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Original Article

The water extract of Angelica Sinensis Radix protects cultured PC12 cells against oxidative stress: Suppression of reactive oxygen species and activation of antioxidant response elements

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ABSTRACT

Objective: Angelica Sinensis Radix (ASR; Dang Gui; the root of *Angelica sinensis*) is an herbal supplement that has been used in invigorating blood circulation. Here, we provided different lines of evidence to support the beneficial role of ASR against oxidative stress in cultured PC12 cell, a rat pheochromocytoma cell line. **Materials and Methods:** The water extract of ASR inhibited the activity of xanthine oxidase *in vitro*. In cultures, the pre-treatment of ASR water extract reduced the cytotoxic effect of tert-butyl hydroperoxide (tBHP), an oxidative stress inducer. **Results:** The protecting mechanisms of ASR were shown to be mediated by: (i) suppression of tBHP-induced reactive oxygen species (ROS) formation; (ii) induction of caspase-3 and PARP activities; and (iii) stimulation of mRNAs encoding antioxidative genes, glutathione S-transferase A2 and NAD(P)H dehydrogenase quinone oxidoreductase 1 (NQO1), via the transcriptional activation of antioxidant response element (ARE). The outcome was the prevention of tBHP-induced cell apoptosis. **Conclusion:** Interestingly, the protective effect of ASR extract in PC12 cells was insignificant when challenging by insults of β-amlyoid (for Alzheimer's disease) and 1-methyl-4-phenylpyridinium (for Parkinson's disease). Taken together, we revealed a neurobeneficial role of ASR in protecting neuronal cells against oxidative damage, which might be useful in developing health food supplements for disease prevention in the future.

KEY WORDS: Angelica Sinensis Radix, neuroprotection, oxidative stress, reactive oxygen species

INTRODUCTION

Neurodegenerative diseases, including the most well-known Alzheimer's and Parkinson's diseases, are a collection of diseases that the patients are suffering from progressive loss of brain functions, resulting in difficulty and/or defect in performing routine activities for daily life. Pathologically, the deterioration of brain functional integrity is caused by a significant loss of neurons [1,2]. Molecular targets for these diseases have been proposed, e.g. the aggregation of β-amyloid (to form senile plaque) and Tau protein (to form neurofibrillary tangle) in cholinergic neurons for Alzheimer's disease, as well as the accumulation of α-synuclein (to form Lewy bodies) in dopaminergic neurons for Parkinson's disease [3]. Unfortunately, most of the current treatments can only slow down the disease progression while the effective cure for different types of neurodegenerative diseases is almost absent. One of the difficulties is attributing to neuronal damage, occurred in post-mitotic neurons: The division of neuron is rare to be triggered [4,5]. Thus, numerous efforts are focusing on the prevention of neurodegenerative diseases, i.e. protecting neurons to die.

To search for new therapeutic drugs, herbal material is a good source, owing to its huge supply, great diversity and historical record in disease prevention and therapy in China and Southeast Asia. More importantly, different lines of evidence support the effectiveness of herbal material for such a purpose [6-8]. Gastrodiae Rhizoma was shown to modulate the cleavage of amyloid precursor protein and the expression of neuroprotective genes in neuronal cells [9]. In addition, berberine, an isoquinoline alkaloid compound isolated from Coptidis Rhizome, was reported to exert beneficial effects in treating Alzheimer's disease [10]. The intake of an herbal decoction called Bushen Huoxue Yin inhibited NF-κB activation and nitric oxide formation in a mouse model of Parkinson's disease [11]. A flavonol aglycone, isorhamnetin, derived from Ginkgo Folium, potentiated the effect of nerve growth factor in stimulating neurite outgrowth

in cultured PC12 cells [12]. In Notoginseng Radix, a flavonol glycoside named quercetin 3-O-β-D-xylopyranoside-β-D-galactopyranoside was identified to possess a strong activity against β-amyloid-induced cytotoxicity and apoptosis in PC12 cells and cortical neurons, as well as to improve the learning and memory ability in scopolamine-induced amnesia rat model [13]. These results therefore greatly support the potential role of chemicals deriving from herbs in drug identification and development against neurological disorders.

Here, we identified a neurobeneficial role of Angelica Sinensis Radix (ASR; Dang Gui; the root of *Angelica sinensis*) against cellular oxidation in cultured neurons. ASR is a well-known Chinese herb that has long been used to harmonize the blood, regulate menstruation, relieve pain, moisten dryness and lubricate the intestines [14-17], and which is commonly consumed in Asia and Europe. Our findings have revealed the neuroprotective effects of ASR against oxidative stress and delineated part of molecular mechanisms in understanding how ASR is suppressing ROS formation and apoptosis. In addition, ASR stimulated the expression of two antioxidative genes, glutathione S-transferase A2 (GSTA2) and NAD(P)H dehydrogenase quinone oxidoreductase 1 (NQO1), via the transcriptional activation of antioxidant response element (ARE) in PC12 cells. These results clearly supported antioxidative roles of ASR in protecting neuronal injury from oxidative stress.

MATERIALS AND METHODS

Preparation and Quality Assessment of ASR Extracts

Fresh roots of A. *sinensis* were obtained from Minxian of Gansu in China, which was demonstrated to produce the best quality of ASR [18]. The authentication of herbs was confirmed morphologically by one of the authors, Dr. Tina Dong, at The Hong Kong University of Science and Technology during the field collection. ASR was stored under 4°C, humidity below 60%; the shelf life was <2 years. In ASR extraction, 10 g of ASR crude herb was ground into powder, then boiled in 80 ml of water for 2 h and extracted twice. The extract was filtered and then lyophilized into dry powder. The ASR powder was dissolved in water to form 20 mg/ml as stock concentration and filtered sterilized for subsequent chemical and biological analyses. The quality of ASR extracts was determined by high-performance liquid chromatography (HPLC) fingerprinting, and the content of two chemical markers, ligustilide, and ferulic acid, were quantified, as described previously [19].

Xanthine Oxidase Activity Assay

Enzymatic activity of xanthine oxidase was assayed as described previously [20]. In brief, Vitamin C (10, 30, 100 and 300 μM; Sigma, St Louis, MO, USA) or ASR extract (0.01, 0.03, 0.1, 0.3 and 1 mg/ml) were pre-mixed with 0.01 U/ml xanthine oxidase (Sigma) for 20 min. Afterward, xanthine (0.3 mM) and hydroxylamine (0.24 mM) were incubated for another 20 min at 37°C. Reactions were stopped by adding 0.1% sodium dodecyl sulfate (SDS) to the mixture and measured at 550 nm absorbance. Vitamin C at various concentrations served as a positive control of antioxidation.

Cell Culture

Pheochromocytoma PC12 cells, a cell line derived from rat adrenal medulla, were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells maintained in Dulbecco's modified Eagle's medium supplemented with 6% fetal calf serum, 6% horse serum, 100 units/ml penicillin and 100 mg/ml streptomycin in a humidified (7.5%) CO_2 incubator at 37°C. Fresh medium was supplied every other day. All culture reagents were purchased from Invitrogen Life Technologies (Grand Island, NY, USA).

Neuroprotection Assay

PC12 cells were seeded in a 96-well plates and incubated for 24 h. Afterward, cultures were pre-treated with ASR extract, or vitamin C (if applicable), for 24 h before the challenge of β-amyloid (Aβ, 1-40 with aging process) [21], or 1-methyl-4-phenylpyridinium iodide (MPP^+; Sigma) for another 24 h, or tert-butyl hydroperoxide (tBHP; Sigma) for another 3 h. Cell viability was assessed by MTT (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide) assay (Sigma) [12]. In brief, the MTT solution was added to cell cultures and incubated for 1 h at 37°C. Absorbance was measured at 570 nm in a microplate reader (Thermo Scientific, Waltham, MA, USA).

Measurement of Reactive Oxygen Species (ROS)

The amount of ROS in PC12 cells was determined as described previously [22]. In brief, PC12 cells in 96-well plates were pre-treated with ASR extracts for 24 h, and then labelled by a ROS fluorescent indicator, dichlorofluorescin diacetate (DCFH-DA, 100 μM; Sigma), in Hank's Balanced Salt Solution for 1 h at 25°C. Afterward, the cultures were challenged by tBHP for 0-3 h. The amount of intracellular ROS, induced by tBHP, was detected with excitation at 485 nm and emission at 530 nm by a fluorescent plate reader (SpectraMax® Gemini XS, Molecular Devices Corporation, Sunnyvale, CA, USA).

Activation of Caspase-3 and poly(ADP-ribose) Polymerase

PC12 cells were pre-treated with ASR extract or vitamin C (if applicable), for 24 h. Afterward, cultures were serum starved for 3 h, and then challenged by tBHP for another 3 h. Cells were then solubilized in lysis buffer containing 125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 2% 2-mercaptoethanol, and analyzed by Western blotting, using anti-cleaved caspase-3 and anti-cleaved poly(ADP-ribose)polymerase (PARP) antibodies (1:1000; Cellular Signaling Technology, Danvers, MA, USA). The immune complexes were visualized using the enhanced chemiluminescence (ECL) method (GE Healthcare; Fairfield, CT, USA). The intensities of bands in control and different samples, run on the same gel and under strictly standardized

ECL conditions, were compared on an image analyzer, using a calibration plot constructed from a parallel gel with serial dilutions of one of the samples.

Real-time Quantitative Polymerase Chain Reaction (PCR)

Cultured PC12 cells were treated with ASR extract (0.1, 0.3 and 1 mg/ml) for 16 h. Total RNA was extracted using TRIzol® reagent (Invitrogen). RNA purity and quantity were determined by UV absorbance at 260 nm, and 5 μg of the RNA was reverse-transcribed by Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen) in the presence of oligo-d(T) primers. Real-time PCR was performed using SYBR green master mix and Rox reference dye according to the product description (Roche Life Science; Indianapolis, IN, USA). The specific primers were as follows:

GSTA2: 5'- CCT GGG CAT CTG AAA CCT TTT GAG AC -3' (forward) and 5'- GCG AGC CAC ATA GGC AGA GAG C -3' (reverse);

NQO1: 5'- GAC CTT GCT TTC CAT CAC CAC CGG -3' (forward) and 5'- GTA GAG TGG TGA CTC CTC CCA GAC -3' (reverse);

glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5'-AAC GGA TTT GGC CGT ATT GG-3' (forward) and 5'-CTT CCC GTT CAG CTC TGG G-3' (reverse).

SYBR green signal was detected by an Applied Biosystems 7500 Fast machine (Applied Biosystems; Carlsbad, CA, USA). Transcript levels were calculated using the ΔΔCt value method, where the values were normalized by an internal control GAPDH in the same sample. PCR products were examined as above and using melting curve analysis to confirm specific amplification.

Transcriptional Activation of Antioxidant Response Element

A luciferase reporter having four repeats of ARE called ARE-Luc was purchased from Promega (pGL4.37 [luc2P/ARE/Hygro]; Madison, WI, USA). This reporter was transiently transfected into PC12 cells by Lipofectamine 2000 (Invitrogen). Cultures were then treated with ASR extract (0.1, 0.3 and 1 mg/ml) for 24 h, and then collected to perform luciferase activity assay according to the product description (Applied Biosystems). The luciferase activity was normalized by protein amount in each sample.

Other Assay and Statistical Analysis

Protein concentrations were measured routinely by Bradford's method (Bio-Rad Laboratories; Hercules, CA, USA). Statistical analyses were performed using one-way Analysis of Variance followed by the Student's *t*-test. Statistically significant changes were classed as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

RESULTS

Before the examination of biological effect of ASR, it was critical to ensure the preparation of ASR extract could be reproducible for subsequent analysis. Two major ingredients of ASR, including ferulic acid and ligustilide, were quantified by HPLC analysis; these two chemicals are the markers for quality assurance of ASR. The quantities of two markers in ASR extracts were determined by measuring the peak area according to the regression equation method [19]. The standardized ASR extract contained at least 0.23 mg ferulic acid and 0.28 mg ligustilide in 1 g of dried ASR extract: these values were similar to our previous findings [19].

To evaluate the antioxidation effect of ASR, xanthine oxidase was firstly chosen as a target. This intracellular enzyme catalyzes the oxidation of hypoxanthine to xanthine with the production of ROS [23]. ROS are well-known to be associated with various human diseases [24,25]. An *in vitro* anti-xanthine oxidase assay was employed for such a purpose. A concentration-dependent curve of xanthine oxidase in catalyzing the oxidation of hypoxanthine was shown in Figure 1a. In the presence of vitamin C, the activity of xanthine oxidase was suppressed accordingly [Figure 1b]; this served as a positive control for the inhibition assay. Interestingly, the pre-incubation of ASR extract with xanthine oxidase also possessed similar response as vitamin C in reducing enzymatic activity of xanthine oxidase in a dose-dependent manner [Figure 1c]. These results suggested that ASR water extract was capable in inhibiting xanthine oxidase activity *in vitro*, and which provided a preliminary data in supporting the antioxidative effect of ASR.

The oxidative insult is known to associate with different neurodegenerative diseases [26]. Oxidative stress could be commonly mimicked by tBHP, a relatively stable organic peroxide to generate free radical. Cultured PC12 cells were treated with ASR extract for 48 h, and then subjected to cell viability to evaluate the drug cytotoxicity. The ASR extract at 0.3-1 mg/ml did not exhibit any cytotoxicity [Figure 2a]. For antioxidation, cultured PC12 cells were treated with different concentration of tBHP for 3 h, and then subjected to cell viability assays. The cytotoxic effect of tBHP in PC12 cells was promising [Figure 2b], with almost 75% of cell lose at 150 μM concentration. Based on the result, a dose of tBHP at 150 μM was chosen for the protection assay. Serving as a positive control, the pre-treatment of different concentrations of vitamin C for 24 h reduced the tBHP-induced cell death in a dose-dependent manner [Figure 2c]. The same antioxidation effect was also observed in the pre-treatment of ASR extract at 0.3-1 mg/ml [Figure 2d]. At 1 mg/ml dose of ASR extract, the protection effect reached up to ~70%. These results clearly indicated a function of ASR in protecting neuronal cells against oxidative injury. The role of two major chemicals within ASR extract was tested for its antioxidative effect. Ligustilide at high concentration of about 25 μM, a concentration much higher than that in 1 mg/ml ASR extract, was revealed to prevent tBHP-induced cell death [Figure 2e]. In contrast, ferulic acid even at 100 μM did not show any effect in cell protection [Figure 2f].

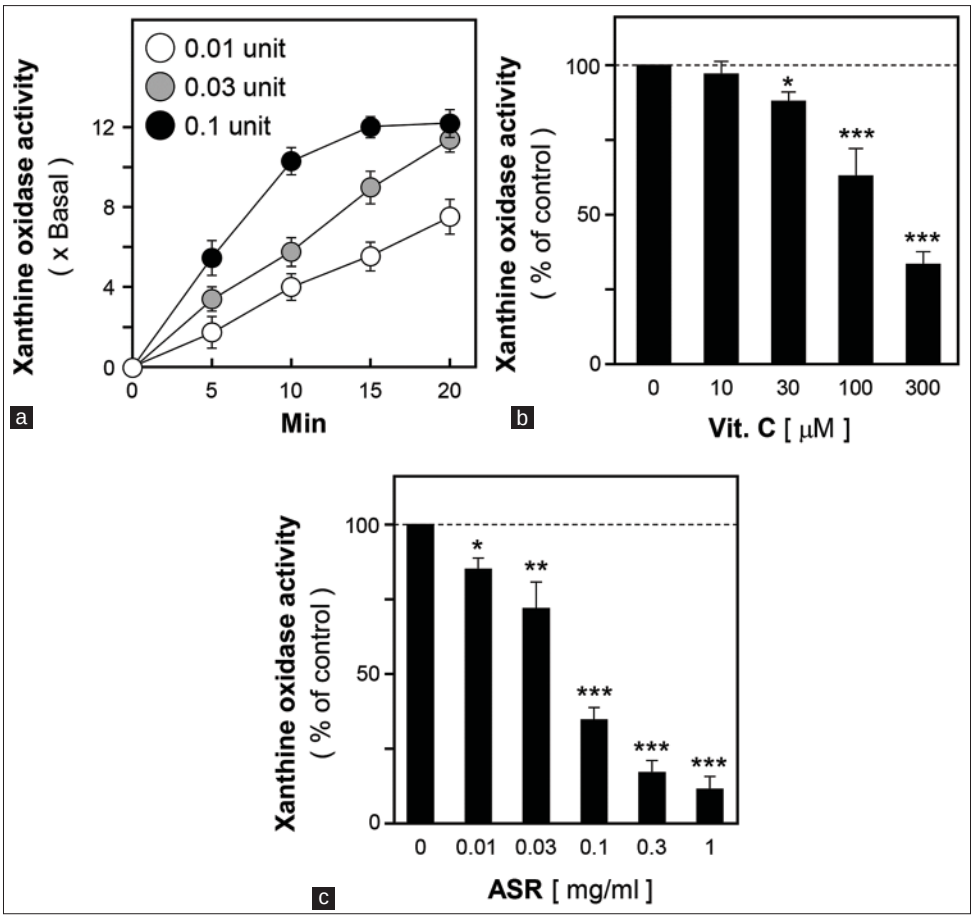

Figure 1: The *in vitro* antioxidant effect of Angelica Sinensis Radix (ASR) extracts against xanthine oxidase. (a) Hypoxathine was incubated with xanthine oxidase (0.01-0.1 unit) and subjected to the measurement of enzymatic activity as described in methods section. (b) Xanthine oxidase was pre-incubated with vitamin C (0-300 μM) for 30 min, and then performed xanthine oxidase activity as in (a). (c) Experiment was performed as in (a) except the use of ASR extracts (0.01-1 mg/ml) in pre-incubation step. Data were expressed as % of control where the value of control was set as 100%; mean ± standard error of the mean; $n = 4$; *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$

ROS is a lethal mediator to cause the oxidation of lipid, protein and DNA, resulting in severe cellular damage in the brain [27]. Here, we speculated that ASR could reduce the formation of ROS, induced by oxidative stress, in PC12 cells. To address this issue, cultured PC12 cells were pre-labeled with a fluorometric ROS indicator, DCFH-DA, and then challenged with different doses of tBHP. The generation of ROS triggered by tBHP (indicated by the fluorescent intensity) in PC12 cells was in a time-dependent manner [Figure 3a]. The reduction of tBHP-induced ROS formation in PC12 cells could be achieved by pre-treatment of vitamin C for 24 h [Figure 3b]. Vitamin C at high dose (500 μM) was effective in suppressing the ROS formation. The pre-treatment of ASR extract for 24 h could also produce the same effect in reducing the amount of intracellular ROS raised by tBHP in PC12 cells. When PC12 cells were pre-treated with ASR at 0.1 mg/ml, the suppression effect was absent, while ASR at 0.3 and 1 mg/ml exerted the suppression effect on tBHP-induced ROS formation, with maximum decrease of ~45% [Figure 3c]. Therefore, the suppression effect of ASR on ROS generation would be one of the molecular explanations to account for the neuroprotection effect of ASR in neurons.

Since ASR could suppress the tBHP-induced ROS formation, we speculated that apoptosis induced by oxidative stress could be inhibited by ASR extract. To support this hypothesis, cultured PC12 cells were challenged with water and tBHP for 3 h, and then collected to examine the activation of apoptosis by detecting the cleaved forms of two well-known apoptotic markers, caspase-3 and PARP [28]. The amounts of cleaved caspase-3 (~20 kDa) and PARP (~90 kDa) were increased upon the application of tBHP [Figure 4a], which indicated that oxidative stress, caused by tBHP, could activate apoptosis in our culture model. Staurosporine (a well-known apoptotic inducer) served as a positive control of apoptotic stimulation while β-actin (~40 kDa) served as an internal control for equal loading [Figure 4a]. To evaluate the ability of ASR extract against apoptosis, PC12 cells were pre-treated with water or ASR extract for 24 h, and then challenged with tBHP for 3 h. The pre-treatment of ASR extract suppressed tBHP-induced apoptosis via reduction of active forms of caspase-3 and PARP [Figure 4b]. Quantification plot [Figure 4b] lower panel indicated that the anti-apoptotic effects of ASR extract against tBHP-induced caspase-3 and PARP activation were rather significant. Therefore, this anti-apoptotic effect of ASR might serve as part of molecular

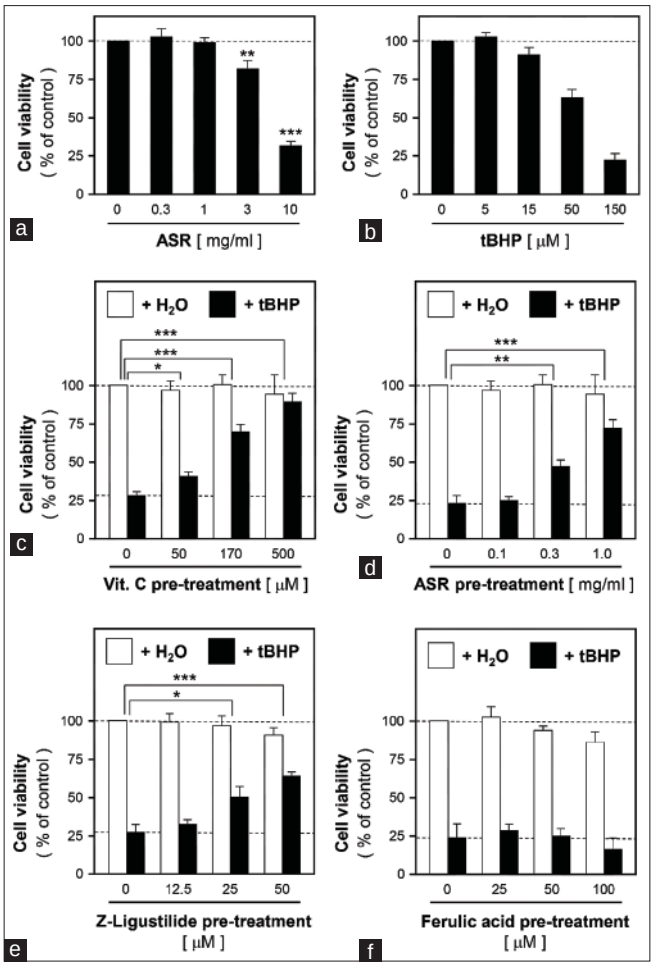

Figure 2: Pre-treatment of Angelica Sinensis Radix (ASR) extracts reduces the cytotoxic effect of oxidative stress in PC12 cells. (a) PC12 cells were treated with different concentrations (0-10 mg/ml) of ASR extracts for 48 h. Afterward, cultures were subjected to cell viability assay using MTT-based colorimetric method. (b) PC12 cells were challenged with tert-butyl hydroperoxide (tBHP) (0-150 μM) for 3 h, followed by the cell viability assay. (c) PC12 cells were pre-treated with vitamin C (0-500 μM) for 24 h before the application of tBHP (150 μM) for another 3 h. Cell viability was then assayed. (d) PC12 cells were pre-treated with the ASR extracts (0.1-1.0 mg/ml) for 24 h and subjected to the same assay as in (b). (e) PC12 cells were pre-treated with Z-ligustilide (12.5-50 μM) for 24 h, and then challenged with tBHP (150 μM) for 3 h, followed by the cell viability assay. (f) The treatment for ferulic acid (25-100 μM) was performed as in (e). Data were expressed as % of control where the value of untreated culture was set as 100; mean ± standard error of mean; $n = 4$; $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$

mechanisms in explaining the neuroprotective effect of ASR against neuronal oxidation.

In addition to suppression of ROS formation and apoptosis, we also determined the regulatory role of ASR on a cellular defense mechanism. In order to ensure the survival against different chronic and acute exposure to a wide array of oxidative stress all the times, cells should acquire an adaptive mechanism for this purpose, e.g. the Phase II defense system. This system mediates the biotransformation of xenobiotics and drugs, which involves different enzymes, including NQO1 and GSTA2 [29,30]. NQO1 functions in catalyzing the detoxification of quinones and their derivatives, which prevents the generation of reactive semiquinones and ROS [31]; while GSTA2 functions in conjugating hydrophobic electrophiles and ROS with glutathione which assists their excretion [32]. Therefore, the up-regulation of these two genes may be an indicator for antioxidant effect. Here, cultured PC12 cells were treated with ASR extract for 16 h and subjected to RNA extraction and real-time PCR analysis. The application of ASR extract in PC12 cultures stimulated the mRNA expression of NQO1 and GSTA2 in dose-dependent manners [Figure 5]; the stimulation effect of ASR at 1 mg/ml concentration in NQO1 (with ~4.5 fold of induction) was greater than that of GSTA2 (~2.8 fold of induction). Serving as a positive control, a potent antioxidant, tBHQ was used, and which increased the mRNA expressions of NQO1 and GSTA2 [Figure 5a].

The expressions of NQO1 and GSTA2 are primarily controlled by a special signaling pathway. This cascade includes a trans-regulatory element called the cap 'n' collar family that comprises four members of transcription factors namely Nrf1, Nrf2, Nrf3, and p45 NF-E2 [33], and a corresponding cis-regulatory element of ARE with core consensus sequence of GTG CAN NNG C on the promoter regions of the genes [29,30]. The transcriptional activation of ARE could explain partly the up regulation of NQO1 and GSTA2 expression in neurons. To determine whether or not ASR stimulates this pathway,

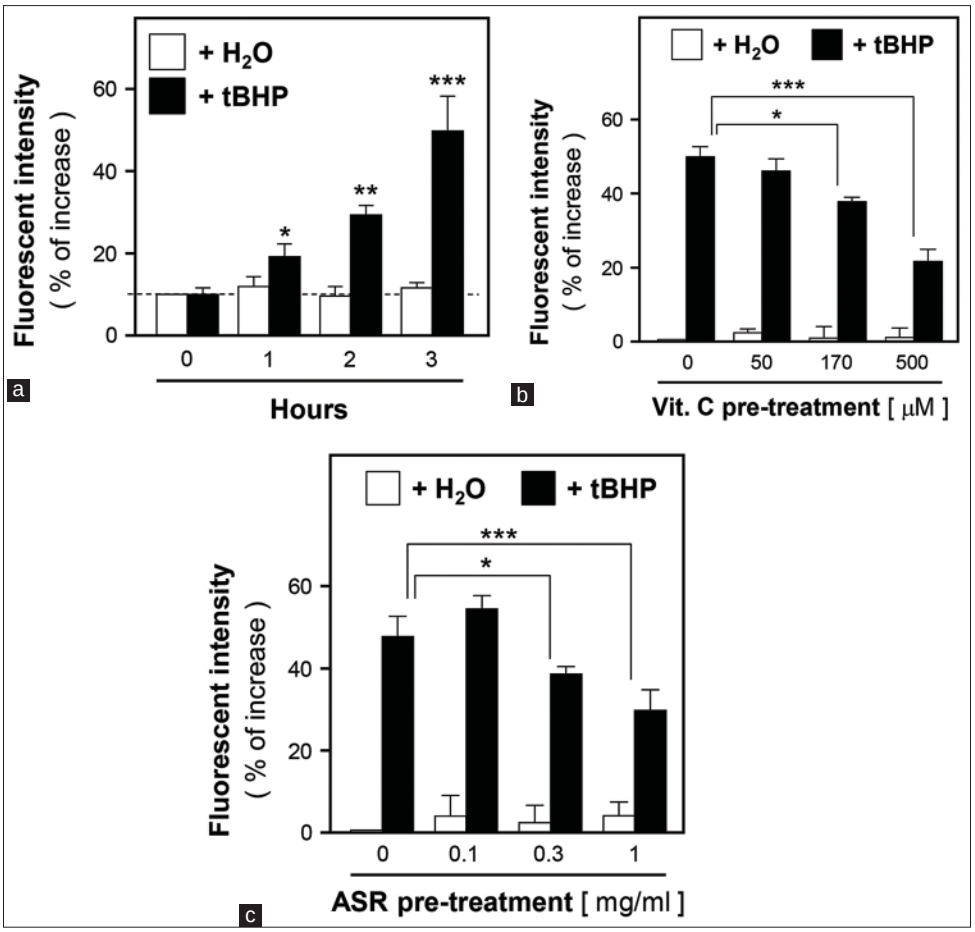

Figure 3: The tert-butyl hydroperoxide (tBHP)-induced reactive oxygen species (ROS) formation is reduced by Angelica Sinensis Radix (ASR) pre-treatment in PC12 cells. (a) PC12 cells were labeled by DCFH-DA (ROS fluorescent indicator) for 1 h, and then challenged by tBHP (150 μM) for 0-3 h. Fluorescent intensity was measured in each hour. (b) PC12 cells were pre-treated with vitamin C (0-500 μM) for 24 h, and then labeled by DCFH-DA as in (a). Finally, cultures were challenged by tBHP (150 μM) for 3 h and subjected to fluorometric measurement. (c) Experiment was performed as in (b) except the using of the ASR extracts (0.1-1 mg/ml) in the pre-treatment. Data were expressed as % of control where the value of untreated culture was set as 100; mean ± standard error of the mean; $n = 4$; *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$

a luciferase reporter having the repeats of ARE was used as a study tool [Figure 5b]. Cultured PC12 cells were transfected with this reporter and then treated with different concentrations of tBHQ (anti-oxidant, as a positive control) and ASR extract for 24 h. Lysates were collected and subjected to luciferase activity for quantification. The application of tBHQ dose-dependently stimulated the transcriptional activity of ARE in transfected PC12 cells [Figure 5b], indicating the appropriate responsiveness of measuring transcriptional activity of ARE by this luciferase reporter system. Similarly, the treatment of ASR extract increased the luciferase activity in a dose-dependent response [Figure 5c], with the maximum induction of ~5.6-fold at 1 mg/ml of ASR extract. These results provided another line of evidence to support antioxidative effect of ASR, via the activation of NQO1 and GSTA2 that represented a vital aspect of the cellular defense mechanism against oxidative stress in neurons.

The brain would suffer from different kinds of insult and damage, resulting in the occurrence of various types of neurodegenerative diseases. Here, two models of neurodegenerative diseases, Alzheimer's and Parkinson's diseases, were chosen for evaluation, which the neuronal cell death could be mimicked by Aβ [34] and MPP^+ [35], respectively. Experimentally, cultured PC12 cells were challenged with different doses of Aβ or MPP^+ for 24 h, followed by the cell viability test. PC12 cells were sensitive to the cytotoxic inducers, i.e. application of Aβ or MPP^+ killed the cells in a dose-dependent manner [Figure 6a and b]. The cytotoxic effects of Aβ or MPP^+ were similar, with ~50% and ~60% reduction in cell number, respectively, which were different from that of tBHP with ~80% decrease in cell number. For the protection of ASR, the results were different from that of tBHP. The pre-treatment of ASR extracts at all tested concentrations in PC12 cells for 24 h did not prevent the cell death against cytotoxic effects of Aβ or MPP^+ [Figure 6c and d]. These results indicated that ASR could protect neurons against cellular damage only from tBHP-induced oxidative stress, but not from Aβ or MPP^+, suggesting the selectivity of ASR on the neuroprotection. In addition, the cell death induced by tBHP, Aβ, and MPP^+ were reported to be triggered by different signaling pathways, which could, therefore, account for this discrepancy.

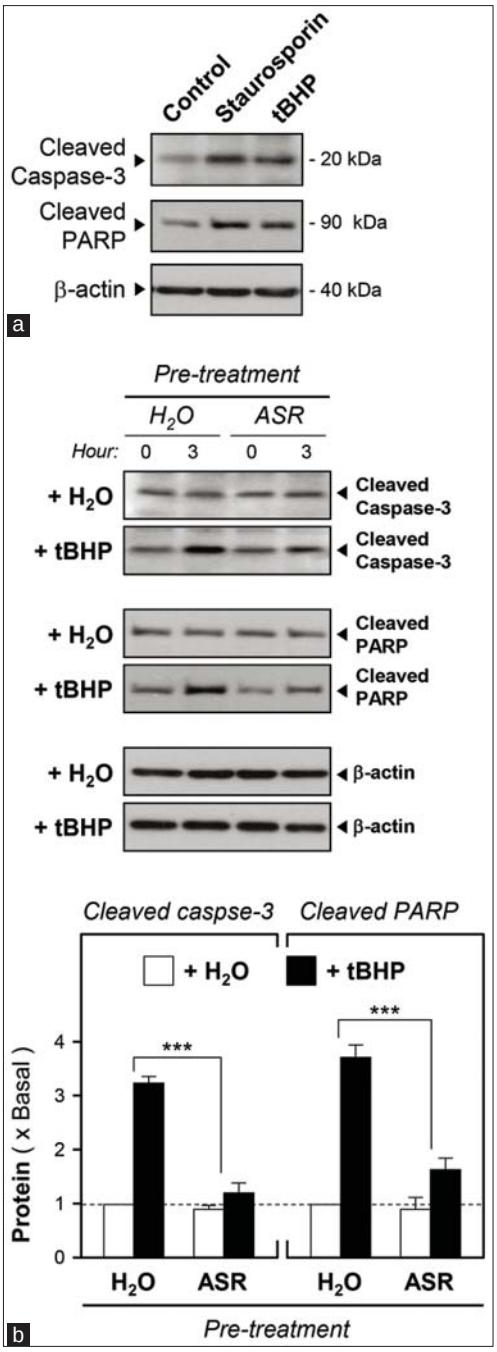

Figure 4: The neuroprotection effect of Angelica Sinensis Radix (ASR) is involved in the suppression of apoptosis. (a) PC12 cells were serum starved for 3 h before application of tert-butyl hydroperoxide (150 μM) for 3 h. Cultures were collected and analyzed for activation of two apoptotic markers, cleaved caspase-3 (~20 kDa) and cleaved PARP (~90 kDa) by Western blotting. Staurosporin served as a positive control of apoptotic stimulation, while β-actin (~40 kDa) served as an internal control for equal loading. Representative gel images were shown. (b) PC12 cells were pre-treated with ASR extracts (1 mg/ml) for 24 h. Cultures were then serum starved and subjected to tert-butyl hydroperoxide treatment as in (a) for measuring the change of cleaved caspase-3 and cleaved PARP (upper panel). Quantification of band intensity was performed (lower panel). Data were expressed as % of control where the value of target protein at 0 min was set as 100; mean ± standard error of the mean; $n = 4$; $^{***}P < 0.001$

DISCUSSION

Our previous research provided different lines of evidence in supporting the traditional functions of ASR. The water extracts of ASR have been shown to possess estrogenic activity in MCF-7 breast cells by stimulating the transcriptional activity of estrogen responsive element, which might account for a beneficial impact to women [19,21]. In blood circulatory system, ASR extract inhibited platelet aggregation *in vitro*, which might reduce the chance of having vascular blockage [19]. In addition, the immuno-modulating activity of ASR was proven by its stimulatory effects on cell proliferation and IL-8 secretion in T-lymphocytes, which might improve the immune system for health strengthening [36]. In osteogenic system, the enzymatic activity of alkaline phosphatase was increased by ASR extract in osteosarcoma MG-63 cells, which might promote bone differentiation process against osteoporosis [20]. On the other hand, its regulatory effect on the nervous system is rarely reported. In the current study, we found that the ASR extracts contained antioxidative activities against xanthine oxidase *in vitro* and tBHP-induced cytotoxicity in PC12 neuronal cells. Regarding the molecular mechanisms of ASR-mediated neuroprotection, the pre-treatment of ASR extracts was able to suppress ROS formation induced by tBHP. This reduction of intracellular oxidative stress by ASR finally led to inhibition of apoptosis, resulting in cell survival. In addition, ASR could activate a specialized cellular defense mechanism involving ARE-mediated NQO1 and GSTA2 expressions against cellular oxidation. A summarized diagram regarding the dual beneficial roles of ASR is illustrated in Figure 7. ASR could protect oxidation-induced cytotoxicity via: (i) the suppression effect of oxidative stress-stimulated ROS formation and apoptosis; and (ii) the activation of Nrf2-ARE-mediated self-defense mechanism against oxidation. Serving as an antioxidant, ASR should be a better agent as compared to vitamin C: the main reason is the multi-components of ASR that could work synergistically on the cellular responses to stress. Owing to the popularity of ASR in herbal decoctions and food diets, the current data in supporting the neuroprotective role of ASR might facilitate the development of health food supplement, or even therapeutic drug, against oxidative stress and neurodegenerative diseases in future.

The methanol extract of ASR has been reported to attenuate the neurotoxicity of Aβ and Tau hyperphosphorylation in cortical neurons via the stimulation of Akt and inhibition of GSK-3β [37]. In addition, the Aβ-induced cytotoxicity could also be reduced by the ethanol extract of ASR in cultured Neuro 2A cells, via decrease of ROS and prevented depletion of glutathione (GSH; an antioxidant) level [38]. In contrast to these studies, the neuroprotective effect of ASR extract, as shown here, was not effective for Aβ for Alzheimer's disease and MPP^+ for Parkinson's disease. This discrepancy might be explained by the choice of extraction solvents, i.e. water vs. methanol/ethanol, resulting in the difference in chemical compositions. The organic acids and polysaccharides were the components within ASR water extract while the phthalides could be more abundant within ASR methanol/ethanol

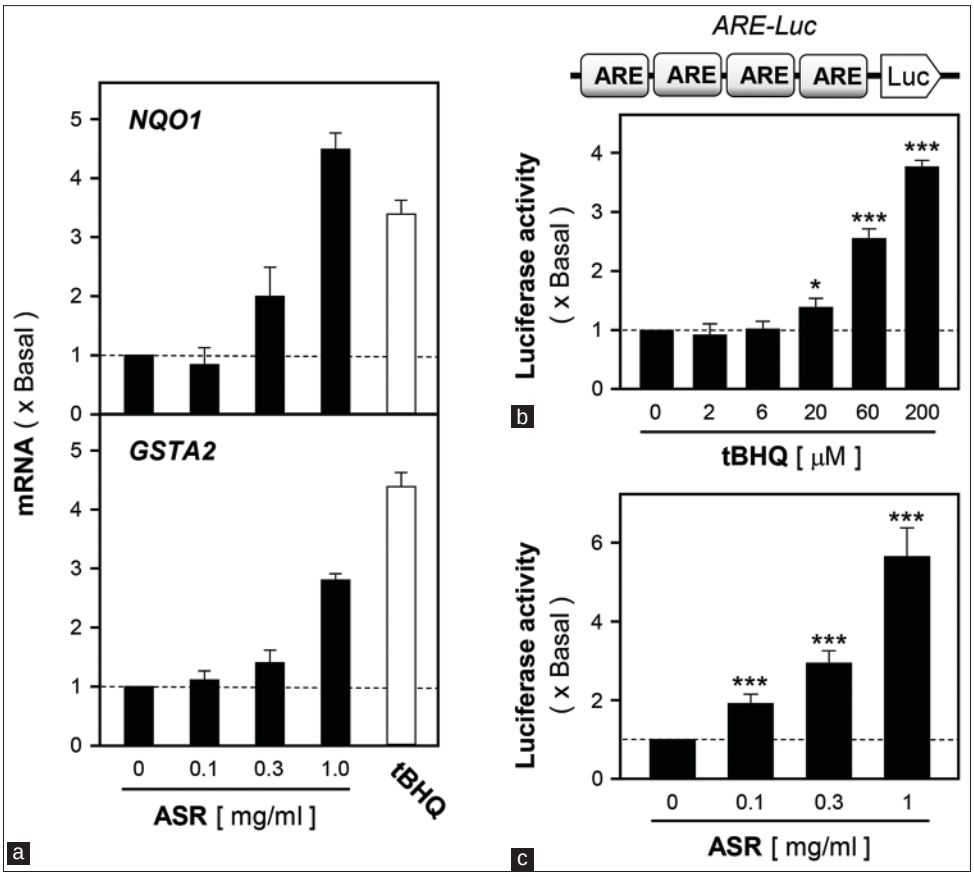

Figure 5: Angelica Sinensis Radix (ASR) extracts activate a cellular defense mechanism involving ARE-mediated NQO1 and GSTA2 expression in PC12 cells. (a) PC12 cells were treated with ASR extracts (0.1-1 mg/ml) for 16 h, and then collected to quantify the change of NQO1 mRNA expression by real-time PCR analysis. Application of tert-butyl hydroperoxide (tBHQ) (200 μM) served as a positive control. (b) The expression of GST2A was performed as in (a). (c) A commercial luciferase-reporter having 4 repeats of ARE (ARE-Luc) was used as a study tool. PC12 cells were transfected with this reporter and then treated with tBHQ (2-200 μM) for 24 h for luciferase activity measurement. Data were expressed as x basal where the value of water-treated control was set as 1, mean ± standard error of the mean, $n = 4$. $^{*}P < 0.05$; $^{***}P < 0.001$

extract [39]. In addition, the amount of Z-ligustilide was dropped dramatically after boiling in water [40]. This chemical distinction might govern the neuroprotective ability of ASR; the effect of ASR in protecting neurons against oxidative insult would be from the water extract while that against Aβ-induced cytotoxicity would be from organic extract. However, the exact biological components of ASR for this differential protection might require further analysis.

Over 80 chemicals so far have been identified in ASR, including organic acids and their esters, e.g. ferulic acid, polyacetylenes, and essential oils (e.g. ligustilide, senkyunolide A, and phthalide) [39,41]. Among them, Z-ligustilide has been demonstrated to exert a pronounced protective effect against oxidative stress through the signaling of ROS, Bax, caspase-3 and cytochrome c in PC12 cells [42]. In rat model, administration of Z-ligustilide decreased the Aβ-induced cytotoxicity via inhibition of tumor necrosis factor-α-mediated NF-κB cascade [43]. Such neuroprotection effect of ligustilide could also be found in our culture system. Even though ligustilide was organic in nature with little abundance, the ASR sample prepared by water extraction still relatively contained a larger amount of ligustilide when compared with other herbs, i.e. at least 0.28 mg/g of dried ASR extract. Therefore, ligustilde might be one of the active ingredients to account for the neuroprotective effect of ASR in our study. Ferulic acid is another major active component found in non-aromatic fractions in ASR, which has been demonstrated to mediate a variety of cellular responses, including anti-cancer formation, antioxidant, anti-inflammation and anti-thrombosis [44-47]. In the nervous system, long-term administration of ferulic acid alleviated the memory impairment caused by Aβ in mice model [48] as well as to reduce the cytotoxicity of Aβ in Neuro 2A cells [38]. Z-ligustilide is a non-polar compound showing lipophilic nature. Ferulic acid is a phenolic compound, which is a slightly polar compound. Z-ligustilide and ferulic acid have been shown to cross the blood-brain-barrier [49,50]. These results therefore suggested that the neurobeneficial effect of ferulic acid on neuroprotection might be cell-type specific and insult-dependent.

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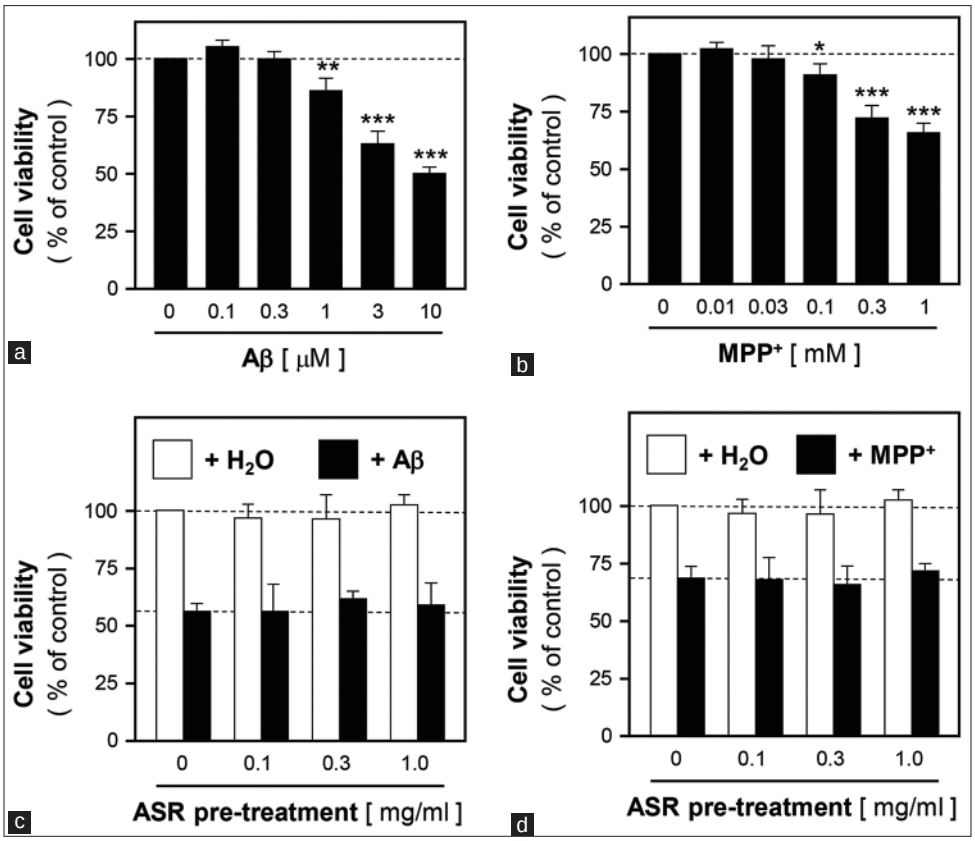

Figure 6: Angelica Sinensis Radix (ASR) extracts do not exert any protection effect in PC12 cells against Aβ and MPP+- induced neuronal cytotoxicity, (a,b): PC12 cells were challenged with β-amyloid (0-10 μM) and MPP+ (0-1 mM) for 24 h, followed by the cell viability assay. (c,d): PC12 cells were pre-treated with ASR extracts (0.1-1.0 mg/ml) for 24 h before the application of β-amyloid (10 μM) or MPP+ (1 mM) for another 24 h. Cell viability was then tested as in (a). Data were expressed as % of control where the value of untreated cultures was set as 100; mean ± standard error of the mean; $n = 4$; $^{*}P < 0.05$ and $^{***}P < 0.001$

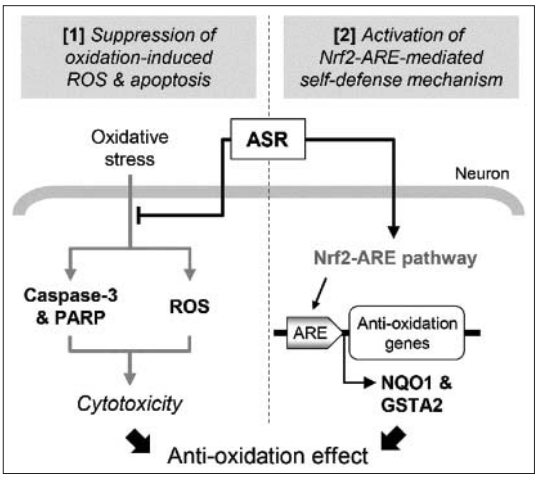

Figure 7: The proposed model of the Angelica Sinensis Radix (ASR)-mediated neuroprotection against oxidative stress. ASR protects oxidation-induced cytotoxicity in neurons via: (1) The suppression effect of oxidative stress-stimulated ROS formation and apoptosis; and (2) the activation of Nrf2-ARE-mediated self-defense mechanism against oxidation

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