

# N-Acetylcysteine Induces Apoptotic, Oxidative and Excitotoxic Neuronal Death in Mouse Cortical Cultures

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N-acetylcysteine (NAC) has been used as an antioxidant to prevent oxidative cell death. However, we found NAC itself to induce neuronal death in mouse cortical cultures. Therefore, the current study was performed to investigate the mechanism of neuronal death caused by NAC. Cell death was assessed by measuring lactate dehydrogenase efflux to bathing media after 24-48 h exposure to NAC. NAC (0.1-10 mM) induced neuronal death in a concentration- and exposure time-dependent manner. However, NAC did not injure astrocytes even at a concentration of 10 mM. Also, 10 mM NAC markedly attenuated oxidative astrocyte death induced by 0.5 mM diethyl maleate or 0.25 mM H<sub>2</sub>O<sub>2</sub>. The NMDA receptor antagonist MK-801 (10 μM) markedly attenuated the neuronal death caused by 10 mM NAC, while NBQX did not affect the neuronal death. Cycloheximide (a protein synthesis inhibitor, 0.1 μg/mL) and z-VAD-FMK (a caspase inhibitor, 100 μM) also significantly attenuated neuronal death. Apoptotic features such as chromatin condensation, nuclear fragmentation, and caspase 3 activation were observed 1 h after the NAC treatment. The neuronal death induced by 1 or 10 mM NAC was significantly attenuated by the treatment with 100 μM Trolox or 1 mM ascorbic acid. NAC induced the generation of intracellular reactive oxygen species (ROS), as measured by the fluorescent dye 2',7'-dichlorofluorescein diacetate. The ROS generation was almost completely abolished by treatment with Trolox or ascorbic acid. These findings demonstrate that NAC can cause oxidative, apoptotic, and excitotoxic neuronal death in mouse neuronal cultures.

**Key Words:** *N-acetylcysteine; Apoptosis; Reactive Oxygen Species*

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## INTRODUCTION

N-acetylcysteine (NAC) is a representative thiol antioxidant that inhibits oxidative damage.<sup>1</sup> NAC has been used clinically as an antidote for acetaminophen overdose, a mucolytic for chronic obstructive pulmonary disease, a renal protectant in contrast-induced nephropathy, and a therapeutic agent in the management of HIV.<sup>2</sup> Recent evidence suggests that NAC may also have therapeutic benefits in multiple neuropsychiatric disorders.<sup>3</sup>

NAC reportedly inhibits oxidative neuronal death of spinal motor neurons,<sup>4</sup> rat sympathetic neurons,<sup>5</sup> and primary dopaminergic neurons.<sup>6</sup>

We found that NAC failed to prevent glutathione-depletion-induced oxidative neuronal death and induced neuronal

death in mouse cortical cultures. In the present study, to delineate the mechanisms of the NAC-induced neuronal death, we investigated the effects of some anti-excitotoxic agents, anti-apoptotic agents, and antioxidants on NAC-induced neuronal death. As a result, we have demonstrated that NAC induces oxidative and apoptotic neuronal death and excitotoxic neuronal death in mouse neuronal cultures.

## MATERIALS AND METHODS

### 1. Cortical cell cultures

Mixed cortical cell cultures including both neurons and glia were prepared from the pregnant ICR mice at 15-17 days of gestation as follows.<sup>7</sup> Fetal mouse brains were excised and then rinsed in cold Ca<sup>2+</sup>/Mg<sup>2+</sup>-free Hanks' bal-

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anced salt solution supplemented with 5 mg/mL glucose, 7 mg/mL sucrose, and 0.35 mg/mL sodium bicarbonate (DM). The meninges were carefully removed from the brain tissue under a stereomicroscope using fine-tipped forceps and a microsurgical knife. The cerebral cortex was dissected free and minced into 1-2 mm<sup>3</sup> sized pieces with a sterile scalpel. The cortex pieces were incubated for 15 min in DM containing 0.25% trypsin at 37°C and centrifuged at 1,000×g for 5 min. After removal of the supernatant, the tissue pellet was suspended in 1-2 mL of plating medium composed of Eagle's minimal essential medium (MEM) containing 2 mM glutamine, 5% fetal bovine serum (FBS), and 5% horse serum (HS). Cells were separated by 8 or 10 trituration passages using a flame-narrowed pipette. Dissociated cortical cells were plated onto the previously established glial layer in 24-well plates at a density of 3 hemispheres/plate (approximately 2.5×10<sup>5</sup> cells per well). The plates were placed in an incubator (Forma, USA) containing 5% CO<sub>2</sub> in humidified air at 37°C. Cytosine arabinoside was added to produce a final concentration of 10 μM at 5 days in vitro (DIV) and maintained for 2 days to halt non-neuronal cell division. The culture medium was changed twice a week after 7 DIV. Cultures were used at 13 or 14 DIV for the experiments. Cortical glial cultures were prepared from 1-2 days old postnatal ICR mice. Dissection and dissociation were performed as described above for mixed cortical cell cultures; cells were plated in 24-well plates at a 0.5 hemisphere/plate density. The plating medium was supplemented with Eagle's MEM containing 2 mM glutamine, 10% FBS, 10% HS, and 10 ng/mL epidermal growth factor. The plates were maintained in the same incubator. The culture medium was changed once a week after 14 DIV. Glial cultures were used for the plating of mixed cortical cell cultures between 18 and 24 DIV.

## 2. Drug treatment

After washing with MEM (with Earle's salts), cultures were exposed to NAC and selected test drugs for 24 or 48 h. Each row of the 24-well plates had 4 wells that received the same treatment. The four wells in the first row were treated with a sham wash, NMDA (500 μM) was used to kill all neurons in the second row, and the third to the sixth rows were treated with drugs at 4 to 8 μL in each well with culture media.

## 3. Measurement of cell death

Cell death was assessed morphologically under a phase-contrast microscope; cell death was then quantitatively assessed by measuring the activity of lactate dehydrogenase (LDH) in bathing media at 24 or 48 h after treatment. Medium samples (25 μL) were taken and diluted by mixing with 125 μL of reaction buffer and 100 μL of 0.3 mg/mL NADPH and 30 μL of 22.7 mM pyruvate solutions. Absorbance changes at 340 nm were monitored at 3 min after mixing with the pyruvate solution using a microplate reader (Molecular Devices, USA). The standard enzyme was purchased from Sigma-Aldrich (USA). After subtracting the

mean background value in control non-treated cultures (=0), each LDH value was scaled to the mean value in positive control cultures treated with 500 μM NMDA for 24 h (=100) that induced near-complete neuronal death in the absence of glial damage. The data are expressed as percentages (mean±SEM) of the activity values in the NMDA-treated cell group (complete kill).

## 4. SYTOX Green staining

For morphological evaluation of nuclei, SYTOX Green (Invitrogen, USA) staining was used. After fixing cells with 4% paraformaldehyde for 40 min at room temperature, the cells were permeabilized with 0.5% Triton X-100 for 10 min, stained with 1 μM SYTOX Green for 15 min, and mounted with a SlowFade Antifade kit (Molecular Probes, USA). Changes in nuclear shapes and patterns were observed, and photographs were taken under a fluorescent microscope (Ex/Em=504/523 nm).

## 5. Western blot analysis

Cortical cells were lysed in RIPA buffer (PBS containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 mM sodium fluoride, 2 mM tetrasodium pyrophosphate, 2 mM orthovanadate, 5 mM EDTA, 1 mM PMSF, 10 μg/mL pepstatin, 10 μg/mL aprotinin, 10 μg/mL leupeptin) and centrifuged at 13,000 rpm for 15 min. The pellet was discarded, and the supernatant was used for protein quantification. Equal amounts of protein from total cell lysates were electrophoresed on 12% SDS-PAGE and transferred to nitrocellulose membranes using Towbin buffer. The membranes were then incubated with anti-caspase-3 antibody (Santa Cruz Biotechnology, Inc.) overnight at 4°C. After washing with TBST (0.05% Tween 20 in Tris-buffered saline), the membranes were incubated with HRP-conjugated anti-rabbit secondary antibodies (Amersham; 1:2000) for 1 h at RT. Immunoreactive proteins were detected with luminol for ECL, and the signals were analyzed by quantitative densitometry using the LAS-3000 system (Fuji Photofilm Co.).

## 6. ROS measurement

Intracellular reactive oxygen species (ROS) were examined using 2,7-dichlorofluorescein diacetate (DCF, Invitrogen, USA). Cells were treated with 10 μM DCF for 30 min and then with NAC alone or in combination with Trolox and ascorbic acid. After treatments, ROS generation was monitored in a fluorescent multiwell plate reader (Fluoreskan Ascent FL, Labsystem, USA) with excitation at 485 nm and emission at 530 nm. Each fluorescence value was corrected by subtracting the mean background value of sham-treated control cultures. In some cases, cultures were viewed under fluorescence microscopy and photographed.

## 7. Reagents

Media for cell culture were purchased from Gibco BRL (Rockville, MD, USA). HEPES (acid), glucose, NaHCO<sub>3</sub>, NaCl, KCl, MgCl<sub>2</sub>, CaCl<sub>2</sub>, NaOH, phenol red, trypsin, cyto-

sine arabinoside, epidermal growth factor, sucrose, ascorbate, cycloheximide, N-acetylcysteine, Trolox, and ascorbic acid were purchased from Sigma-Aldrich. N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-FMK) and MK-801 (RBI, USA) were purchased. Fetal bovine and horse sera from Hyclone (Logan, UT, USA) were inactivated by keeping at 55°C for 30 min before use.

## 8. Data analyses

Results are expressed as mean±SEM values; differences between effects were statistically evaluated using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls post hoc test. Statistical significance was accepted at p-values<0.05.

## RESULTS

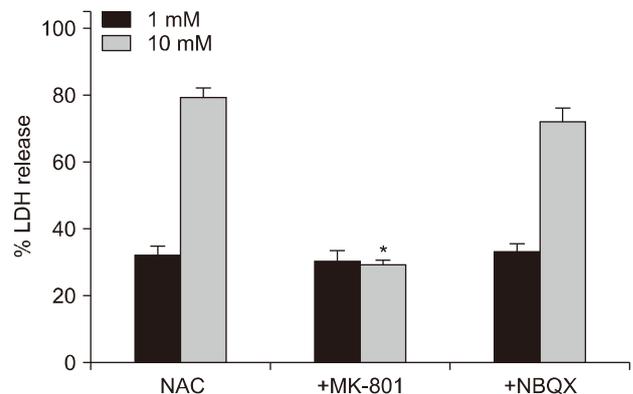
To determine the effect of NAC on cortical neuronal cell survival, mixed cortical cell cultures were treated with NAC at three concentrations (0.1, 1, 10 mM) for 24 and 48 h. Cell death was quantitated by measuring LDH activity in media. Treatment with 0.1 mM NAC for 24 h induced minor neuronal death (11±2.3%; n=12), while increasing the concentrations to 1 and 10 mM increased neuronal death to 41±4.9% and 81±3.5%; n=12, respectively. After 48 h, cell death increased slightly at 0.1 mM (22±4.6%; n=12), and almost all neurons were damaged at 1 mM and 10 mM (89±6.1% and 96±5.4 %; n=12, respectively) (Fig. 1A).

In cortical glial cultures, treatment with 10 mM or even higher concentrations of NAC failed to injure astrocytes. However, treatment with 10 mM NAC almost completely inhibited oxidative astrocyte death induced by 0.5 mM diethyl maleate (DEM) or 0.25 mM H<sub>2</sub>O<sub>2</sub> (% cell death by DEM and H<sub>2</sub>O<sub>2</sub> from 84±2.0% and 79±4.6% to 2±0.7% and

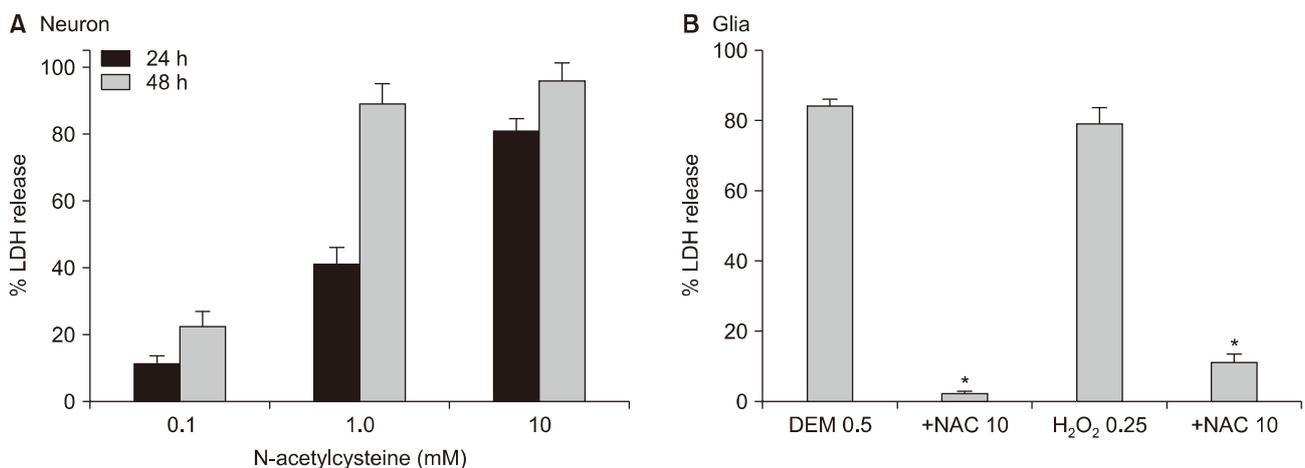
11±2.4%; n=8-12, respectively) (Fig. 1B).

To examine whether excitotoxicity contributes to the NAC-induced neuronal death, the effects of MK-801 (10 μM), an NMDA receptor antagonist, and NBQX (10 μM), an AMPA receptor antagonist, were investigated. Neuronal death by 1 mM NAC (32±2.7%; n=12) was not affected by MK-801 or NBQX treatment (30±3.2%; 33±2.4%; n=8 respectively). However, neuronal cell death by 10 mM NAC (79±3.1%; n=12) was significantly suppressed by the treatment with MK-801 (29±1.4%; n=8) but was not affected by the treatment with NBQX (72±3.9%; n=8) (Fig. 2).

SYTOX green nuclear staining and western blotting for caspase 3 were used to examine the involvement of apopto-



**FIG. 2.** Excitotoxicity through NMDA receptor involved in 10 mM N-acetylcysteine-induced neuronal death. Effects of NMDA receptor antagonist, MK-801 (10 μM), or AMPA receptor antagonist, NBQX (10 μM), on the 1 mM and 10 mM N-acetylcysteine (NAC)-induced neuronal death in cultured mouse cortical cells. Mean±SEM from 8-12 wells. \*Significantly different from the corresponding NAC-treated group (p<0.01).



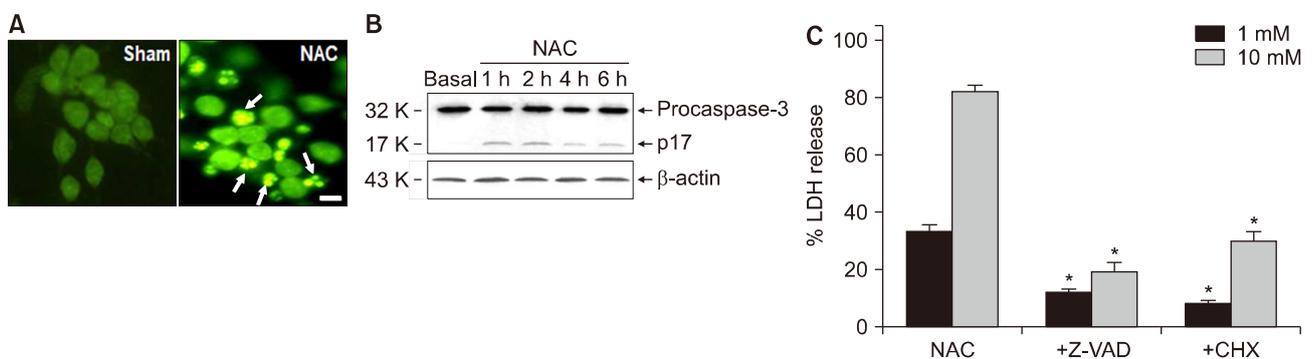
**FIG. 1.** N-acetylcysteine induced selective neuronal death without any glial cell death. (A) Concentration- and exposure time-dependent neurotoxic effects of N-acetylcysteine (0.1, 1, 10 mM) in cultured mouse cortical cells. Cell death was assessed by measuring culture media's lactate dehydrogenase (LDH) activity at the end of 24 h or 48 h exposure. Each column and vertical bar represent the mean±SEM from 8-24 wells. (B) Effects of 10 mM N-acetylcysteine (NAC) on the glial death induced by 0.5 mM diethyl maleate (DEM) or 0.25 mM H<sub>2</sub>O<sub>2</sub> at the end of 24 h exposure in cortical glial cultures. Mean±SEM from 8-12 wells. \*DEM or H<sub>2</sub>O<sub>2</sub> results significantly different from the control group (p<0.01).

sis in neuronal death. Chromatin condensation and nuclear fragmentation, a morphologic hallmark of apoptosis, and the activation of caspase 3, an important marker of apoptosis, were observed 1 h after treatment with 10 mM NAC (Fig. 3A, B). Furthermore, treatment with anti-apoptotic agents such as cycloheximide (CHX, 0.1  $\mu$ g/mL), a protein synthesis inhibitor, and z-VAD-FMK (100  $\mu$ M), a non-specific caspase inhibitor, also significantly attenuated the NAC-induced neuronal death (% cell death by 1 and 10 mM NAC from 33 $\pm$ 2.5% and 82 $\pm$ 2.0% to 8 $\pm$ 1.0% and 30 $\pm$ 3.1% by CHX and 12 $\pm$ 1.0% and 19 $\pm$ 3.4% by z-VAD-FMK; n=8-12, respectively) (Fig. 3C).

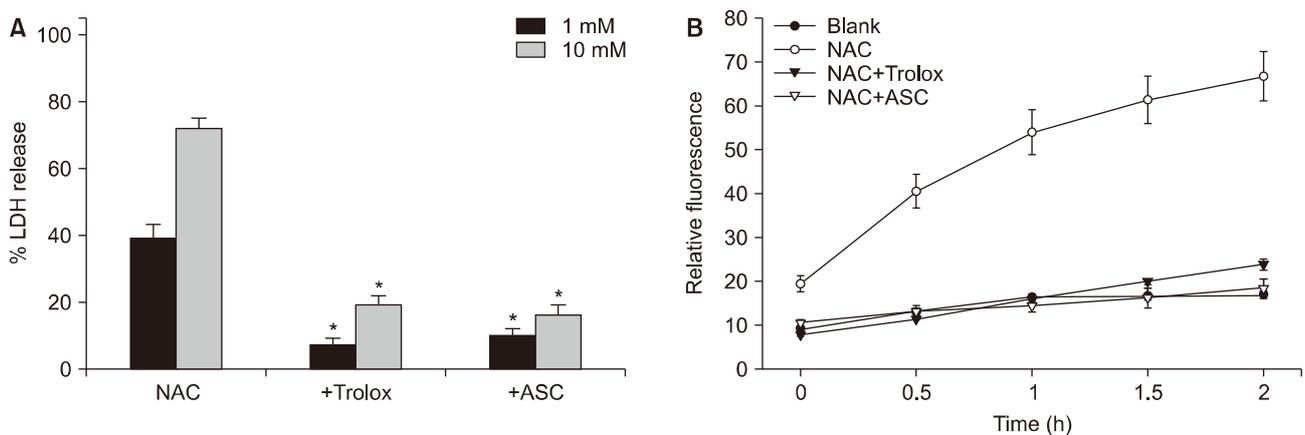
To examine whether oxidative damage contributes to the NAC-induced neuronal death, the effects of anti-oxidants, Trolox (100  $\mu$ M) and ascorbic acid (1 mM) were

examined. The neuronal death by 1 and 10 mM NAC (39 $\pm$ 4.1% and 72 $\pm$ 2.9%; n=8, respectively) were inhibited significantly by treatment with Trolox (7 $\pm$ 2.1% and 19 $\pm$ 2.8%; n=8, respectively) and ascorbic acid (10 $\pm$ 1.8% and 16 $\pm$ 3.1%; n=8, respectively) (Fig. 4A).

To examine whether NAC generates reactive oxygen species (ROS) in cells, ROS were measured using DCF, a fluorescent indicator for intracellular ROS. The relative fluorescence generation of 19.3 $\pm$ 5.0 (n=8) before NAC treatment increased significantly at 30 min after NAC treatment (40 $\pm$ 3.8; n=8) and gradually increased with time (1 h, 1.5 h, 2 h; 53.9 $\pm$ 5.2, 61.4 $\pm$ 5.3, 66.8 $\pm$ 5.6, respectively; n=8). ROS generation was almost abolished by the treatment with Trolox or ascorbic acid (Fig 4B).



**FIG. 3.** N-acetylcysteine induced apoptotic neuronal death in cortical cultures. (A) Photomicrographs showing chromatin condensation and nuclear fragmentation with SYTOX Green nuclear staining in cultured mouse cortical cells. Sham: sham wash, NAC: 1 h after exposure to 10 mM N-acetylcysteine. Arrows indicate the fragmented and condensed chromatin stained with SYTOX green. (B) Effect of N-acetylcysteine on the activation of caspase 3 in cultured cortical cells. Cell extracts were prepared from cultures treated with 10 mM N-acetylcysteine for the indicated times and subjected to SDS-PAGE and western blot analysis. Caspase 3 levels were detected using the caspase 3 antibody. (C) Effects of the caspase inhibitor z-VAD-FMK (100  $\mu$ M) or the protein synthesis inhibitor cycloheximide (CHX, 0.1  $\mu$ g/mL) on 1 mM and 10 mM N-acetylcysteine (NAC)-induced neuronal death in cultured mouse cortical cells. Mean $\pm$ SEM from 8-12 wells. \*Significantly different from the corresponding NAC-treated group ( $p < 0.01$ ).



**FIG. 4.** N-acetylcysteine induced oxidative neuronal death in cortical cultures. (A) Effects of Trolox (100  $\mu$ M) and ascorbic acid (ASC, 1 mM), on the 1 mM and 10 mM N-acetylcysteine (NAC)-induced neuronal death in cultured mouse cortical cells. Mean $\pm$ SEM from 8 wells. \*Significantly different from the corresponding NAC-treated group ( $p < 0.01$ ). (B) Time-course of ROS generation by 10 mM N-acetylcysteine (NAC) and the effects of treatment with Trolox (100  $\mu$ M) and ascorbic acid (ASC, 1 mM) on the NAC-induced ROS generation in cultured mouse cortical cells. Cells were exposed to 11 mM 2',7'-dichlorofluorescein diacetate (DCF-DA) for 1 h at 37 $^{\circ}$ C. DCF fluorescence was measured by a fluoro-spectrophotometer (Ex, 485 nm; Em, 538 nm). Mean $\pm$ SEM from 8 wells.

## DISCUSSION

In the present study, NAC unexpectedly induced concentration-dependent neuronal death (Fig. 1A). Some reports have suggested that NAC can cause neuronal death in rat cortical culture,<sup>8</sup> copper-dependent neuronal death in rat cerebellar culture,<sup>9</sup> and 6-OHDA-induced neuronal death in cultured dopaminergic neurons.<sup>10</sup>

In contrast, NAC treatment did not induce any glial cell death. Furthermore, NAC treatment completely inhibited the oxidative glial cell death caused by DEM or H<sub>2</sub>O<sub>2</sub> (Fig. 1B). NAC is known to inhibit oxidative damage in various types of cells.<sup>11-14</sup> Our result also indicates that NAC acts as a powerful antioxidant in glial cells in this study.

In the present study, treatment with NBQX, an AMPA antagonist, failed to inhibit the neuronal death induced by NAC. However, treatment with MK-801, an NMDA receptor antagonist, significantly inhibited the neuronal death caused by 10 mM NAC, although it did not affect the neuronal death by 1 mM NAC (Fig. 2). This result suggests that the activation of the AMPA receptor is not involved in the neuronal cell death induced by NAC, but the activation of NMDA receptor may be involved in the neuronal death caused by NAC; due to the conversion of NAC to cysteine, NAC and cysteine can act as weak NMDA receptor agonists.<sup>8,15</sup>

Apoptotic features such as chromatin condensation, nuclear fragmentation, and caspase 3 activation were observed in NAC-induced neuronal death (Fig. 3A, B). Furthermore, anti-apoptotic agents CHX and z-VAD-fmk significantly inhibited NAC-induced neuronal death (Fig. 3C). These findings demonstrate that NAC induces neuronal apoptosis in cortical cultures.

Treatment with ascorbic acid and Trolox antioxidants significantly inhibited the NAC-induced neuronal death (Fig. 4A). ROS generation after exposure to 10 mM NAC (as measured by cellular DCF fluorescence) was also significantly reduced by treatment with either Trolox or ascorbic acid (Fig. 4B). This finding is difficult to interpret because NAC, a representative thiol antioxidant, caused oxidative damage by generating ROS. However, some reports have reported that antioxidants can act as pro-oxidants under certain conditions.<sup>16-18</sup> Interestingly, there is some evidence that NAC can modulate the levels of essential trace elements such as zinc and copper,<sup>19</sup> and that it can potentiate oxidative cell death in cancer cells<sup>20</sup> and cultured neurons.<sup>9</sup> Even though we cannot delineate the mechanisms of oxidative cell death induced by NAC in our cortical cultures, our findings and other reported data have shown NAC can cause oxidative neuronal death.

In conclusion, our findings suggest that NAC can cause not only oxidative and apoptotic but also excitotoxic neuronal death in mouse neuronal cultures.

## CONFLICT OF INTEREST STATEMENT

None declared.

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