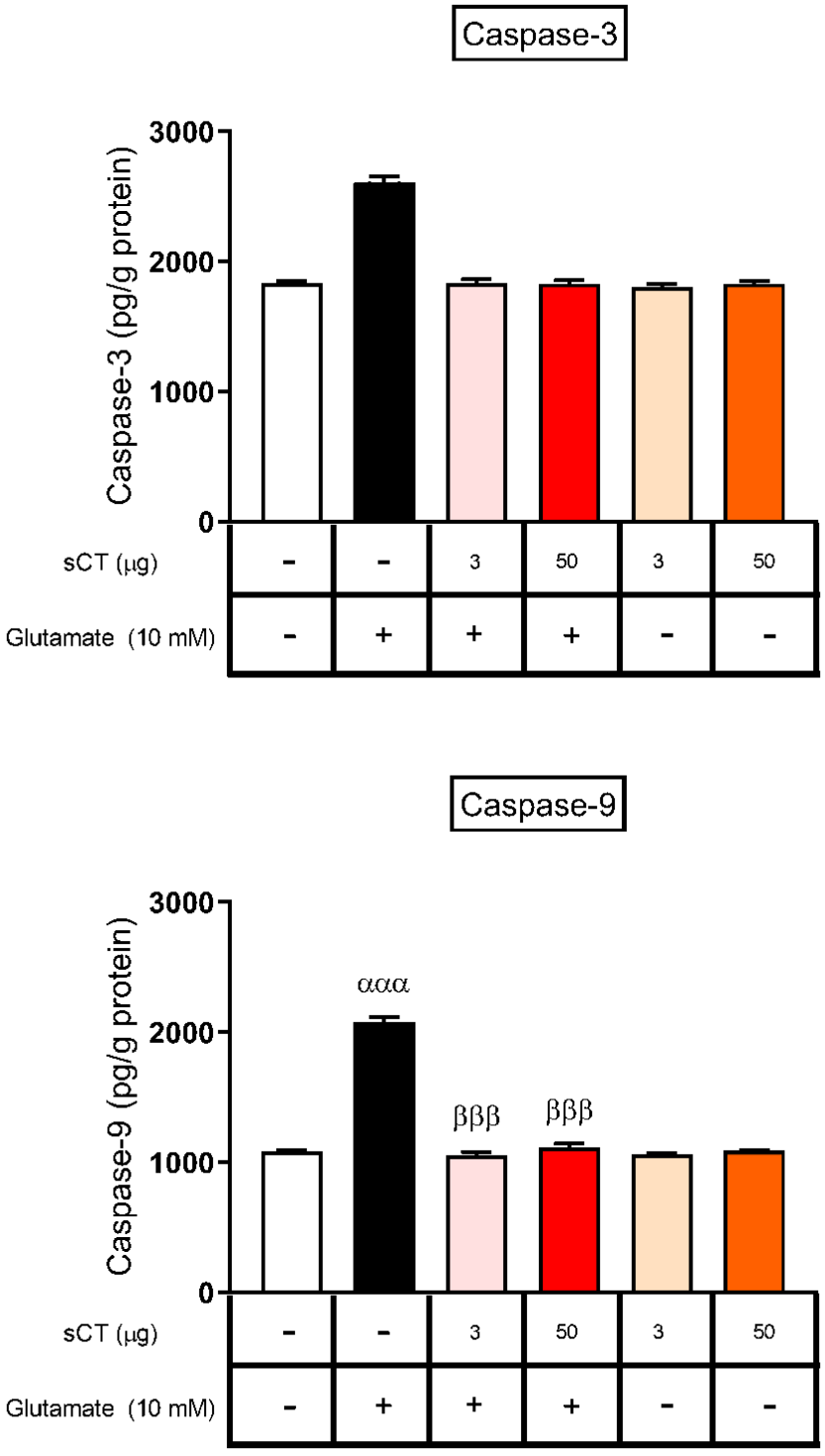

Figure 4

Effect of sCT on apoptosis after glutamate-induced cytotoxicity in C6 cells. The data are expressed as mean ± SEM. ααα$p < 0.001 as compared to the untreated control group; βββ$p < 0.001 compared to the glutamate-treated group.

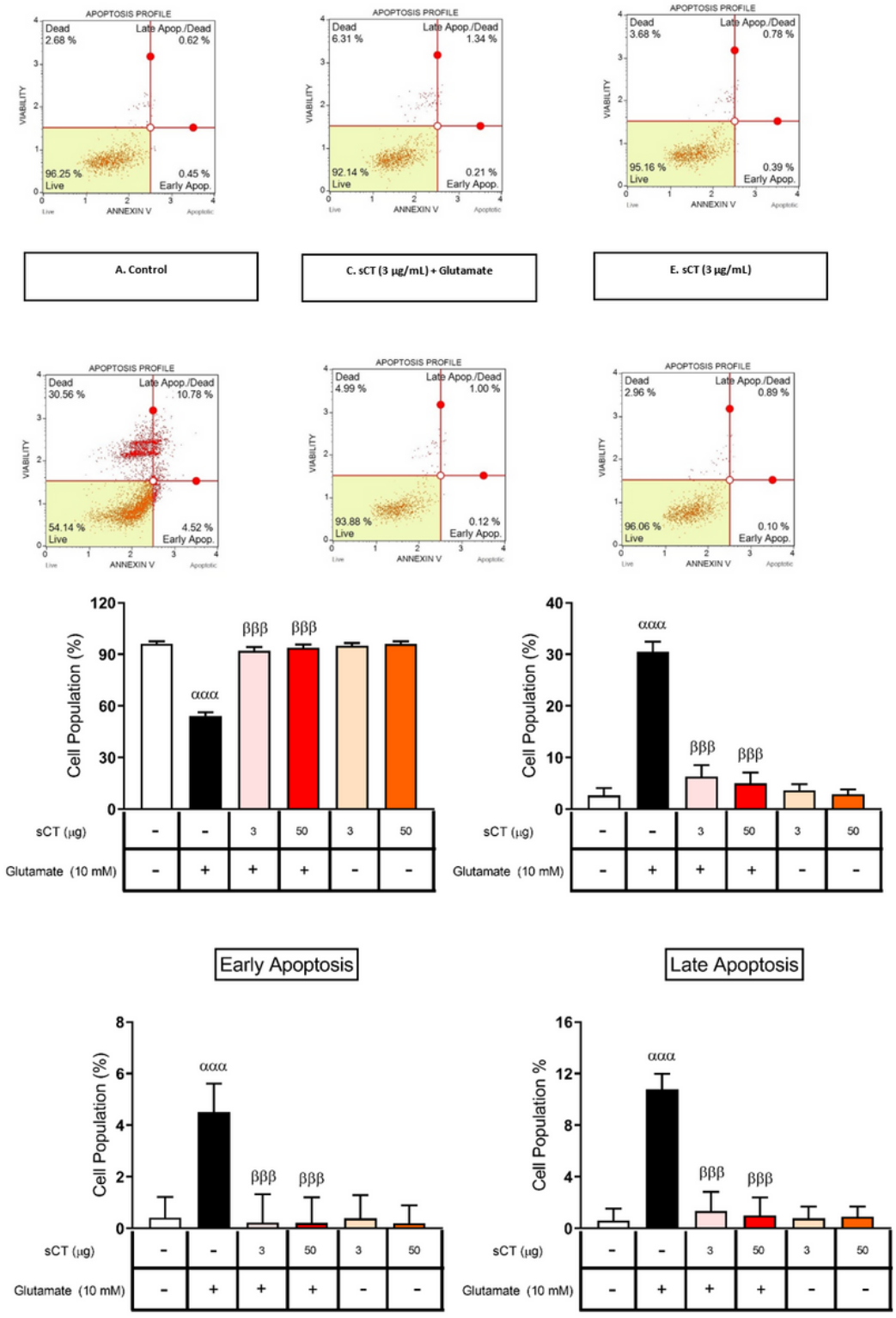

Figure 5

Effect of sCT on caspase-3 and caspase-9 levels after glutamate-induced cytotoxicity in C6 cells. The data are expressed as mean ± SEM. αααp < 0.001 as compared to untreated controlgroup; βββp < 0.001 compared to the glutamate-treated group.

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