Resveratrol attenuates neurological deficit and neuroinflammation following intracerebral hemorrhage

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Abstract. Resveratrol (RESV) improves histopathological and behavioral outcomes in diseases of the central nervous system. However, to the best of our knowledge, there have been no studies investigating its neuroprotective effects on secondary brain injury following intracerebral hemorrhage (ICH). The aim of the present study was to evaluate the neuroprotective function of resveratrol following ICH. Male Sprague-Dawley rats were randomly divided into 3 groups: Sham, ICH and ICH+RESV groups. Rats underwent ICH and received an intraperitoneal injection of RESV daily. Rotarod and open field tests were performed to evaluate improvements in motor disturbance pre- and post-surgery. Rats were sacrificed following the final behavioral test; subsequently, neuron injury and cell death in the hippocampus and microglia activation in the cortex were determined using Nissl staining and ionized calcium binding adaptor molecule 1 immunofluorescence staining, respectively. Compared with the ICH group, rats treated with resveratrol for 2 weeks performed significantly better in behavioral tests. Furthermore, less neural damage in the hippocampus and decreased activation of microglia was observed in the ICH+RESV group. The results of the present study therefore indicate that resveratrol may alleviate secondary brain injury following ICH.

Introduction

Spontaneous intracerebral hemorrhage (ICH) is a particularly devastating cerebral vascular disease with a high mortality rate. Complications, including enlarged hematoma volumes, edema exacerbation and secondary brain injury, often develop following ICH. Craniotomy to clear blood clots, antioxidants, antithrombin, neutrophil infiltration inhibitors and heme oxygenase inhibitors are used to treat ICH (1-5). However, there are still few effective methods able to prevent secondary brain injury following ICH.

Resveratrol (RESV) is a natural, non-flavonoid, polyphenol compound found in grapes and other berries that can induce pleiotropic effects in vertebrates (6-9). It has been demonstrated that resveratrol exhibits a neuroprotective function, as it ameliorates kainate-induced excitotoxicity (10). Previous studies have revealed that RESV improves neurological functions in various diseases of the central nervous system, including cerebral ischemia/reperfusion (11), acute and secondary spinal injury (12), neurodegenerative diseases (13) and depression (14). It has been suggested that the protective effects of RESV may be mediated through the sirtuin 1, adenosine 5’-phosphate-activated kinase and nuclear factor erythroid 2-related factor pathways (15-17).

The autologous blood perfusion model is used in physiological, pathomorphological and therapeutic research into ICH, as it mimics hypertensive cerebral hemorrhage (18). Therefore the present study investigated the neuroprotective function of RESV using autologous blood perfusion in a rat model of ICH.
Rat motor disturbance and neural damage/inflammation were assessed using behavioral tests and immunohistochemistry, respectively.

Materials and methods

Animals. A total of 30 male Sprague-Dawley rats, weighing 300-350 g and aged 55 days, were purchased from the Guangdong Medical Laboratory Animal Center (Guangzhou, China). Rats were cared for in accordance with the Guideline for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publications no. 8023, revised 1978) (19). Rats were housed conventionally (room temperature, 20±2°C; humidity, 55%; 15 air changes per h and a 12-h light-dark cycle) in polycarbonate cages on hardwood bedding and acclimatized for at least 7 days prior to study initiation. Rats were provided with tap water and rodent chow ad libitum. All experimental procedures were approved by the Animal Care and Use Committee of Peking University Shenzhen Graduate School (Shenzhen, China).

Establishing a hypertensive cerebral hemorrhage rat model. Rats were randomly divided into 3 groups: A sham-dimethyl sulphoxide (DMSO) group, an ICH+DMSO group and an ICH+RESV group (all n=10). ICH was induced using the autologous blood perfusion model. Rats were anesthetized with 1% sodium pentobarbital (60 mg/kg), administered intraperitoneally (Shanghai Longsheng Chemical Co., Ltd., Shanghai, China) and were immobilized in a stereotactic apparatus frame (RWD Life Science Co., Ltd., Shenzhen, China). A 1-mm bur hole was punctured into rat skulls (1 mm anterior and 3 mm lateral to bregma). Fresh blood (100 µl) was drawn from rat caudal arteries using a microsyringe and was injected into the caudate putamen (5.5 mm deep to bregma; Fig. 1A). Autologous blood was infused over 10 min using a microinfusion pump (RWD Life Science Co., Ltd.). The needle was slowly withdrawn 40 min following injection to prevent the backflow of infused blood and to allow for hematoma formation. The burr hole was then sealed with bone wax and the wound was sutured. The needle of an empty syringe was inserted into rats in the sham group. Rat body temperature was maintained at 36±0.5˚C with a 12-h light-dark cycle, 20±2°C temperature, 20±2°C; humidity, 55%; 15 air changes per h and a 12-h light-dark cycle) in polycarbonate cages on hardwood bedding and acclimatized for at least 7 days prior to study initiation. Rats were provided with tap water and rodent chow ad libitum. All experimental procedures were approved by the Animal Care and Use Committee of Peking University Shenzhen Graduate School (Shenzhen, China).

Rotarod test. Rats underwent a rotarod test on the ZB-200 (Chengdu Techman Software Co., Ltd., Chengdu, China) at a velocity of 5 rpm, which was subsequently increased to 60 rpm over 55 sec. All rats were trained 20 times with a 20 sec interval. The mean duration of the 20 trials was recorded to assess the daily performance of rats. Rats were trained for 8 days prior to ICH surgery. The performance of rats on the final 3 days was recorded and the mean duration was used as a baseline value. The recovery of motor impairment was examined 3, 7 and 14 days following ICH surgery.

Open field test. Rats were exposed to a circular arena (100x40 cm) constructed from black plywood and the floor was divided into 25 sections. Rats were individually placed in the center of the apparatus and their behavior was recorded for 10 min using a digital camera situated above their head. Tests were performed 1, 7 and 14 days following surgery. The total distance travelled, average speed, number of total rotations and line crossings were analyzed using ANY Maze software (version 4.84; Stoelting Co., Wood Dale, IL, USA).

Tissue processing. Rats were euthanized 14 days following surgery with an intraperitoneal injection of 120-150 mg/kg sodium pentobarbital and then were perfused intracardially with 4% paraformaldehyde in PBS. Brains were removed and fixed in 10% neutral formalin buffer (Sigma-Aldrich; Merck KGaA) at room temperature for 24 h. Following dehydration with graded ethanol and xylene, brains were embedded in paraffin wax. A rotary microtome (RM2255; Leica Microsystems GmbH, Wetzlar, Germany) was utilized to cut 5-µm tissue sections, which were subsequently stored at 4°C.

Nissl staining. To quantify brain injuries induced via ICH, Nissl staining was performed following the manufacturer's protocol (Beyotime Institute of Biotechnology, Shanghai, China). Paraffin-embedded sections were deparaffinized, rehydrated and stained with Cresyl violet (C0117; Beyotime Institute of Biotechnology). Images were captured using an Olympus fluorescence microscope (CKX41; Olympus Corporation, Tokyo, Japan) at a magnification of x400 or x100 and a cooled charge-coupled camera (QICAM 12-bit), and were processed using the QCapture Pro 6.0 program (both QI imaging, Surrey, Canada). To assess whether the number of living neurons differed significantly between the groups, cells in three independent microscopic fields were examined. The mean ratio of normal neurons was calculated from three independent counts and plotted with error bars representing standard deviation. Neurons with round and pale staining nuclei were regarded as surviving, while shrunken neurons with condensed nuclei were regarded as damaged (20).

Immunostaining. To determine the anti-inflammatory effects of RESV, immunofluorescence with anti-ionized calcium binding adaptor molecule 1 (Iba-1) antibodies was utilized to assess microglial activation in the cortex as previously described (21). Slides were deparaffinized, rehydrated (100, 90, 80, 70 and 50% ethanol, and ddH₂O), and immersed in 3% H₂O₂/methanol for 10 min at room temperature to inactivate endogenous peroxidase. Antigen retrieval was performed using a microwave in sodium citrate buffer (pH 6.0). Endogenous peroxidase was blocked using 3% hydrogen peroxide for 10 min. Antigen retrieval was performed with a microwave in sodium citrate buffer (pH 6.0). The slides were then washed in PBS and blocked with 1% bovine serum albumin in PBS for 10 min. The slides were incubated with rabbit anti-Iba-1 antibodies (cat. no. 019-19741; 1:500; Wako Pure Chemical Industries, Ltd., Osaka, Japan) overnight at 4°C. Sections were washed in PBS and incubated with a fluorescein isothiocyanate-conjugated secondary antibody diluted 1:100 for 1 h at room temperature. Following washing, sections were incubated in 10% normal donkey serum in PBS for 1 h and then incubated with a fluorescein isothiocyanate-conjugated secondary antibody (1:100) for 1 h at room temperature. Following washing, sections were incubated in 10% normal donkey serum in PBS for 1 h and then incubated with a fluorescein isothiocyanate-conjugated secondary antibody (1:100) for 1 h at room temperature. Following washing, sections were incubated in 10% normal donkey serum in PBS for 1 h and then incubated with a fluorescein isothiocyanate-conjugated secondary antibody (1:100) for 1 h at room temperature.
secondary goat anti-rabbit Immunoglobulin G antibody (cat. no. 111-095-003; 1:50; Jackson ImmunoResearch Europe, Ltd., Newmarket, UK) at room temperature for 1 h. Subsequently, sections were mounted in mowiol mounting medium containing 1 µg/ml DAPI for DNA staining. Images were captured using a fluorescence microscope at a magnification of x400 or x100 and a cooled charge-coupled camera, and processed using the QCapture Pro 6.0 software. The images were analyzed using ImageJ 1.42q software (32-bit; National Institutes of Health, Bethesda, MD, USA). To quantify neuroinflammation, cortex Iba-1 positive cells were counted and compared between different groups.

Statistical analysis. The results are presented as the mean ± standard deviation and all experimental data were analyzed using Prism 5.0 Software (GraphPad Software, Inc., La Jolla, CA, USA). Statistical differences among sham, ICH and ICH+RESV groups were assessed using one-way analysis of variance followed by the Tukey's test for the comparison of multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

RESV improves motor ability following ICH. To investigate the neuroprotective effects of RESV following ICH, the recovery of rat motor abilities were assessed following ICH (Fig. 2A).

Rotarod tests. The time spent on the rod during the rotarod test was markedly decreased 1 day post-surgery in the ICH and ICH+RESV groups, compared with their performance prior to surgery (Fig. 2B). No significant differences in performance...
were identified between the ICH and ICH+RESV groups on day 1. A recovery was observed 7 and 14 days post-surgery in the ICH+RESV and ICH groups (Fig. 2B). Sham rats exhibited a small increase in relative duration 1 day post-surgery but subsequently remained steady between 3 and 14 days post-surgery. Although the ICH and ICH+RESV groups exhibited a significant recovery 14 days post-surgery, the ICH+RESV group exhibited a significant increase in relative duration compared with the ICH group (P<0.05; Fig. 2C). The results indicate that RESV accelerates the recovery of rat motor abilities following ICH.

**Open field test.** Parameters, including the total distance traveled (Fig. 3A), average speed (Fig. 3B), number of lines crossed (Fig. 3C) and total body rotations (Fig. 3D) were evaluated in an open field test 1, 7 and 14 days following ICH and RESV treatment. Compared with the sham group, the distance traveled, average speed and total body rotations were significantly decreased 1 day post-surgery in the ICH and ICH+RESV groups (all P<0.05; Fig. 3A, B and D). Rats in the ICH group travelled shorter distances and moved at slower speeds than the sham group 1-7 days post-surgery (P<0.05; Fig 3A and B). No significant differences between the ICH and ICH+RESV groups were identified in any of the tests conducted 1 day post-surgery. In addition, no significant differences in the total number of lines crossed were identified among any of the groups (Fig. 1C). These data indicate that the motor ability of rats is significantly decreased following ICH.

Following RESV administration for 7 days post-surgery, the total distance travelled and average speed of rats in the ICH+RESV group were significantly increased compared with ICH rats (P<0.05; Fig. 3A and B). However, no significant differences between these groups were identified in the number of lines crossed and total body rotations. These results indicated that the motor abilities of rats had partially recovered following RESV treatment. However, no significant differences were identified in any groups 14 days post-surgery. This may have been due to the habituation of rats to the experimental environment following test repetition. These data demonstrate that RESV treatment may improve the motor abilities of rats following ICH.

**RESV alleviates damage to neurons in the hippocampus.** Nissl staining was performed to evaluate the morphological changes of neurons in the hippocampus following ICH. Integrated bluish-violet neuronal cells with an articulate structure of mottled nuclei and cytoplasm were observed in the sham group, while condensed and irregular cytons of injured neurons, combined with few normal neurons, were identified in the ICH groups (Fig. 4A). The number of normal neurons significantly decreased following ICH surgery compared with the sham group (P<0.001; Fig. 4A). The number of normal neurons significantly decreased following ICH surgery compared with the sham group (P<0.001; Fig. 4B). Following RESV treatment, the number of normal neurons significantly increased (P<0.001; Fig. 4B). These results suggest that RESV treatment alleviates the neuronal damage induced by ICH.

**Neuroinflammatory responses are ameliorated by RESV following ICH.** Microglia are the resident macrophages of the central nervous system and are the primary form of active immune defense available (22). Following activation...
by neuroinflammation, the overall size and soma of microglia increase and the thickness of axons increases. Iba-1, a biomarker of microglia, stains microglia green color and revealed that the neurons comprise of a soma and several axons (23). Furthermore, Iba-1 identifies the disperse axons involved in nerve conduction (24). The morphological and proliferative changes of microglia following ICH were determined using Iba-1 to assess whether the neuroprotective effects of RESV were mediated by the anti-inflammatory response of glial cells. Iba-1-stained microglia were activated by the formation of an intracerebral hematoma following ICH (Fig. 5A). The number of microglia and length of microglial axons were significantly increased following ICH (P<0.01; Fig. 5B). However, the administration of RESV post-surgery significantly reversed the upregulation of ICH induced Iba-1 (P<0.05; Fig. 5B). These data indicate that the neuroinflammatory response induced by ICH is ameliorated following RESV treatment.

Discussion

Previous studies have demonstrated that secondary brain injury, rather than primary mechanical injury, contributes to
Table I. Comparison of drugs used to treat ICH.

| Treatment       | CEGI improves neurobehavioral outcomes 7 days PS (modified Garcia scale) (41) | GM-1 improves neurobehavioral outcomes 14 days PS (corner turn test) (41) | F3. BDNF improves neurobehavioral outcomes 8 days PS (mouse ICH model, rota-rod) (42) | RESV improves neurobehavioral outcomes 7 days PS (open field)/14 days PS (Rota-rod) |
|-----------------|---------------------------------------------------------------------------------|------------------------------------------------------------------------|---------------------------------------------------------------------------------|---------------------------------------------------------------------------------|
| Neurological    |                                                                                  |                                                                        |                                                                                 |                                                                                 |
| outcome         |                                                                                  |                                                                        |                                                                                 |                                                                                 |
| ICH, intracerebral hemorrhage; CEGI, cattle encephalon glycoside and ignotin; GM-1 monosialotetrahexosyl ganglioside; F3. BDNF, human neural stem cells expressing brain-derived neurotrophic factor; RESV, resveratrol; PS, post-surgery. |
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Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to the regulations of the researched funding but are available from the corresponding author on reasonable request.

Authors’ contributions

JCC, WL and FL proposed the hypothesis, analyzed the results, and wrote the manuscript. JCC designed and executed the majority of the experiments. WKB, XXZ, PM, CXL and YW assisted in the execution of some experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experimental procedures were approved by the Animal Care and Use Committee of Peking University Shenzhen Graduate School (Shenzhen, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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