



Polysaccharides from *Angelica sinensis* alleviate neuronal cell injury caused by oxidative stress

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Abstract

Angelica sinensis has antioxidative and neuroprotective effects. In the present study, we aimed to determine the neuroprotective effect of polysaccharides isolated from *Angelica sinensis*. In a preliminary experiment, *Angelica sinensis* polysaccharides not only protected PC12 neuronal cells from H₂O₂-induced cytotoxicity, but also reduced apoptosis and intracellular reactive oxygen species levels, and increased the mitochondrial membrane potential induced by H₂O₂ treatment. In a rat model of local cerebral ischemia, we further demonstrated that *Angelica sinensis* polysaccharides enhanced the antioxidant activity in cerebral cortical neurons, increased the number of microvessels, and improved blood flow after ischemia. Our findings highlight the protective role of polysaccharides isolated from *Angelica sinensis* against nerve cell injury and impairment caused by oxidative stress.

Key Words: nerve regeneration; cerebral ischemia; *Angelica sinensis*; polysaccharides; antioxidation; reactive oxygen species; mitochondrial membrane potential; apoptosis; microvessels; NSFC grant; neural regeneration

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Introduction

Cerebral ischemia, induced by cerebral artery occlusion, dramatically decreases local cerebral blood flow, leading to cell death and functional deficits in the brain^[1-4]. Reactive oxygen radicals are involved in cerebral ischemia and reperfusion^[5]. During reperfusion, reoxygenation from restored blood flow provides oxygen for enzymatic oxidation reactions in neurons and their subcellular organelles^[6-7], including mitochondria^[8-9]. Reactive oxygen species are constantly produced in both cerebral ischemia and reperfusion processes^[10], and it has been well documented that in both processes reactive oxygen species cause cell death, both directly, through oxidative damage, and indirectly, by reactive oxygen species signaling pathways^[11]. Although reperfusion may cause neuronal damage^[12], it is able to rescue cells and tissues. The rescue processes, which include preservation of surviving cells, restoration of impaired tissues and recuperation of brain functions, largely depend on restoration of local blood supply. Microvessels play an important role in providing blood supply to brain tissues^[13]. But therapeutic trials of agents that protect against reperfusion injury have not yet shown consistent benefit^[14-15], and there remains a need for

the development of novel pharmacologic treatments.

According to traditional Chinese medicine, the roles of *Angelica sinensis* correlate with tonifying the blood and promoting its circulation^[16]. Recent studies have shown that extracts of *Angelica sinensis* have antioxidative and neuroprotective effects^[17-18]. Injection of preparations from *Angelica sinensis* alleviate sciatic nerve crush injury and diabetic peripheral neuropathy^[19-20], and recent evidence also suggests that a number of pharmacological effects of *Angelica sinensis* are closely associated with its polysaccharide fractions^[21]. The polysaccharides from *Angelica sinensis* roots have immunomodulatory^[22], antitumor^[23-24], and hematopoietic effects^[16]; however, the anti-oxidative function of *Angelica sinensis* polysaccharide (ASP) has rarely been addressed. The present study aims to determine whether ASPs exert protective effects against oxidative damage *in vitro* and, if so, whether antioxidant activity would also be observed *in vivo*, in rats with focal cerebral ischemia.

Results

ASP inhibited H₂O₂-induced cytotoxicity in PC12 cells

We performed a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl

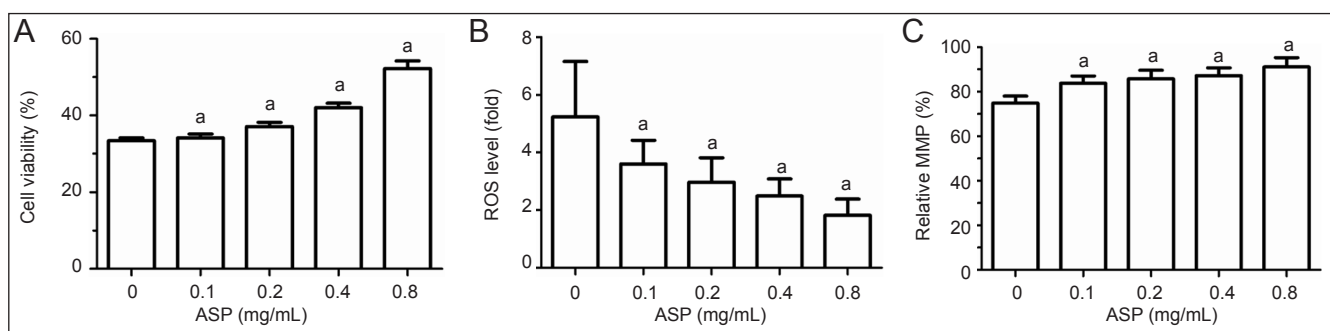


Figure 1 Effect of pretreatment of *Angelica sinensis* polysaccharide (ASP) on H₂O₂-induced cytotoxicity, reactive oxygen species (ROS) accumulation, and reduction in mitochondrial membrane potential (MMP) in PC12 cells.

(A) Pretreatment of PC12 cells with ASP reduces H₂O₂-induced cytotoxicity (MTT assay). Relative cell viability is calculated as the percentage of viable cells in the experimental groups over that in the control group (no H₂O₂ or ASP; taken as 100%, not shown). (B) Pretreatment of PC12 cells with ASP inhibits H₂O₂-induced ROS accumulation. The level of intracellular ROS is presented as the fold change of fluorescence intensity in ASP-pretreated cells compared with control cells not exposed to H₂O₂ or ASP (taken as 1, not shown). (C) Pretreatment with ASP inhibits H₂O₂-induced reduction in MMP in PC12 cells. The mean rhodamine 123 fluorescence intensity of 1×10^4 cells was measured on a flow cytometer with the excitation wavelength at 488 nm and the emission wavelength at 520 nm. Mean fluorescence intensity of ASP-pretreated cells is expressed as a percentage of that of control cells (no exposure to H₂O₂ or ASP; taken as 100%, not shown). Data are expressed as mean \pm SEM, and the experiment was repeated in triplicate. Data were analyzed by one-way analysis of variance and the least significant difference test for pairwise comparison. ^a*P* < 0.05, vs. 0 mg/mL ASP.

Table 1 Effect of *Angelica sinensis* polysaccharides on antioxidant enzyme activity (U/mg) and malondialdehyde content (nmol/mg) in rats with middle cerebral artery occlusion

Group	Antioxidant enzyme activity		
	Superoxide dismutase	Glutathione peroxidase	Malondialdehyde content
Control	2.10 \pm 0.13	4.91 \pm 0.21	1.14 \pm 0.06
Model	0.64 \pm 0.04 ^a	1.25 \pm 0.11 ^a	1.84 \pm 0.08 ^a
<i>Angelica sinensis</i> polysaccharides	1.38 \pm 0.06 ^b	3.19 \pm 0.12 ^b	1.56 \pm 0.06 ^b

Data are expressed as mean \pm SEM, *n* = 6 rats per group. Data were analyzed by one-way analysis of variance and the least significant difference test for pairwise comparison. ^a*P* < 0.05, vs. control group; ^b*P* < 0.05, vs. model group.

tetrazolium bromide (MTT) assay in the PC12 neuronal cell line. As expected, treatment with H₂O₂ reduced cell viability to about 33% of that of untreated cells. However, pre-incubation with 0.1, 0.2, 0.4 or 0.8 mg/mL of the < 20 kDa ASP fraction increased the viability of H₂O₂-treated cells to 34%, 37%, 42% and 52% of that of untreated cells, respectively (*P* < 0.05; Figure 1A). When the cells were pretreated with the > 20 kDa ASP fraction at the same concentrations and under the same conditions, the viability of H₂O₂-treated PC12 cells remained unchanged (data not shown). These results demonstrate that the < 20 kDa ASP fraction is capable of protecting PC12 cells from H₂O₂-induced injury. Therefore, this fraction was used in the subsequent experiments.

ASP reduced H₂O₂-induced reactive oxygen species accumulation in PC12 cells

Fluorescence results showed that treatment with H₂O₂ significantly increased intracellular reactive oxygen species levels to 5.2-fold those of untreated cells. However, when the cells were pretreated with 0.1, 0.2, 0.4 or 0.8 mg/mL ASP, the level of H₂O₂-induced reactive oxygen species accumulation was reduced, respectively, to 3.6-, 3.0-, 2.5- and 1.8-fold that of control cells (*P* < 0.05), in a concentration-dependent manner (Figure 1B).

ASP inhibited H₂O₂-induced reduction of mitochondrial membrane potential (MMP) in PC12 cells

As mitochondria are a critical target of oxidative damage^[25], we tested the effect of ASP on H₂O₂-induced MMP changes using rhodamine 123, a fluorescent dye highly specific for mitochondria, which actively accumulates in living cells in direct proportion to the MMP^[26]. MMP was significantly reduced to 75% of that of normal cells after exposure to H₂O₂ alone. ASP pretreatment significantly inhibited this H₂O₂-induced decrease in MMP at all concentrations tested (0.1–0.8 mg/mL), resulting in MMP values of 84–91% of those of control cells (*P* < 0.05; Figure 1C).

ASP protected PC12 cells against H₂O₂-induced apoptosis

MMP is an early indicator of apoptosis^[27]. We determined the effect of ASP on H₂O₂-induced apoptosis in PC12 cells using propidium iodide staining and flow cytometry. The apoptosis rate of control cells was 1.0% (Figure 2A) while that of the cells treated with H₂O₂ alone was 10.9% (Figure 2B). When the cells were pre-incubated with 0.1, 0.2, 0.4 or 0.8 mg/mL ASP and then stressed with H₂O₂, apoptosis rates were reduced to 7.0%, 5.9%, 5.7% and 3.3%, respectively (Figure 2C–F). These data demonstrate that ASP is capable of protecting PC12 cells from H₂O₂-induced apoptosis.

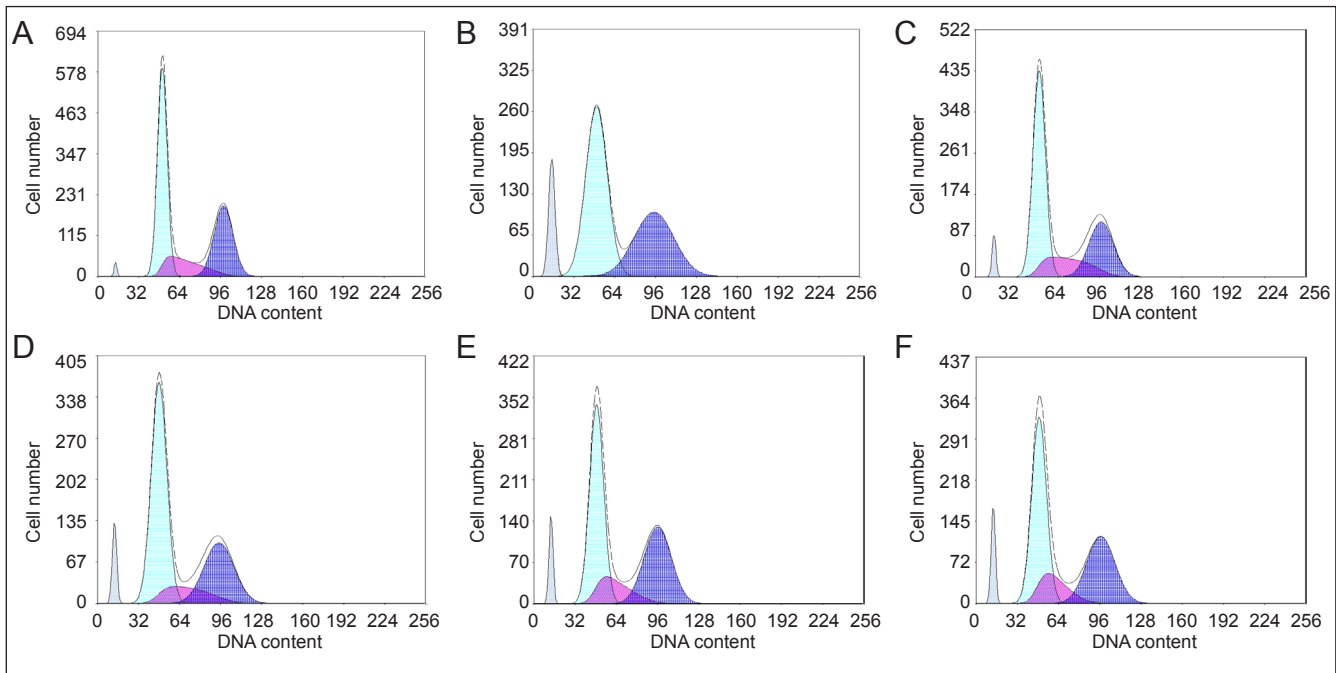


Figure 2 Pretreatment with *Angelica sinensis* polysaccharide (ASP) prevented H₂O₂-induced apoptosis in PC12 cells, as measured using flow cytometry.

(A) Control cells; (B) cells exposed to H₂O₂ alone; (C–F) cells treated with 0.1 (C), 0.2 (D), 0.4 (E) or 0.8 (F) mg/mL ASP before incubation with H₂O₂.

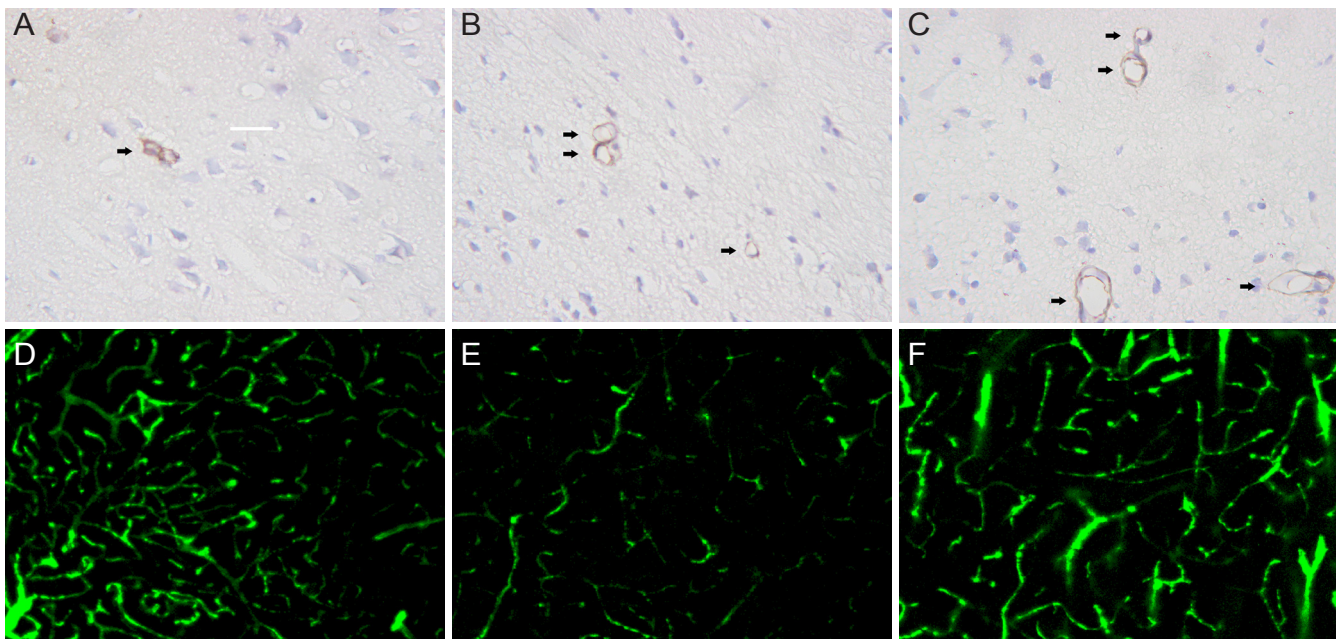


Figure 3 Effect of *Angelica sinensis* polysaccharide (ASP) on CD31-positive cerebral microvessels and FITC-dextran-perfused cerebral microvessels in focal cerebral ischemia rats (× 400).

(A–C) CD31-positive cerebral microvessels (immunohistochemical staining). Control (A), model (B) and ASP-treated (C) groups. The number of microvessels (arrows) in rats treated with ASP was higher than that in the model group. The control group had fewest microvessels. (D–F) FITC-dextran-perfused cerebral microvessels (laser scanning confocal microscopy). Green fluorescence shows perfused microvessels, which were abundant in normal rats (D), sparse after focal cerebral ischemia (E), and abundant after treatment with ASP (F).

Effect of ASP on antioxidant enzyme activity and lipid peroxidation level in cortical tissue of rats with focal cerebral ischemia

The above results clearly demonstrate the antioxidant activity of ASP *in vitro*. To examine the antioxidative effect

of ASP *in vivo*, we determined the activities of antioxidant enzymes and the level of lipid peroxidation in a rat model of focal cerebral ischemia^[28]. At 7 days after cerebral artery occlusion, the activities of the antioxidant enzymes superoxide dismutase and glutathione peroxidase were significantly

lower in the cortical tissue of rats with cerebral ischemia than in that of control rats ($P < 0.05$). When middle cerebral artery-occluded rats were injected with ASP for 7 days, both superoxide dismutase and glutathione peroxidase activities were significantly greater than those in the model rats that were not injected with ASP ($P < 0.05$). Conversely, the level of malondialdehyde, a lipid oxidation product, was significantly greater in model rats compared with control rats ($P < 0.05$). When the rats with focal cerebral ischemia were injected with ASP, the level of malondialdehyde was lower than that in the model group ($P < 0.05$; Table 1).

ASP increased the number of microvessels in the brain of rats with cerebral ischemia

Immunohistochemical staining showed that the number of microvessels in the brain of ASP-treated rats was much higher than that in the focal cerebral ischemia model rats, which in turn was higher than that in the control rats (Figure 3A–C).

Under a laser scanning confocal microscope, the perfused microvessels in normal rat brain were abundant and homogeneously distributed. After focal cerebral ischemia, they became sparse and heterogeneously distributed. However, when ASP was administered to rats with focal cerebral ischemia, the density and distribution of perfused microvessels in the brain were noticeably protected (Figure 3D–F).

Discussion

The polysaccharides from *Angelica sinensis* have a number of pharmacological activities. For example, a polysaccharide fraction from *Angelica sinensis* root has a strong immunomodulatory effect, facilitating the growth of murine peritoneal macrophages and regulating the expression of Th1 and Th2 related cytokines^[22]. ASPs are also cardioprotective, limiting ischemia/reperfusion-induced myocardial injury^[29]. Three polysaccharides isolated from *Angelica sinensis* are all found to have antioxidative activities in H₂O₂-injured macrophages^[30]. Here, using PC12 cells, we found that the < 20 kDa ASP was capable of protecting cells from H₂O₂-induced injury, MMP reduction, and apoptosis. We further demonstrated the antioxidative effect of ASP on a rat model of middle cerebral artery occlusion; activities of antioxidant enzymes (superoxide dismutase and glutathione peroxidase) were significantly greater and the level of the lipid peroxidation product malondialdehyde was lower than in the model group, similarly to the effects of ASP previously shown in middle-aged women and in rabbits with cerebral ischemia reperfusion injury^[31-32]. Together, these data indicate that ASP not only protects PC12 neuronal cells from H₂O₂-induced oxidative and apoptotic injury, but also promotes recovery of middle cerebral artery-occluded rats from cerebral ischemia and reperfusion damage, suggesting that ASP has potential as a neuroprotective agent.

It has previously been shown that the polysaccharide from the brown marine alga *Sargassum stenophyllum* can inhibit the activity of heparin-binding vascular growth factors during microvessel formation^[33]. The polysaccharides from *Antrodia cinnamomea* also have anti-angiogenic activity

through immunomodulation^[34]. Therefore, this type of polysaccharide is likely to have anticancer activity. Conversely, low molecular weight fucoidan stimulates therapeutic revascularization in critical hindlimb and ischemia migration^[35], and the polysaccharide isolated from *Bletilla striata* induces proliferation of endothelial cells and expression of vascular endothelial growth factor *in vitro*^[36]. Interestingly, the hot-water extract of *Angelica sinensis* root has shown angiogenic potential by stimulating the expression of vascular endothelial growth factor in myocardium^[37]. In the present study, we found that polysaccharides isolated from hot-water extract of *Angelica sinensis* root promoted recovery of microvessels from cerebral ischemia. This evidence demonstrates the angiogenesis-promoting potential of these polysaccharides. However, a crude extract of *Angelica sinensis* root also has an angiogenesis-inhibiting effect^[38]. Other extracts, such as resveratrol from red grapes, have seemingly contradictory effects, promoting or inhibiting angiogenesis, depending on different disease contexts^[39-40]. Therefore, it will be of great interest to further investigate the underlying mechanisms of ASP in the promotion or inhibition of angiogenesis under specific disease conditions.

Brain cells die rapidly when they are deprived of blood supply^[41], and their high degree of specialization makes it difficult for the remaining live cells to assume the functions of the dead cells in the brain^[42-43]. Surgical decompression may be used within 48 hours from stroke onset to reduce fatality and improve clinical outcome, but many survivors are left with disabilities and depression^[44]. Therefore, efforts have been made to find therapeutic strategies to tackle such problems. For example, recombinant tissue plasminogen activators are used to break down blood clots, indicating that intravenous thrombolysis is a promising approach to improve clinical outcome after acute ischemic stroke. However, this type of treatment is only effective and safe for patients within a few hours of onset of acute stroke^[45]. Growth factors are also used in the recovery process after ischemic stroke because of their angiogenesis- and neurogenesis-promoting capacities, although further studies are still needed^[3]. Recent research has revealed that ASPs promote blood production by stimulating secretion of interleukin-6, granulocyte-macrophage colony-stimulating factor and CD34⁺ cells^[46-47]. In the present study, we found that ASP significantly prevented the reduction in microvessel quantity after middle cerebral arterial occlusion in rats. Using laser scanning confocal microscopy, we further found that ASP improved the perfusion and distribution of microvessels in rats with middle cerebral arterial occlusion. These findings suggest that ASP not only helps increase the quantity of microvessels in rats with middle cerebral arterial occlusion, but also improves microvascular perfusion, indicating its potential to promote recovery from cerebral ischemia.

The limitation of this study is lack of the quantification on the microvessels and the investigation on the mechanism.

In conclusion, polysaccharides isolated from *Angelica sinensis* root inhibit the increase of intracellular reactive oxygen species and prevent the decrease in mitochondrial

membrane potential in H₂O₂-treated PC12 cells, as well as protecting cells from H₂O₂-induced cytotoxicity and apoptosis. Importantly, we also showed that ASP has an antioxidant effect *in vivo* and increases the quantity and perfusion of microvessels in rats with middle cerebral arterial occlusion. This evidence reveals the potential of ASP in facilitating recovery from cerebral ischemia and reperfusion injury. Together, our results demonstrate the neuroprotective effects of ASPs and provide an insight into the potential of these and other polysaccharides as treatment for ischemic stroke.

Materials and Methods

Design

A randomized, controlled *in vitro* and *in vivo* study.

Time and setting

The *in vitro* experiment was carried out at the School of Pharmaceutical Sciences, Wuhan University, China, and the animal experiment was performed at the Experimental Animal Center of Wuhan University, China from March 2012 to May 2013.

Materials

Cells

The rat pheochromocytoma cell line PC12 was obtained from the American Type Culture Collection (ATCC).

Animals

Healthy male Sprague-Dawley rats, aged 3–4 months, weighing 200–250 g, were obtained from the Experimental Animal Center of Wuhan University, China (No. 4200500164). The animals were housed at 22°C and 55 ± 5% relative humidity with a regular 12-hour light/dark cycle, and standard diet and water available *ad libitum*. All animals were acclimatized to housing conditions for 1 week before the experiment. The protocols were conducted in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China^[48]. A total of 55 rats were included in the study. The animals were randomly divided into three groups: control ($n = 15$), focal cerebral ischemia model ($n = 20$) and focal cerebral ischemia model with ASP treatment ($n = 20$). The latter group received ASP intraperitoneally (i.p.) after focal cerebral ischemia. An equivalent volume of normal saline was administered to the two other groups. Five rats in each of the model and ASP groups died during the experiment.

Drugs

ASP was extracted and isolated as described previously^[49]. Sliced *Angelica sinensis* roots were purchased from Tongrentang Group (Beijing, China), identified by Professor Zebo Huang (Guangdong Pharmaceutical University, China), ground and passed through a 0.9 mm mesh sieve. The powder was extracted with 80% ethanol at 70°C, and, after removal of the solvent, the materials were immersed overnight in distilled water at room temperature. Water extraction was then performed at 80°C, and the extract was centrifuged at

1,000 r/min for 10 minutes. The supernatant was collected and concentrated under reduced pressure at 45°C, and the concentrated solution was precipitated with four volumes of ethanol. The precipitate was redissolved in distilled water and precipitated with ethanol another three times to further remove small molecules. After collection by filtration, the materials were redissolved in distilled water and potential protein contaminants were removed using the Sevag method. The polysaccharide solution was freeze-dried, redissolved in water and dialysed (20 kDa molecular weight cut-off). The dialysate and retentate were collected to obtain polysaccharides of < 20 kDa and > 20 kDa, respectively, by concentration under reduced pressure and then lyophilization.

Methods

PC12 cell culture

PC12 cells were maintained at 37°C in an atmosphere of 5% CO₂ in RPMI 1640 medium supplemented with 5% fetal bovine serum, 10% horse serum (Hyclone, Logan, Utah, USA), 100 U/mL penicillin and 100 mg/mL streptomycin^[50]. The culture medium was replaced with fresh medium every other day, and the PC12 cells were passaged by trypsinization when the confluence reached about 80%.

PC12 cell viability determined by MTT assay

The viability of PC12 cells was determined using the MTT assay as previously described^[51]. Briefly, 100 µL of cells (1×10^5 /mL) were incubated at 37°C in 96-well plates (Nest, Wuxi, Jiangsu Province, China) for 24 hours, and treated with 10 µL of polysaccharide at various concentrations (0.1–0.8 mg/mL) for 15 minutes. After the addition of 10 µL H₂O₂ (final concentration, 150 µmol/L), the cells were further incubated for 24 hours at 37°C. To determine cell viability, 10 µL of 5 mg/mL MTT (Amersco, Solon, Ohio, USA) was added to each well and the plates were incubated at 37°C for 3 hours. The medium was then carefully removed, and the formazan crystals were dissolved in 150 µL of dimethyl sulfoxide by gentle shaking of the plate. Absorbance was determined with a spectrometer (Thermo Fisher, Waltham, MA, USA) at a wavelength of 570 nm.

Fluorescence detection of intracellular reactive oxygen species levels in PC12 cells

2 mL of PC12 cells (3×10^5 /mL) were seeded in 6-well plates and incubated at 37°C for 24 hours. After the medium was removed, the cells were washed twice with PBS and incubated with 200 µL of polysaccharide at various concentrations (0.1–0.8 mg/mL) in serum-free RPMI 1640 medium for 15 minutes. H₂O₂ (20 µL, final concentration 150 µmol/L) containing dichloro-dihydro-fluorescein diacetate (DCFH-DA; final concentration 10 µmol/L; Beyotime, Nantong, Jiangsu Province, China) was added to each well, and the cells were incubated for 30 minutes. The cells were collected carefully and washed twice with ice cold PBS. The fluorescence intensity was measured on a flow cytometer (Epics Altra II, Beckman, Brea, CA, USA) with the excitation wavelength at 488 nm and the emission wavelength at 520 nm.

The relative reactive oxygen species level (fold change) was expressed as the ratio of fluorescence intensity at 520 nm in an experimental group to that in the normal group^[52].

MMP in PC12 cells determined by rhodamine 123

The MMP in PC12 cells was assessed using the fluorescent cationic dye rhodamine 123 as previously described^[53]. The PC12 cells were treated with polysaccharide as above, and then incubated with 10 $\mu\text{mol/L}$ of rhodamine 123 (Beyotime) for 30 minutes. The intensity of rhodamine 123 fluorescence was measured using flow cytometry. MMP was expressed as relative mean fluorescence intensity, which was the percentage of the mean fluorescence intensity (per 1×10^4 cells) of an experimental group over that of normal cells.

Detection of PC12 cell apoptosis using flow cytometry

Apoptotic cells were quantified using propidium iodide staining^[54]. In brief, about 6×10^5 cells in 2 mL of medium were cultured in 6-well plates at 37°C for 24 hours. Then 200 μL of polysaccharides were added to the wells at the indicated concentrations (0.1–0.8 mg/mL) and incubated for 15 minutes. After addition of 200 μL of H_2O_2 (final concentration 150 $\mu\text{mol/L}$), the cells were further incubated for 24 hours. The cells were then collected, resuspended in ice cold 70% ethanol, and placed at 4°C for 16 hours. The cells were collected by centrifugation, washed twice with PBS, resuspended in 1 mL of PBS containing 50 $\mu\text{g/mL}$ propidium iodide (Sigma, St. Louis, MO, USA), and incubated at 4°C for 30 minutes. The red fluorescence for DNA was measured in about 1×10^4 cells using flow cytometry with the excitation wavelength set at 488 nm.

Preparation of focal cerebral ischemia model

Focal cerebral ischemia was introduced in the rats using the middle cerebral arterial occlusion model as previously described^[55]. After the rats were anesthetized with chloral hydrate (350 mg/kg, i.p.), the right middle cerebral artery was occluded by inserting a nylon suture through the right common carotid artery into the internal carotid artery, up to about 17 mm from the bifurcation of the artery. The nylon suture was withdrawn after 2 hours. During the surgical procedure, body temperature was kept constant at $37.0 \pm 0.5^\circ\text{C}$ using a heat pad connected to a rectal probe. After revival from anesthesia, animals were returned to their home cages.

Injection of ASP

The polysaccharides were dissolved in 1 mL normal saline and a dose of 200 mg/kg i.p. was administered to animals in the ASP group at 2, 26, 50, 74, 98, 122 and 146 hours after middle cerebral artery occlusion, while an equal volume (1 mL) of normal saline was administered to animals in the model group.

Determination of antioxidant enzyme activity and lipid peroxidation level in cortex tissue of focal cerebral ischemic rats

Seven days after middle cerebral artery occlusion, six rats from each group were anesthetized and perfused transcar-

dially with 250 mL of 0.9% saline, and the cortex around the penumbra was dissected. The cortical tissue (20 mg) was then homogenized in 2 mL of ice cold PBS for 10 minutes using a glass homogenizer and centrifuged at $12,000 \times g$ for 10 minutes. The supernatant was collected to determine the activities of superoxide dismutase and glutathione peroxidase as well as the content of malondialdehyde, using colorimetry as previously described^[56]. Protein content was determined using the Bradford method^[57]. The Total Superoxide Dismutase Assay Kit, Cellular Glutathione Peroxidase Assay Kit, Lipid Peroxidation MDA Assay Kit and Bradford Protein Assay Kit were purchased from Beyotime.

Immunohistochemical visualization of microvessels

At 7 days after ischemia, six rats from each group were anesthetized and perfused transcardially with 250 mL of 0.9% saline and 250 mL of 4% paraformaldehyde. The brains were removed and immersed in 4% paraformaldehyde overnight, embedded in paraffin, and sectioned (6 μm thickness). Immunohistochemistry was performed using a streptavidin peroxidase kit (Maixin Bio, Fuzhou, Fujian Province, China). Goat anti-platelet endothelial cell adhesion molecule-1 (CD31) polyclonal antibody (1:100; Santa Cruz Biotechnology, Dallas, TX, USA) was used to detect blood vessels. The brain sections were incubated overnight with CD31 primary antibody at 4°C, and then with anti-goat IgG-TRITC antibody (1:400; Maixin Bio) at room temperature. For each animal, CD31-positive microvessels were quantified from 15 different fields of view (400 \times magnification) around the marginal zone of the infarct region according to Weidner's counting methods^[58].

Observation of microvessels by laser scanning confocal microscopy

Seven days after ischemia, the remaining rats were anesthetized with chloral hydrate and then injected with 2 mL of 50 mg/mL FITC-dextran (Sigma) into the left ventricle. After 1 minute, the brains were rapidly removed and placed in 4% paraformaldehyde at 4°C for 48 hours, before being transferred to PBS. Coronal sections (300 μm thickness) close to the optic chiasma were cut while the brains remained immersed in PBS. The cut coronal sections were analyzed using a laser scanning confocal microscope (Leica TCS-SP2-AOBS-MP, Wetzlar, Germany) at 488 nm (excitation wave). The areas of ischemic penumbra in the infarct cortex were located under 10 \times magnification, and scanned along the z-axis with a 1 μm step under 40 \times magnification with a 512×512 matrix in the x–y direction. The area analyzed each time was $750 \times 750 \mu\text{m}^2$ ^[59].

Statistical analysis

Data are expressed as mean \pm SEM and were analyzed by one-way analysis of variance with the least significance difference test for pairwise comparison. A value of $P < 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS 15.0 for Windows (SPSS, Chicago, IL, USA).

Author contributions: Liao WJ was responsible for the funds, and also conceived and designed the study. Lei T, Li HF, Fang Z, Lin JB, Wang SS, Xiao LY, and Yang F wrote the manuscript, and provided and integrated data. Liu X, Zhang JJ, and Huang ZB analyzed data and revised the manuscript. All authors approved the final version of the manuscript.

Conflicts of interest: None declared.

Peer review: We chose a common method to identify the micro-molecule, non-simple component polysaccharide extracted from *Angelica sinensis*. Both *in vivo* and *in vitro* experiments showed that, polysaccharide improved neuronal injury caused by oxidative stress and had potential effect in the studies addressing neuronal impairment in human brain.

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