The Extract of Inflamed Rabbit Skin Induced by Inoculation of Vaccinia Virus Possesses Antioxidant and Neuroprotective Effects in Acute Ischemic Stroke

Zhihang Chen, MD, PhD,*† Jinjing Che, PhD,*† Yunan Hou, PhD,*† Yuanguo Cheng, PhD,*† and Paul Tzongpai Lin, PhD‡

Acute cerebral ischemia remains a major cause for death and disability but current therapeutic options are limited. A mixture of biological agents extracted from the inflamed rabbit skin induced by inoculation of vaccinia virus has been shown to reduce ischemia-induced cerebral edema in vivo. In the current study we show that treatment with such a mixture can also significantly reduce the infarct volume and ameliorate the neurologic deficits in animals after acute occlusion of the middle cerebral artery. Further studies demonstrate that this mixture possesses antioxidant capacities that can decrease the levels of lactic acidosis and increase the activities of superoxide dismutase in the lesional brain. It can also preserve the viability of neuronal cells under local hypoxia and hypoglycemia environments or after exposure to hydrogen peroxide in vitro. Such extract, therefore, may become a potential treatment regimen with promising therapeutic value in human subjects. **Key Words:** Acute cerebral ischemia—antioxidant—neuroprotection—skin extracts.

Acute cerebral ischemia remains a leading cause for death and disability worldwide; however, its therapeutic options are still limited.[1] One of the most recognized treatment regimens is delivery of the tissue plasminogen activator, which needs to be administered within a short period of time after disease onset,[2,3] but risks of hemorrhagic complications and exclusion criteria reduce the number of patients that can benefit from this management module.[4] It would, therefore, be desirable to develop further treatment options with a wider therapeutic window that could be used alone or in combination with current effective pharmaceuticals and other treatment modalities.

In acute ischemia stroke, the volume of the infarct area is critical and often correlates with postischemia neurologic deficits and physical disability.[5] For example, disruption of the cerebral perfusion immediately initiates tissue hypoxia, local hypoglycemia, cellular acidosis, glutamate release, the increase of intracellular Ca^{2+} levels, and defects in the cellular respiratory chains, collectively referred as oxidative stress, which may result in the further depletion of the cellular energy reserve and neuronal necrosis. Moreover, lactic acidosis may facilitate anaerobic reactions and oppose the reoxidation of lactate to pyruvate in the neuron.[6,7] The observation that lactic acidosis increases the neuronal injury in the infarct region of animals further suggests a more direct harmful effect of the lactate on neuronal cells.[8,9] Excessive glutamate accumulating outside the cells after acute ischemia and the increased intracellular calcium ions can also lead to neuronal damage and eventual cell death, known as excitotoxicity.[10] Aiming at opposing these ischemia-induced insults, some regimens such as superoxide dismutase (SOD) and free radical scavengers have recently been used. Among them, edaravone (3-mathyl-1-phenyl-2-pyrazolin-5-one) possesses

From the *State Key Laboratory of Pathogen and Biosecurity, Beijing, †Department of Pharmacology, Institute of Microbiology and Epidemiology, Beijing; and ‡Department of Research and Development, Vanworld Pharmaceutical (Rugao) Co Ltd, Rugao, Jiangsu, China.

Received April 21, 2009; revision received June 8, 2009; accepted June 16, 2009.

Address correspondence to Yuanguo Cheng, PhD, Department of Pharmacology, Institute of Microbiology and Epidemiology, No. 20, Dongda St, Fengtai District, Beijing, China. E-mail: chengyg@nic.bmi.ac.cn.

1052-3057/$—see front matter

doi:10.1016/j.jstrokecerebrovasdis.2009.06.006

antioxidant actions through increased prostacyclin production,[11] inhibition of lipoxygenase metabolism of arachidonic acid by trapping hydroxyl radicals,[12] and quenching of active oxygen leading to protection of various cells such as endothelial cells and myocardial cells against damage by reactive oxygen species (ROS).[13-16] Furthermore, the effect of edaravone has been successfully verified in human subjects.[15]

A mixture of bioactive agents extracted from skin tissue of rabbits with inflammation elicited by inoculation of the virus *V variolae*, which contain inhibitors against the kallikrein-kinin system, has been used for treatment of chronic pain or allergic disease for decades.[17,18] This extract has also been reported to regulate intracerebral Ca^{2+} metabolism in mice and to reduce cerebral edema in both mice and human beings,[19,20] although the active components have not been clearly delineated yet. It is still unknown and remains to be investigated whether this extract can also alleviate the functional consequences after acute cerebral ischemia. The mechanism(s) for its potential neuroprotective effects also awaits illustration. In this report, we designed a series of in vivo and in vitro experiments to investigate the role of this mixture in acute cerebral ischemia and show that treatment with this mixture significantly reduces the infarct volume and preserves the neurologic function after acute stroke. Furthermore, these effects are mediated by, at least in part, its antioxidant activities antagonizing the ischemia-induced oxidative stress subsequent to the cerebral artery occlusion.

Methods

Drugs and Reagents

The mixture of the bioactive agents extracted from skin tissues of rabbits with inflammation elicited by inoculation of the virus *V variolae* was prepared as described[21] and was provided by the Vanworld Pharmaceutical Co Ltd, Rugao, China, with a trade name of AGC (10 U/mL, 25 mL/vial). Edaravone (10 mg/5 mL, Shensen Dongyuen Pharmaceutical Co Ltd, Nanjing, China) was used as a positive control in some experiments. 2,3,5-Triphenyl-tetrazolium chloride (Sigma, St. Louis, MO) solution for brain tissue staining contained 1.5 mL of 4% TCC, 3.4 mL of distilled water, and 0.1 mL of 1 mol/L K_2HPO_4. RPMI-1640 media, glucose-free Earle media, hydrogen peroxide (H_2O_2), cobaltous chloride, collagenase, and MTT were all from Sigma.

Animals and Acute Cerebral Ischemia Model

Intraluminal occlusion of the left middle cerebral artery (MCA) was conducted as modified from a previous description,[22] and with institutional ethical approval. Briefly, male rats (Wistar) with body weight ranging from 280 to 300 g (Weitonglihua Ltd, Beijing, China) were anesthetized with intraperitoneal injection of chloral hydrate. After a V-opening was created at the bifurcation of left external and internal carotid arteries, a heat-blunted nylon monofilament surgical suture (6-0) was intraluminally inserted into the internal carotid artery. Occlusion of the left MCA was achieved by advancing the nylon thread inward for 18.5 ± 0.5 mm until resistance was sensed, the tip of which was wedged into the circle of Willis to obstruct the origin of MCA. The filament was left in place until sacrifice (i.e., 48 hours after surgery). The arteries and skin were subsequently closed in layers and about 1 cm of the nylon thread was left outside the skin. The core temperature in all animals was maintained between 36.5°C and 37.5°C during surgery and for 4 hours after the procedures, and at 20 and 47 hours after surgery. Animals were randomized into 5 groups receiving (1) sham operation, (2) ligation of left MCA with no treatment, (3) treatment with 10 U/kg of AGC, (4) treatment with 40 U/kg of AGC, and (5) treatment with 3 mg/kg of edaravone. Treatment in groups 3, 4, and 5 were given at 2, 6, 20, and 47 hours after left MCA occlusion. All animals were killed by neck dislocation at 48 hours and the brain tissues were harvested for analysis. One hour before surgery and at 6, 20, and 47 hours postoperatively, blood (3-4 μL) was also taken from the tail vein and the levels of serum glucose and blood gas were monitored.

Measurement of Infarct Volume

As described in detail elsewhere,[23-25] 48 hours after the induction of acute cerebral ischemia the rats were neck-dislocated and the brains were removed, frozen, and coronally sectioned (thickness, 2 mm) in a cryostat, starting at the mid-point between anterior pole and optic chiasma, and subsequently at the level of optic chiasma, followed by at infundibulum, and finally at mid-point between infundibulum and caudate nucleus. The sections were immediately immersed in 5 mL of 2,3,5-triphenyl-tetrazolium chloride solution, by which normal brain tissue was stained as rose-red and infracted tissues were stained as white in color, respectively. Infarct area on the sectioned slides was determined using an image analyzer (MCID, Imaging Research Inc, St. Catharines, Ontario Canada) and the infarct volume was measured by timing the infarct area by 2 mm. To eliminate the contribution of postischemic edema to the volume of injury, infarct volumes were corrected for swelling according to the method described by others.[26] The infarct volume was calculated as [(volume of the right hemisphere – noninfarct volume of left hemisphere) ÷ volume of the right hemisphere] × 100%.

Assessment of Neurologic Deficits after Acute Cerebral Ischemia

The evaluation for neurologic deficits was conducted before animals were killed. The operator administering the tests was not aware of the modes of treatment.