Research Article

Pamukkale Medical Journal
doi:https://dx.doi.org/10.31362/patd.1758959

In vitro investigation of caspase-3 dependent and independent apoptotic processes in cortical and hippocampal neurons triggered by S-sulfocysteine-induced cell death

S-sülfosistein ile tetiklenen hücre ölümünde kortikal ve hipokampal nöronlarda kaspaz-3 bağımlı ve bağımsız apoptotik süreçlerin in vitro incelenmesi

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Posted date:05.08.2025 Acceptance date:25.09.2025

Abstract

Purpose: S-sulfocysteine (SSC), a toxic byproduct of sulfur-containing amino acid metabolism, accumulates in conditions such as molybdenum cofactor deficiency (MoCD) and isolated sulfite oxidase deficiency (iSOD), leading to severe neurodegeneration. Despite evidence of SSC's neurotoxicity, the apoptotic mechanisms it triggers remain unclear, particularly in different brain regions. This study aimed to investigate the region-specific apoptotic pathways induced by SSC in hippocampal HT-22 and primary cortical neurons, focusing on caspase-dependent and -independent mechanisms.

Materials and methods: Neuronal cells were treated with increasing doses of SSC, and cell viability, glutathione (GSH) levels, and apoptosis-related proteins (AIF, calpain, cytochrome c, caspase-3) were assessed using CCK-8 assay, GSH enzymatic assay, and ELISA. The protective effects of specific inhibitors targeting AIF, calpain, and cytochrome c were also evaluated.

Results: SSC reduced cell viability in both neuronal types with half-maximal lethal dose (LD_{50}) values of 150 µM (HT-22) and 155 µM (cortical neurons). In both models, SSC elevated AIF and calpain levels, whereas cytochrome c and caspase-3 were significantly increased only in cortical neurons. GSH levels initially rose at 2–8 hours and declined by 16 hours. Inhibitors of AIF, calpain, and cytochrome c partially restored viability, with combined administration offering the most robust protection.

Conclusion: SSC induces both caspase-independent and caspase-dependent apoptosis in a region-specific manner: HT-22 cells predominantly activate AIF and calpain, while cortical neurons engage additional cytochrome c and caspase-3 pathways. These findings suggest distinct molecular vulnerabilities and offer potential targets for therapeutic intervention in sulfite-related neurodegenerative diseases.

Keywords: S-sulfocysteine, apoptosis, primary cortical neurons, oxidative stress, sulfite oxidase deficiency.

Alphan A, Adiguzel E, Kucukatay V, Cort A, Tunc Ata M, Kilic Toprak E. In vitro investigation of caspase-3 dependent and independent apoptotic processes in cortical and hippocampal neurons triggered by S-sulfocysteine-induced cell death. Pam Med J 2025;18:913-925.

Öz

Amaç: Kükürt içeren amino asit metabolizmasının toksik bir yan ürünü olan S-sülfosistein (SSC), molibden kofaktör eksikliği (MoCD) ve izole sülfit oksidaz eksikliği (iSOD) gibi durumlarda birikir ve ciddi nörodejenerasyona yol açar. SSC'nin nörotoksisitesi kanıtlanmış olsa da özellikle farklı beyin bölgelerinde tetiklediği apoptoz mekanizmaları tam olarak açıklığa kavuşmamıştır. Bu çalışma, SSC'nin hipokampal HT-22 ve primer kortikal nöronlarda oluşturduğu bölgeye özgü apoptoz yollarını, kaspaz-bağımlı ve bağımsız mekanizmalar açısından araştırmayı amaçlamaktadır.

Gereç ve yöntem: Nöronal hücreler artan dozlarda SSC ile muamele edilmiştir. Hücre canlılığı, glutatyon (GSH) düzeyleri ve apoptoza ilişkin proteinler (AIF, kalpain, sitokrom c, kaspaz-3) sırasıyla CCK-8 testi, enzimatik GSH testi ve ELISA yöntemi ile analiz edilmiştir. AIF, kalpain ve sitokrom c'yi hedef alan spesifik inhibitörlerin koruyucu etkileri de değerlendirilmiştir.

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Bulgular: SSC, her iki hücre modelinde de canlılığı azaltmış; yarı maksimal letal doz (LD_{50}) değerleri HT-22 hücrelerinde 150 µM, kortikal nöronlarda ise 155 µM olarak belirlenmiştir. SSC, her iki modelde AIF ve kalpain düzeylerini artırmış, ancak sitokrom c ve kaspaz-3 düzeyleri yalnızca kortikal nöronlarda anlamlı olarak yükselmiştir. GSH düzeyleri 2–8 saat arasında artmış, 16. saate gelindiğinde azalmıştır. AIF, kalpain ve sitokrom c inhibitörleri hücre canlılığını kısmen geri kazandırmış; kombinasyon tedavisi en güçlü koruyucu etkiyi göstermiştir.
Sonuç: SSC, bölgeye özgü şekilde hem kaspaz-bağımsız hem de kaspaz-bağımlı apoptozu tetiklemektedir: HT-22 hücrelerinde ağırlıklı olarak AIF ve kalpain mekanizmaları devreye girerken, kortikal nöronlarda buna ek olarak sitokrom c ve kaspaz-3 de aktive olmaktadır. Bu bulgular, bölgesel moleküler hassasiyetleri ortaya koymakta ve sülfite bağlı nörodejeneratif hastalıklar için potansiyel tedavi hedefleri sunmaktadır.

Anahtar kelimeler: S-sülfosistein, apoptoz, primer kortikal nöronlar, oksidatif stres, sülfit oksidaz eksikliği.

Alphan A, Adıgüzel E, Küçükatay V, Çört A, Tunç Ata M, Kılıç Toprak E. S-sülfosistein ile tetiklenen hücre ölümünde kortikal ve hipokampal nöronlarda kaspaz-3 bağımlı ve bağımsız apoptotik süreçlerin in vitro incelenmesi. Pam Tıp Derg 2025;18:913-925.

Introduction

Sulfur-containing amino acid metabolism is essential for maintaining redox balance and neuronal function. Disruptions in this pathway, particularly within the transsulfuration route, have been implicated in various neurodegenerative disorders, including Alzheimer's and Huntington's diseases [1]. Cysteine catabolism generates sulfite, a toxic intermediate normally detoxified by sulfite oxidase (SO), which requires the molybdenum cofactor (Moco). Deficiencies in SO—either due to mutations in SuOX or defects in Moco biosynthesis—cause molybdenum cofactor deficiency (MoCD) and isolated sulfite oxidase deficiency (iSOD) [2]. These conditions are characterized by early-onset seizures, feeding difficulties, progressive cerebral atrophy, and early mortality [3]. Biochemical hallmarks include elevated sulfite and S-sulfocysteine (SSC) and reduced levels of cystine and homocysteine [4]. Sulfite can disrupt protein structure by cleaving disulfide bonds, forming S-sulfonated derivatives like SSC [5]. In MoCD and iSOD, SSC is markedly elevated and exerts glutamate-like excitotoxic effects through NMDA receptor activation, promoting calcium influx, calpain activation, and neuronal death [6]. Despite clear evidence of SSC toxicity, the underlying apoptotic mechanisms remain incompletely defined [6, 7]. Both caspase-dependent and caspase-independent pathways—mediated by cytochrome c, calpain, and apoptosis-inducing factor (AIF)—are implicated in excitotoxic neuronal death [8-10]. However, regional differences in the activation of these pathways remain to be clarified. Recent studies support the concept of region-specific vulnerability to excitotoxicity, highlighting distinct calcium dynamics and oxidative pathways in cortical versus hippocampal neurons [11, 12]. Moreover, NMDA receptor-mediated excitotoxicity has been shown to differentially affect hippocampal subfields, suggesting that subtle regional differences may underlie distinct apoptotic responses [13]. Studies suggest that glutamate-induced neuronal death varies by brain region; in cortical neurons, caspase-3, calpain, and AIF are activated, whereas hippocampal HT-22 cells primarily involve calpain and AIF, with minimal caspase-3 activity [14]. Given SSC's glutamate-mimetic activity, it may similarly induce region-specific apoptotic responses.

To date, the exact pathways involved in SSC-induced cell death in these brain regions remain unclear. It is plausible that both caspase-dependent and caspase-independent mechanisms are differentially activated in cortical and hippocampal neurons. The rationale for investigating these two regions stems from the high density of NMDA receptors in both the cortex and hippocampus. Since SSC is a glutamate agonist, its preferential interaction with NMDA receptors in these regions may underlie the observed selective vulnerability. Understanding the mechanisms underlying this selective vulnerability is crucial for elucidating region-specific neurodegenerative processes. Further research into the apoptotic signaling cascades triggered by SSC in distinct brain regions could contribute to the development of more targeted therapeutic approaches for sulfite-induced neurodegeneration and other excitotoxic conditions.

Materials and methods

Cell culture and treatments

The murine hippocampal neuronal cell line HT-22 was kindly provided by the Department of Medical Biology, Pamukkale University. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 1% penicillin/streptomycin (Gibco), and incubated at 37°C in a humidified 5% CO_2 atmosphere. Primary cortical neurons (PCNs) were commercially obtained (Gibco™, #A15585) as cryopreserved preparations and cultured in Neurobasal medium supplemented with 2% B-27, 1% GlutaMAX, and 1% penicillin/streptomycin. HT-22 cells were treated with increasing concentrations of S-sulfocysteine (SSC; 0–300 μM), while PCNs received 0, 50, 75, or 100 μM SSC. In certain experiments, cells were co-treated with inhibitors targeting apoptotic pathways: calpain-1 inhibitor (Cayman Chemical, #14921), methazolamide (Sigma, SML0720; 0–20 μM), and N-phenylmaleimide (Santa Cruz, sc-250486; 1–50 nM). SSC concentrations were determined based on prior cytotoxicity assessments (data not shown). Control groups received vehicle treatment. Based on pilot toxicity testing, SSC was applied at 0–300 μM in HT-22 cells and 0–150 μM in PCNs. The narrower range in cortical neurons reflected limited cell yield and rapid cytotoxicity at concentrations above ~100 μM. Non-toxic effective concentration (NTEC) was defined a priori as a dose yielding ≥90% of control viability and showing no significant difference vs. control (one-way ANOVA with Dunnett's multiple comparisons, α=0.05). Dose–response data are presented as mean ± SD with value labels; a dashed line at 90% indicates the NTEC threshold.

Cell viability assay

Cell viability was assessed using the Cell Counting Kit-8 (CCK-8; Dojindo), which measures mitochondrial dehydrogenase activity. Cells were seeded in 96-well plates (1×10^4 cells/well) and treated for 24 hours with various concentrations of SSC. After treatment, 10 μL of WST-8 solution was added to each well, and plates were incubated at 37°C for 2 hours. Absorbance was measured at 450 nm using a microplate reader (Thermo Scientific Multiskan GO). Viability was expressed as a percentage of the untreated control group. In pilot experiments, HT-22 cells tolerated higher concentrations of SSC; therefore, a treatment range of 0–300 μM was selected. In contrast, PCNs were markedly more sensitive, and concentrations above ~100 μM resulted in extensive cytotoxicity and limited cell yield, so a narrower range of 0–150 μM was used. Dose–response data from both models were analyzed using Probit regression (AAT Bioquest LD_{50} calculator, https://www.aatbio.com/tools/ld50-calculator) to determine the half-maximal lethal dose (LD_{50}), expressed in micromolar concentrations (μM). For cortical neurons, the LD_{50} estimate (155 μM) lay near the upper boundary of the tested range; therefore, a 150 μM concentration point was included in the revised plot to improve transparency.

Glutathione assay

Total glutathione (T-GSH) and the GSH/GSSG ratio were quantified using a Glutathione Assay Kit (Abcam, ab239709) according to the manufacturer's instructions. HT-22 cells were treated with SSC at 0–300 μM and PCNs at 0–150 μM, consistent with the viability testing ranges described above. Cells were harvested at 2, 8, 16, and 24 hours following SSC exposure. Due to limited availability of PCNs, untreated control groups could not be included in the GSH assay. The main aim of this experiment was to monitor the temporal dynamics of GSH changes following SSC exposure. In HT-22 cells, GSH levels were measured at 2, 8, 16, and 24 hours, with a decline after 8 hours suggesting the onset of apoptosis. Similar trends were observed in PCNs at 2, 8, and 16 hours. To standardize apoptosis assessment across both models, the 24-hour time point was selected as the reference for subsequent analyses. After incubation, cells were lysed, and deproteinized supernatants were subjected to enzymatic recycling reactions. Absorbance was measured at 405 nm using a microplate reader, and GSH levels were normalized to total protein concentration.

ELISA for apoptotic markers

Levels of apoptosis-related proteins—including apoptosis-inducing factor (AIF), calpain, caspase-3, and cytochrome c—were measured using mouse-specific ELISA kits (BT LAB, China), following the manufacturer's

instructions. After 24-hour SSC and/or inhibitor treatments, cells were lysed using RIPA buffer supplemented with protease inhibitors and centrifuged at 10,000×g for 10 minutes at 4°C. Supernatants were analyzed in duplicate. Absorbance was measured at 450 nm using a microplate reader. Results were normalized to total protein content and expressed relative to control values.

Statistical analysis

Data were analyzed using GraphPad Prism 9.0 (GraphPad Software). All experiments were performed in triplicate. One-way ANOVA followed by Bonferroni's post hoc test was used for multiple comparisons. Data are presented as mean ± standard deviation (SD), and *p*-values <0.05 were considered statistically significant.

Results

1. Determination of LD_{50} values for SSC in neuronal cultures

The LD_{50} values of S-sulfocysteine (SSC) were calculated based on neuronal viability in PCNs and HT-22 hippocampal neurons (Figure 1). Probit analysis indicated an LD_{50} of 155 µM for PCNs and 150 µM for HT-22 cells.

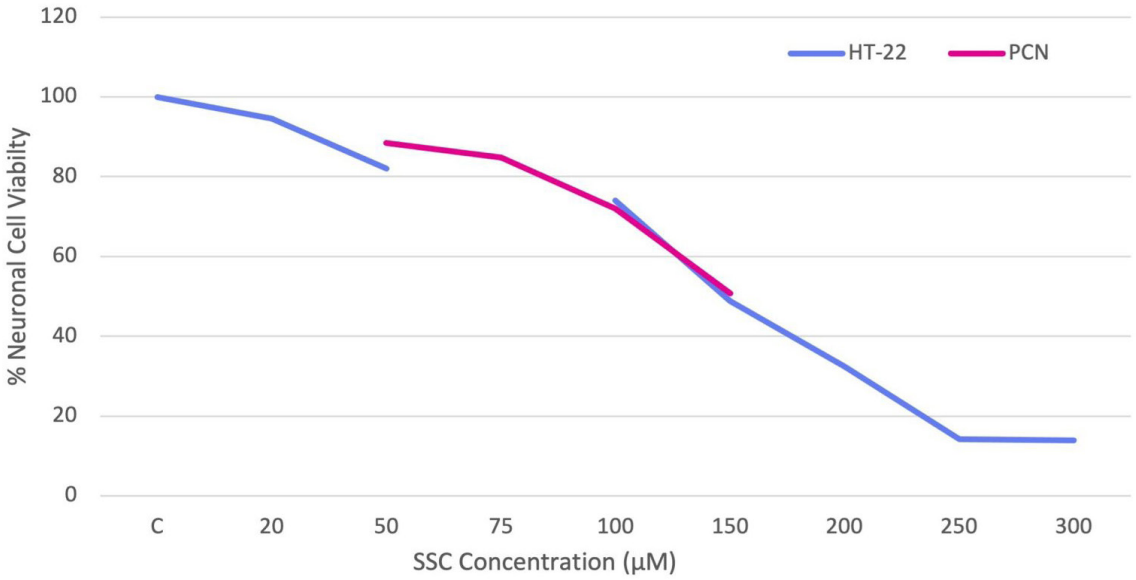


Figure 1. LD_{50} determination of SSC in neuronal cultures

Dose–response curves of SSC in HT-22 hippocampal neurons and primary cortical neurons (PCNs). Neuronal viability (%) is presented as mean ± standard deviation (SD). LD_{50} values were calculated as 150 µM for HT-22 cells and 155 µM for PCNs using Probit analysis (https://www.aatbio.com/tools/ld50-calculator). (n=6) C= Control; n= number of wells × 2 independent experiments

2. Determination of non-toxic effective concentrations of inhibitors

Exposure to different concentrations of calpain-1 inhibitor, methazolamide (MA), and N-phenylmaleimide (NF) demonstrated that specific ranges were non-toxic in both neuronal models (Figure 2A–C). For the calpain-1 inhibitor, HT-22 cells maintained >90% viability at 10 µM, which was considered a non-toxic effective concentration. In PCNs, 0.1 µM and 10 µM met the non-toxic effective concentration (NTEC) criterion, whereas 5 µM fell below the 90% viability threshold and differed significantly from controls; therefore, it was classified as toxic and excluded from subsequent co-treatments (Figure 2A). MA preserved viability at 10 µM in HT-22 cells and up to 20 µM in cortical neurons without significant cytotoxicity (Figure 2B). NF maintained >90% viability at 10 nM in both models (Figure 2C). These concentrations were selected for subsequent co-treatment experiments with SSC.

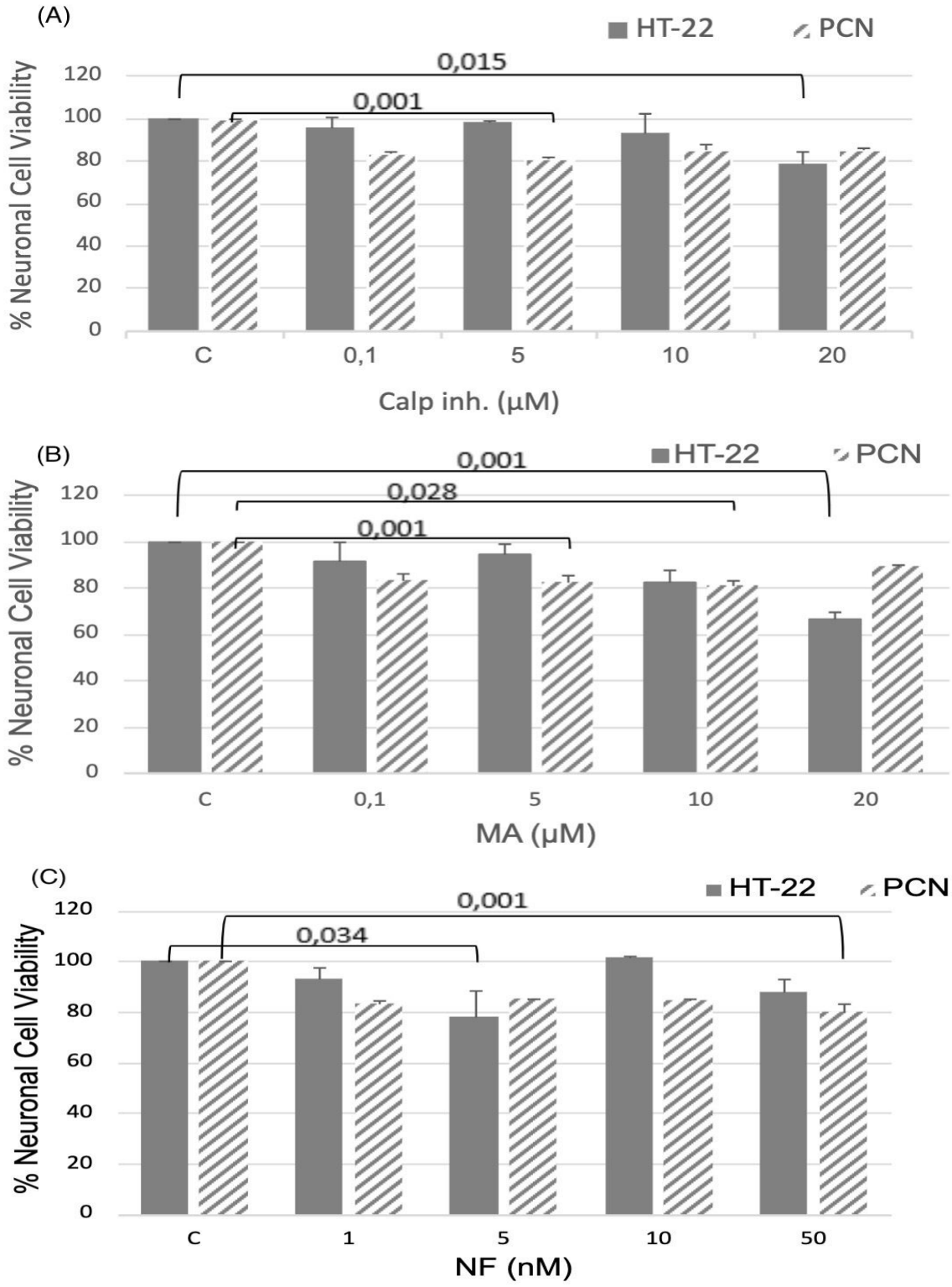


Figure 2. Determination of non-toxic effective concentrations of inhibitors in neuronal cultures

(A) Calpain-1 inhibitor: In HT-22 cells, 10 μM maintained >90% viability and was considered non-toxic. In PCNs, 0.1 μM and 10 μM met the non-toxic effective concentration (NTEC) criterion (≥90% viability and not significantly different from control, C), whereas 5 μM fell below this threshold and was classified as toxic (p<0.05 vs. C). Results are mean ± SD of neuronal viability (%).
(B) Methazolamide (MA); Cytochrome c inhibitor: non-toxic effective concentrations were 10 μM for HT-22 neurons and 20 μM for PCNs (p>0.05 vs. C).
(C) N-phenylmaleimide (NF); Apoptosis-inducing factor (AIF) inhibitor: non-toxic effective concentration was 10 nM for both HT-22 and PCNs (p>0.05 vs. C).
Results are mean ± SD. n= number of wells × 2 independent experiments. Control groups are denoted as C

3. Effects of SSC on glutathione levels

In HT-22 cells, SSC (150 μM) significantly increased GSH levels at 2 h (p=0.010) and 8 h (p=0.038) compared with controls, whereas the increases at 16 and 24 h were not statistically significant (Figure 3A). In PCNs, SSC (155 μM) increased GSH levels from 2 to 8 h (p=0.05) and then significantly decreased at 16 h (p=0.019) compared with controls (Figure 3B).

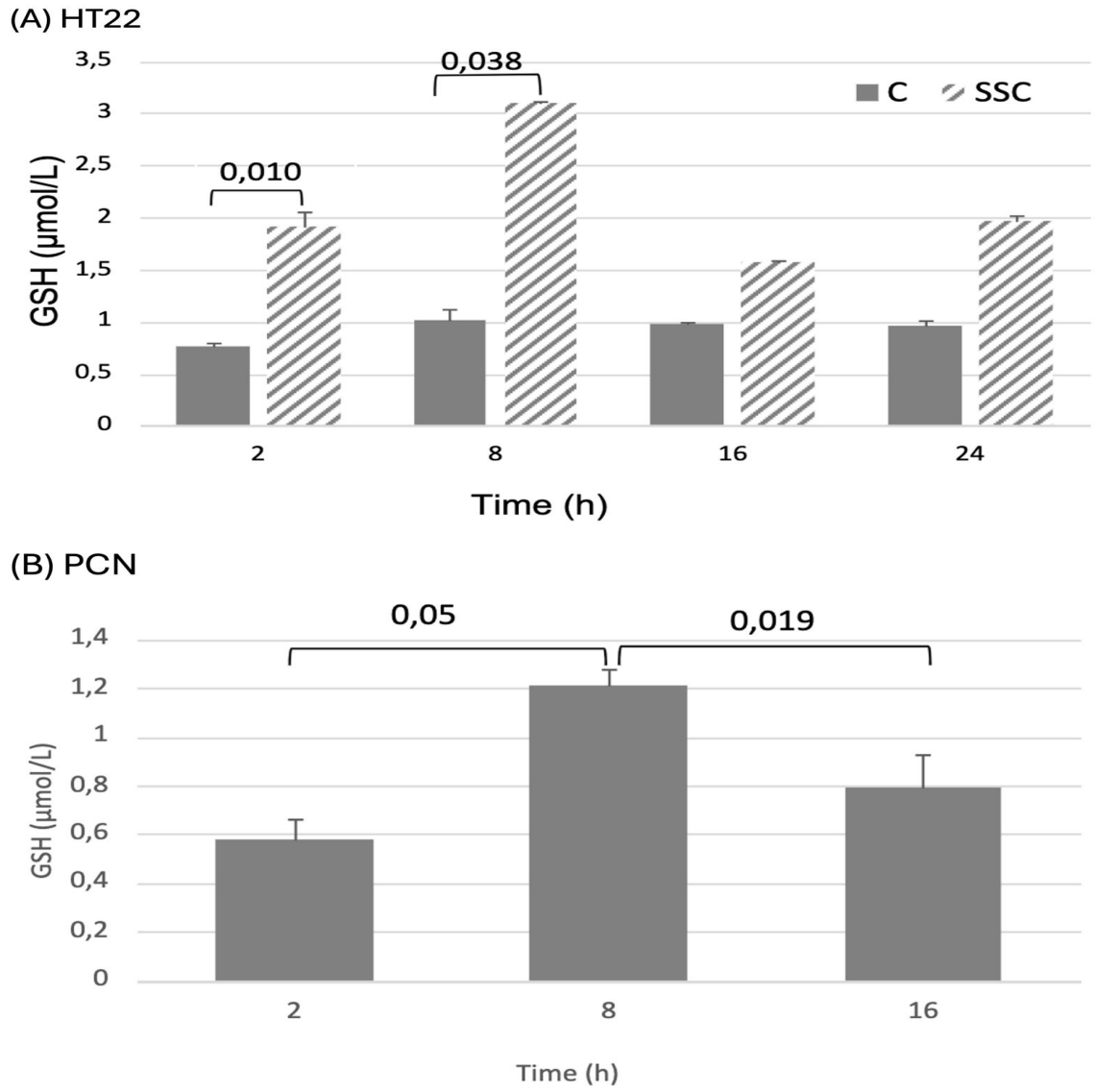
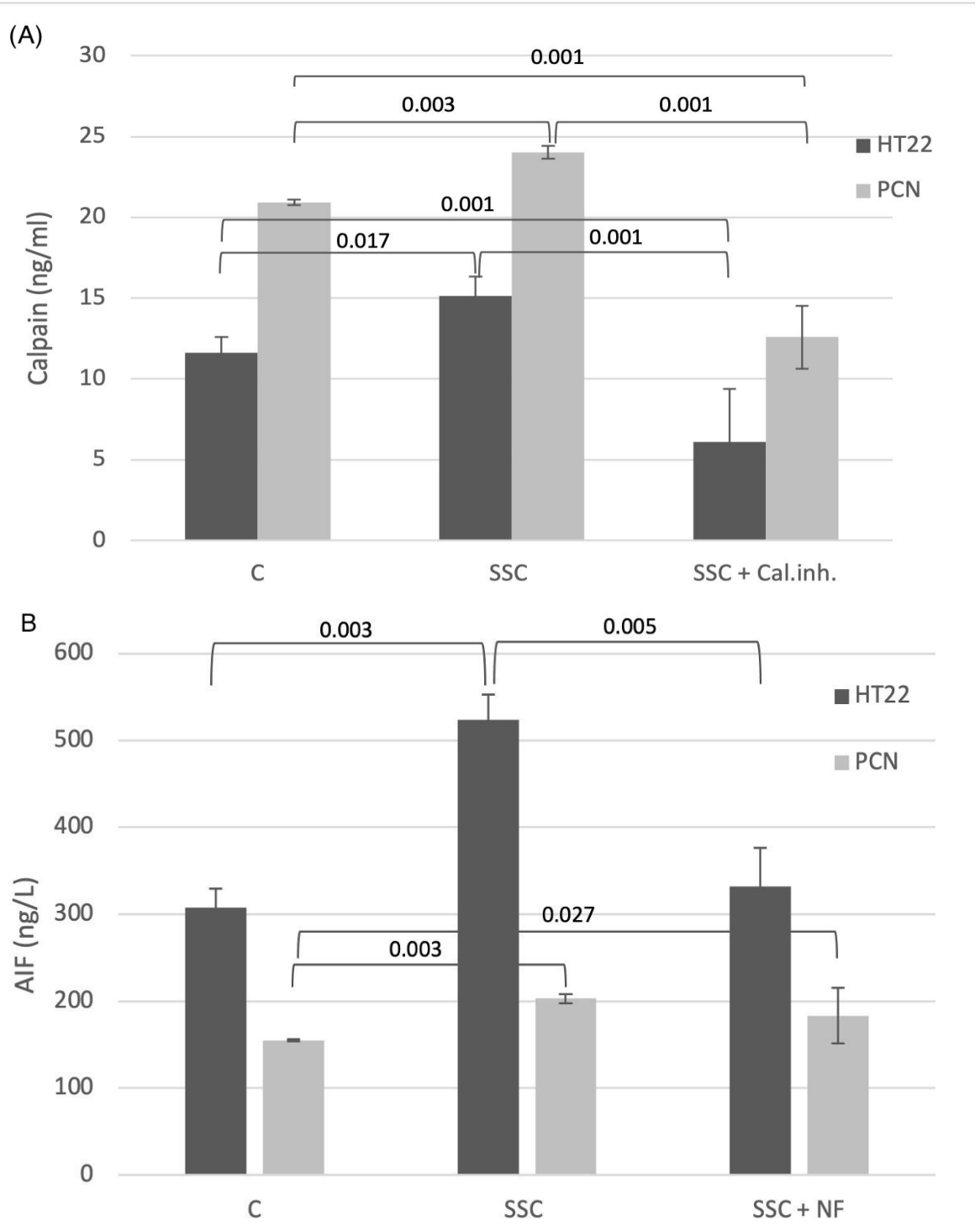


Figure 3. Time-dependent changes in GSH levels

(A) HT-22 hippocampal neurons: SSC (150 μM) significantly increased glutathione (GSH) levels at 2 h (p=0.010) and 8 h (p=0.038) compared with the control (C) group. No significant differences were observed at 16 h and 24 h.
(B) PCNs: SSC (155 μM) increased GSH levels from 2 to 8 h (p=0.05) and significantly decreased them at 16 h (p<0.05). Due to material limitations, only SSC-treated groups were included; untreated controls were not shown in the figure.
All results are presented as mean ± SD; n= number of pellets × number of measurements. Control groups are denoted as C

4. SSC-induced changes in apoptotic markers

In HT-22 neurons, SSC significantly increased calpain (p=0.017) and AIF (p=0.003) levels, which were reduced by calpain-1 inhibitor (p<0.001) and NF (p=0.005), respectively (Figures 4A–B). No significant changes were observed in cytochrome c or caspase-3 levels (p>0.05; Figures 4C–D). In PCNs, SSC significantly increased calpain (p=0.003), AIF (p=0.003), and caspase-3 (p=0.030) levels (Figures 4A–C). Calpain was reduced by the calpain-1 inhibitor (p<0.001), and cytochrome c levels were significantly reduced by MA (p<0.001) (Figure 4C). In contrast to PCNs, HT-22 cells did not show a significant change in caspase-3 levels after SSC exposure (p>0.05; Figure 4D).



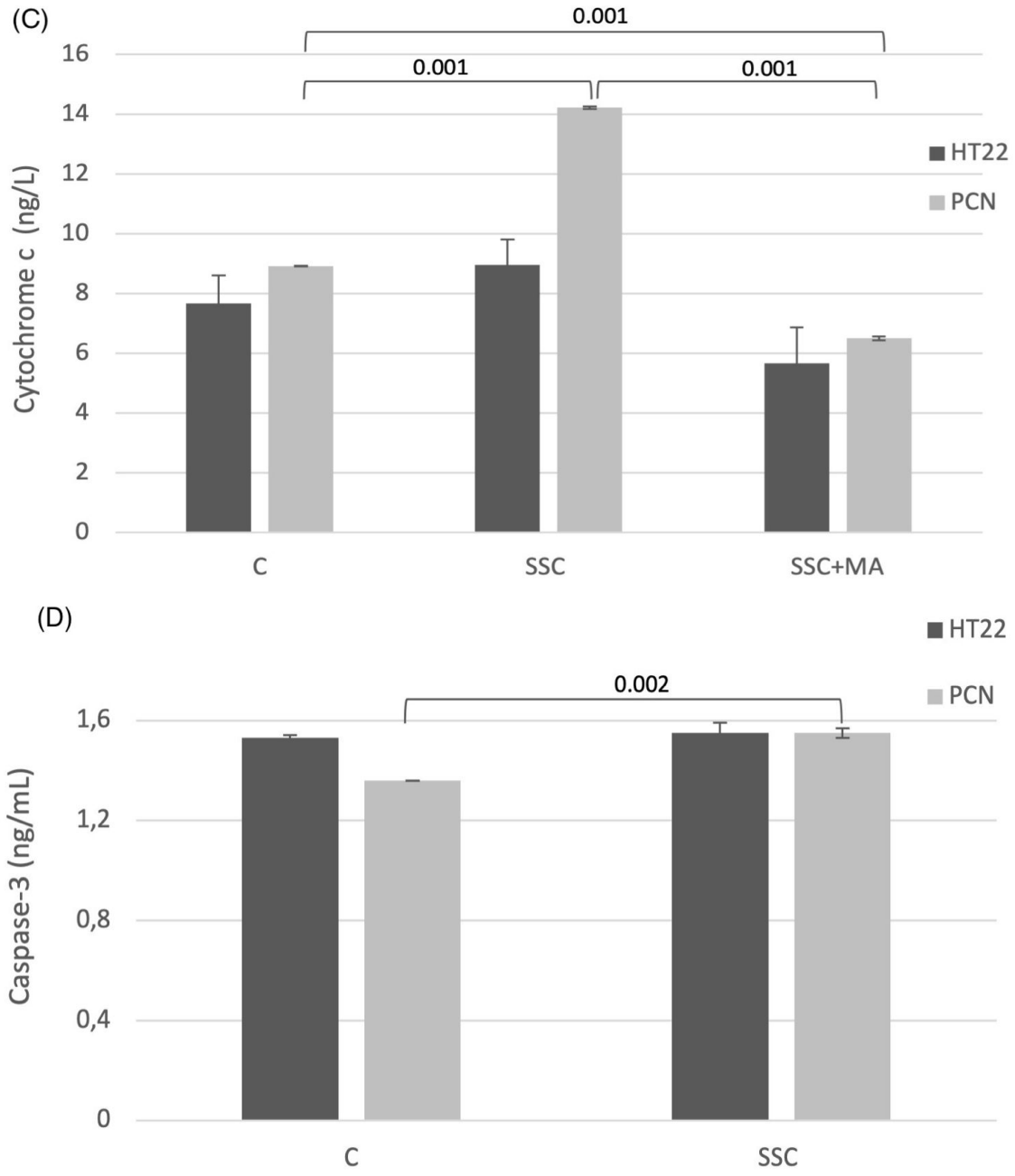


Figure 4. Effects of SSC and inhibitors on apoptotic pathway markers

(A) Calpain levels in HT-22 and PCNs: Significant differences were observed among control (C), SSC, and SSC + calpain-1 inhibitor groups (p<0.05) in HT-22. Significant differences were observed among control (C), SSC, and SSC + calpain 1 inhibitor groups (p<0.05) in PCNs.
(B) AIF levels in HT-22 and PCNs: SSC treatment significantly increased AIF levels compared with control (C), and this was reduced by NF co-treatment (p<0.05) in HT-22. SSC treatment significantly increased AIF levels compared with control (C), and NF co-treatment partially reduced these levels (p<0.05) in PCNs.
(C) Cytochrome c levels in HT-22 and PCNs: No significant differences were observed between groups in HT-22. Significant differences were observed among control (C), SSC, and SSC + MA groups (p<0.05) in PCNs.
(D) Caspase-3 levels in HT-22 and PCNs: No significant differences were observed between SSC and control (C) groups (p>0.05) in HT-22. SSC (155 µM) treatment significantly increased caspase-3 compared with control (C) (p<0.05) in PCNs.
All results are presented as mean ± SD; n= number of pellets × number of measurements. Control groups are denoted as C

5. Protective effects of inhibitors against SSC toxicity

Co-treatment with calpain 1 inhibitor, MA, and NF significantly improved % viability compared to SSC alone in both HT-22 and cortical neurons (p<0.05) (Figures 5A–B).

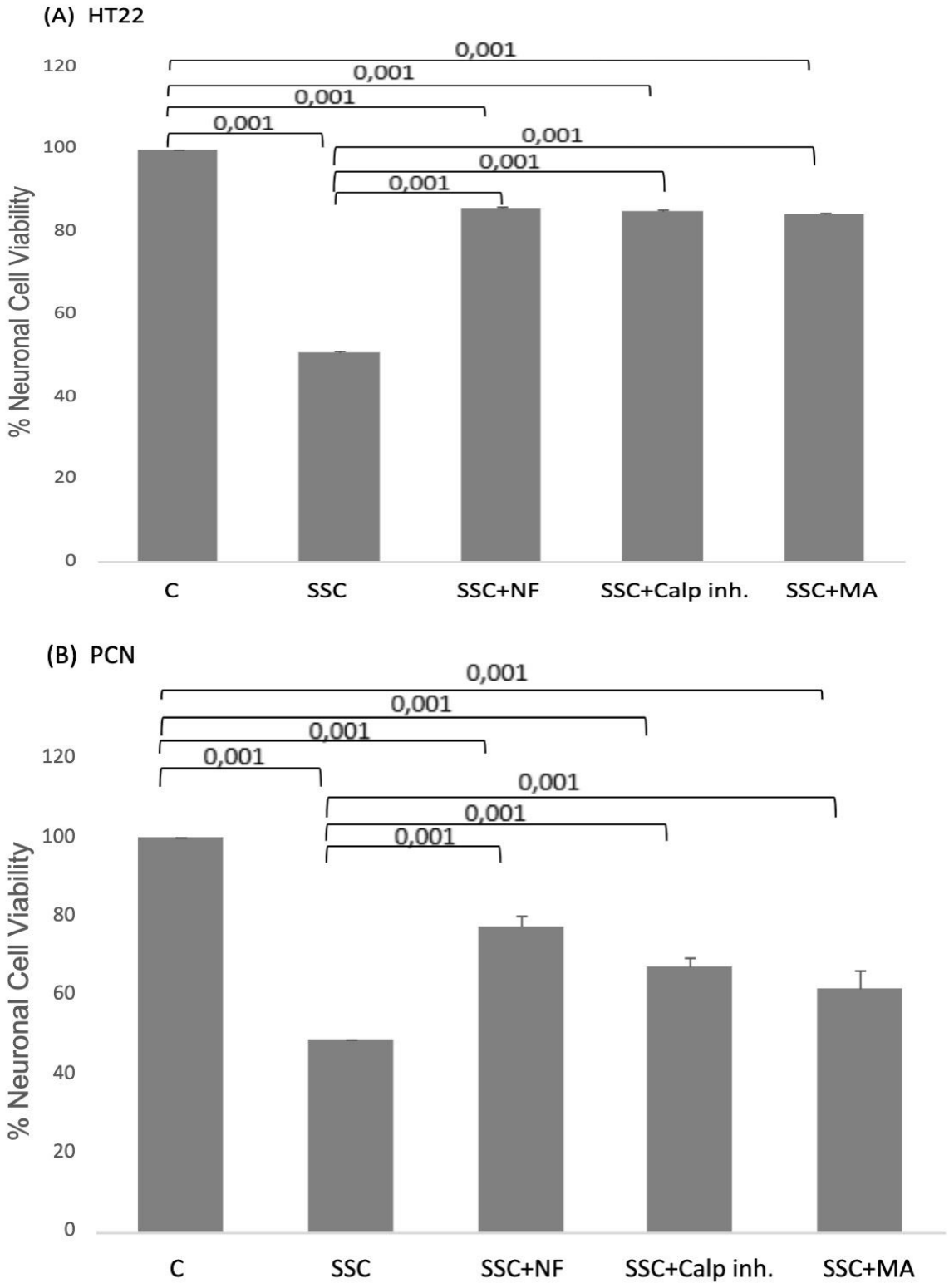


Figure 5. Protective effects of inhibitors against SSC-induced neurotoxicity

(A) HT-22 hippocampal neurons: SSC (150 μM) significantly decreased viability compared with control (C) (p=0.001). Co-treatment with NF (10 nM, AIF inhibitor), Calpain-1 inhibitor (10 μM), or MA (methazolamide, 10 μM) significantly improved cell viability compared with SSC alone (p=0.001).
(B) PCNs: SSC (155 μM) significantly decreased viability compared with C (p<0.05). Co-treatment with NF (10 nM), Calpain-1 inhibitor (10 μM), or MA (20 μM) significantly improved viability compared with SSC alone (p<0.05).
Control group: n= 8 (4 wells × 2 independent experiments); other groups: n= 6 (3 wells × 2 independent experiments). Results are presented as mean ± SD. Control groups are denoted as C (control)**.** All abbreviations are defined at first mention

Discussion

Since the 1970s, glutamate-induced excitotoxicity has been recognized as a critical mechanism contributing to neuronal death in various neurodegenerative diseases [15-17]. Although SSC, a glutamate analog, is known to exhibit neurotoxic effects, the precise apoptotic pathways it activates have not been fully elucidated. This study aimed to investigate whether SSC induces neuronal death primarily via caspase-independent mechanisms in HT-22 hippocampal neurons and through both caspase-dependent and -independent pathways in PCNs. Our findings confirm that SSC activates the caspase-independent apoptotic pathway in both cell types, as indicated by increased AIF levels. AIF is a key mediator of mitochondrial apoptosis, as it translocates from the mitochondria to the nucleus and initiates caspase-independent cell death [18, 19]. Previous studies have demonstrated that AIF plays a central role in glutamate-induced toxicity in HT-22 neurons [20, 21], which is in line with our observations. In the present study, SSC significantly increased AIF release in both neuronal models. Notably, AIF inhibition in HT-22 neurons markedly reduced AIF levels and improved cell viability. In cortical neurons, however, viability was improved despite no significant change in AIF release, suggesting that the protective effect of AIF inhibition may be partial or dose-dependent. SSC shares several mechanistic features with glutamate-induced excitotoxicity, including NMDA receptor overactivation, calcium overload, oxidative stress, and mitochondrial dysfunction. However, SSC appears to favor caspase-independent pathways to a greater extent than glutamate, as indicated by prominent AIF translocation observed in our study. While glutamate toxicity in HT-22 neurons has been strongly associated with ROS generation and mitochondrial impairment, SSC induced both overlapping mechanisms and distinct features, particularly an enhanced contribution of AIF-mediated cell death. These similarities and differences may help explain the region-specific vulnerabilities we observed between hippocampal and cortical neurons.

SSC concentrations used in this study are modeled on pathophysiological exposures observed in patients with molybdenum cofactor deficiency (MoCD) and isolated sulfite oxidase deficiency (iSOD), in which elevated SSC levels in urine and serum have been documented [2-4]. Although precise central nervous system SSC concentrations in these disorders are not well established, the plasma/urine metabolite levels in these reports reach micromolar ranges far above physiological baseline. Thus, using SSC at 100-300 μM in our in vitro models aims to mimic toxic SSC accumulation rather than normal levels. Given SSC's role as a glutamate agonist, it likely acts via NMDA receptor stimulation. Previous studies suggest that NMDA receptor–mediated activation of PARP-1 can lead to AIF translocation and caspase-independent cell death [22, 23]. In discussing SSC-induced caspase-independent apoptosis, we referred to the potential involvement of PARP-1. This statement is intended as a hypothesis based on prior literature linking NMDA receptor activation to PARP-1–dependent AIF release and subsequent cell death. Since we did not directly assess PARP-1 activity in the present study, these remarks remain speculative. Future work in our laboratory will include PARP-1 inhibition assays to validate its contribution experimentally to SSC-induced apoptosis. Calpain, a calcium-dependent cysteine protease, also plays a key role in SSC-induced apoptosis. Its activation—triggered by calcium influx following excitotoxic insult—has been widely associated with neuronal injury [24, 25, 6]. Our data show increased calpain levels in both neuronal types after SSC treatment, and calpain inhibition significantly reduced its activation and improved neuronal survival, in agreement with previous neuroprotection studies [26]. Cytochrome c, a hallmark of caspase-dependent apoptosis, was significantly elevated only in cortical neurons, suggesting SSC triggers this pathway in the cortex but not in the hippocampus. Our findings are in line with recent evidence demonstrating that cortical and hippocampal neurons display differential susceptibility to NMDA receptor-mediated excitotoxic stress [13]. Consistent with our results, newer reports emphasize that oxidative stress and calcium overload contribute to regional differences in excitotoxic cell death [11, 27]. Methazolamide (MA), a cytochrome c release inhibitor, effectively reduced cytochrome c levels and cytotoxicity in cortical neurons. Although cytochrome c was not elevated in HT-22 neurons, MA still improved viability, possibly via indirect effects on mitochondrial function

or AIF modulation. An interesting observation was that methazolamide (MA) improved cell viability in HT-22 neurons, despite no significant cytochrome c elevation in this model. This effect can be explained by MA's broader mitochondrial protective actions beyond cytochrome c inhibition. Previous studies have demonstrated that MA can reduce mitochondrial oxidative stress, stabilize mitochondrial membrane potential, and indirectly influence AIF-dependent apoptosis. Therefore, the beneficial effect of MA in HT-22 cells may reflect these pleiotropic mitochondrial actions rather than a direct effect on cytochrome c release. Similarly, caspase-3 was activated only in cortical neurons, corroborating literature that supports regional differences in susceptibility to glutamate toxicity [28, 14]. In the present study, apoptotic mechanisms were primarily investigated using ELISA-based quantification of key proteins (AIF, calpain, cytochrome c, and caspase-3). While these assays provide quantitative and reproducible evidence of pathway activation, they remain indirect compared to imaging- or blot-based methods. Confirmatory assays such as TUNEL staining, Annexin-V flow cytometry, or Western blotting for cleaved caspase-3 would further validate the mechanistic conclusions. Although these experiments could not be included due to resource limitations, our findings are consistent with previously reported patterns of excitotoxic neuronal apoptosis, supporting the reliability of the present results. Future studies will integrate complementary molecular assays to strengthen the mechanistic framework of SSC-induced neuronal apoptosis. Time-course analysis of glutathione (GSH) levels provided further support for SSC-induced oxidative stress. In both cell types, GSH levels initially rose (2–8 h), likely reflecting a compensatory antioxidant response, followed by a decline (8–16 h), coinciding with apoptotic activation. This biphasic GSH pattern aligns with oxidative stress–mediated apoptosis and supports the timing of apoptotic marker assessment at 24 hours. SSC LD_{50} values were consistent with previous studies [6], and dose-finding for AIF, calpain, and cytochrome c inhibitors established effective concentrations. NF at 10 nM preserved neuronal viability in both cell types, contrasting with prior studies using μM doses in other systems [29, 30]. Calpain inhibitor MDL-28170 was effective at 10 μM, consistent with literature on excitotoxicity prevention [31]. Likewise, MA at 10–20 μM aligned with established neuroprotective ranges [32, 33]. Rationale for Cell Line Selection. HT-22 and PCNs were selected based on their regional relevance and differing susceptibility to excitotoxicity: Comparative Vulnerability: SSC increased AIF and calpain levels in both cell lines, but only cortical neurons showed significant elevation of caspase-3 and cytochrome c, suggesting greater vulnerability. NMDA Receptor Density: These receptors are highly expressed in the hippocampus and cortex, the primary targets of glutamate analogs like SSC. Differentiation Capacity: Both lines can be differentiated, and N2 supplement was used in this study. Although not directly compared here, future work should assess the impact of differentiation on SSC sensitivity.

Study limitations

This study has several limitations that should be acknowledged. First, HT-22 cells at passage 16 were used, which may carry the risk of phenotypic drift; using lower-passage or primary cells would have been preferable. Similarly, the PCNs had been cryopreserved prior to use, which might have influenced their baseline viability. Another limitation is that apoptotic protein measurements were restricted to a single main time point, as material constraints prevented the assessment of multiple intervals. Importantly, confirmatory apoptosis assays such as TUNEL staining, Annexin-V assays, or Western blot/RT-PCR analyses were not performed. These techniques would provide additional mechanistic validation beyond ELISA-based detection of apoptotic proteins. Their absence reflects financial and technical constraints rather than a conceptual omission, and we acknowledge that incorporating such assays in future investigations will be essential to fully confirm the apoptotic signaling pathways identified here. Antioxidant analysis was also confined to glutathione levels, and additional enzymes such as superoxide dismutase and catalase should be evaluated in future studies. Finally, dose–response analyses and caspase-3 inhibition experiments could not be conducted due to budgetary constraints, which should be addressed in subsequent investigations. Looking ahead, future studies should extend these findings into in vivo models to better capture the complexity of neuronal

microenvironments and excitotoxic injury. Moreover, genetic approaches such as targeted knockdown of AIF, caspase-3, or calpain could provide direct mechanistic evidence to complement the current ELISA-based protein measurements. Combining pharmacological inhibition with gene silencing strategies may offer a more comprehensive understanding of the relative contributions of caspase-dependent and caspase-independent pathways in SSC-induced neurotoxicity.

In conclusion, this study provides novel insight into the differential apoptotic mechanisms activated by SSC in neuronal cells. SSC induces caspase-independent apoptosis via AIF and calpain in HT-22 neurons and both caspase-dependent and -independent apoptosis in PCNs. These findings suggest region-specific vulnerability and therapeutic potential for AIF, calpain, and cytochrome c inhibitors in SSC-related neurodegeneration.

Key findings:

1. SSC LD_{50} was 150 μM (HT-22) and 155 μM (cortical neurons).

2. Non-toxic Effective concentrations for AIF, calpain-1, and cytochrome c inhibitors were identified.

3. SSC elevated GSH at 2–8 h and reduced it at 8–16 h in both cell types.

4. SSC increased AIF and calpain (HT-22); AIF, calpain, cytochrome c, and caspase-3 (cortical neurons).

5. Calpain inhibitor reduced calpain levels in both cell types.

6. Methazolamide suppressed cytochrome c in cortical neurons only.

7. AIF inhibitor reduced AIF levels in HT-22 neurons; viability improved in both types.

8. Co-treatment with all three inhibitors significantly reduced SSC-induced cytotoxicity in both neuronal models.

Acknowledgments: This study was supported by the Scientific Research Projects Coordination Unit of Pamukkale University (project no: 2020SABE17) and by the TÜBİTAK 1002 Rapid Support Program (project no: 120Z825).

Author contributions: A.A., E.A. and V.K. contributed to the conceptualization, design, funding and supervision of the study. A.A., A.C., M.T.A., E.K.T. conducted all experiments and wrote the first draft of the manuscript. A.A., E.A., V.K., A.C., M.T.A. and E.K.T. collected, analyzed and interpreted the data. All authors contributed to the critical revision of the manuscript and have read and approved the final version.

Conflict of interest: The authors have no conflicts of interest.

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