RESEARCH ARTICLE

Selenocysteine antagonizes oxygen glucose deprivation-induced damage to hippocampal neurons

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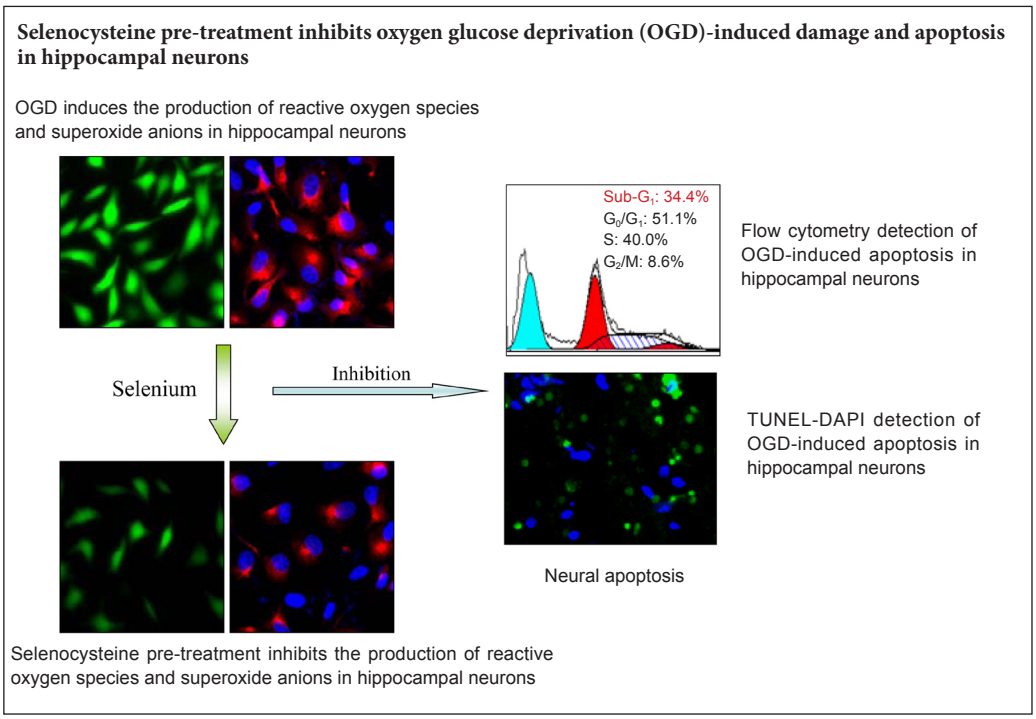
Graphical Abstract



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Abstract

Designing and/or searching for novel antioxidants against oxygen glucose deprivation (OGD)-induced oxidative damage represents an effective strategy for the treatment of human ischemic stroke. Selenium is an essential trace element, which is beneficial in the chemoprevention and chemotherapy of cerebral ischemic stroke. The underlying mechanisms for its therapeutic effects, however, are not well documented. Selenocysteine (SeC) is a selenium-containing amino acid with neuroprotective potential. Studies have shown that SeC can reduce irradiation-induced DNA apoptosis by reducing DNA damage. In this study, the *in vitro* protective potential and mechanism of action of SeC against OGD-induced apoptosis and neurotoxicity were evaluated in HT22 mouse hippocampal neurons. We cultured HT22 cells in a glucose-free medium containing 2 mM $Na_2S_4O_2$, which formed an OGD environment, for 90 minutes. Findings from MTT, flow cytometry and TUNEL staining showed obvious cytotoxicity and apoptosis in HT22 cells in the OGD condition. The activation of Caspase-7 and Caspase-9 further revealed that OGD-induced apoptosis of HT22 cells was mainly achieved by triggering a mitochondrial-mediated pathway. Moreover, the OGD condition also induced serious DNA damage through the accumulation of reactive oxygen species and superoxide anions. However, SeC pre-treatment for 6 hours effectively inhibited OGD-induced cytotoxicity and apoptosis in HT22 cells by inhibiting reactive oxygen species-mediated oxidative damage. Our findings provide evidence that SeC has the potential to suppress OGD-induced oxidative damage and apoptosis in hippocampal neurons.

Key Words: *selenium; selenocysteine; ischemic stroke; oxygen glucose deprivation; hippocampal neuron; mitochondria; reaction oxygen species; superoxide anion; oxidative damage; apoptosis*

Introduction

Treating human stroke represents one of the biggest challenges in clinical practice. Approximately 80% of strokes are due to cerebral ischemia, which can lead to severe neurological deficits and eventually death (Sveinsson et al., 2014; Mozaffarian et al., 2016; Wang et al., 2017a; Chang et al., 2018). The human brain is rich in polyunsaturated fatty acids, but contains low levels of endogenous antioxidant enzymes. Neurons are especially vulnerable to oxidative damage after cessation of blood flow to brain tissue (Juurlink and Sweeney, 1997; Li et al., 2007). Excess accumulation of reactive oxygen species (ROS) causes oxidative damage and can even lead to cell apoptosis (Fan et al., 2017a, b). Oxygen glucose deprivation (OGD) is the key condition underlying the pathogenesis of cerebral ischemia, resulting in oxidative damage and apoptosis. Therefore, designing and/or searching for effective novel antioxidants against OGD-mediated oxidative damage represents an effective strategy in the treatment of cerebral ischemia.

Selenium is an indispensable trace element, which is a fundamental component of selenoproteins and antioxidant enzymes in animals, including humans. It exhibits novel antioxidant properties by maintaining redox homeostasis (Fan et al., 2014). Considerable evidence indicates that taking a selenium supplement can effectively reduce the risk of human diseases (Gupta et al., 2003; Ozbal et al., 2008; Mehta et al., 2012; Fan et al., 2014; Pillai et al., 2014; Wang et al., 2016a, b; Zhao et al., 2017). Selenium inhibits the formation of ROS, which can harm synapses, block cell communication, and even result in neuronal apoptosis (Wang et al., 2012, 2016c; Singh et al., 2013). Selenocystine (SeC), a selenium-containing amino acid, exhibits novel protective potential (Zhang et al., 2008; Chen and Wong, 2009). Kunwar et al. reported that SeC can prevent γ-radiation-induced genotoxicity by reducing DNA damage (Kunwar et al., 2011). They also reported that 3,3′-diselenodipropionic acid (DSePA), a SeC derivative, attenuates radiation-induced DNA apoptosis by reducing DNA damage (Kunwar et al., 2010). Our previous study also showed that DSePA has the potential to suppress oxidative damage-induced neurotoxicity by inhibiting ROS generation and apoptosis in PC12 cells (Wang et al., 2016c). However, whether and how SeC might antagonize OGD-induced neurotoxicity in HT22 mouse hippocampal neurons has not been investigated. Therefore, in the present study, we evaluated the protective potential and mechanisms of action of SeC against OGD-induced neurotoxicity in an *in vitro* cell model.

Materials and Methods

Chemicals

SeC, sodium hydrosulfite ($Na_2S_4O_2$), MTT, dimethylsulfoxide (DMSO), propidium iodide (PI) and all other reagents were bought from Sigma (St. Louis, MO, USA). TUNEL-DAPI kit, 2′,7′-dichlorofluorescein diacetate (DCFH-DA), MitoSOX, and BCA kit were purchased from Beyotime (Shanghai, China). Caspase substrates for caspase-3, -8 and -9 and all antibodies were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA).

Cell culture and drug treatment

HT22 mouse hippocampal neurons were bought from KeyGen Biotech (Nanjing, China) and were cultured with Dulbecco's modified Eagle medium-Ham's nutrient mixture F-12 (DMEM-F12) with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) at 37°C and 5% CO_2. Glucose deprivation was achieved using a glucose-free Earle's solution (Invitrogen). Experiments were carried out on four groups: the control group (untreated cells), the OGD group (104 cells in 96-well plates treated with glucose-free medium + 2 mM $Na_2S_4O_2$ for 90 minutes), the SeC-treated group (cells treated with SeC for 7.5 hours) and the protective group (cells pre-treated with SeC for 6 hours, then treated with OGD for 90 minutes). Subsequently, the MTT method was used to detect cell viability, as described previously (Fan et al., 2014).

Flow cytometry analysis

After measuring cell viability, cell apoptosis was assessed using flow cytometry (BD Biosciences, Bedford, MA, USA) (Fan et al., 2017a). HT22 cells were incubated with 0.4 or 0.6 μM SeC for 6 hours and/or cultured under OGD (glucose-free medium + 2 mM $Na_2S_4O_2$) for 90 minutes. After treatment, cells were harvested, fixed and loaded with PI. The Sub-G1 peak was used to quantify the number of apoptotic cells. Cell cycle distribution was analyzed using ModFit software (BD Biosciences). About 1×10^4 cells per sample were recorded. Optical density was used to measure cell viability, expressed as a percentage of control values.

TUNEL-DAPI assay

After flow cytometry analysis, OGD-induced apoptosis in HT22 cells was measured using the TUNEL-DAPI kit (Beyotime), as previously described (Fan et al., 2017a). Briefly, cells were pre-treated with 0.6 μM SeC for 6 hours and/or cultured under OGD (glucose-free medium + 2 mM $Na_2S_4O_2$) for 90 minutes. Then, cells were fixed, permeabilized, and incubated with TUNEL working solution and DAPI solution, respectively. After washing, cells were imaged under an Eclipse80i fluorescence microscope (magnification, 200×; Nikon, Tokyo, Japan). The number of TUNEL-positive cells was manually counted and expressed as a percentage of the control count.

Evaluation of caspase activation

After quantifying cell apoptosis using flow cytometry and the TUNEL-DAPI assay, caspase activation was measured using specific caspase substrates (Ac-DEVD-AMC for caspase-3, Ac-IETD-AMC for caspase-8, and Ac-LEHD-AMC for caspase-9; all obtained from CST). HT22 cells were pre-treated with 0.4 and 0.6 μM SeC for 6 hours and/or cultured under OGD (glucose-free medium + 2 mM $Na_2S_4O_2$) for 90 minutes. Cells were then collected and total protein was prepared. 100 μg of total protein per well was incubated with caspase substrates at 37°C for 2 hours. Caspase activa-

tion was determined using a microreader, as described previously (Fan et al., 2014). These procedures were performed at least three times, and the mean caspase activation was expressed as a percentage of control values.

Detection of ROS and superoxide anions

After measuring cell apoptosis, the intracellular oxidative status of HT22 cells was evaluated using specific fluorescent substrates. Briefly, cells were pre-labeled with 10 μM DCFH-DA or 10 μM MitoSOX. Then, cells were washed and pre-treated with 0.6 μM SeC for 6 hours and/or cultured under OGD (glucose-free medium + 2 mM $Na_2S_4O_2$) for 90 minutes. The production of ROS and superoxide anions was then quantified under a fluorescence microscope (Olympus, Tokyo, Japan).

Western blot assay

After assessing cell viability and apoptosis, the underlying mechanisms were examined by western blotting. Cells were pre-treated with 0.6 μM SeC for 6 hours or/and cultured under OGD (glucose-free medium + 2 mM $Na_2S_4O_2$) for 90 minutes. Total protein was prepared and 40 μg was loaded per lane for separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein was then transferred from the blot onto a nitrocellulose membrane, blocked and incubated overnight with anti-rabbit monoclonal primary antibodies (1:1000; CST) at 4°C. Primary antibodies included cleaved-PARP, active-caspase-3, active-caspase-7, active-caspase-9, Ser1981-ATM, Ser428-ATR, Ser15-p53, total-p53 and Ser139-H2A (all obtained from CST). The membrane was then incubated with goat-anti monoclonal second antibodies IgG (1:2000; CST), at 37°C for 2 hours. The target protein of interest was imaged on X-ray films using an enhanced chemiluminescence system (Kodak, Japan). β-actin was used as the reference band. Protein expression (relative optical density) was quantified by Quantity-One Software (Bio-Rad, CA, USA) based on the optical density of β-actin.

Statistical analysis

All data and images were obtained from at least three experiments and mean values were used for all statistical analyses. SPSS 17.0 software (SPSS, Chicago, IL, USA) was used to perform the statistical analysis. Data shown are presented as the mean ± SD. Intergroup comparisons were performed using a one-way analysis of variance, followed by the least significant difference test with a significance level of $\alpha = 0.05$.

Results

SeC alleviates OGD-induced cytotoxicity in HT22 cells

As shown in **Figure 1A**, SeC (0.2–1.0 μM) treatment alone was not toxic to HT22 cells. In contrast, cells cultured under OGD conditions (glucose-free medium + 2 mM $Na_2S_4O_2$) showed a time-dependent decline in cell viability (**Figure 1B**). For instance, cells cultured under OGD for 60, 90 and 120 minutes significantly decreased in cell viability to 75.3%, 64.2% and 51.8%, respectively. However, SeC pre-treatment markedly alleviated OGD-induced cytotoxicity in HT22 cells. Pre-treatment of cells with 0.4 and 0.6 μM SeC for 6 hours (**Figure 1C**) significantly increased survival in OGD-treated cells from 68.5% (treated with $Na_2S_4O_2$) to 76.9% and 89.4%, respectively. Moreover, SeC pre-treatment also improved measures of cell morphological change. As shown in **Figure 1D**, SeC effectively reduced cell shrinkage, increased cell numbers and increased cell-to-cell contact in HT22 cells under the OGD condition. These results demonstrate that SeC alleviates OGD-induced cytotoxicity *in vitro*.

SeC suppresses OGD-induced apoptosis in HT22 cells

The OGD-induced cell death model system was explored using flow cytometry analysis. As shown in **Figure 2A**, cells cultured under OGD conditions showed significant cell apoptosis, as revealed by the increase of the Sub-G1 peak. However, SeC pre-treatment dramatically suppressed OGD-induced apoptosis. Pre-treatment of cells with 0.4 and 0.6 μM SeC for 6 hours significantly suppressed cell apoptosis from 34.4% (OGD condition) to 23.8% and 10.2%, respectively. SeC treatment alone did not differ in levels of apoptosis in HT22 cells compared with control. Statistical analysis of the Sub-G1 peak (quantifying apoptotic cells) confirmed this protective effect (**Figure 2B**). There was no significant effect on cell cycle distribution (**Figure 2C**). Furthermore, TUNEL-DAPI assay results suggested that cells cultured under OGD exhibited obvious HT22 cell apoptosis, as indicated by the increased green fluorescence of TUNEL-positive cells (**Figure 2D, E**). As expected, SeC pre-treatment significantly reduced apoptosis in HT22 cells (**Figure 2D, E**). Taken together, both of the cell viability methods used indicate that SeC has the potential to antagonize OGD-induced HT22 cell apoptosis.

Role of mitochondrial-mediated apoptosis

Apoptosis can be triggered by mitochondrial (intrinsic) or death receptor (extrinsic) pathways, which are widely accepted as the two most important cell death pathways (Yagami et al., 2014). To characterize the specific cell death mechanism induced by OGD, the status of intracellular caspase activation was evaluated with three specific caspase substrates. As shown in **Figure 3A**, cells cultured under OGD conditions (glucose-free medium + 2 mM $Na_2S_4O_2$) showed significant activation of caspase-3, -8 and -9, indicating that OGD triggered mitochondrial and death receptor apoptosis pathways. Caspase-9, the main initiator of the mitochondrial-mediated cell death signal, was more active than caspase-8 under OGD conditions. However, SeC pre-treatment significantly inhibited OGD-induced caspase activation. Western blot results further confirmed this protective effect. As shown in **Figure 3B**, cells cultured under OGD showed obvious PARP cleavage and activation of caspase. SeC pre-treatment reduced OGD-induced PARP cleavage and caspase activation. Taken together, these results show that SeC has the potential to antagonize mitochondrial-mediated apoptosis in OGD-treated cells.

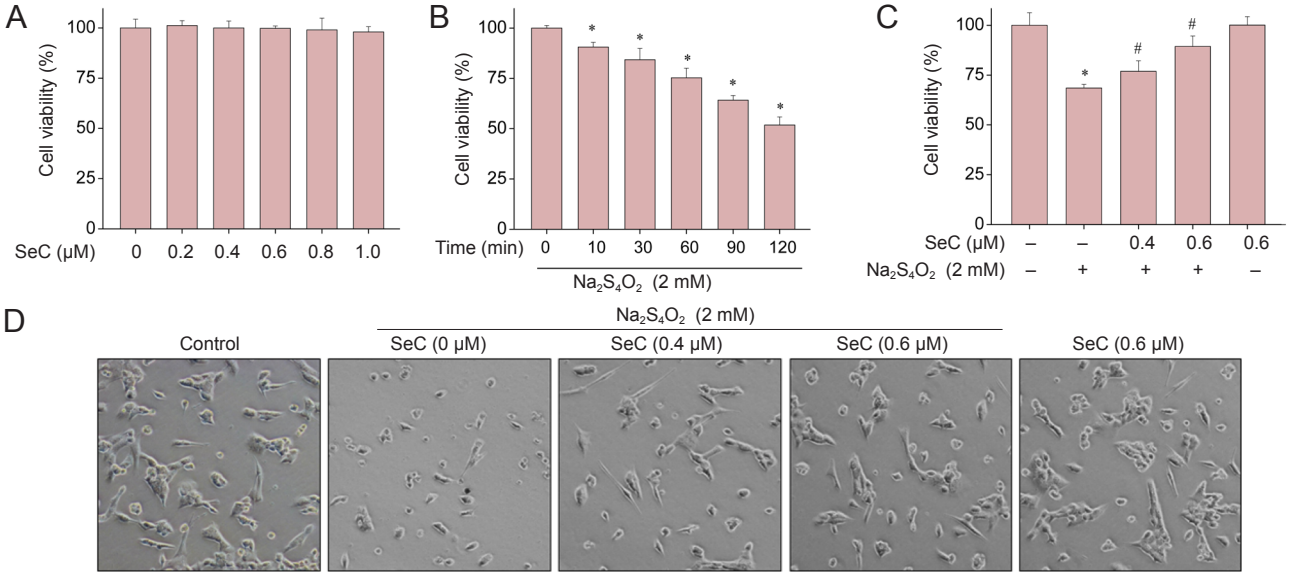

Figure 1 Selenocysteine (SeC) inhibits oxygen glucose deprivation (OGD)-induced cytotoxicity in HT22 cells.
Cytotoxicity of SeC alone (A) and OGD (B) in HT22 cells. (C) SeC reduced OGD-induced neurotoxicity. HT22 cells were pre-treated with 0.4 and 0.6 μM SeC for 6 hours (h) and cultured under OGD condition (glucose-free medium + 2 mM $Na_2S_4O_2$) for 90 minutes (min). Cell viability was measured by the MTT assay. (D) Morphological observation. Cells were imaged by inverted microscopy (original magnification, 200×). ∗P < 0.05, *vs.* control; #P < 0.05, *vs.* OGD treatment (mean ± SD, n = 3, one-way analysis of variance followed by the least significant difference test). Experiments were performed at least three times. The optical density was used as a measure of cell viability, and expressed as a percentage of the control. $Na_2S_4O_2$: Sodium hydrosulfite.

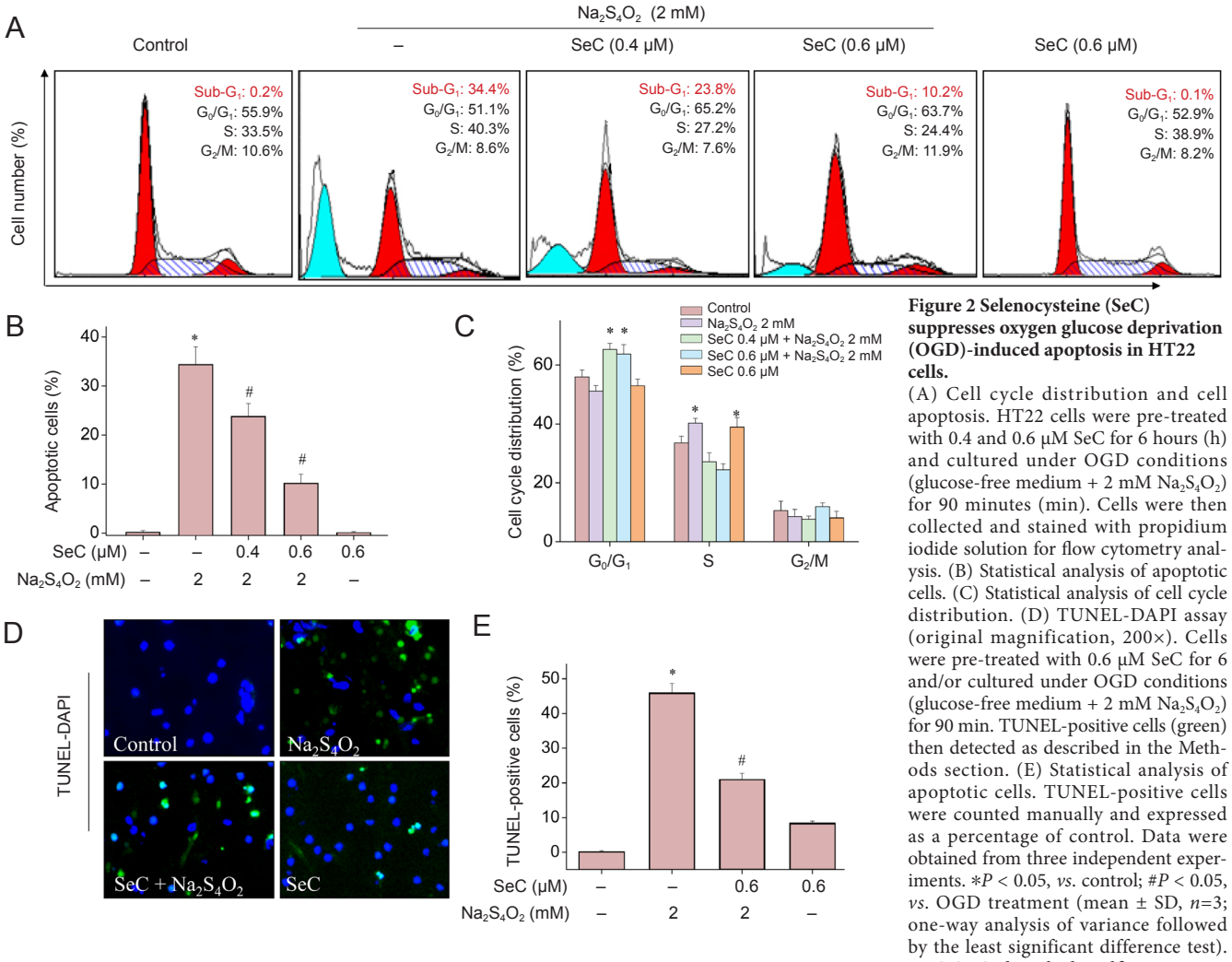

Figure 2 Selenocysteine (SeC) suppresses oxygen glucose deprivation (OGD)-induced apoptosis in HT22 cells.
(A) Cell cycle distribution and cell apoptosis. HT22 cells were pre-treated with 0.4 and 0.6 μM SeC for 6 hours (h) and cultured under OGD conditions (glucose-free medium + 2 mM $Na_2S_4O_2$) for 90 minutes (min). Cells were then collected and stained with propidium iodide solution for flow cytometry analysis. (B) Statistical analysis of apoptotic cells. (C) Statistical analysis of cell cycle distribution. (D) TUNEL-DAPI assay (original magnification, 200×). Cells were pre-treated with 0.6 μM SeC for 6 and/or cultured under OGD conditions (glucose-free medium + 2 mM $Na_2S_4O_2$) for 90 min. TUNEL-positive cells (green) then detected as described in the Methods section. (E) Statistical analysis of apoptotic cells. TUNEL-positive cells were counted manually and expressed as a percentage of control. Data were obtained from three independent experiments. ∗P < 0.05, *vs.* control; #P < 0.05, *vs.* OGD treatment (mean ± SD, n=3; one-way analysis of variance followed by the least significant difference test). $Na_2S_4O_2$: Sodium hydrosulfite.

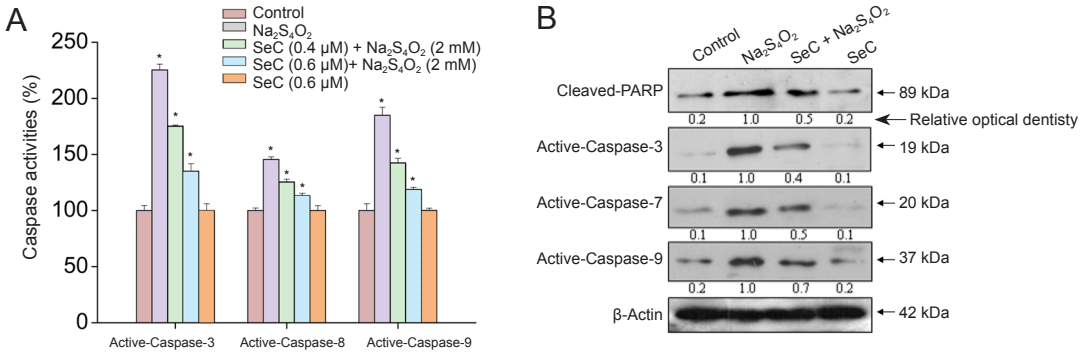

Figure 3 Effect of selenocysteine (SeC) on mitochondrial-mediated apoptosis in HT22 cells.
(A) SeC inhibited oxygen glucose deprivation (OGD)-induced caspase activation. HT22 cells were pre-treated with 0.4 and 0.6 μM SeC for 6 hours (h) in either control or OGD (glucose-free medium + 2 mM $Na_2S_4O_2$) culture conditions for 90 minutes. Caspase activation was assayed using a fluorescent substrate. (B) SeC reduced OGD-induced PARP cleavage and caspase activation, as tested using specific substrates (Ac-DEVD-AMC for caspase-3, Ac-IETD-AMC for caspase-8, and Ac-LEHD-AMC for caspase-9). Protein expression was detected by western blotting and quantified using Quantity-One software. Experiments were performed at least three times, and the mean optical density of the band was expressed as a percentage of control. $*P < 0.05$, *vs.* control (mean ± SD, $n = 3$; one-way analysis of variance followed by the least significant difference test). $Na_2S_4O_2$: Sodium hydrosulfite.

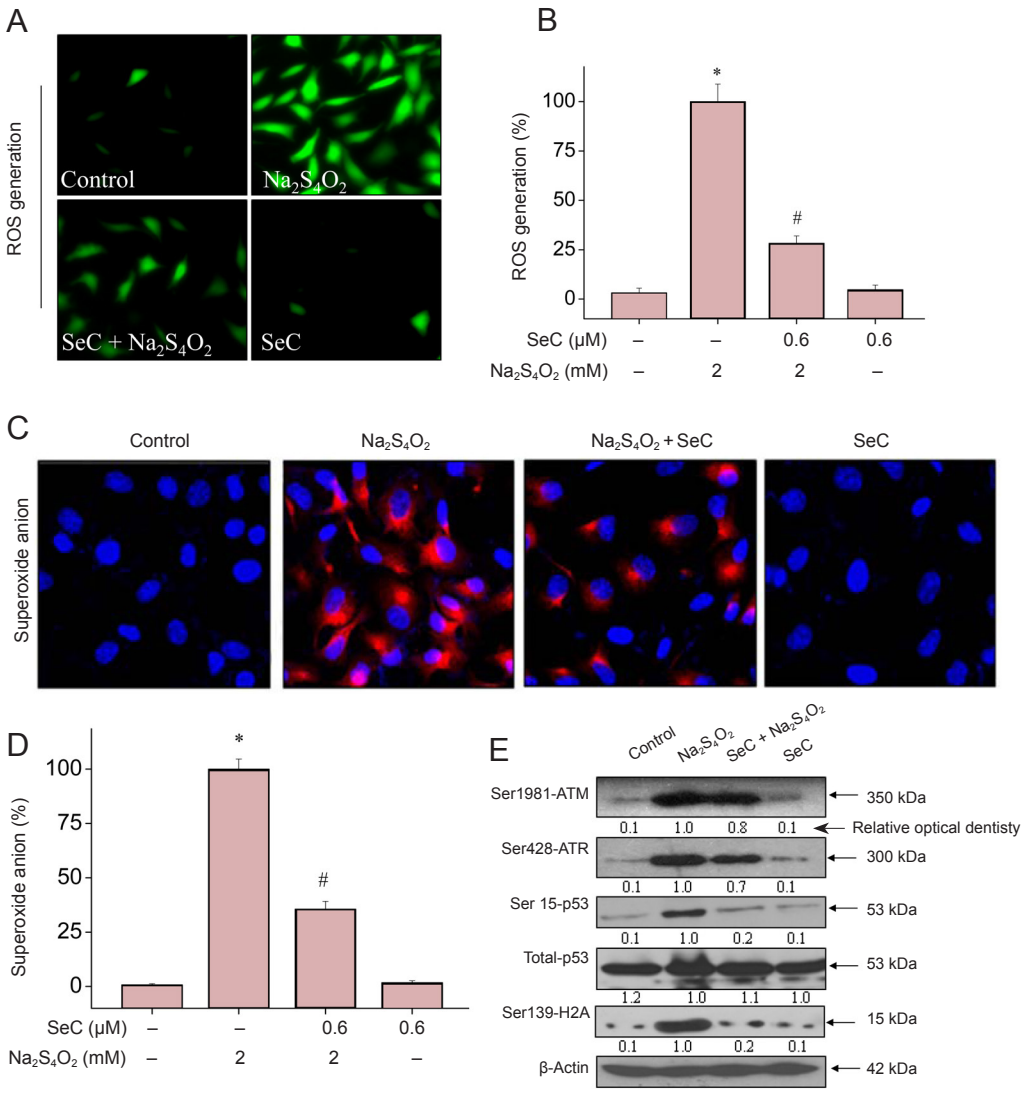

Figure 4 Selenocysteine (SeC) attenuates oxygen glucose deprivation (OGD)-induced oxidative damage by inhibiting reactive oxygen species (ROS) generation in HT22 cells.
(A) SeC attenuated OGD-induced accumulation of ROS. Cell with green fluorescence indicated the ROS generation. (B) Statistical analysis of ROS generation. (C) SeC attenuated OGD-induced accumulation of superoxide anion. Cell with green fluorescence indicated the surperoxide anion generation. (D) Statistical analysis of superoxide anion generation. Cells were pre-loaded with DCFH-DA or Mito-SOX probe for 15 minutes (min), and cultured under OGD conditions (glucose-free medium + 2 mM $Na_2S_4O_2$) for 90 min, with or without pre-treatment with 0.6 μM SeC for 6 hours (h). Generation of ROS or superoxide anions over a 2 h period was observed using a fluorescence microscope (original magnification, 200×). (E) SeC attenuated OGD-induced DNA damage. Protein expression was quantified by Quantity-One software. The expression rate is indicated under each band. All data were obtained over at least three independent experiments. $*P < 0.05$, *vs.* control; $\#P < 0.05$, *vs.* OGD treatment (mean ± SD, $n = 3$; one-way analysis of variance followed by the least significant difference test). $Na_2S_4O_2$: Sodium hydrosulfite.

SeC attenuates OGD-induced oxidative damage in HT22 cells

Oxidative damage acts as an important cell death mechanism in response to OGD treatment. Therefore, we also evaluated intracellular oxidative status in the current study. As shown in **Figure 4A**, **B**, cells cultured under OGD conditions (glucose-free medium + 2 mM $Na_2S_4O_2$) showed a marked increase in ROS generation. Furthermore, accumulation of the superoxide anion was also detected in OGD-treated cells (**Figure 4C**, **D**).

Furthermore, we found that ROS overproduction subsequently triggered DNA damage. As shown in **Figure 4D**, cells cultured under OGD conditions exhibited substantial DNA damage, as shown by the increased phosphorylation level of Ser1981-ATM, Ser428-ATR, Ser139-H2A and Ser15-p53. However, SeC pre-treatment significantly attenuated OGD-induced DNA damage by inhibiting ROS and superoxide anion generation (**Figure 4E**). Taken together, these results indicate that SeC has the potential to attenuate OGD-induced oxidative damage by inhibiting ROS generation.

Discussion

In the present study, HT22 mouse hippocampal neurons were cultured and employed to investigate the neuroprotective effect of SeC against OGD-induced neurotoxicity. Compared with 95% N_2 and 5% CO_2, the use of $Na_2S_4O_2$ in a glucose-free medium is an effective way of quickly establishing OGD conditions, and is therefore commonly used in basic research. Selenium, an essential trace element that is beneficial in the chemoprevention and chemotherapy of cerebral ischemic stroke, is incorporated into. SeC, a selenium-containing amino acid that also exhibits novel biological potential (Zhang et al., 2008; Chen and Wong, 2009). However, to date, it had not been investigated whether SeC antagonizes OGD-induced neurotoxicity in HT22 mouse hippocampal neurons. In the current study, the *in vitro* protective potential and mechanism of selenocystine against oxygen glucose deprivation (OGD) condition-induced neurotoxicity were evaluated in HT22 mouse hippocampal neurons. Our findings confirm that SeC has the potential to ameliorate OGD-induced neuronal apoptosis, which has been shown to be the main cause of cell death in response to cerebral ischemia (Charriaut-Marlangue et al., 1998; Snider et al., 1999; Mehta et al., 2012; Wu et al., 2013; Wang et al., 2016c). Apoptosis is an ordered cellular death process which can alter intracellular homeostasis, and is controlled by serial gene and protein expression (Wu et al., 2013; Yuan et al., 2013; Fu et al., 2015, 2016). The extrinsic/death receptor-mediated pathway and the intrinsic/mitochondrial-mediated pathway are the two key pathways responsible for regulating cell apoptosis (Yagami et al., 2014; Fu et al., 2015; Li et al., 2015; Zhu et al., 2016). In this study, OGD-induced activation of caspase-8 and -9 was detected, which was effectively inhibited by SeC pre-treatment. Activation of caspase-9 was greater than that of casepase-8, suggesting that OGD-induced apoptosis was mediated by the intrinsic apoptosis pathway. Moreover, activated caspase-3 evoked a cell apoptosis cascade by inducing PARP cleavage. These mechanisms indicate that SeC interferes with OGD-induced apoptosis mainly by inhibiting the mitochondrial-mediated apoptotic pathway.

Superoxide anion, hydroxyl radical, and hydrogen peroxide are all involved in cerebral ischemia and contribute to ischemic neuronal damage (Floyd and Carney, 1992). Human brain tissue is extremely sensitive to oxidative damage due to its high oxygen consumption, high level of polyunsaturated fatty acids (Islam et al., 2002), and inability of nerve cells to regenerate (Floyd and Carney, 1992). Therefore, suppression of ROS generation is crucial to preventing ischemic injury. P53, a transcription factor, acts as a tumor suppressor protein to regulate apoptosis, cell proliferation, cell cycle checkpoints, and DNA damage repair by regulating multiple signal transduction pathways (Aminzadeh et al., 2014, Renaud et al., 2014; Fan et al., 2017c). Activation of P53 through phosphorylation can induce apoptosis by activating downstream proteins, such as Bax/Bcl2, Fas/Apol, and IGF-BP3 (Jarolim et al., 2017; Wang et al., 2017b; Beyfuss and Hood, 2018; Ma et al., 2018). Our findings show that over-accumulation of ROS and superoxide anion activates DNA damage markers, including Ser139-H2A and Ser15-p53 in HT22 cells after treatment with $Na_2S_4O_2$. However, SeC treatment reduced the production of free radicals and rescued HT22 cells from $Na_2S_4O_2$-induced DNA damage.

In conclusion, our findings provide evidence that SeC inhibits OGD-induced oxidative damage and apoptosis *in vitro*. To our knowledge, this study is the first to fully investigate the molecular mechanisms underlying the action of SeC against OGD-induced neurotoxicity. However, given that the present study focused solely on *in vitro* experiments, future investigations of the *in vivo* effects in animal models are warranted to clarify the mechanisms by which SeC protects against cerebral ischemic damage.

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