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Effect of Angelica polysaccharide on brain senescence of Nestin-GFP mice induced by D-galactose

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ABSTRACT

The incidence of neurodegenerative diseases is severely increasing with the aging. It has been proposed that NSCs (neural stem cells) help to control aging, but the mechanisms responsible remain unclear. Angelica polysaccharide is an active ingredient of Angelica sinensis in traditional Chinese medicine, which possesses versatile pharmacological activities including anti-oxidative and anti-aging effects. In this study, D-gal (D-galactose) was used to construct an aging model of Nestin-GFP transgenic mice brain tissues and NSCs. Mouse model was subcutaneously injected with D-gal, as we observed that mice consistently displayed acceleration of aging-like behavior change, energy metabolism decreased, the expression of aging-related genes was up-regulated. Conversely, aging retardation was achieved in Nestin-GFP mice Induced by D-gal that was locally injected with ASP (Angelica polysaccharide). Mechanistically, we isolated and cultured NSCs in vitro. ASP protected NSCs by increasing the cell proliferation; decreasing the number of SA-β-gal stained neurons; increasing the activity of SOD(superoxide dismutase) and T-AOC(total antioxidant capacity), decreasing the content of MDA(malondialdehyde); decreasing the levels of IL-1b,IL-6,TNF-a and ROS; and down-regulated the expression of cellular senescence associated genes p53, p21 in the aging NSCs. In conclusion, ASP can delay aging speed by protecting NSCs and promote neurogenesis by enhancing the antioxidant and anti-inflammatory capacity, up-regulation of p53/p21 signaling pathway. As to provide theoretical basis for treatment for brain aging related diseases, add new scientific connotation for "qi and blood theory" and "supplement blood and delay aging" of Traditional Chinese Medicine.

1. Introduction

Aging is characterized by progressive declines in the physiology and functionality of adult tissues. Chronic progressive neuronal degeneration, aging, loss and death are significant causes of neurodegenerative diseases and brain aging(Poulose et al., 2017). How to prevent and cure brain aging has become a major issue that is concerned about life sciences and social sciences today. Angelica polysaccharide (ASP) is an important pharmacological component of Angelica (Pan et al., 2018). Our previous work proves that ASP is an important anti-aging ingredient in Angelica, which can slow the effects of aging of hematopoietic stem cell, on the modeling senescence of bone marrow in rats and can antagonize D-gal injury to organs such as the liver, kidney and spleen (Fan et al., 2015; Xia et al., 2016; Xiao et al., 2017). To investigate the inductive effects of ASP, the latest study concluded that ASP has the role of protecting neural stem cell against aging (Jia et al., 2015; Liu et al., 2010; Yao et al., 2016). Relationship between the ASP delaying brain aging mechanism and regulation of NSCs are unclear.

Nestin is an intermediate silk protein, a marker for NSCs, and found mainly in undifferentiated NSCs. The expression of Nestin gradually decreased with the migration and differentiation of NSCs (Jin et al., 2013; Strojnik et al., 2007). Nestin-GFP transgenic mice can detect the number, distribution, differentiation and migration pathways of NSCs in brain tissue using fluorescence-labeled nestin. As a model mouse, Nestin-GFP transgenic mice have been widely used in brain aging and NSC biology (Wang et al., 2016; Zeng et al., 2016).

In this study, the nestin-green fluorescent protein (GFP) mouse aging model was constructed to explore the mechanism of ASP anti-

ABBREVIATIONS: NSC, neural stem cell; D-gal, D-galactose; ASP, Angelica polysaccharide; SOD, superoxide dismutase; T-AOC, total antioxidant capacity; MDA, malondialdehyde; GFP, green fluorescent protein; HE, eosin; DAPI, 4′,6-diamidino-2-phenylindole; PBS, phosphate buffer saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SA-D-gal, senescenceassociated D-galactosidase; CCK, cell count kit; SD, standard deviation; ANOVA, analysis of variance

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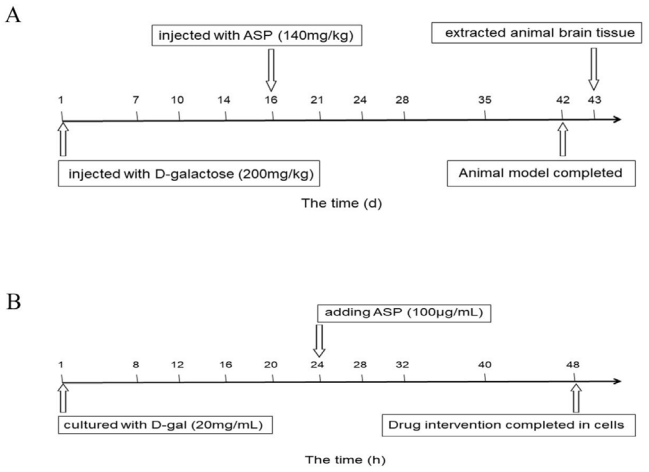

Fig. 1. The experimental protocol of the treatment paradigm (ASP and D-galactose treatment) in mice (A) and in the NSCs (B).

aging and look for a natural drug for the treatment of neurodegenerative diseases.

2. Materials and methods

2.1. Animal treatment

Two months old male Nestin-GFP transgenic mice were purchased from Cyagen Biosciences Inc. (Animal Certificate of Conformity: SYXK Guangdong, 2008-0090). After acclimatized to a mouth under constant condition of temperature and light-controlled room in the Animal Experiment Center of Chongqing Medical University, male Nestin-GFP transgenic mice (n = 60) were randomly divided into normal group, ASP normal group, D-gal plus ASP treatment group and D-gal group. The D-gal group was subcutaneously injected with D-galactose (200 mg/kg), qd × 42. As shown in Fig. 1A, the D-gal plus ASP treatment group was intraperitoneally injected with ASP (140 mg/kg) since the 16th day of the replication in the brain aging model, qd × 27. The ASP normal group was subcutaneously injected with the same amount of saline, qd × 15, and following intraperitoneally injected with ASP (140 mg/kg), qd × 27. The normal group was subcutaneously injected with an equal volume of saline within the same time. Perform the related experiment on the second day after finishing copying the model.

2.2. Reagents

Angelica polysaccharide (CY150407, Purity ≥ 98%) was acquired from Ci Yuan Biological Technology Co., Ltd (Xi'an, China) and dissolved in saline at the concentration of 20 mg/mL and sterilized by ultrafiltration. D-galactose (purity ≥ 99%) was purchased from Solarbio Science&Technology Co., Ltd (Beijing, China). C57BL/6 mouse neural stem cell complete medium was purchased from Cyagen Biosciences Inc. T-AOC kit, SOD kit and MDA kit were purchased from Beyotime Institute of Biotechnology (Shanghai, China). IL-1b kit, IL-6 kit and TNF-a kit were purchased from Neobioscience Biosciences Inc. BCA kit, SA-β-gal staining kit, EDU kit and P53 antibody were purchased from Beyotime Institute of Biotechnology (Shanghai, China).

2.3. Morris water maze performances

After the 42-day treatment, spatial memory of the mouse was assayed by the Morris water maze task. The maze was a water tank which divided into four quadrants, and a hidden platform was placed at the midpoint of a quadrant and submerged 1 cm below the surface of the water. Hidden-platform training: mice were required to place along the wall of the 4 points in the pool in random order, and record the escape latency time and movement track of each mouse within 120 s. If it exceeds 120 s, it is recorded as 120 s, and the mice are guided back to the invisible platform for 10 s for a total of 6 d. Exploration experiment: on day 7, the stealth platform was removed. The mice were placed in two quadrants farther from the platform and recorded their movement trajectories within 120 s. The number of times the mouse crossed the original platform position within the set time and percentage of occupancy time in the original platform quadrant were measured.

2.4. Tissue processing and hematoxylin-eosin staining

Animals were anesthetized and infused with 4% buffered paraformaldehyde solution. Then, the brains were isolated and dehydrated in 20% sucrose at 4 °C for 4 h. The brains were sliced into 20 μM sections using a cryotome. Histological characteristics were assessed using hematoxylin and eosin (HE) staining.

2.5. Detection of the activity of Na^+-K^+-ATPase

After the therapy, hippocampuses were collected and lysed in an ice bath for 30 min. The supernatant was collected after centrifugation (12000 rpm, 4 °C, and 30 min). The activity of Na^+-K^+ -ATPase was detected by chemical colorimetric analysis according to the manufacturer's instructions. ATPase catalyzed the decomposition of ATP into ADP and free phosphate. The amount of 1 μmol of inorganic phosphorus produced by ATPase decomposing ATP per milligram of tissue protein per hour is an ATPase activity unit, that is μmolPi/mgprot/hour. The activity of Na^+-K^+ -ATPase was calculated according to the formula: activity of Na^+-K^+-ATPase (μmolPi/mgprot) = (OD value of the detection group-OD value of the control group)/(OD value of the standard group-OD value of the blank group) × the standard concentration(0.02 μmol/ml) × 6 × 7.8 ÷ the sample protein concentration(mgprot/ml).

2.6. Immunofluorescence

For immunofluorescence analysis, the sections and cells were fixed with 4% PFA for 30min, washed with PBS and permeated with 0.2% Triton X-100 for 10min, then blocked with 10% goat serum for 1 h. Following by incubating with anti-Sox2 antibodies diluted for 24 h and secondary antibodies for 2 h. DAPI(4',6-diamidino-2-phenylindole) stained the nuclei for 15 min. Finally, 20 μL of glycerol was added to each slide and sealed with a cover slip. All slides were analyzed directly under a fluorescence microscope. Because of the transgenic mice, we can observe nestin green fluorescence directly in hippocampus and neural stem cells.

2.7. Western blotting analysis

Hippocampuses and NSCs in each group were treated with PBS and lysate to collect total protein. And the BCA protein assay kit measured the protein concentrations. Protein was separated from SDS-polyacrylamide gels and transferred to PVDF membranes. Membranes were blocked in 5% non-fat milk powder and incubated with primary antibodies overnight. Followed by washing in TBST, Membranes were incubated with secondary antibodies for 1 h. The enhanced chemiluminescence detection system (Pierce, USA) visualized membranes, and quantitative analysis used Image-Quant software.

2.8. Cell culture

The whole brain was dissected from newborn Nestin-GFP transgenic mouse which were post-natal day 1, minced and digested with trypsin for 5 min. After centrifugation to remove the supernatant, the cells grow in the NSC medium in suspension. Two days later, the

neurospheres were collected by centrifugation and trypsinized into single cells. Each cell exchange and passage was replaced with new culture flasks and the adherent cells were discarded, so that the NSCs could be naturally purified until experimental use. Neonatal rats used throughout this study are Nestin-GFP transgenic mice. NSCs have the green fluorescence of nestin, and NSCs can be designated by observing the green fluorescence of nestin.

2.9. CCK-8 assay

Cell proliferation assay was performed using the Cell Counting Kit-8.100 μL of neural stem cell suspension was placed in a 96-well plate. The plate was pre-incubated in the incubator for 24 h. The test substance was added to the culture plate. The plate was incubated in the incubator for an appropriate period of time. 10 μL CCK8 solutions were added to each well and incubated for 4 h. The optical density (OD) at 450 nm was measured with a microplate reader. The cell viability of NSC was calculated according to the formula: a viability rate = OD value of the experimental group/OD value of the control group.

2.10. Effect of ASP on proliferation of D-gal induced aging NSCs

NSCs were seeded in culture flasks which were divided into normal group, ASP normal group, D-gal plus ASP treatment group and D-gal group. The D-gal group was cultured with D-gal (20 mg/mL) for 48 h. As shown in Fig. 1B, D-gal plus ASP treatment group was first cultured with D-gal (20 mg/mL) for 24 h, following by adding ASP (100 μg/mL) with medium containing ASP and D-gal for 24 h. The ASP normal group was routinely cultured for 24 h, and then added ASP (100 μg/mL) for 24 h. The normal group was routinely cultured for 48 h.

2.11. EDU cell proliferation detection

The EDU solution was diluted with the cell culture medium at a ratio of 1000:1. Each well was incubated with 100 μL of EDU medium for 2 h. After washing with PBS, the cells were fixed with 4% PFA for 30 min. After washing, each well was incubated with a glycine for 5 min. The cells were permeated with 0.5% Triton X-100 for 10 min. Each wall added 100 μL of Apollo staining reaction solution, incubated in the dark for 30 min and added 100 μL of penetrant (0.5% TritonX-100). Following by adding methanol, 100 μL of Hoechst reaction solution was added to each well, and the reaction solution was discarded after incubation for 30 min at room temperature. Finally, 20 μL of glycerol was added to each slide and sealed with a cover slip. All slides were analyzed directly under a fluorescence microscope.

2.12. SA-β-gal staining

Sections and neurospheres were fixed at room temperature for 10 min, after washing three times with PBS, and stained with β-galactosidase staining solution for 6 h. Following by staining, the sections and neurospheres were washed three times with PBS and analyzed by optical microscopy. The ROD was measured by multiscanner auto reader. ROD reflected percentage of SA-β-gal staining positive neurospheres. SA-β-gal staining of brain tissue sections is roughly the same as cell staining.

2.13. Measurement of oxidation-associated biomarkers

The levels of intracellular ROS, MDA, SOD, and T-AOC in NSCs were measured. Briefly, cells in each group were processed with 10 μM of dichlorodihydrofluorescein diacetate (DCF-DA) and incubated for 30 min in the dark at room temperature. The reactive oxygen species in cells can oxidize non-fluorescent DCFH to generate fluorescent DCF. The level of reactive oxygen species in the cells can be determined by measuring the fluorescence of DCF. ROS in each group of NSCs was measured by flow cytometry. Each group of cells was lysed and centrifuged to collect the supernatant. The levels of MDA, SOD and T-AOC activity in NSCs were measured by chemical colorimetric analysis according to the manufacturer's instructions.

2.14. Detection of inflammatory cytokines in the NSCs by ELISA

Each group of NSCs was collected and lysed on ice, and the resulting mixture was centrifuged to obtain a supernatant, and levels of pro-inflammatory cytokines IL-1b, IL-6, and TNF-α in each group of NSCs were measured by an ELISA kit according to the manufacturer's instructions.

2.15. Statistical analysis

Experimental data were expressed as the mean ± standard deviation (SD). Statistical analysis was performed using SPSS 20.0 statistical software. Data was compared using one-way analysis of variance (ANOVA) for each group. If $P < 0.05$, the difference is considered significant.

3. Results

3.1. ASP restored cognitive impairment caused by D-gal administration

During the six-day place navigation training, the D-gal-administrated mice had the longer escape latency, while ASP treatment of D-gal-administrated mice significantly shortened the escape latency (Fig. 2A). To assess the spatial memory more directly, we removed the target platform on the next day after the navigation training, D-gal-administrated mice crossed the location fewer times and less time in the target quadrant where the platform once was compared to the D-gal plus ASP treatment group. However the normal group and the ASP normal group showed no significant differences in the escape latency and number of target crossing (Fig. 2B and C). Analysis of the performance in the Morris water maze test showed that D-gal-injected mice manifested a cognitive decline more than the controls did. This physiological change in D-gal-injected mice reflected acceleration in aging, while implantations of ASP can slowdown cognitive impairment.

The Morris water maze can test the spatial learning and memory ability of mice. (A) Effects of ASP on the escape latency in spatial orientation test of brain senescence mice. Latencies to find a hidden platform in the water maze during the six days of placing navigation training. (B) The number of times the mice crossed the target quadrant. (C) The percentage of time that the mice stayed in the quadrant where the platform once was. (D) Hippocampus sections from C57BL/6 Nestin-GFP transgenic mice at indicated months of age were stained for Hematoxylin-eosin(x40). (E) Effects of ASP on Na^+-K^+-ATPase activity in the hippocampus of brain senescence mice. (F) Effects of different concentrations of ASP on the proliferation of neural stem cells. Dose and time optimization of ASP on the viability of NSCs. NSCs (1×10^4 cells) were treated with different doses of ASP (0–160 mg/mL) and incubated for 24, 48 or 72 h in culture medium. At the end of incubation, cell viability was determined by CCK-8 assay. All values are expressed as mean ± SD (n = 10, per group). Different letters $P < 0.05$; *$P < 0.05$ vs. D-gal group; #$P < 0.05$ vs. normal group.

3.2. Effect of D-gal on the morphology of brain tissue

To observe the pathological changes of hippocampal tissue, we investigated Hematoxylin-eosin staining (Yang et al., 2017). This approach resulted in no significant differences in each group (Fig. 2D). So we continue to explore the experiment from other directions of aging research.

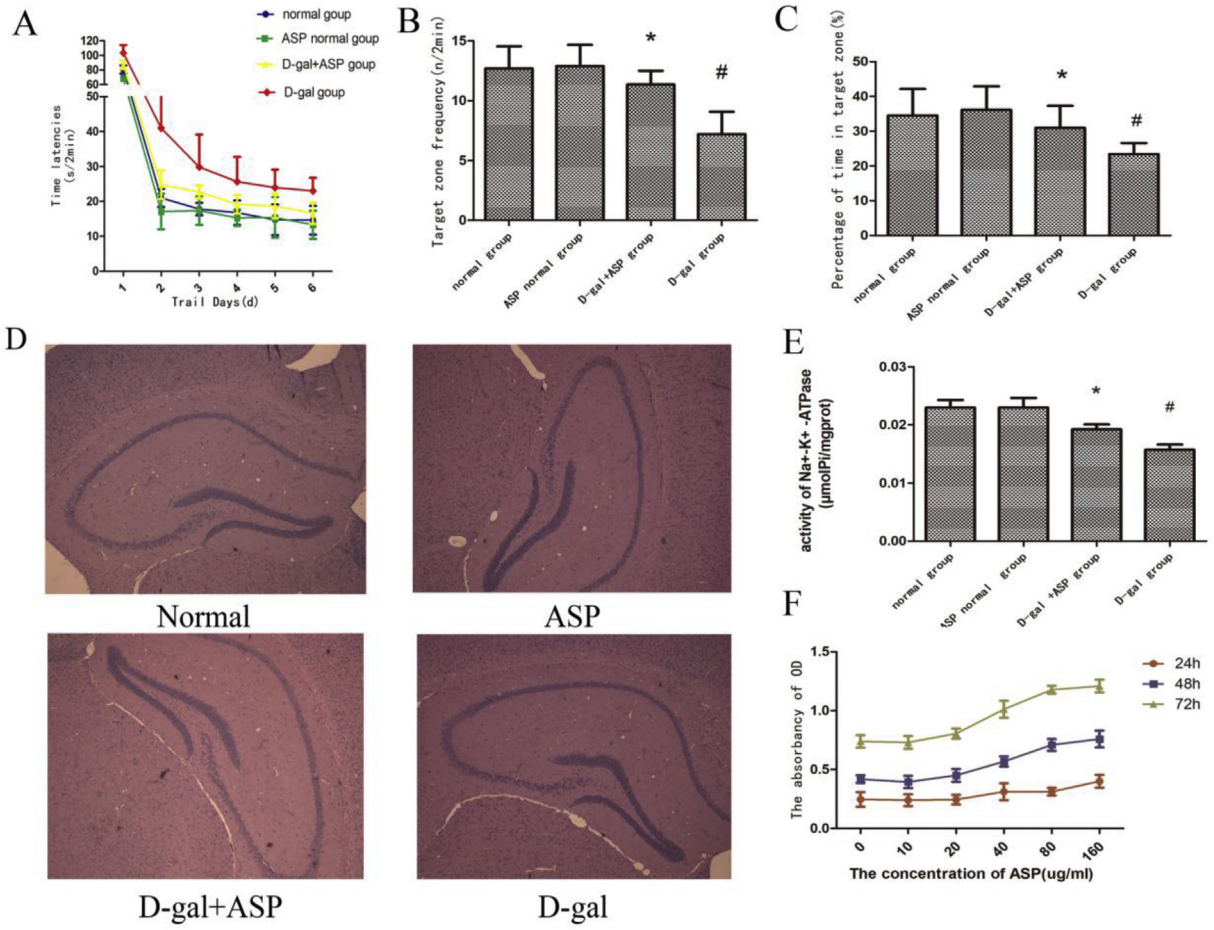

Fig. 2. Effects of ASP on cognitive ability and energy metabolism of brain senescence mice.

3.3. D-gal decreased energy metabolism of cells in the hippocampus of brain aging mice

There was a significant decrease of the activity of Na^+-K^+ -ATPase in D-gal-injected mice compared to the normal group, but the D-gal plus ASP treatment mice increased of the activity of Na^+-K^+ -ATPase, as shown in Fig. 2E. Hence, result from this test consistently indicated that D-gal decreased energy metabolism of cells in the hippocampus of the brain aging mice, which was significantly reversed by ASP.

3.4. ASP promoted NSCs proliferation

We have previously shown that D-gal could accelerate aging and ASP could slow down the speed of aging in Nestin-GFP transgenic mice brain aging model, but the cellular mechanism responsible for aging is still unknown. As we know, stem cell aging theory is one of the important mechanisms for studying aging. Adult hippocampal neurogenesis plays a pivotal role in learning and memory, and the dysfunction of neurogenesis is related to neurodegenerative diseases. Neural stem cells, able to generate neurons and glial cells, reside in the sub-ventricular zone of the lateral ventricle and the subgranular zone of the dentate gyrus of the hippocampus, keep up the production of neurons over the lifespan. Adult neural stem cells (NSCs) are essential for brain homeostasis (Imayoshi et al., 2008; Kempermann et al., 2004; Varma et al., 2016). So we investigated whether NSCs in the hippocampus might be mechanistically responsible for this process.

NSCs which cultured in vitro grew from single cells to neurospheres. We used the inverted microscope observing the process (Fig. 3A). Sox2 is a nuclear transcription factor that is strongly expressed in NSCs and has often been applied to label NSCs. We have concluded that, in adult mice, Sox2 was expressed in a population of cells in the hippocampus (Favaro et al., 2009). We also examined nestin, which, although it is not NSCs specific, is known to be strongly expressed in NSCs. Because of the transgenic mice, we can observe nestin green fluorescence directly in hippocampus and neural stem cells (Fig. 3B).

In order to investigate the effect of ASP treatment for NSCs proliferation viability, NSCs were exposed to various concentrations of ASP for 24 h, 48 h or 72 h, and then analyzed information on a CCK-8 assay. As shown in Fig. 2F, ASP increased the cell proliferation, and proliferation viability of ASP treated NSCs was dose-dependent (0–160 μg/mL). In addition, EDU (5-ethynyl-2 ′-deoxyuridine) is a thymine nucleoside analogue that can replace thymine (T) to infiltrate into the DNA molecules being synthesized during DNA replication, and specific reactions with Apollo fluorescent dyes can directly and accurately detect DNA replication activity. To investigate whether ASP induced cell proliferation, immunofluorescence of EDU was used to observe the EDU^+ positive number of each group. As shown in Fig. 3C, the number of EDU^+ cells in the D-gal group was less than that in the D-gal + ASP group, indicating that ASP delayed brain aging by increasing the number of neural stem cells.

(A) Neural stem cell and neurosphere in primary culture. a. 1d of primary culture; b. 3d of primary culture; c-d. 7d of primary culture; Scale bars, a-c, 100 μm, d, 10 μm (B)Hippocampus and neural stem cell from male C57BL/6 Nestin-GFP transgenic mice were immunostained for Nestin and Sox2. An immunofluorescence showed that nestin in NSCs was expressed mainly in the cytoplasm. Sox2 was expressed in the nucleus. Scale bars, 100 μm. (C) The effect of ASP on cell proliferation. Each group of cells was treated with EUD reagent, red fluorescence was

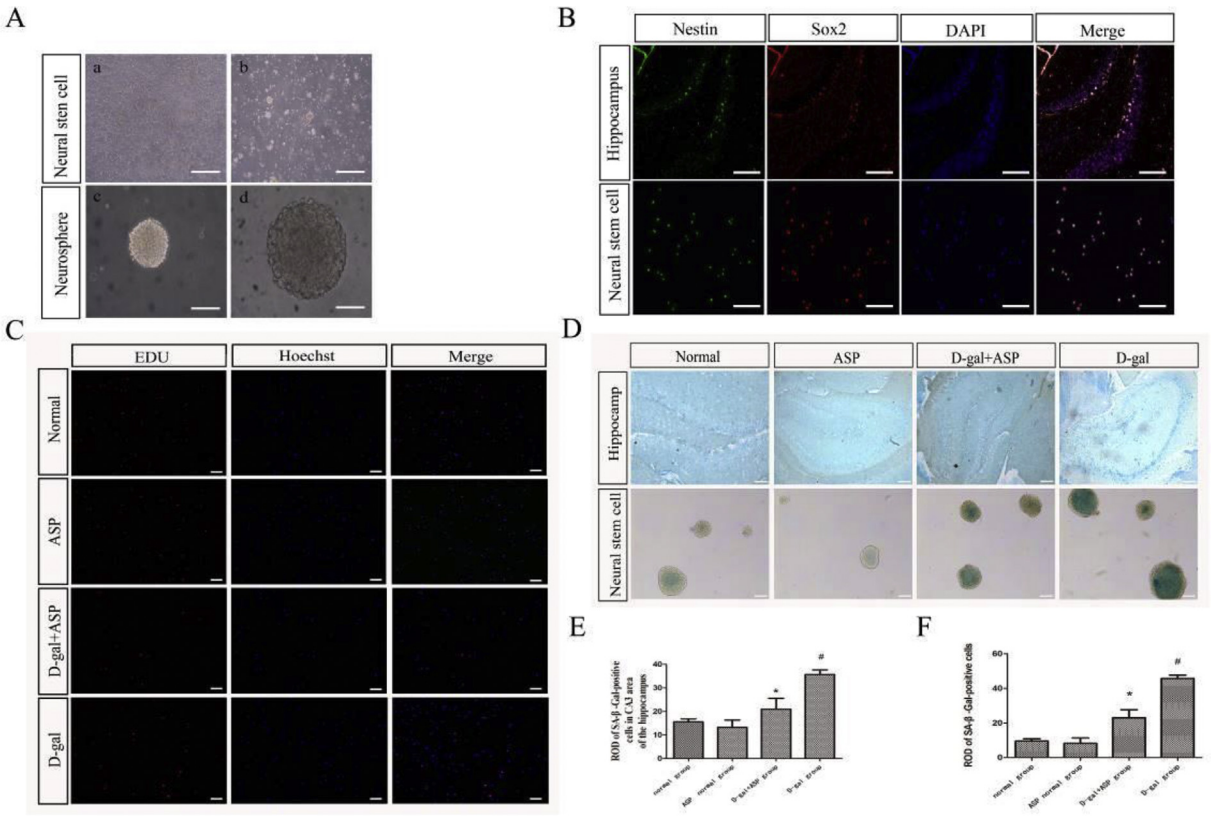

Fig. 3. ASP slowed down the aging process of NSCs.

EDU$^+$ positive cells, and finally the number of EDU$^+$ cells in each group was observed by fluorescence microscope. Scale bars, 100 μm (D) Effects of ASP on SA-β-Gal staining in area CA3 in hippocampus of brain aging mice and in NSCs. Scale bars, 100 μm. After treatment, the NSCs (4×10^3 cells) were cultured in the medium until the 7th day to form the third generation of neurospheres. Then the NSCs were gathered and fixed. SA-β-gal staining was carried out on the cells to assess the aging of NSCs. Aging cells stained blue in the cytoplasm. Scale bars, 10 μm (E) ROD of SA-β-gal-positive cells in CA3 area of the hippocampus. (F) ROD reflected percentage of SA-β-gal staining positive neurospheres. Experiments were repeated three times, and similar results were obtained. All values are expressed as mean ± SD (n = 3, per group). Different letters $P < 0.05$; *$P < 0.05$ vs. D-gal group; #$P < 0.05$ vs. normal group.

3.5. ASP attenuated D-gal induced NSCs senescence

A previous study (Chen et al., 2018; Yao et al., 2016) has shown that D-gal increased the number of senescent cells in the hippocampus of brain aging mice. SA-β-gal is a widely used biomarker for senescent and aging cells, since the over expression and accumulation of the endogenous lysosomal β-galactosidase is specific to senescent cells, only cells in senescence stage stain at pH 6.0(Ke et al., 2018; Singh and Piekorz, 2013). From Fig. 3D, we can see some of the cells in the hippocampus and neural stem cells which stained blue. We found that the percentage of senescence neurospheres in the D-gal group was significantly increased as compared to the normal group according to the statistics of SA-β-gal-positive neurospheres in CA3 area of the hippocampus (Fig. 3E)and in vitro (Fig. 3F). The D-gal plus ASP treatment group decreased the percentage of senescence neurospheres, suggesting that ASP protected NSCs against senescence.

3.6. ASP decreased the level of oxidative stress by enhancing anti-oxidative capacity

ROS is generated during mitochondrial oxidative metabolism. MDA (malondialdehyde) is used as a biomarker to assess the level of oxidative stress in a cell or organism. The antioxidant SOD (superoxide dismutase) is the principal enzymes capable of reducing oxidative stress in the cellular environment. Total antioxidant capacity (T-AOC) also is one of the significant indicators of the body's antioxidant effect. As shown in Fig. 4A–B, D-gal significantly increased levels of MDA and ROS in the D-gal group compared to the normal group. However, ASP reduced the level of MDA and ROS in D-gal plus ASP treatment group. Concomitantly, D-gal plus ASP treatment group increased the activity of SOD and T AOC compared to the D-gal group. The results indicate that ASP reduced oxidative stress in NSCs by enhancing the activity of endogenous anti-oxidative enzymes.

(A) The level of ROS in NSCs. The level of ROS was measured by flow cytometry. (B) The level of MDA and the activity of SOD and T-AOC in NSCs were determined by chemical colorimetric analysis from each group. (C) The NSCs in each group were collected. The inflammatory cytokines levels of IL-1β, IL-6 and TNF-α were measured by ELISA kit. (D and E) The protein of hippocampus (D) and NSCs (E) in each group were collected. Senescence-associated protein expressions were detected by western blotting. Experiments were repeated three times, and similar results were obtained. All values are expressed as mean ± SD (n = 3, per group). Different letters $P < 0.05$; *$P < 0.05$ vs. D-gal group; #$P < 0.05$ vs. normal group.

3.7. ASP decreased the levels of inflammatory cytokines of NSCs

Inflammation cytokine in the cell is associated with natural aging and neurodegenerative disease. High levels of inflammatory cytokines as IL-1b, IL-6 and TNF-α have been detected in flammatory tissue and

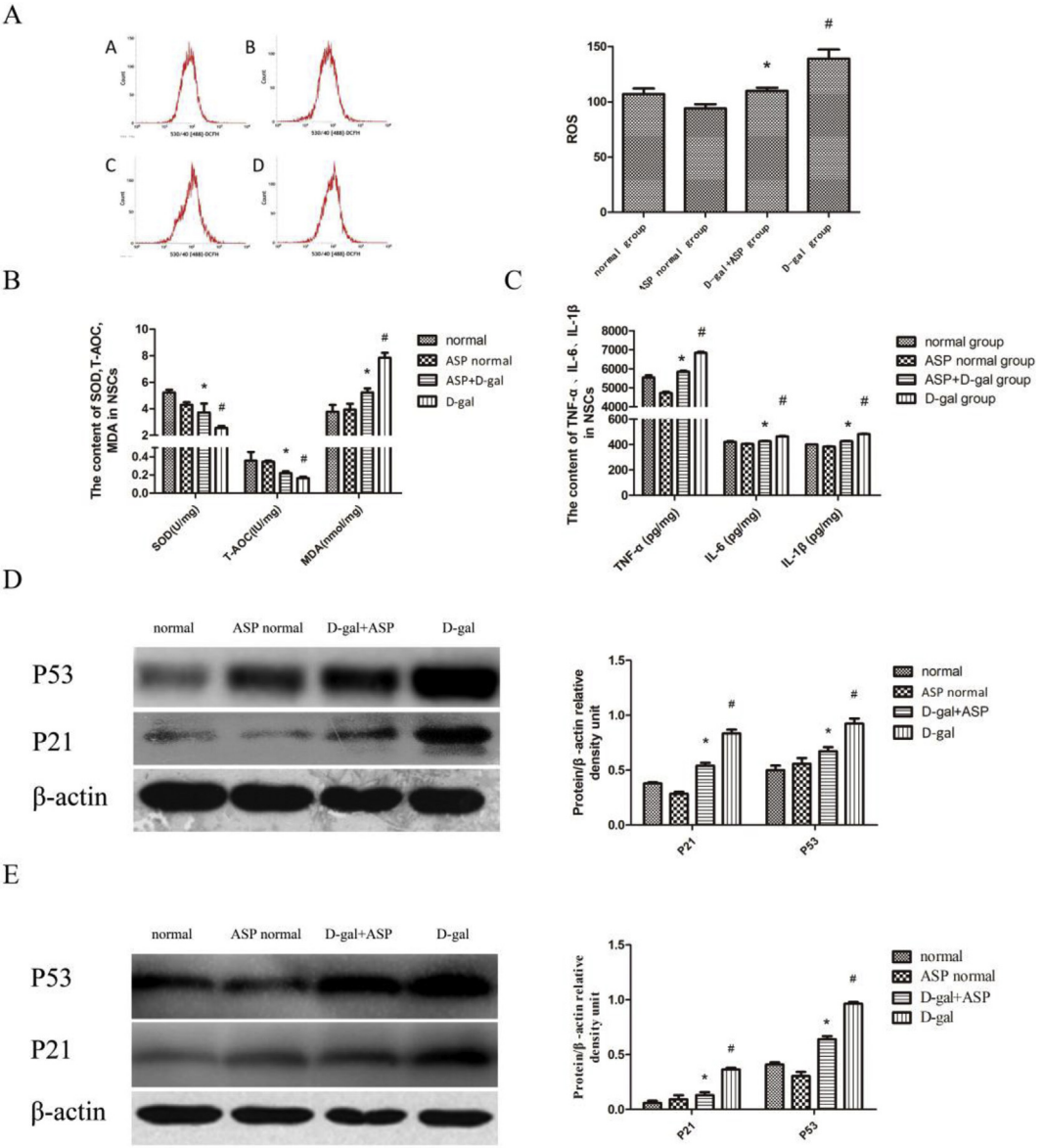

Fig. 4. The mechanism of ASP delaying cell senescence of NSCs.

correlated well with aging cells. The levels of IL-1b, IL-6 and TNF-α increased significantly in NSCs of the D-gal group, compared with the normal group. Meanwhile, the levels of the inflammatory cytokines were reduced in the D-gal plus ASP treatment group, relative to the D-gal group (Fig. 4C). And ASP normal group didn't significantly reduce the levels of IL-1b, IL-6 and TNF-a in the ASP treatment group, compared with the normal group. We can speculate that ASP may protect NSCs against senescence by reducing inflammation.

3.8. ASP down-regulated the protein expression of senescence associated genes in hippocampus of brain aging mice and NSCs

The p53-p21 pathway is a main signal transduction pathway involved in cell aging processes. Therefore, we performed western-blot to explore the protein expressions of p53 and p21, which are in the core position of the pathway. As shown in Fig. 4D and E, the protein expression of p53 and p21 in the D-gal group was violently higher than that of the normal group. However, in the D-gal plus ASP treatment group, the expression of the protein was significantly lower than that of the D-gal group. Meanwhile, there were no differences between the normal group and the ASP normal group. It indicates that ASP can down-regulate the expression of senescence associated genes in the hippocampus of brain aging mice and NSCs.

4. Discussion

With the acceleration of population aging process, the incidence of neurodegenerative diseases is increasing. It will be of great value to find out a new medicine for the treatment of neurodegeneration to delay brain senescence. ASP is the initial extraction solvent of the roots of Angelica Sinensis (Olive) Diels with water. It is a ??-dpyranoid polysaccharide with an average molecular weight of 72,900 Da. In traditional Chinese medicine, Angelica has the effect of “qi and blood theory” and “supplement blood and delay aging”. ASP has been proved to have beneficial effects on multiple disease models including cancer, leukemia, anemia, and inflammation (Chao and Lin, 2011; Yao et al., 2016; Zhou et al., 2015). Previous studies have shown that, ASP has the ability to delay cell aging of hematopoietic stem cell and neural stem cell, and improve the hypoxic injury to stem cell (Jia et al., 2015; Mu et al., 2017). However the mechanism responsible for ASP delaying brain-aging is still unknown.

D-galactose (D-gal) is a physiological nutrient and a reducing sugar

that reacts with free amines of amino acids in proteins to form advanced glycation end products of nonenzymatic glycation. As such, over supply of D-gal could contribute to generation of ROS through oxidative metabolism of D-gal as well as through glycation end products. So D-gal is currently recognized as a degenerative agent. D-gal administration model is similar to natural aging. D-gal has been widely used in the animal model of aging and the replication of cell aging model in vitro (Bei et al., 2018; Chen et al., 2018; Li et al., 2018a; Zhao et al., 2018).

Aging is a complex process that the physiological function of organisms slows decline. Brain aging is an important aspect of the aging process, which shows a decline in cognition and motor skills, and a reduction in neurogenesis. In this study, mouse brain aging model was successfully established for D-gal subcutaneous injection of 42d. The experiment found that the escape latency of mice in brain aging model group was significantly prolonged, while the number of crossing the original platform location and the percentage of stay in the quadrant where the original platform was significantly reduced, suggesting that the spatial learning and memory abilities of mice in brain aging model group decreased significantly. Na + -K + -ATPase is a protease that promotes the exchange of anions and cations, maintains cell homeostasis, and is responsible for maintaining neuronal excitability. We also found a decrease of the activity of Na^+-K^+-ATPase in brain aging model group. Those are consistent with the performance of brain aging (Bomba et al., 2017; Zhang et al., 2017).

The mice were injected intraperitoneally with ASP (140 mg/kg, 27d) during the establishment of brain aging mouse model. The results showed that ASP could significantly improve the spatial learning and memory abilities in the brain of aging mice. The activity of Na^+-K^+-ATPase in D-gal + ASP group was significantly increased. Experimental results suggest that, ASP can delay brain-aging in mice, can be used for clinical prevention and treatment of neurodegenerative diseases. So we further investigated the cellular mechanism responsible for ASP delaying brain senescence.

We know that loss of neural stem cell (NSC) is a major cause of aging in the whole body. Research has been shown that adult NSCs reside in a few brain regions that mediate local neurogenesis and therefore several aspects of brain functioning. More recently, it has been shown that adult NSCs are crucial to the neuroendocrine regulation of the physiological homeostasis of the whole body. So we investigated whether these hypothalamic NSCs might be mechanistically responsible for brain senescence (Zhang et al., 2017).

We extracted primary neural stem cells from the hypothalamus of transgenic mice and purified them in vitro. NCSs were cultured with D-gal to establish cellular aging model. Senescence staining, CCK8 result and EDU result showed that ASP could promote the proliferation of NSCs and protect NSCs against senescence. The hippocampal tissue aging staining experiment can also confirm this result. It is suggested that ASP may antagonize the D-gal on the damage to NSCs, thereby delaying brain aging in mice.

There are many theories of aging research, oxidative damage is part of the mainstream theories of cellular aging. Oxidative stress due to destruction of mitochondria has been proposed to be a major cause of Alzheimer's disease, Parkinson's disease and other neurodegenerative diseases (Bu et al., 2017; Grimm and Eckert, 2017).The mechanism is the increase in reactive oxygen species (ROS) and the decrease to antioxidant capacity. Oxygen metabolism of D-gal produces various reactive oxygen species (ROS). Excess ROS can damage the molecular structure of lipids, proteins, and DNA. In addition, ROS can potently inhibit neurogenesis and particularly NSCs proliferation (Conte-Daban et al., 2018; Ikawa and Yoneda, 2009; Islam et al., 2017; Terron-Camero et al., 2018). MDA is the final product of lipid peroxidation which is a well known indicator for oxidative damage of membranes and its content can reflect the degree of lipid peroxidation in the body. There are many kinds of antioxidant components in normal body. The antioxidant SOD is one of the important antioxidant enzymes which catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide, eliminates endogenous ROS and prevent its induced oxidative stress. Total antioxidant capacity (T-AOC) also is one of the critical indicators of the body's antioxidant effect (Fang et al., 2018; Liu et al., 2018b). In this study, ASP decreased the levels of MDA and ROS in NSCs. We also observed the level of SOD and T-AOC increasing significantly. It is suggested that ASP which delays brain aging mechanism may be related to antioxidant damage. In addition, chronic inflammation is also considered to be one of the major risk factors of aging and neurodegenerative diseases. Oxidative stress is related to inflammatory reactions. (Chhetri et al., 2018). According to the theory of inflammatory aging, aging is a chronic inflammatory state and it is a phenomenon in which innate immunity is activated, accompanied by an increase in the level of pro-inflammatory cytokines. With aging, pro-inflammatory cytokines in cells such as IL-1b, IL-6, and TNF-α are released and the increased levels play an important role (Geng et al., 2017; Liu et al., 2018a; Wang et al., 2018). In the present study, ASP treatment significantly reduced the levels of IL-1b, IL-6, and TNF-α, compared with the D-gal administration group. It suggests that ASP can protect the NCSs from age-induced chronic inflammation.

Cell aging can be induced by a variety of cellular signaling pathways, of which the p53-based signaling pathway is important for cell senescence, and p53 can activate different gene expression profiles to prevent cell proliferation. It can induce transient arrest of G1 and G2 cell cycle. (Shchors et al., 2013). DNA damage can increase the expression of P53 protein through phosphorylation of p53 and induce the transcriptional activity of it. One of the major effectors of P53 activation is p21. (Fang et al., 2017; Li et al., 2018b). In the present study, the reduced protein levels of p53, p21 by ASP indicated that ASP regulates the expression of the genes to delay hippocampus and NSCs senescence.

In summary, ASP can effectively improve the spatial learning and memory ability of brain aging mice, increase the energy metabolism of cells and decrease cell senescence in the hippocampus to delay brain aging in mice. We speculate that ASP may regulate the number and function of neural stem cells in the hippocampus, reduce the oxidative damage to neural stem cells and inhibit the expression of inflammatory cytokines, and down-regulate the expression of aging genes to delay brain aging in mice to retard the aging process.

This experiment combines animal model building technology, the latest theory of stem cells and traditional Chinese medicine anti-aging theory. By constructing the model of mice brain aging model in vivo and vitro, we studied the molecular mechanism of ASP retarding brain aging and regulating senescence of NSCs. The topic explains the new connotation of “qi and blood theory” and “supplement blood and delay aging” of Traditional Chinese Medicine. Modern biological mechanisms that regulate NSCs aging provide an essential basis of the prevention and treatment of brain aging related diseases.

Competing interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Compliance with ethics guidelines

Xiao Cheng, Hui Yao, Yue Xiang, Linbo Chen, Minghe Xiao, Ziling Wang, Hanxianzhi Xiao declare they have no conflict of interest.

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