

Research Article

The Neuroprotective Effects of Astragaloside IV against H₂O₂-Induced Damage in SH-SY5Y Cells are Associated with Synaptic Plasticity

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Received 17 October 2019; Accepted 16 November 2019; Published 13 May 2020

Guest Editor: Shaohui Wang

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The aim of this study was to investigate whether the neuroprotective effects of astragaloside IV (AS-IV) against hydrogen peroxide (H₂O₂)-induced damage on human neuroblastoma cell line (SH-SY5Y) are associated with synaptic plasticity. The concentration screening of AS-IV and H₂O₂ on SH-SY5Y cells and the protective effects of AS-IV on SH-SY5Y cells under H₂O₂ stress were all determined by MTT assay. The expression of postsynaptic density 95 (PSD-95) and growth-associated protein 43 (GAP-43) were measured by western blot (WB) and immunofluorescence staining assay under the same treatment conditions. According to the MTT results, the concentration of H₂O₂ at 50 μmol/L for 3 h was used for the cell damage model, and various concentrations of AS-IV (0.1, 0.2, 0.3, and 0.4 μmol/L) were used to affect SH-SY5Y cells. The MTT results showed that pretreatment of SH-SY5Y cells with AS-IV (0.1, 0.2, 0.3, and 0.4 μmol/L) attenuated the damage induced by H₂O₂ (50 μmol/L, 51.62% cell viability) and increased cell viability to 64.19, 63.48, 65.86, and 65.81%, respectively. Western blot analysis and immunofluorescence staining showed that the protective effects of AS-IV against SH-SY5Y cell damage caused by H₂O₂ resulted in reduced expression of PSD-95 and increased expression of GAP-43 in comparison with the H₂O₂ treatment group. The conclusion shows that AS-IV protected SH-SY5Y cells and enhanced their viability under H₂O₂ stress. AS-IV may facilitate presynaptic and postsynaptic plasticity to exert protective effects against oxidative damage of SH-SY5Y cells.

1. Introduction

Synaptic plasticity is the process by which neurons and neural circuits undergo adaptive changes to maintain relative stability in the external environment, as well as the molecular basis for learning and memory [1, 2]. Changes in synaptic plasticity, including those associated with particular diseases, can affect signal transduction in the nervous system [3]. Many important functions of the nervous system are dependent on synaptic plasticity.

Synaptic plasticity can occur as presynaptic plasticity and postsynaptic plasticity [4]. Recent research on the mechanisms of synaptic plasticity has been focused mainly on the functions of proteins involved in presynaptic and postsynaptic plasticity, as well as neural cytoskeletal proteins in key signaling pathways. Growth-associated protein 43

(GAP-43) and postsynaptic density 95 (PSD-95) are two synaptic protein markers distributing on presynaptic and postsynaptic membranes, respectively. Research had confirmed that these marker proteins could directly reflect the changes of synaptic morphology and functions of synaptic biology. Compelling evidence demonstrates that high expression levels of GAP-43 facilitate the construction of new synaptic connections and axonal growth during the process of neuronal growth coneformation [5]. PSD-95 is a prominent organizing protein in PSD complexes, which couple the C terminus of the modulatory NMDAR [6] subunit to various cytoplasmic proteins and enzymes [7].

Synaptic plasticity reflects the ability of the nervous system to adapt to internal and external environmental stimuli via dynamic changes in structure or function, which often involve compensation for damaged nerves and neural

circuits, as well as repair of nerve injuries, such as those caused by oxidative stress. Oxidative stress caused by the accumulation of reactive oxygen species can overwhelm the capacity of the cell to eliminate toxic reactive intermediates and repair the damage induced by such intermediates [8]. In the laboratory, oxidative stress is induced *in vitro* by adding H_2O_2 to the cell culture medium [9, 10].

Emotional and cognitive disorders seriously affect human life and health [11, 12]. In recent years, increasing attention has been paid to the neuroprotective effects of natural substances extracted from plants on the central nervous system. For example, natural substances such as curcuma [13], resveratrol [14], and ginsenosides [15] have been the focus of studies on their antioxidant properties and effects on synaptic plasticity. Astragaloside IV (AS-IV) is extracted from *Astragalus membranaceus* and frequently used as an herbal medicine. Based on its history as a traditional Chinese medicine and recent studies, AS-IV is known to be a free radical scavenger with many positive pharmacological effects, including antioxidant, anti-inflammatory, and antihypertensive effects [16–20]. Although AS-IV is used as a traditional therapy for degenerative diseases in China, a few studies have investigated the effects of AS-IV on synaptic plasticity. Moreover, further research is required in order to fully examine the neuroprotective effects of AS-IV.

Therefore, the aim of the present study was to evaluate the effects of AS-IV on synaptic plasticity *in vitro* using SH-SY5Y cells exposed to H_2O_2 , as well as to measure changes in the protein expression levels of PSD-95 and GAP-43 associated with the protective effects of AS-IV. We found that AS-IV might protect cells from oxidative damage by regulating synaptic plasticity. Our experiments suggest that the protective effects of AS-IV are mediated by downregulation of PSD-95 expression and upregulation of GAP-43 expression. Our findings provide a foundation for further research on the neuroprotective effects of AS-IV, which could lead to the development of new therapies for diseases associated with changes in synaptic plasticity using AS-IV or similar molecules.

2. Methods

2.1. Cell Culture. SH-SY5Y cells were stored in liquid nitrogen, thawed rapidly at 37°C , and centrifuged at 1000 rpm for 4 min. The medium was removed and replaced by DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, HyClone, New Zealand) and 0.2% penicillin/streptomycin/amphotericin B solution (Sangon Biotech, Shanghai). The cells were grown in an incubator (ThermoFisher, USA) at 37°C with an atmosphere containing 5% CO_2 . The cells were transferred to new plates after 1–2 generations to restore growth.

2.2. Assay of Cell Viability. Firstly, the toxicity of AS-IV and H_2O_2 was assessed by the MTT assay. The SH-SY5Y cell line (5000 cells/well) was plated in 96-well microplates, adhered for 24 h, and then treated with H_2O_2 (25–300 $\mu\text{mol/L}$) for 2,

3, and 4 h and AS-IV (EDQM, European) (0–5 $\mu\text{mol/L}$) for 24 h, separately. The medium was replaced with 180 μL of fresh medium containing MTT (Sigma, USA) solution (5 mg/mL). The cells were incubated in an incubator (37°C , 5% CO_2) for 4–6 h, after which formazan was dissolved in 150 μL DMSO (Sigma, USA). The optical density of the samples was detected at 490 nm by a microplate reader (BioTek, USA). Untreated cells were used as a control group.

Secondly, the neuroprotective effects of different concentrations of AS-IV (0.1–0.4 $\mu\text{mol/L}$) on cell viability under H_2O_2 (50 $\mu\text{mol/L}$) stress were also measured by the MTT assay. SH-SY5Y cells (5000 cells/well) were plated in 96-well microplates and allowed to adhere for 24 h. Various concentrations of AS-IV (0.1–0.4 $\mu\text{mol/L}$) were used to pretreat the cells for 2 h, after which H_2O_2 (50 $\mu\text{mol/L}$) was added for 3 h. Cell viability was assessed using the MTT assay. Untreated SH-SY5Y cells were used as a control group, whereas H_2O_2 -treated cells were used as a negative control group [21].

2.3. Cell Morphological Observation. Various concentrations of AS-IV (0.1–0.4 $\mu\text{mol/L}$) were used to pretreat SH-SY5Y cells for 2 h, followed by treatment with 50 $\mu\text{mol/L}$ H_2O_2 for 3 h. Finally, the cells were observed under a microscope (OLYMPUS, Japan).

2.4. Western Blot Analysis. SH-SY5Y cells (3×10^4 cells/mL) were seeded in 12-well microplates for 24 h and pretreated with AS-IV (0.1–0.4 $\mu\text{mol/L}$) for 2 h prior to exposure to H_2O_2 (50 $\mu\text{mol/L}$) for 3 h. The treated cells were lysed in RIPA buffer with phosphatase inhibitor tablets and protease inhibitor cocktail on ice for 30 min. The resulting suspension was centrifuged at 12000 rpm for 15 min at 4°C , and the supernatant was collected. The protein in each sample was resuspended in an equal volume of $2 \times$ loading buffer, followed by protein denaturing in boiling water for 10 min. Finally, the protein samples were cooled at room temperature and stored at -80°C .

Equal amounts of protein were subjected to 12% sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) microporous membranes (MERCK). The samples were blocked with 5% nonfat dry milk in TBS containing 0.1% Tween-20 for 1 h at room temperature, followed by incubation with GAP-43 (Abcam, Britain), PSD-95 (Abcam, Britain), and GAPDH antibodies at a 1/800, 1/1000, and 1/2000 dilutions, respectively, for 20 h at 4°C . Next, the membranes were incubated with rabbit IgG secondary antibodies (Promega, USA) for 1 h at room temperature. Proteins were detected using enhanced chemiluminescence solution (ECL, BIO-RAD) with an Image Ware System.

2.5. Immunofluorescence Staining. The treated SH-SY5Y cells were fixed with 4% paraformaldehyde for 15 min on the ice and permeabilised with 0.3% triton X-100 and 0.3% H_2O_2 in PBS for 30 min at room temperature. The background was blocked with 5% donkey serum in PBS for 1 h

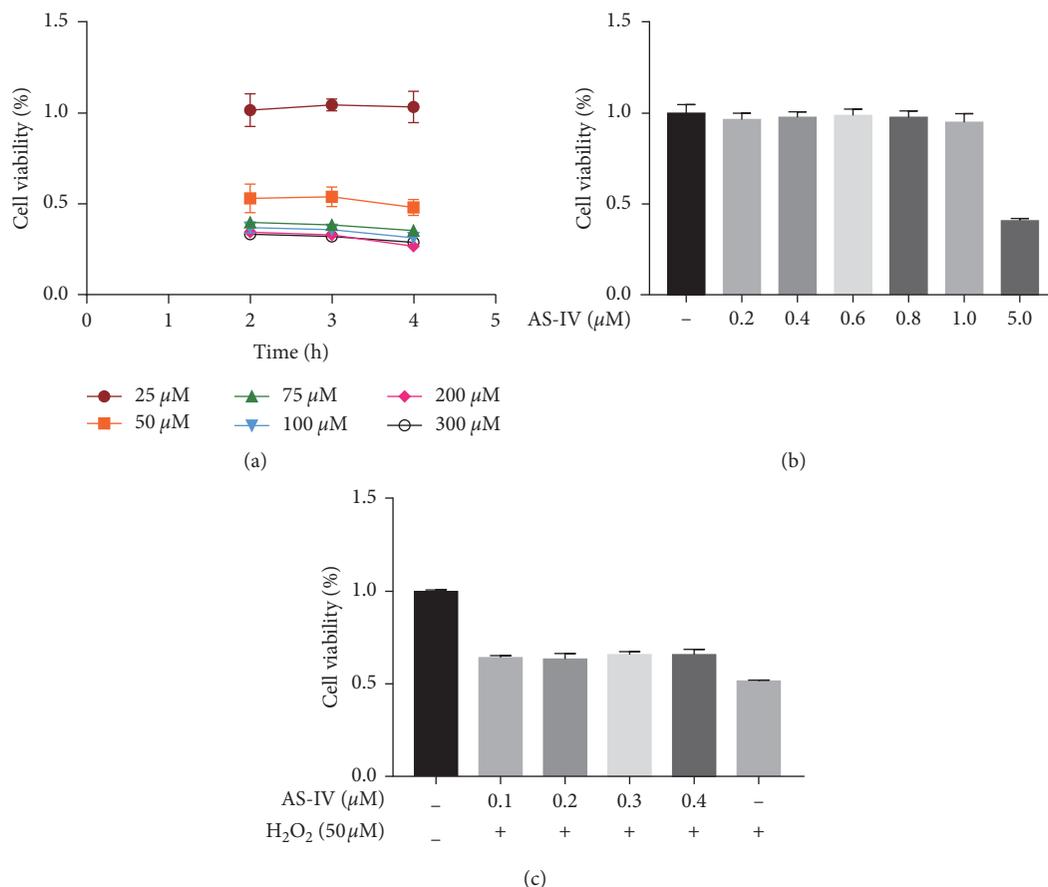


FIGURE 1: MTT assay for SH-SY5Y cells viability: (a) viability detection of SH-SY5Y cells treated with H₂O₂ (25~300 μmol·L⁻¹) for 2, 3, and 4 h. (b) Viability detection of SH-SY5Y cells treated with AS-IV (0.2~5 μmol·L⁻¹) for 24 h (c) Viability detection of SH-SY5Y cells under H₂O₂ (50 μmol·L⁻¹) treatment for 3 h after AS-IV (0.1~0.4 μmol·L⁻¹) pretreatment for 2 h.

beforeincubation with the primary antibodies. The primary antibodies for PSD-95 (1/1000) and GAP-43 (1/800) were diluted with PBS, followed by incubation with the cells for 90 min at 37°C and at 4°C overnight. The secondary antibodies (green) (Alexa Fluor 488 donkey-anti-rabbit, 1/200, Jackson ImmunoResearch Laboratories) were diluted in PBS for 1 h at room temperature. Finally, the cells were stained with DAPI (1/1000) for 10 min, mounted with 80% glycerol, and stored at 4°C for confocal microscopy (OLYMPUS, Japan) detection.

3. Results

3.1. The Protective Effects of AS-IV against H₂O₂-Induced Damage in SH-SY5Y Cells. Firstly, we detected the viability of SH-SY5Y cells by MTT assay (Figure 1) after treating the cells with H₂O₂ for 2, 3, or 4 h in the presence or absence of AS-IV for 2 h. As shown in Figure 1(a), the cell viability of SH-SY5Y cells treated with H₂O₂ showed a gradual decreasing trend that was time-dependent and dose-dependent. The cell viability was approximately 53.97% of the value of the control group following treatment with 50 μmol/L H₂O₂ for 3 h. Therefore, for the subsequent experiments, treatment with 50 μmol/L H₂O₂ for 3 h was used to induce cell damage.

As shown in Figure 1(b), SH-SY5Y cells were not affected by treatment with 0.2, 0.4, 0.6, 0.8, and 1 μmol/L AS-IV for 24 h. As shown in Figure 1(c), SH-SY5Y cells were pretreated with different concentrations of AS-IV (0.1, 0.2, 0.3, and 0.4 μmol/L) for 2 h, followed by treatment with H₂O₂ (50 μmol/L) for 3 h. The cell viability of the AS-IV treatment group was increased in comparison with that of the H₂O₂ treatment group, but it was lower than that of the control group. The cell viability of the group treated with H₂O₂ (50 μmol/L) for 3 h was 51.62% of that of the control group. However, cell viability of the cells pretreated with AS-IV (0.1, 0.2, 0.3, and 0.4 μmol/L) for 2 h prior to H₂O₂ increased to 64.19, 63.48, 65.86, and 65.81%, respectively. So, as a conclusion, AS-IV was effective in reducing the damage of H₂O₂ on SH-SY5Y cells to protect cells.

3.2. Morphology of Treated SH-SY5Y Cells. As shown in Figure 2, treatment with H₂O₂ (50 μmol/L) for 3 h resulted in shrinkage and aggregation of cell bodies. However, treatment with AS-IV (0.1~0.4 μmol/L) prior to H₂O₂ treatment significantly attenuated the morphological manifestations of cell damage. These results suggest that AS-IV can inhibit or prevent the morphological changes shown by SH-SY5Y cells under H₂O₂ stress.

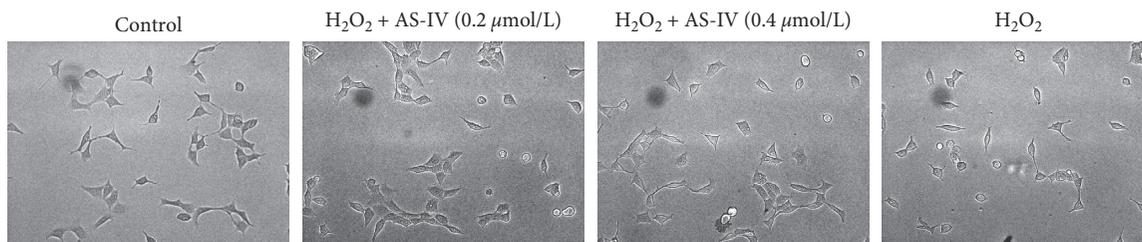


FIGURE 2: Morphological observation of SH-SY5Y cells under H_2O_2 ($50 \mu\text{mol}\cdot\text{L}^{-1}$) treatment 3 h after AS-IV ($0.1\sim 0.4 \mu\text{mol}\cdot\text{L}^{-1}$) pretreatment for 2 h.

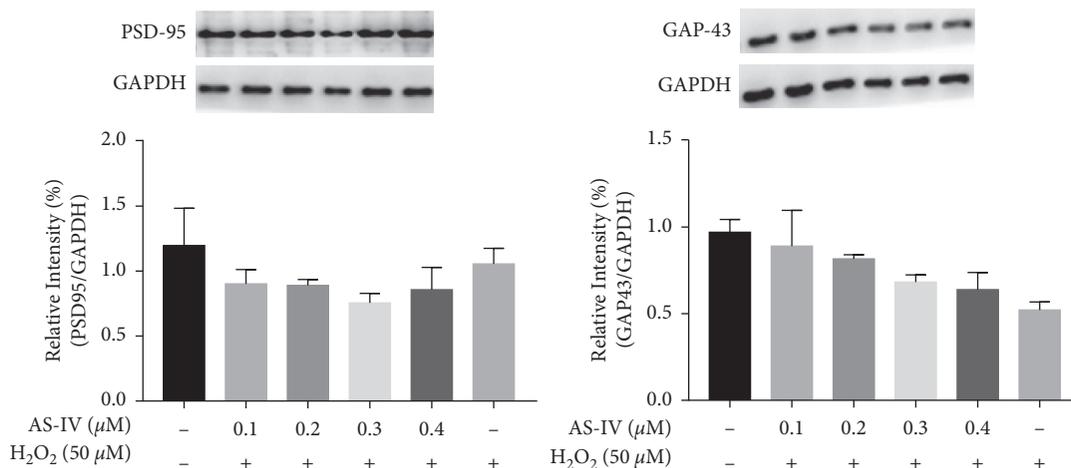


FIGURE 3: Protein expression of PSD-95 and GAP-43 in SH-SY5Y cells under stress conditions. (a) Expression of PSD-95 under H_2O_2 ($50 \mu\text{mol}\cdot\text{L}^{-1}$) treatment for 3 h after AS-IV ($0.1\sim 0.4 \mu\text{mol}\cdot\text{L}^{-1}$) pretreatment for 2 h. (b) Expression of GAP-43 under H_2O_2 ($50 \mu\text{mol}\cdot\text{L}^{-1}$) treatment 3 h after AS-IV ($0.1\sim 0.4 \mu\text{mol}\cdot\text{L}^{-1}$) pretreatment for 2 h untreated, and H_2O_2 -treatment cells were used as blank and negative controls, respectively.

3.3. Analysis of PSD-95 and GAP-43 Expression by Western Blot. We detected the expression levels of PSD-95 and GAP-43 proteins by western blot analysis to determine the relationship between the neuroprotective effects of AS-IV under H_2O_2 stress and synaptic plasticity. Western blot analysis was performed using a polyclonal rabbit antibody against PSD-95 and a monoclonal rabbit antibody against GAP-43. As shown in Figure 3(a), when SH-SY5Y cells were treated with H_2O_2 ($50 \mu\text{mol}/\text{L}$) for 3 h, PSD-95 expression was decreased by 11.84% in comparison with that of the control group. In contrast, following AS-IV ($0.1, 0.2, 0.3$ and $0.4 \mu\text{mol}/\text{L}$) pretreatment for 2 h, PSD-95 protein expression levels were decreased by 24.67%, 25.66%, 39.96%, and 28.35%, respectively.

As shown in Figure 3(b), the protein expression level of GAP-43 was decreased to 46% of that of the control group following treatment with H_2O_2 ($50 \mu\text{mol}/\text{L}$) for 3 h. In comparison, pretreatment with $0.1, 0.2, 0.3$, or $0.4 \mu\text{mol}/\text{L}$ AS-IV increased the expression level of GAP-43 by 36.95%, 30.42%, 11.54%, and 12.62%, respectively, in comparison with that of the H_2O_2 -exposed cells. Therefore, we speculate that the neuroprotective effects of AS-IV under H_2O_2 stress were mediated by reducing the protein expression level of PSD-95 and increasing that of GAP-43 in comparison with the H_2O_2 -exposed group.

3.4. Analysis of PSD-95 and GAP-43 Expression by Immunofluorescence Assay. We performed immunofluorescence

assays using a polyclonal rabbit antibody against PSD-95 and a monoclonal rabbit antibody against GAP-43 to further investigate the neuroprotective effects of AS-IV in the context of regulation of synaptic plasticity. As shown in Figure 4, the immunofluorescence analysis was consistent with the western blot analysis. However, DAPI staining revealed that nuclear condensation and fragmentation occurred after the SH-SY5Y cells were treated with H_2O_2 ($50 \mu\text{mol}/\text{L}$) for 3 h; however, pretreatment with AS-IV prevent the nuclear damage induced by H_2O_2 .

4. Discussion

Synaptic plasticity plays a role in learning and memory and is the basis for many important functions of the nervous system. Recent results have shown that oxidative stress is involved in the pathological mechanisms of several neurodegenerative diseases, including Alzheimer's disease and Parkinson's disease [22–24]. Treatment with H_2O_2 is widely used in cellular models to induce oxidative stress [25, 26], and the SH-SY5Y cell line is extensively used as a cell model for studying neuronal cell death [27]. In our study, SH-SY5Y cells were subjected to H_2O_2 -induced oxidative stress to induce H_2O_2 -induced neurotoxicity and reveal the relationship between the neuroprotective effects of AS-IV and synaptic plasticity.

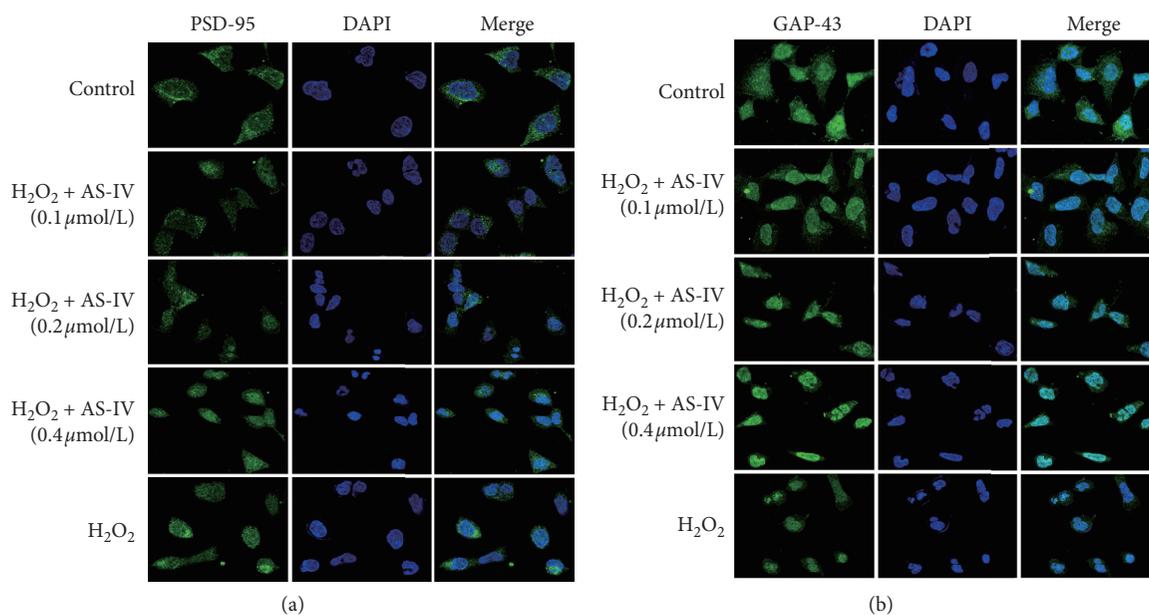


FIGURE 4: Immunofluorescence expressions of synaptic markers PSD-95 and GAP-43. (a) Expression of PSD-95 under H_2O_2 ($50 \mu\text{mol}\cdot\text{L}^{-1}$) treatment 3 h after AS-IV ($0.1\sim 0.4 \mu\text{mol}\cdot\text{L}^{-1}$) pretreatment for 2 h. (b) Expression of GAP-43 under H_2O_2 ($50 \mu\text{mol}\cdot\text{L}^{-1}$) treatment 3 h after AS-IV ($0.1\sim 0.4 \mu\text{mol}\cdot\text{L}^{-1}$) pretreatment for 2 h.

Although antioxidant agents represent potentially effective treatments for neurodegenerative diseases [28], the relationship between their beneficial effects and synaptic plasticity are unclear. AS-IV, the main active component of astragalus, has several beneficial pharmacological effects that are useful for treating particular pathophysiological conditions. Recent studies of AS-IV have demonstrated its antioxidant and antiaging effects [29–31], as well as positive effects on learning and memory [32]. In this study, we demonstrated that AS-IV protected SH-SY5Y cells from H_2O_2 -induced cell damage and increased their cell viability in comparison with that of the H_2O_2 -exposed group.

In our study, H_2O_2 was used to stimulate cells to allow us to explore the relationship between synaptic plasticity and the neuroprotective effects of AS-IV. We found that the protective effects of AS-IV against H_2O_2 -induced damage might be mediated by decreased expression of PSD-95. PSD-95, a scaffold protein of the PSD, plays a role in signal transduction by linking the NMDAR to downstream enzymes and regulating the activity of signaling enzymes through its binding domains [33]. NMDA receptors function in synaptic plasticity by regulating the concentration of Ca^{2+} in postsynaptic cells, mediating some intracellular signaling pathways, and ultimately affecting long-term potentiation and long-term depression of synaptic transmission. However, overactivation of NMDA receptors is a major cause of ischemic cell injury [34]. NMDA receptors consist of principal NR1 and NR2 subunits [35], and tyrosine phosphorylation of NR2 (particularly NR2A and NR2B) upregulates the function of NMDA receptors [36]. Recently, it has been reported that NR2A-PSD-95-Src is a signal transduction module that promotes overactivation of NR2A and NMDA receptors [37]. In addition, excessive activation of NMDA receptor activated another signaling molecule,

neuronal nitric oxide synthase (nNOS), which binding to the PDZ2 domain of PSD-95 [38], subsequently catalyzed the excessive production of highly reactive nitric oxide (NO) and induced NO toxicity [39]. Our results show that AS-IV decreased the expression level of PSD-95, which may have prevented the interaction of NR2A-PSD-95-Src that leads to NMDA receptor phosphorylation and increased the phosphorylation level of nNOS, thus protecting SH-SY5Y cells under H_2O_2 -induced stress [40].

GAP-43 is a specific neuronal phosphoprotein. Expression of GAP-43 promotes the neuron growth, development and axon regeneration during development of the nervous system, as well as regeneration after injury [41]. GAP-43 is a repository of calmodulin that releases calmodulin due to the action of a second messenger molecule, leading to phosphorylation of GAP-43 and ultimately affecting the release of synaptic transmitters. Dephosphorylated GAP-43 recombines with calmodulin to form a feedback loop. Activated GAP-43 interacts with cytoskeletal components to regulate the activity of nerve endings, thereby altering cell morphology [42]. In this study, we found that AS-IV increased the expression level of GAP-43 in comparison with that of cells treated with H_2O_2 . Therefore, we speculate that H_2O_2 exposure leads to GAP-43 phosphorylation, whereas AS-IV leads to dephosphorylation of GAP-43, which facilitates repair of oxidative damage, thereby protecting SH-SY5Y cells.

In conclusion, AS-IV protects cells under H_2O_2 -induced injury in a manner that may be related to changes in the expression levels of PSD-95 and GAP-43. Furthermore, the neuroprotective effects of AS-IV against H_2O_2 -induced damage in SH-SY5Y cells may be associated with synaptic plasticity. Our study may provide a therapeutic strategy for nervous system diseases, although further research into the

mechanisms through which AS-IV mediates synaptic plasticity is necessary.

Data Availability

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

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

This work was supported by the National Undergraduate Innovation and Entrepreneurship Project (201712216034), Outstanding Young and Middle-Aged Talents in Colleges and Universities (gxfzD2016295), School-level Scientific Research Projects (2017zr010), and the Natural Science Research Foundation of the Department of Education of Anhui Province (nos. KJ2019A0874 and KJ2018A0583).

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