

The p38-MAPK Pathway Is Involved in Neuroprotection of Artemisinin against H₂O₂-induced Apoptosis in PC12 Cells

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Abstract: Oxidative stress, owing to the excessive production of ROS (reactive oxygen species), is one of the leading causes for the progression of AD (Alzheimer's disease). Increasing evidences suggested that oxidative stress insult impaired the physiological functioning of neuronal cells by inducing cell apoptosis. The search for drug candidates that can effectively protect neurons from oxidative stress insult might hold therapeutic potential for AD. In the present study, we tested the neuroprotective effects and the related action mechanisms of artemisinin, a FDA-approved anti-malarial drug, against H₂O₂ induced oxidative damage in PC12 cells. It was found that artemisinin reduced cell viability loss caused by hydrogen peroxide (H₂O₂) in PC12 cells. In addition, data from Flow cytometry displayed that artemisinin significantly decreased the apoptosis of PC12 cells induced by H₂O₂. Furthermore Western blot analysis displayed that artemisinin stimulated the p38MAPK signaling, while treatment of PC12 cells with specific p38MAPK pathway inhibitor SB203580 blocked the neuroprotective effect of artemisinin. These results together indicated that artemisinin is a potential protectant, and it protects PC12 cells against H₂O₂ injury through activation of the p38MAPK pathway.

Key words: Artemisinin, oxidative stress, AD, PC12 cells, p38MAPK.

1. Introduction

AD (Alzheimer's disease) is the most commonly occurring neurodegenerative disease in the elderly people, which has devastating effects in terms of morbidity and mortality [1]. The clinical features of AD present dementia as well as cognitive impairment [2]. Despite the fact that specific pathologies of AD are still unclear, oxidative stress, caused by the over production of ROS, which further induces neuronal cell apoptosis and impairs the normal brain functions, contributes majorly to the development of this disease [3, 4]. AD is an incurable condition, and even worse the disabling effects may last for years, representing an enormous disease load, with regard to human suffering and economic cost [5, 6]. H₂O₂, the common chemical used to produce ROS, can cause cell membrane injury as well as lipid peroxidation and DNA damage in

neuronal cells, and is also considered as one of the risk factors for AD [7, 8]. In cultured neuronal cells, exposure to H₂O₂ leads to cell apoptosis and other alterations similar to what happens in the brains of AD patients [9, 10]. However, some chemicals with antioxidant effects, which is able to protect neuronal cells against H₂O₂-induced cell death via decreasing ROS production, may confer benefits in AD therapy [1, 11, 12].

In the recent past, increasing attention has been paid towards harnessing the antioxidant potential of natural products and compounds extracted from plants which has higher efficacy and lower side effects in general [11, 13]. Artemisinin, a sesquiterpene lactone, was originally extracted from the qinghao by a female Chinese scientist, and has become one of the most famous chemical in the world [14]. In clinical relevance, artemisinin is among the most effective therapies for malaria with great safety and the preferred first-line treatment for the disease [15]. Beside these

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effects, previous studies including some from our group, have shown that artemisinin and its derivatives have neuroprotective effects suggesting that artemisinin might be potential candidate for AD therapy [16-19].

It has been recognized that artemisinin-mediated neuroprotection against neuronal apoptosis from various insults is regulated by ERK1/2 survival/apoptotic signaling pathway rather than the Akt pathway [16, 18, 19]. In addition, researchers also found that artemisinin was able to stimulate the p38MAPK pathway [16, 20]. Furthermore, our group have recently reported that artemisinin can successful protect PC12 and primary neurons from β -amyloid insult [18]. Different studies have shown that artemisinin inhibited superoxide anions and had radical scavenging activity against the highly reactive hydroxyl radicals, while the understanding of mechanisms that participated in its antioxidant effects is still very limited [19, 21]. PC12 cell line with most of the typical features of primary neuron, originally derived from rat pheochromocytoma, is a useful neuronal model for the study of AD as well as other neurodegenerative disease, and was also widely used to investigate free ROS biochemical pathways related to cell apoptosis and neuroprotection [18, 22, 23]. Therefore, this cell line is a suitable model for studying oxidative stress-induced neuronal injury.

In the present study, we found that artemisinin was capable of protecting PC12 cells from H₂O₂-induced oxidative stress through inhibition of the apoptosis. We also revealed that the neuroprotective effect of artemisinin is mediated by p38MAPK pathway. These findings further sustained the hypothesis that artemisinin administration might be a potential therapeutic approach for the treatment of AD.

2. Materials and Methods

2.1 Materials

Analytical grade artemisinin was purchased from Chengdu Kangbang Biotechnology Ltd. China. DMSO

(Dimethyl sulfoxide) and bovine serum albumin were ordered from Sigma-Aldrich (St. Louis, MO, USA). FBS (fetal bovine serum), penicillin-streptomycin antibiotics (PS), DMEM and trypsin were purchased from Invitrogen (Carlsbad, USA). MTT (Methyl thiazolyl tetrazolium) was obtained from Molecular Probes (Eugene, OR, USA). P38MAPK inhibitor SB203580 was obtained from Calbiochem (La Jolla, CA, USA). Anti- β -actin and phospho-p38MAPK antibodies were obtained from Cell Signaling Technology (Woburn, USA). Annexin V-FITC/PI Apoptosis Detection Kit was purchased from BD Biosciences (San Diego, CA, USA).

2.2 Cell Culture and Treatment

The rat pheochromocytoma PC12 cell line was kindly provided by Dr. Gordon Guroff (National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD, USA). Cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% heat-inactivated horse serum, 5% FBS (fetal bovine serum) and antibiotics (100 U/mL penicillin; 100 μ g/mL streptomycin) as previous described [24]. Cells were incubated at 37 °C with 5% CO₂ humidified atmosphere. All the experiments were carried out 24 h after the cells were seeded. For the protection assay, PC12 cells were pretreated with artemisinin for 2 h and then treated with H₂O₂ for 24 h in all experiments. In addition, artemisinin was removed from the cell culture medium before H₂O₂ exposure.

2.3 MTT Assay

The cell viability was measured by the MTT assay as previously described [19, 25]. Briefly, PC12 cells were seeded in 96-well plates at a density of 5×10^3 cells/well. After desired treatment, MTT (0.5 mg/mL) was added to each well for an additional 2 h. Subsequently, medium was aspirated, and DMSO (100 μ L) was added to each well to solubilize the formazan crystals. Absorbance was measured at 490 nm by

Infinite M200 PRO Multimode Microplate (Tecan, Switzerland). The relative cell viability was presented as a percentage compared with the control group.

2.4 Apoptosis of PC12 Cells Was Measured by Flow-cytometry

The apoptosis of PC12 cells after desired treatment was analyzed by flow cytometer [25]. Briefly, the PC12 cells were harvested by centrifugation (1000 rpm for 5 min) and washed with PBS twice. Cells were suspended in 400 μ L of 1X Binding Buffer. 5 μ L Annexin V-EGFP mix was added to each sample followed by the addition of 10 μ L Propidium Iodide; mixed and kept away from light at room temperature for 20 min. Data acquisition and analysis were performed using BD C Sample plus.

2.5 Western Blot Analysis

The western blotting was performed as described previously [22]. Briefly, collected cells after desired treatment were washed with ice-cold PBS for once, then lysed in RIPA buffer with different inhibitors for 15 minutes on ice. The protein concentrations of each sample were measured by BCA protein assay kit following the manufacturer's instructions. For western blotting, samples (20 μ g protein/lane) were separated on SDS-PAGE gel with a pre-stained protein ladder (5 μ L) as a molecular weight marker, then transferred to PVDF membranes. The p-p38MAPK was measured by phospho-specific antibody while β -actin was used as a loading control. Immuno-reactive bands were visualized by ECL kit according to the manufacturer's instructions. The intensity of band was quantified using Image J software. The experiments were repeated at least 3 times by using independent cultures.

2.6 Statistical Analysis

Statistical analysis and data handling were carried out by using SPSS version 16.0. All experiments were performed in triplicates. The data were expressed as the mean \pm standard deviation ($X \pm SD$). Statistical

analysis was performed using one-way ANOVA followed by Tukey's multiple comparison. The null hypothesis was rejected at $p < 0.05$.

3. Result

3.1 Artemisinin Decreased the Cell Viability Loss Caused by H₂O₂ in PC12 Cells

Firstly, we tested the cytotoxicity of H₂O₂. PC12 cells were exposed to different concentrations of H₂O₂ for 24 h and the cell viability was measured by MTT assay. As shown in Fig. 1B, 24 h H₂O₂ exposure significantly reduced the cell viability of PC12 cells in a concentration-dependent manner. H₂O₂ at concentration of 200 μ M lead to 20% cell viability reduction, thus this concentration of H₂O₂ was chosen in the further experiments. To examine the toxicity of artemisinin, PC12 cells were treated with different concentrations of artemisinin for 24 h, and the cell viability was determined by MTT assay. As shown in Fig. 2C, artemisinin itself did not show any cytotoxicity between 3 μ M and 90 μ M, so these concentrations were chosen for further experiments. To examine the protective effects of artemisinin, PC12 cells were treated with artemisinin for 2 h, then treated with H₂O₂ (200 μ M) for 24 h. The data from MTT assay displayed that artemisinin at the concentration from 3 μ M (75.435 ± 2.344) to 30 μ M (77.479 ± 2.85) significantly attenuated H₂O₂-induced cell viability loss.

3.2 Artemisinin Reduced H₂O₂-induced Apoptosis in the PC12 Cells

Cell apoptosis was the main type of cell death caused by H₂O₂ exposure [21]. It was reported previously that artemisinin successfully protected neuronal cells from apoptosis in various cell types [19, 21]. Therefore, we investigated whether artemisinin could reduce the cell apoptosis induced by H₂O₂ in PC12 cells. Cell apoptosis was measured by flow cytometry, and the data demonstrated that H₂O₂ exposure markedly increased apoptosis in PC12 cells, while artemisinin

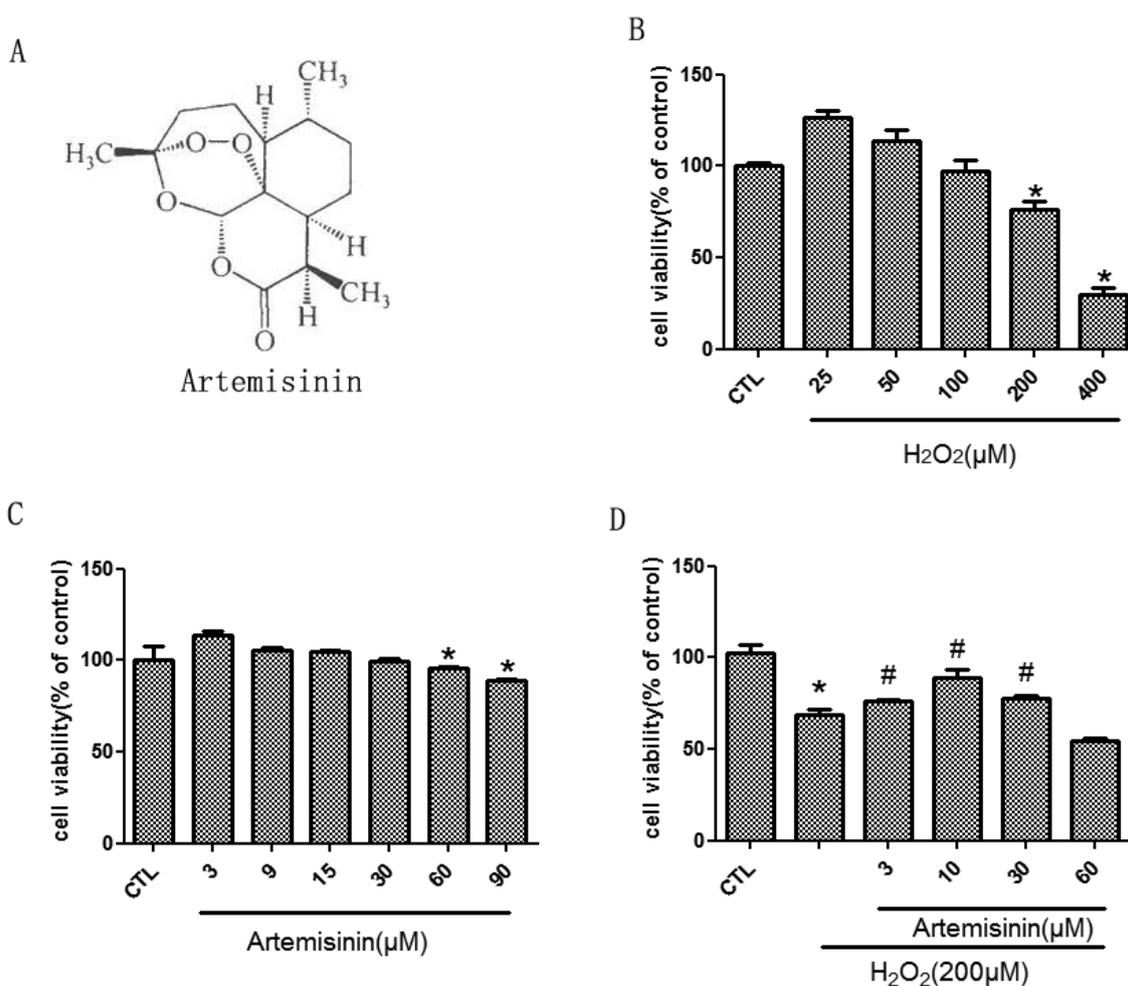


Fig. 1 Artemisinin attenuated the decrease in cell viability induced by H₂O₂ in PC12 cells.

(A) The structure of artemisinin; (B) Cells were treated with H₂O₂ (25–400 μM) or 0.1% DMSO (vehicle control) for 24 h and cell viability was measured using the MTT assay; (C) Cells were treated with artemisinin (3–90 μM) or 0.1% DMSO (vehicle control) for 24 h and cell viability was measured using the MTT assay; (D) Cells were pretreated with artemisinin at indicated concentrations and then incubated with or without 200 μM H₂O₂ for further 24 h. Data represent means ± SD, * *p* < 0.05, versus control group; # *p* < 0.05 versus the H₂O₂-treated group.

pretreatment significantly decreased the cell apoptosis caused by H₂O₂ (Fig. 2).

3.3 Artemisinin Stimulated the p38MAPK Signaling Pathway in PC12 Cells

p38MAPK pathway plays a crucial role in the cell survival and apoptosis [26]. In addition, alteration of this signaling pathway was also found to contribute to the disease progression in Alzheimer's disease mouse models [20, 27]. Artemisinin was able to activate p38MAPK signaling in cells as reported earlier [16, 20]. Therefore, we tested whether p38MAPK

pathway is involved in artemisinin-induced neuroprotective effects in PC12 cells. As shown in Fig. 3, phosphorylation of p38MAPK was gradually enhanced in a concentration-dependent manner after administration of artemisinin (*F* = 136.4, *df* = 5). This data suggested that p38MAPK pathway might make a contribution to the neuroprotective action of artemisinin.

3.4 The p38MAPK Inhibitor SB203580 Attenuated the Neuroprotective Effects of Artemisinin

To further clarify whether p38MAPK signaling

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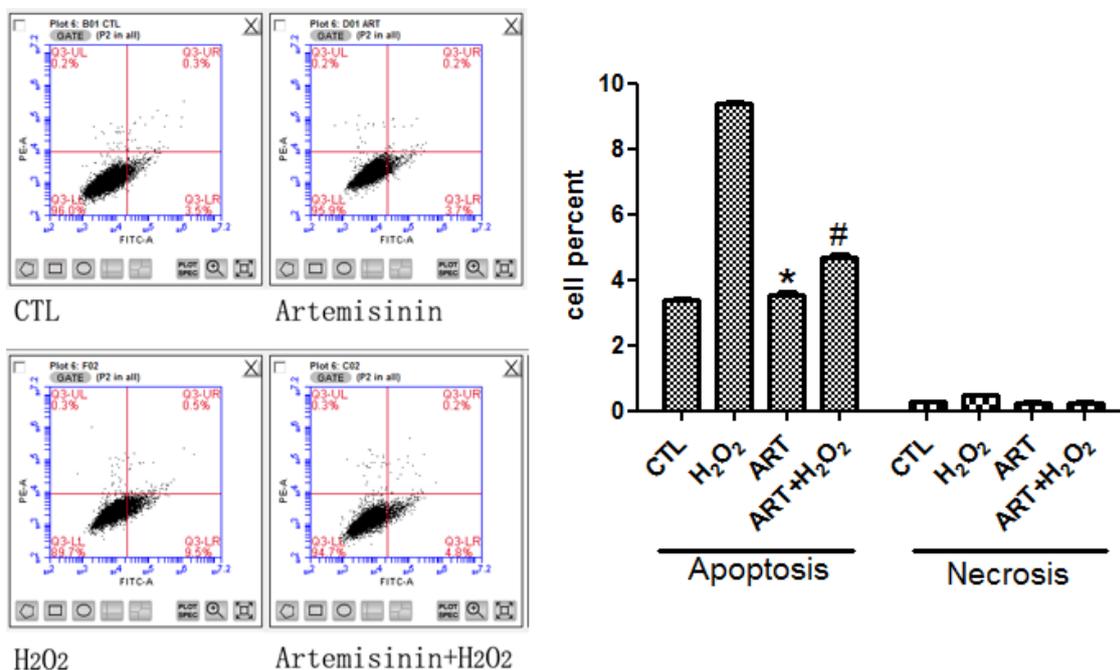


Fig. 2 Protective effect of artemisinin on H₂O₂-induced apoptosis in the PC12 cells. Photographs of representative cultures measured by flow cytometer. Data represent means \pm SD, * $p < 0.05$ versus control group; # $p < 0.05$, versus the H₂O₂-treated group.

pathway was involved in the neuroprotective effect of artemisinin, PC12 cells were pretreated with 10 μ M SB203580 (a specific inhibitor of p38MAPK), and the neuroprotective effect of artemisinin on H₂O₂-induced injury was examined by MTT assay. As shown in Fig. 4, pretreatment with SB203580 significantly attenuated the neuroprotective effect of artemisinin on H₂O₂-induced cell viability loss. These results further confirmed that the neuroprotective effect of artemisinin was mediated by p38MAPK pathway.

4. Discussion

AD is one of the common neurodegenerative diseases in the aging population which lacks any effective treatments currently [28]. Oxidative stress, caused by the overproduction of ROS leads to lipid peroxidation, protein oxidation, plasma membrane breakage as well as cross-linking of cytoskeletal biomolecules, which play a key role in the pathogenesis of AD [29-32]. Increasing evidences support that antioxidants extracted from the plants can reduce or delay oxidation process [1]. In the present

study, we explored the possible neuroprotective effects of artemisinin on H₂O₂-induced oxidative injury and the underlying mechanisms. The data from MTT assay and flow cytometry revealed that 200 μ M H₂O₂ significantly increased the cell apoptosis in PC12 cells, while pretreatment with artemisinin was able to remarkably reduce the cell viability loss caused by H₂O₂ insult. Further data from the western blotting and specific pharmacological inhibitor suggested that the neuroprotective effect of artemisinin was mediated by the p38MAPK pathway.

Artemisinin, currently the most popular drug in the world, was successfully extracted from the herbs by a Chinese scientist [14]. Though it was the first line drug used for treatment of malaria, artemisinin has abundant other pharmacological activities [33, 34]. One of the attractive pharmacological activities of artemisinin is its super antioxidant effects. Previous research by our group has well proved that artemisinin was able to inhibit ROS production and also conferred neuroprotection against various insults in cultured cells [18, 19, 21, 35]. Our present study here further supported

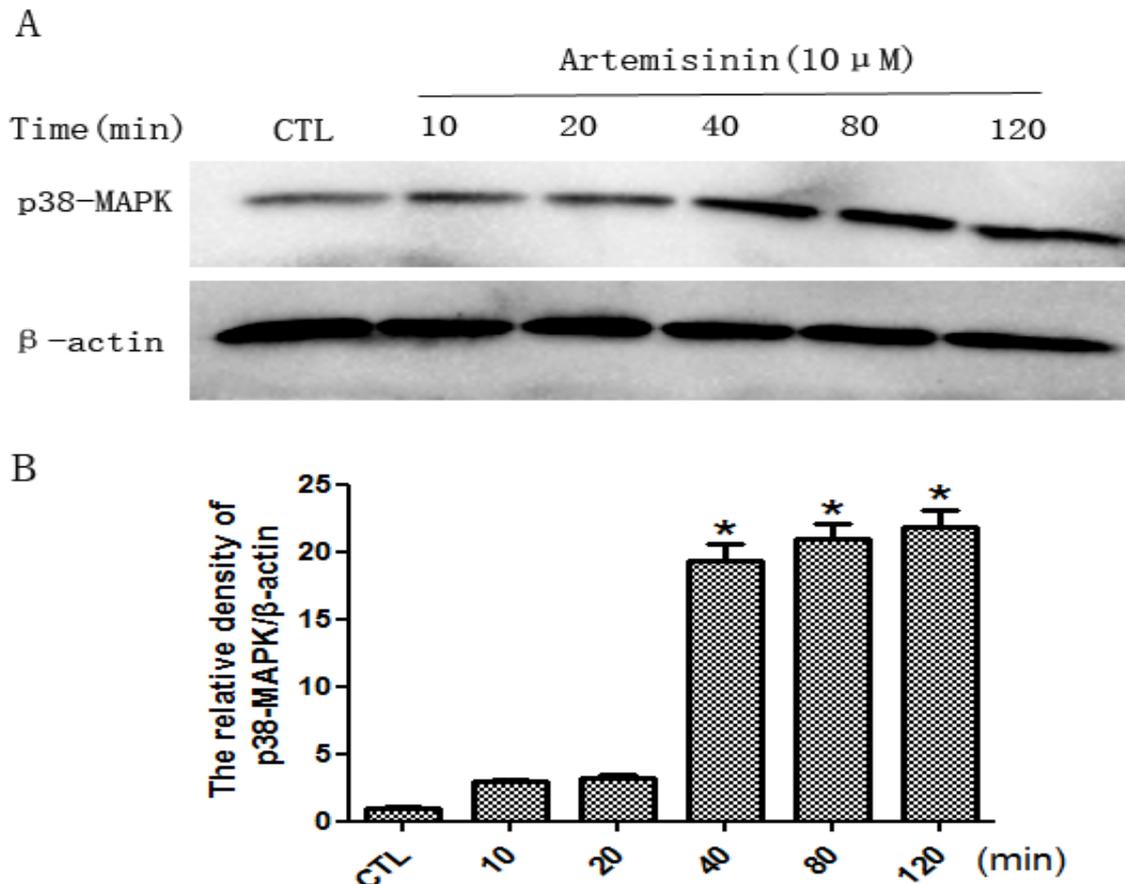


Fig. 3 Involvement of p38MAPK signaling in the cyto-protective effect of artemisinin.

(A) PC12 cells were treated with artemisinin for different time points. The expression of p38-MAPK and beta-actin was detected by Western blotting with specific antibodies; (B) Quantification of representative protein bands from Western blotting. * $p < 0.05$, versus control group.

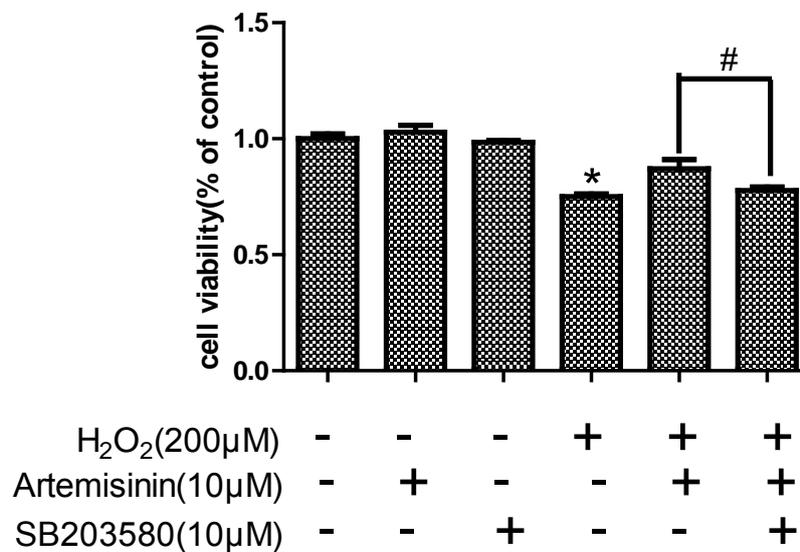


Fig. 4 The p38-MAPK inhibitor SB203580 attenuated the protective effects of artemisinin.

PC12 cells were pre-treated with 10 μM SB203580 for 15 min, then incubated with 10 μM artemisinin for 2 h, and exposed to 200 μM H₂O₂ for a further 24 h. Cell viability was measured by MTT assay. * $p < 0.05$, versus the control group; # $p < 0.05$ versus the artemisinin & H₂O₂-treated group.

the antioxidant effects of artemisinin and indicated the possibility that artemisinin administration might be a potential therapeutic strategy for the treatment of AD. Biologically, it has been found that artemisinin-mediated neuroprotection against neuronal apoptosis is regulated by several pathways including ERK1/2, p38MAPK and Nrf2/HO-1 survival/apoptotic signaling pathway [17, 19, 21, 35]. Our results provided mechanistic evidences to support that artemisinin protected PC12 cells from H₂O₂-induced oxidative damage via p38MAPK activation.

5. Conclusions

In summary, our data together indicated that artemisinin was able to protect PC12 cells from H₂O₂-induced oxidative damage similar to other cells, and more interestingly, we found that this protective effect is mediated, at least in part by activated p38MAPK signaling pathway.

Conflict of Interest

None

Acknowledgements

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