Angelica Sinensis Extract Protects Against Memory Deficits by Promoting P38 MAPK-mediated Hippocampal Neurogenesis and Neuronal Survival in the Chronic Phase of Transient Global Cerebral Ischemia in Rats

Yu-Chen Lee

China Medical University

Hui-Chi Huang

China Medical University

Shung-Te Kao

China Medical University

Chin-Yi Cheng (✉ c5398@ms22.hinet.net)

School of Post-baccalaureate Chinese Medicine, College of Chinese Medicine, China Medical University, Taichung 40402, Taiwan

Research

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Abstract

Background: Reversible cardiac arrest and diseases related to reduced blood flow to the brain cause transient global cerebral ischemia (GCI), which may induce long-term cognitive deficits. In addition, hippocampal cornu ammonis 1 (CA1) pyramidal neurons are selectively vulnerable to ischemic insults and play an important role in memory. This study aimed to determine the effects of the *Angelica sinensis* (Oliv.) Diels (ASD) extract on ischemia-induced memory deficits at 28 d after transient global cerebral ischemia (GCI) and to investigate the precise mechanisms underlying the p38 mitogen-activated protein kinase (MAPK)-related signaling pathway's involvement in hippocampal neurogenesis. Rats underwent 25 min of four-vessel occlusion. The ASD extract was intragastrically administered at doses of 0.25 g/kg (ASD-0.25 g), 0.5 g/kg (ASD-0.5 g), 1 g/kg (ASD-1 g), 1 g/kg after dimethyl sulfoxide administration (D+ASD-1 g), or 1 g/kg after SB203580 (a p38 MAPK inhibitor) administration (SB+ASD-1 g) at 1, 3, 7, 10, 14, 17, 21, and 24 d after transient GCI.

Results: *ASD-0.5 g, ASD-1 g, and D+ASD-1 g treatments effectively attenuated memory deficits and had the following effects: upregulation of bromodeoxyuridine (BrdU) and Ki67 expression, and BrdU/neuronal nuclei (NeuN) co-expression in the hippocampal dentate gyrus (DG); upregulation of microtubule-associated protein 2 /NeuN co-expression in the hippocampal CA1 region; upregulation of phospho-p38 MAPK (p-p38 MAPK), phospho-cAMP response element-binding protein (p-CREB), brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), and vascular endothelial growth factor A (VEGF-A) expression in the hippocampus. Furthermore, most BrdU-positive cells colocalized with NeuN and Ki67 in the hippocampal DG. SB+ASD-1 g treatment abrogated the effects of ASD-1 g on the expression of these proteins and exacerbated memory deficits.*

Conclusion: *ASD-0.5 g and ASD-1 g treatments provided neuroprotective effects against memory deficits by enhancing hippocampal neurogenesis and dendritic stability. The effects of the ASD extract on hippocampal neurogenesis and neuronal survival are caused by the activation of p38 MAPK-mediated CREB/BDNF, GDNF, and VEGF-A signaling pathways in the hippocampus at 28 d after transient GCI.*

Background

Various clinical conditions, including reversible cardiac arrest and diseases related to reduced blood flow to the brain, induce transient global cerebral ischemia (GCI). Transient GCI frequently causes long-lasting cognitive impairments, principally memory and learning dysfunction, and contributes to the development of neurological disorders, such as vascular dementia [1, 2]. Hippocampal cornu ammonis 1 (CA1) neurons are selectively vulnerable and undergo delayed degeneration in response to ischemic injury. Furthermore, the degeneration of these pyramidal neurons results in cognitive impairment [3]. However, the pharmacological rescue of post-ischemic hippocampal neurogenesis enhances cognitive function recovery [4, 5]. The process of adult hippocampal neurogenesis includes the proliferation, differentiation, and survival of neural progenitor cells, which are regulated by different signaling pathways following ischemic brain injury [6]. Activation of mitogen-activated protein kinase (MAPK) cascades, including

extracellular signal-regulated kinase 1/2 and p38 MAPK, plays crucial roles in neurogenesis in the hippocampal dentate gyrus (DG) after cerebral ischemia [7]. Furthermore, various ischemic stroke models have indicated that p38 MAPK plays a dual role in the regulation of adult hippocampal neurogenesis. Studies have reported that activation of p38 MAPK suppresses neurogenesis in the ischemic cortex and hippocampus in the chronic phase of cerebral ischemia [8, 9], whereas other studies have reported that activation of p38 MAPK-mediated signaling promotes the differentiation of neural stem/progenitor cells (NSPCs) and neurogenesis in the ischemic area after transient [10] and permanent [11] middle cerebral artery occlusion. During neurogenesis, the MAPK signaling pathway activates the downstream transcription factor cAMP response element-binding protein (CREB), which binds to DNA, thus inducing target gene transcription, and enhancing neural progenitor cell proliferation, differentiation, and survival in the hippocampal DG, which reduces memory impairment after GCI [12].

The adult mammalian brain is known to continuously generate new neurons in the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the hippocampal DG. Adult hippocampal progenitor cells are generated at the border between the dentate granule cell layer (GCL) and the hilus [13]. Moreover, trophic factors, such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), glial cell line-derived neurotrophic factor (GDNF), and vascular endothelial growth factor (VEGF), are crucial in facilitating neuronal proliferation, differentiation and survival [14, 15]. Studies have reported that the upregulation of neurotrophic factors is responsible for the effects of MAPK signaling cascades on ischemia-induced hippocampal neurogenesis [15, 16]. Neurogenesis principally occurs in the peri-injured brain region (SVZ and SGZ), where the newborn neurons migrate, along angiogenic niches, into the existing hippocampal network and the damaged cortex. The newborn neurons subsequently provide cell replacement, thereby exerting a compensatory effect after ischemic stroke [10, 17]. Thus, neurogenesis from progenitor cells occurs in the hippocampal DG as a compensatory action to brain insults, such as GCI [1, 18].

Bromodeoxyuridine (BrdU) is a synthetic analog of thymidine that can be incorporated into DNA in the synthesis phase of the cell cycle, and it is widely used as an indicator of cell proliferation in living tissues and as a marker of neurogenesis [18]. BrdU is expressed in the SGZ of the hippocampal DG after GCI [19]. Ki67 is a cell cycle-related protein, and it has been identified as a cell proliferation marker. Ki67 is primarily expressed in the hippocampal DG after cerebral ischemic injury [20]. Therefore, after GCI, hippocampal progenitor cell proliferation is identified based on BrdU and Ki67 expression in the DG. The pharmacological upregulation of BrdU expression in the hippocampal DG indicates that the therapeutic intervention has triggered the self-renewal of NSPCs after ischemic stroke [21]. Microtubule-associated protein 2 (MAP-2), located in the neuronal perikarya and dendrites, is crucial in plasticity and in stabilizing the dendrite shape during neuronal development [22]. MAP-2, a neuronal dendritic marker, can be used as an early indicator for measuring the development of mature neurons in neurogenesis after cerebral ischemia [23].

Angelica sinensis (Oliv.) Diels (ASD), commonly known as Dang Gui, is a popular Chinese herb that has long been used to treat gynecological diseases, anemia, and ischemic stroke [24, 25]. The major bioactive

compounds of ASD are ferulic acid and ligustilide [26]. Ferulic acid protects against memory deficits by downregulating the toll-like receptor 4/myeloid differentiation factor 88 signaling pathway after transient GCI [27]. A study in a rat model of chronic cerebral hypoperfusion (CCH) revealed that ligustilide exerts neuroprotective effects on memory by inhibiting apoptosis in the hippocampus [2]. The ASD extract enhances adult hippocampal neurogenesis by activating CREB/BDNF signaling in the hippocampus after CCH [28]. In a previous study, we determined that the ASD extract exerts neuroprotective effects against cerebral ischemia/reperfusion (I/R) injury after transient GCI by activating the p38 MAPK-mediated 90-kDa ribosomal S6 kinase (p90RSK)/phospho-Bad and p90RSK/CREB/BDNF signaling pathways in the hippocampus [29]. Based on the aforementioned findings, we speculated that the ASD extract protects against ischemia-induced memory impairment by regulating p38 MAPK-related signaling in the hippocampus in the chronic phase of transient GCI.

Therefore, we aimed to determine the effects of the ASD extract on hippocampal neurogenesis and neuronal survival at 28 d after transient GCI and to investigate the precise mechanisms underlying p38 MAPK-related signaling in the hippocampus.

Methods

Experimental animals

Adult male Sprague-Dawley rats (300–350 g) were purchased from Lasco Co. (Taiwan). All rats were housed under standard laboratory conditions and were maintained in a constant 12:12 h light–dark cycle, at a controlled temperature of 22–24 °C, and in relative humidity of 55%. All study procedures were performed following the guidelines approved by the Institutional Animal Care and Use Committee of China Medical University (Permit Number: 2018-075). All surgical procedures were performed under appropriate anesthesia to avoid or minimize discomfort, pain, and stress in the animals.

ASD extract preparation

ASD extracts, with ASD powders (Batch Number A1306801), were purchased from Chuang Song Zong Pharmaceutical Co., Ltd. (Kaohsiung, Taiwan). ASD extract powder was prepared as previously described [29]. In brief, the dried roots of ASD were extracted using boiling water for 1.5 h and then the aqueous extract and essential oils from ASD were collected. The concentrated aqueous extract, essential oils, and excipients including microcrystalline cellulose and corn starch were placed in the fluidized bed dryer and granulated using fluidized bed granulation techniques. Each gram of the ASD powder consists of 0.5 g of the DG extract, 0.4 g of microcrystalline cellulose, and 0.1 g of corn starch. In this study, 2 g of the ASD extract powder was dissolved in 8 mL of normal saline. The supernatant fraction of the ASD extract was subsequently obtained by centrifugation at $1000 \times g$ at 4 °C for 10 min. The final concentration of the ASD aqueous extract was 0.125 g/mL.

Assessment of ASD extract indicators using ultra-performance liquid chromatography

The indicator compounds of the ASD extract, including ferulic acid and ligustilide, were detected using an Agilent ultra-performance liquid chromatography (UPLC) system (Agilent 1260 Infinity II, Agilent Technologies, Inc.). Ferulic acid (purity > 99%, Sigma-Aldrich, St. Louis, MO, USA) and ligustilide (purity > 98%, Adooq BioScience, Irvine, CA, USA) were accurately weighted and subsequently dissolved in methanol to prepare the standard solutions. A series of standard solutions were prepared with concentrations of 1, 0.5, 0.25, 0.1, 0.05, 0.025, and 0.01 mg/mL. The aqueous extract of ASD (0.125 g/mL) was prepared as the test solution. One microliter of the standard solution or 3 μ L of the test solution was injected using an autosampler (Agilent 1260 Infinity II Vialsampler). The UPLC profile of the ASD extract was performed using a RP-18 column (Poroshell 120 EC-C18, 3.0 $\times$ 100 mm. I.D., 2.7 μ m). The mobile phase consisted of a mixture of acetonitrile (A) and 0.03% phosphoric acid (B) solution. Gradient elution was performed as follows: 0–12 min (phase A: 5%–70%), 12–13 min (phase A: 70%–80%), and 13–15 min (phase A: 80%–100%). The flow rate was 0.5 mL/min and the ultraviolet detection wavelength was set at 320 nm.

Transient GCI

GCI was induced in the rats using the 4-vessel occlusion (4-VO) method as previously described [30]. In brief, the rats were anesthetized by inhalation of 5% isoflurane, and the anesthesia was maintained using 2% isoflurane. A dorsal neck midline incision was performed to expose the alar foramina of the first cervical vertebrae, and the bilateral vertebral arteries were permanently occluded using electrocautery. Twenty-four hours later, another incision was performed on the ventral neck to expose the bilateral common carotid arteries (CCAs), which were occluded for 25 min using vascular clips. The rats that displayed the loss of righting reflex and whose pupils were dilated during the ischemic period were validated as successful 4-VO models and were included in this study.

Passive avoidance task

A passive avoidance task was performed to evaluate 4-VO-induced memory deficits; the task was a modification of previously described procedures [31]. The passive avoidance apparatus (San Diego Instruments, San Diego, CA, USA) consisted of a box with two equal-sized light/dark compartments (25 $\times$ 20 $\times$ 17 cm³ each), which were separated by a guillotine-type door (9 $\times$ 7 cm²). The floor of each compartment consisted of 14 stainless steel rods. The floor rods of the dark compartment administered a brief electric shock. The memory evaluation test consisted of training and retention protocols. The training protocol comprised two acquisition tests 1 h before surgery. In the first acquisition test, the rats were individually placed in the light compartment for a 30-s habituation period. The guillotine door was then raised; when the rat entered the dark compartment, the door was immediately closed and a brief electric foot shock (50 Hz, 0.5 mA, 3 s) was delivered. The training was completed when the rats no longer entered the dark compartment before the cut-off time (120 s). The retention protocol was performed without foot shock at 7, 14, 21, and 28 d after reperfusion. The latency to enter the dark compartment (step-through latency [STL]) was measured, and the maximum score was recorded as 300 s.

Experiment A

Grouping

The rats were randomly divided into seven groups ($n = 4-5$): Sham, Control, ASD-0.25 g, ASD-0.5 g, ASD-1 g, D+ASD-1 g, and SB+ASD-1 g groups. The rats in the ASD-0.25 g, ASD-0.5 g, and ASD-1 g groups were intragastrically administered 0.25 g/kg, 0.5 g/kg, and 1 g/kg of the ASD extract, respectively, at 1, 3, 7, 10, 14, 17, 21, and 24 d after transient GCI. Furthermore, to determine the rate of progenitor proliferation, the rats received daily intraperitoneal injections of BrdU (50 mg/kg, Sigma-Aldrich) from day 14 to day 27 after reperfusion. On day 28 after reperfusion, the rats underwent CO₂ euthanasia until 1 min after breathing had stopped and their brains were quickly removed. The rats in the D+ASD-1 g group underwent the same experimental procedures as those in the ASD-1 g group, but they were intracerebroventricularly (ICV) administered 1% dimethyl sulfoxide (DMSO) before the bilateral CCA occlusion. The rats in the SB+ASD-1 g group underwent the same experimental procedures as those in the D+ASD-1 g group, but they were ICV administered SB203580, a selective inhibitor of p38 MAPK, instead of 1% DMSO. The rats in the Control group underwent the same experimental procedures as those in the ASD-1 g group, but the ASD extract was substituted by saline solution. The rats in the Sham group underwent the same experimental procedures as those in the Control groups, but their bilateral CCAs were only exposed instead of occluded.

ICV administration of SB203580 or 1% DMSO

The rats were ICV administered SB203580 or 1% DMSO after anesthesia maintenance through isoflurane inhalation, and two symmetrical burr holes were drilled on both sides of the parietal bone. The burr holes were 2 mm lateral, 3 mm posterior to the bregma, and 3.5 mm under the cortical surface. The rats were ICV administered 10 μ L SB203580 (2 mM, #S1076 Selleckchem.com) or 1% DMSO solution through each burr hole. ICV administration of the solution was performed using a microliter syringe (10 μ L, Hamilton Company, Reno, NV, USA).

Assessment of immunofluorescence staining

The rats were euthanized by inhalation of CO₂ at 28 d after reperfusion, and their brains were rapidly removed. The brain tissue sections were prepared as previously described [29]. Coronal brain sections were fixed with 4% paraformaldehyde for 15 min at room temperature (RT). After the brain sections were washed thrice with phosphate-buffered saline/0.01% Tween 20 (PBST), the sections were incubated with 1% Triton X-100 for 30 min at RT. The sections were incubated with 1% bovine serum albumin for 1 h at RT and were subsequently exposed to rabbit (1:100 dilution) and mouse (1:100 dilution) primary antibodies (listed in Table 1) overnight at 4 °C. After the sections were washed thrice with PBST, they were incubated with anti-rabbit (1:200 dilution) and anti-mouse (1:200 dilution) secondary antibodies for 1.5 h at 37 °C. The sections were then counterstained with 4',6-diamidino-2-phenylindole (DAPI, ab4139 abcam) and mounted using an aqueous mounting medium. The immunopositive cells in the selected

hippocampal SGZ or CA1 regions were detected in each of three 400× magnification fields under a fluorescence microscope (CKX53, Olympus, Tokyo, Japan).

Assessment of immunohistochemistry

The brain tissue sections were prepared, and subsequent immunohistochemistry (IHC) evaluations were performed as previously described [32]. Anti-ki67 antibody used in the IHC evaluation was listed in Table 1. Immunopositive cells in the hippocampal SGZ region were calculated in each of three 400× magnification fields using a light microscope (Axioskop 40, Zeiss, Oerzen, Germany). For the negative control, the brain section in the ASD-1 g group was incubated without Ki67 primary antibody.

Experiment B

Grouping

The rats were randomly divided into seven groups ($n = 4$): Sham, Control, ASD-0.25 g, ASD-0.5 g, ASD-1 g, D+ASD-1 g, and SB+ASD-1 g groups. These groups underwent the same procedures as those performed in Experiment A.

Evaluation of Western blot

The rats underwent CO₂ euthanasia at 28 d after reperfusion, and their brains were immediately removed. The right and left hippocampal tissues were carefully separated, collected, and homogenized on ice. The protein concentrations of the hippocampal tissues were measured using a Bio-Rad protein assay. The proteins separated by gel electrophoresis, transfer onto nitrocellulose membranes, and densitometric analysis were performed as previously described [33]. The primary antibodies used in the Western blot analyses were listed in Table 1.

Statistical analysis

All the results are expressed as means $\pm$ standard deviations. Statistical analysis of the data obtained for the experiment groups from SLT, immunofluorescence (IF), IHC, and Western blot analyses was performed using one-way analysis of variance followed by Scheffé's post-hoc test. Significant differences were accepted for $P < 0.05$.

Results

UPLC analysis of the ASD extract

After the detection of the standard and ASD extract solutions through UPLC, the retention times (T_R) of ferulic acid and ligustilide were revealed at 5.87 min and 12.81 min, respectively. The concentrations of ferulic acid and ligustilide in the ASD extract solution were 0.234 mg/mL and 0.027 mg/mL, respectively (Figs. 1A–1C).

Effects of ASD extract treatments on memory deficits

The STL was lower in the Control group than in the Sham group (all $P < 0.05$) and was greater in the ASD-0.5 g, ASD-1 g, and D+ASD-1 g groups than that in the Control group (all $P < 0.05$; Fig. 2) at 7, 14, 21, and 28 d after reperfusion. However, the STL did not differ significantly between the Control, ASD-0.25 g, and SB+ASD-1 g groups ($P > 0.05$).

Effects of ASD extract treatments on BrdU expression and BrdU/neuronal nuclei co-expression

Fewer BrdU-positive and BrdU/neuronal nuclei (NeuN)-positive cells were identified in the SGZ of the hippocampal DGs (Fig. 3B) of the Control group compared with the Sham group (both $P < 0.05$) and more positive cells were identified in the ASD-0.5 g, ASD-1 g, and D+ASD-1 g groups compared with the Control group at 28 d after reperfusion (all $P < 0.05$; Figs. 3A, 3C, and 3D). The numbers of BrdU-positive and BrdU/NeuN-positive cells in the hippocampal SGZs did not differ significantly between the Control, ASD-0.25 g, and SB+ASD-1 g groups (all $P > 0.05$). The majority of BrdU-positive cells colocalized with NeuN in the hippocampal SGZ.

Effects of ASD extract treatments on Ki67 expression

Fewer Ki67-positive cells were identified in the hippocampal SGZ (Fig. 4C) of the Control group compared with the Sham group ($P < 0.05$), and higher numbers were observed in the ASD-0.5 g, ASD-1 g, and D+ASD-1 g groups compared with the Control group at 28 d after reperfusion (all $P < 0.05$; Figs. 4A and 4D). However, the number of Ki67-positive cells in the hippocampal SGZ did not differ significantly between the Control, ASD-0.25 g, and SB+ASD-1 g groups (all $P > 0.05$). Furthermore, BrdU-positive cells colocalized with Ki67 in the hippocampal SGZ (Figs. 4E-1–E-3).

Effects of ASD extract treatments on MAP-2/NeuN co-expression

Fewer MAP-2/NeuN-positive cells were identified in the CA1 region (Fig. 5B) of the Control group compared with the Sham group ($P < 0.05$), and more MAP-2/NeuN-positive cells were identified in the ASD-0.5 g, ASD-1 g, and D+ASD-1 g groups compared with the Control group at 28 d after reperfusion (all $P < 0.05$; Figs. 5A and 5C). The number of MAP-2/NeuN-positive cells in the CA1 region did not differ significantly between the Control, ASD-0.25 g, and SB+ASD-1 g groups (all $P > 0.05$).

Effects of ASD extract treatments on p-p38 MAPK, p38 MAPK, p-CREB, CREB, BDNF, NGF, GDNF, and VEGF-A expression

The ratios of p-p38 MAPK/p38 MAPK, p-CREB/CREB, BDNF/actin, GDNF/actin, and VEGF-A/actin in the hippocampus were lower in the Control group (0.4-fold, 0.4-fold, 0.3-fold, 0.4-fold, and 0.4-fold, respectively) compared with the Sham group (all $P < 0.05$) and were greater in the ASD-0.5 g (2.5-fold, 2.3-fold, 4.2-fold, 2.4-fold, and 2.7-fold, respectively), ASD-1 g (2.9-fold, 2.1-fold, 4.1-fold, 2.4-fold, and 2.6-fold, respectively), and D+ASD-1 g (2.6-fold, 2.3-fold, 4.2-fold, 2.6-fold, and 2.8-fold, respectively) groups compared with the Control group at 28 d after reperfusion (all $P < 0.05$; Figs. 6A–6D, 7A, 7C, and 7D).

However, the ratios of these proteins did not differ significantly between the Control, ASD-0.25 g, and SB+ASD-1 g groups ($P > 0.05$). Furthermore, no significant differences were identified in the hippocampal NGF/actin ratios in the experimental groups ($P > 0.05$; Figs. 7A and 7B).

Discussion

In a previous study, we determined that ASD extract treatments protect against I/R injury by activating p38 MAPK-mediated signaling in the hippocampus at 7 d after transient GCI [29]. In the present study, we extended our research to the chronic phase (28 d) of GCI to clarify the relationship between p38 MAPK-related signaling and adult hippocampal neurogenesis. Various ischemic models have indicated that the p38 MAPK-mediated signaling pathway plays diverse roles in ischemia-induced cognitive impairment. Studies have reported that pharmacological treatments promote functional recovery of memory deficits by suppressing p38 MAPK-mediated inflammatory cascades in the acute [34] and chronic [9] phases of transient cerebral ischemia. However, our previous findings revealed that activation of p38 MAPK/p90RSK-related signaling pathways in the hippocampus provides neuroprotective effects against I/R injury in the subacute phase of transient GCI [29]. In the present study, the rats underwent 25 min of 4-VO, resulting in significant memory deficits at 7–28 d after transient cerebral ischemia. The ASD extract administered at doses of 0.5 g/kg (ASD-0.5 g), 1 g/kg (ASD-1 g), and 1 g/kg after ICV injections of 1% DMSO (D+ASD-1 g) effectively attenuated memory impairment; administering 0.25 g/kg (ASD-0.25 g) did not attenuate memory impairments. However, ICV injections of SB203580, a selective inhibitor of p38 MAPK, (SB+ASD-1 g) completely abrogated the effects of the ASD extract (ASD-1 g) on promoting the functional recovery of memory. Therefore, activation of p38 MAPK in the hippocampus may have a key function in restoring memory loss in the chronic phase of transient GCI. Increasing evidence indicates that hippocampal neurogenesis originating from progenitor cells in the DG region is closely related to the restoration of the ischemia-induced memory deficits following transient GCI [3, 35]. Furthermore, activation of the p38 MAPK-related pathways enhances the survival and differentiation of newborn neurons, inducing increased neurogenesis in the ischemic areas after ischemic stroke [10, 11]. Therefore, the effects of ASD extract treatments (ASD-0.5 g and ASD-1 g) on memory dysfunction may be, at least partially, caused by the promotion of p38 MAPK-mediated adult hippocampal neurogenesis in the chronic phase of transient GCI.

Adult hippocampal neurogenesis is a complex multistep process that includes the proliferation and differentiation of neurons to generate new excitatory granule cells; the process originates from progenitor cells in the SGZ of the DG and culminates in various signaling events triggered by stimuli [36]. During neurogenesis, progenitor cells proliferate in the SGZ of the hippocampus and subsequently differentiate into neurons or glial cells [37]. The thymidine analog BrdU is incorporated in the newly synthesized DNA of replicating cells and is commonly used to detect neural progenitor cell proliferation in the central nervous system (CNS) [38]. Studies have demonstrated that most BrdU-labeled cells colocalize with the neuronal marker NeuN in the hippocampal SGZ in the chronic phase of transient GCI. Furthermore, BrdU/NeuN double-labeled cells represent newborn neurons, which are closely associated with recovery of hippocampal function [19, 39]. Ki67, a mitosis-related protein expressed in dividing cells, exhibits a

similar expression pattern to BrdU. Therefore, Ki67 is also widely used as a cell proliferation marker. Moreover, Ki67 is primarily applied as a diagnostic tool in the study of CNS tumors [20, 40]. Studies have reported that the pharmacological promotion of BrdU-associated and Ki67-associated neural progenitor cell proliferation in the SGZ of the DG exerts neuroprotective effects against memory dysfunction in the chronic phase of GCI [41, 42]. The IF, IHC, and Western blot results of the present study indicated that the majority of BrdU-positive cells were double-labeled with NeuN and Ki67, and that the numbers of BrdU-, BrdU/NeuN- and Ki67-positive cells were downregulated in the SGZ of the DG. ASD extract treatments effectively restored the reduced numbers of these immunopositive cells in the SGZ of the DG at 28 d after GCI. However, SB+ASD-1 g treatment significantly reduced the effects of ASD-1 g on p38 MAPK activation, and increased BrdU, BrdU/NeuN, and Ki67 expression in the hippocampus in the chronic phase of GCI. These results demonstrated that ASD extract treatments effectively promote newborn neuron and BrdU/Ki67-mediated progenitor cell proliferation in the SGZ of the DG at 28 d after transient GCI by activating the p38 MAPK-related signaling pathway in the hippocampus.

MAP-2, a marker of neuronal dendrites, stabilizes the microtubule cytoskeleton of dendrites and is a sensitive and early indicator for evaluating dendritic integrity, neuronal damage, and neuron maturity in post-ischemia neurogenesis [2, 23, 43]. In the process of neurogenesis, BrdU/MAP-2 double-labeled cells are generated in the SGZ and migrate to the GCL, where they become mature neurons, whereas reduced MAP-2 expression in the hippocampal CA1 region indicates neuronal damage or death [43, 44]. Increased MAP-2 expression in the CA1 region promotes hippocampal dendrite and synapse stability, which alleviates memory dysfunction in the chronic phase of GCI [43, 45]. In the hippocampal CA1 region, MAP-2-positive cells co-labeling with NeuN indicate dendritic outgrowth of hippocampal pyramidal neurons. In addition, NeuN, a marker of mature neuron, can be used to determine the survival of neuron following ischemic insults [46]. In the present study, IF double staining revealed that the expression of MAP-2/NeuN was downregulated in the hippocampal CA1 regions. However, 28 d after transient GCI, ASD extract treatments effectively restored MAP-2/NeuN expression. These findings further indicate that ASD treatments alleviate neuronal damage and enhance the dendritic outgrowth recovery of injured neurons in the CA1 region, thereby promoting neuronal survival. However, SB+ASD-1 g treatment markedly reduced the effects of ASD-1 g on hippocampal neuronal survival. These results indicate that ASD extract treatments provide neuroprotective effects against memory deficits by promoting dendritic stability in hippocampal pyramidal neurons. Furthermore, the effects of ASD extract treatments on hippocampal neuronal survival and dendritic stability at 28 d after transient GCI are caused by the activation of the p38 MAPK-mediated signaling pathway in the hippocampus.

Studies have demonstrated that the p38 MAPK signaling pathway activates the transcription factor CREB, which regulates several downstream genes and provides anti-apoptotic neuroprotective effects in the penumbra following transient focal cerebral ischemia [47, 48]. Our previous study indicated that pharmacological activation of p38 MAPK/CREB signaling in the hippocampus protects against I/R injury in the subacute phase of transient GCI [29]. CREB activation plays a crucial role in adult hippocampal neurogenesis, including cell proliferation, differentiation, maturation, and survival, and hippocampal dendritic-synaptic plasticity, which facilitate memory recovery after cerebral ischemia [49, 50]. After GCI,

BDNF and NGF, the downstream targets of CREB, enhance the proliferation and differentiation of progenitor cells, accelerate the maturation of newborn neurons, increase neuronal survival, and promote hippocampal synaptic formation and plasticity, resulting in an increase in adult hippocampal neurogenesis and the recovery of hippocampus-dependent memory capacity [49, 51, 52]. Increased GDNF expression promotes the proliferation of glial and neuronal precursors in the hippocampus, and the effects of GDNF on progenitor cell proliferation may be crucial for stimulating neurogenesis in the hippocampal DG and for facilitating memory recovery [53]. VEGF family members include VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor. VEGF-A exerts beneficial effects on angiogenesis and neurogenesis during cerebral ischemia [54]. Increases in VEGF-A levels promote newborn neuron proliferation and survival in the SGZ of the hippocampal DG and facilitates the migration of nascent neurons to the penumbral cortex, resulting in improvements to memory and brain injury recovery in the chronic phase of ischemic stroke [54, 55]. Our experimental data revealed that the expression of p-p38 MAPK, p-CREB, BDNF, GDNF, and VEGF-A was considerably downregulated in the hippocampus, whereas ASD extract treatments considerably upregulated the expression of these proteins in the hippocampus at 28 d after transient GCI. Moreover, SB+ASD-1 g treatment abrogated the effects of ASD-1 g upregulation on p-p38 MAPK, p-CREB, BDNF, GDNF, and VEGF-A expression in the hippocampus. These results suggest that ASD extract treatments promote hippocampal neurogenesis and neuronal survival, thereby reducing memory impairments, by activating p38 MAPK-mediated CREB/BDNF, GDNF, and VEGF-A, but not CREB/NGF, signaling pathways in the hippocampus at 28 d after transient GCI.

Conclusions

ASD extracts administered at doses of 0.5 and 1 g/kg effectively attenuated memory deficits during GCI. ASD extract treatments exhibited neuroprotective effects against ischemia-induced memory impairment by promoting hippocampal neurogenesis and dendritic stability. Moreover, the effects of ASD extract treatments on adult hippocampal neurogenesis and neuronal survival were revealed to be caused by activation of the p38 MAPK-mediated CREB/BDNF, GDNF, and VEGF-A signaling pathways in the hippocampus at 28 d after transient GCI. Therefore, *A. sinensis* could be a therapeutic candidate for the treatment of memory deficits in the chronic phase of ischemic stroke. However, additional studies are needed to clarify the interactions of the BDNF, GDNF, and VEGF-A signaling pathways during transient GCI.

Abbreviations

GCI

global cerebral ischemia; CA1:cornu ammonis 1; MAPK:mitogen-activated protein kinase; DG:dentate gyrus; NSPC:neural stem/progenitor cell; CREB:cAMP response element-binding protein; SVZ:subventricular zone; SGZ:subgranular zone; GCL:granule cell layer; BDNF:brain-derived neurotrophic factor; NGF:nerve growth factor; GDNF:glial cell line-derived neurotrophic factor; VEGF:vascular endothelial growth factor; BrdU:bromodeoxyuridine; MAP-2:microtubule-associated protein 2;

ASD:*Angelica sinensis* (Oliv.) Diels; CCH:chronic cerebral hypoperfusion; I/R:ischemia/reperfusion; p90RSK:90-kDa ribosomal S6 kinase; UPLC:ultra-performance liquid chromatography; 4-VO:4-vessel occlusion; CCA:common carotid artery; STL:step-through latency; ICV:intracerebroventricularly; DMSO:dimethyl sulfoxide; RT:room temperature; PBST:phosphate-buffered saline/0.01% Tween 20; IHC:immunohistochemistry; IF:immunofluorescence; T_R:retention time; NeuN:neuronal nuclei; CNS:central nervous system; mAU:mini absorbance unit.

Declarations

Ethics approval and consent to participate

All study procedures were performed following the guidelines approved by the Institutional Animal Care and Use Committee of China Medical University (Permit Number: 2018-075).

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author upon request.

Conflicts of interest

The authors declare that they have no competing interests.

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Authors' Contributions

YCL and CYC designed the study protocols. CYC performed the experiments, collected and analyzed the experimental data, and wrote the manuscript. HCH and STK participated in the discussions of the research works and helped to draft the manuscript. All authors read and approved the final manuscript.

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Author details

¹ Department of Chinese Medicine, China Medical University Hospital, Taichung 42056, Taiwan

² Research Center for Chinese Medicine & Acupuncture, China Medical University, Taichung 40402, Taiwan

³ Graduate Institute of Acupuncture Science, China Medical University, Taichung 40402, Taiwan

⁴ Department of Chinese Pharmaceutical Sciences and Chinese Medicine Resources, College of Chinese Medicine, China Medical University, Taichung 40402, Taiwan

⁵ School of Chinese Medicine, College of Chinese Medicine, China Medical University, Taichung 40402, Taiwan

⁶ School of Post-baccalaureate Chinese Medicine, College of Chinese Medicine, China Medical University, Taichung 40402, Taiwan

⁷ Department of Chinese Medicine, Hui-Sheng Hospital, Taichung 42056, Taiwan

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Tables

Table 1. Primary antibodies used in this study

Host	Primary antibody	IHC (Dilution)	IF (Dilution)	WB (Dilution)	Source/catalog No.
Rabbit	BrdU		1:100		abcam/ab152095
Mouse	NeuN		1:100		Sigma-Aldrich/MAB377
Mouse	BrdU		1:100		CST/#5292
Rabbit	Ki67	1:200	1:100		abcam/ab15580
Rabbit	MAP-2		1:100		abcam/ab32454
Rabbit	p-p38 MAPK			1:1000	CST/#9211
Rabbit	P38 MAPK			1:1000	CST/#9212
Mouse	p-CREB			1:500	Millipore/05-807
Rabbit	CREB			1:1000	CST/#9197
Rabbit	BDNF			1:1000	abcam/ab108319
Rabbit	NGF			1:1000	abcam/ab6199
Rabbit	GDNF			1:500	abcam/ab18956
Rabbit	VEGF-A			1:1000	Proteintech/#19003-1-AP
Mouse	Actin (loading control)			1:5000	NB/NB600-501

IHC, immunohistochemistry; IF, immunofluorescence; WB, Western blotting; CST, Cell Signaling Technology; NB, Novus Biologicals.

Figures

Figure 1

UPLC chromatograms concerning ferulic acid and ligustilide in the ASD extract. (A) The chemical profile of the ASD extract was performed using a C-18 column and detected at 320 nm. UPLC chromatograms reveal the peak areas of (B) ferulic acid (tR = 5.87 min) and (C) ligustilide (tR = 12.81 min) standards. mAU, mini absorbance unit.

Figure 2

Effects of ASD extract treatments on memory impairment. The STLs of the Sham, Control, ASD-0.25 g, ASD-0.5 g, ASD-1 g, D+ASD-1 g, and SB+ASD-1 g groups ($n = 4$) were evaluated at 7, 14, 21, and 28 d after transient GCI. STL, step-through latency. Data are presented as mean $\pm$ standard deviation. * $P < 0.05$ versus the Sham group; # $P < 0.05$ versus the Control group.

Figure 3

Effects of ASD extract treatments on hippocampal neurogenesis in the SGZ of the hippocampal DG. (A) The representative images reveal BrdU and BrdU/NeuN expression in the hippocampal SGZ in the Sham, Control, ASD-0.25 g, ASD-0.5 g, ASD-1 g, D+ASD-1 g, and SB+ASD-1 g groups ($n = 4-5$) at 28 d after transient GCI. (B) The representative image provides a DAPI-stained section of the hippocampus. The tilted rectangle indicates the selected hippocampal SGZ, in which the immunopositive cells were calculated. The bar graphs illustrate the numbers of (C) BrdU-positive and (D) BrdU/NeuN-positive cells in the hippocampal SGZ in the experimental groups. * $P < 0.05$ versus the Sham group; # $P < 0.05$ versus the Control group. Arrows and arrowheads indicate BrdU-positive and BrdU/NeuN-positive cells, respectively. Scale bars in (A) and (B) are equal to 50 μm and 200 μm , respectively.

Figure 4

Effects of ASD extract treatments on Ki67 and BrdU/Ki67 expression in the hippocampus. (A) The representative images reveal ki67 expression in the hippocampal SGZ in the Sham, Control, ASD-0.25 g, ASD-0.5 g, ASD-1 g, D+ASD-1 g, and SB+ASD-1 g groups ($n = 4-5$) at 28 d after transient GCI. (B) The representative image reveals the negative control for Ki67 antibody. (C) The tilted rectangle indicates the selected hippocampal SGZ, in which Ki67-positive cells were calculated. (D) The bar graph illustrates the number of Ki67-positive cells in the hippocampal SGZ in the experimental groups. * $P < 0.05$ versus the Sham group; # $P < 0.05$ versus the Control group. (E) The representative images indicate BrdU-positive cells co-labeling with Ki67. Arrows in (A), (E-1), (E-2), and (E-3) indicate Ki67-positive, BrdU-positive (red), Ki67-positive (green), and BrdU/Ki67-positive (yellow orange) cells, respectively. Scale bars in (A), (B), (C), and (E-3) are equal to 10 μm , 10 μm , 500 μm , and 50 μm , respectively.

Figure 5

Effects of ASD extract treatments on MAP-2/NeuN expression in the hippocampus. (A) The representative images reveal MAP-2/NeuN expression in the CA1 region in the Sham, Control, ASD-0.25 g, ASD-0.5 g, ASD-1 g, D+ASD-1 g, and SB+ASD-1 g groups ($n = 4-5$) at 28 d after transient GCI. Intact MAP-2-positive dendrites were found in the Sham, ASD-0.5 g, ASD-1 g, and D+ASD-1 groups, whereas discontinuous patterns of MAP-2-positive dendrites were found in the Control, ASD-0.25 g, and SB+ASD-1 g groups. (B) The rectangle indicates the selected CA1 region, in which the MAP-2/NeuN-positive cells were calculated. (C) The bar graph illustrates the number of MAP-2/NeuN-positive cells in the CA1 region in the experimental groups. * $P < 0.05$ versus the Sham group; $\#P < 0.05$ versus the Control group. Arrows on the left (first and fourth columns), middle (second and fifth columns), and right (third and sixth columns) sides of the representative images indicate the MAP-2-positive (green), NeuN-positive (red), and MAP-2/NeuN-positive (yellow orange) cells, respectively. Scale bars in (A) and (B) are equal to 50 μm and 200 μm , respectively.

Figure 6

Effects of ASD extract treatments on p-p38 MAPK, p38 MAPK, p-CREB, CREB, and BDNF expression in the hippocampus. (A) The representative Western blot images reveal p-p38 MAPK, p38 MAPK, p-CREB, CREB, and BDNF expression in the hippocampus in the Sham, Control, ASD-0.25 g, ASD-0.5 g, ASD-1 g, D+ASD-1 g, and SB+ASD-1 g groups ($n = 4$) at 28 d after transient GCI. The ratios of (B) p-p38 MAPK/p38 MAPK, (C) p-CREB/CREB, and (D) BDNF/actin expression were evaluated in the hippocampus in the experimental groups. * $P < 0.05$ versus the Sham group; # $P < 0.05$ versus the Control group.

Figure 7

Effects of ASD extract treatments on NGF, GDNF, and VEGF-A expression in the hippocampus. (A) The representative Western blot images reveal NGF, GDNF, and VEGF-A expression in the hippocampus in the Sham, Control, ASD-0.25 g, ASD-0.5 g, ASD-1 g, D+ASD-1 g, and SB+ASD-1 g groups ($n = 4$) at 28 d after transient GCI. The ratios of (B) NGF/actin, (C) GDNF/actin, and (D) VEGF-A/actin expression were evaluated in the hippocampus in the experimental groups. * $P < 0.05$ versus the Sham group; # $P < 0.05$ versus the Control group.