Isosteviol Sodium Inhibits Astrogliosis after Cerebral Ischemia/Reperfusion Injury in Rats

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Previous reports have indicated that isosteviol sodium (STVNa) has neuroprotective effects against acute focal cerebral ischemia in rats; however, the exact underlying mechanisms and ideal treatment paradigm are not known. To find a reasonable method for STVNa administration and to determine its possible therapeutic mechanisms, we characterized the protective effects of single-dose and multiple-dose STVNa in cerebral ischemia/reperfusion (I/R) injury in rats. Single and multiple treatments with 10 mg/kg STVNa were administered intraperitoneally after injury to investigate its neuroprotective effects. Neurobehavioral deficits and infarct volume were assessed 7 d after ischemia. Both STVNa treatments reduced infarct volumes, improved neurological behaviors, preserved cellular morphology, enhanced neuronal survival, and suppressed cell apoptosis. Multiple treatments performed better than single treatment. Reactive astrogliosis was apparent at 7 d after injury and was significantly inhibited by multiple STVNa treatments but not single treatment. These results indicate that STVNa exerts neuroprotection by different mechanisms in the acute and delayed phases of I/R. Specifically, STVNa neuroprotection in the delayed phase of injury was found to be accompanied with the inhibition of astrogliosis.

Key words isosteviol sodium; cerebral ischemic/reperfusion injury; astrogliosis; rat

Ischemic stroke ranks among the leading causes of death and adult disability worldwide, and accounts for 70% of all stroke events. 1 There are 2 major approaches for the treatment of acute ischemic stroke: recanalization and neuroprotection. Thrombolysis with tissue plasminogen activator is effective for recanalization when administered early after stroke onset, but has several disadvantages including a narrow therapeutic time window and a risk of hemorrhagic complications. 2 In the United States, tissue plasminogen activator is only used to treat 3–5% of patients with ischemic stroke. 3 Alternatively, neuroprotective strategies aim to protect the penumbra brain tissue and attenuate cellular sequelae of stroke.

Astrocytes are the most abundant glial cell population in the central nervous system (CNS) and play an important role in the pathogenesis of cerebral ischemia. Astrocytes activated by cerebral ischemia/reperfusion (I/R) exhibit changes including the over-expression of intracellular glial fibrillary acidic protein (GFAP) and cellular hypertrophy. Reactive astrocytes begin to proliferate 2 d after I/R. 4 At 3–5 d, there is a significant increase in the number of reactive astrocytes at the ischemic boundary. 5 Over time, these astrocytes become hypertrophied and form a glial scar in the border zone of the injured tissue by 7–10 d after I/R. 6–8

In recent years, research has uncovered both beneficial and detrimental consequences of astrocyte reactivity. 9 Reactive astrocytes generally exert beneficial effects on neurons through actions including lesion sequestering, neuroprotection, and counteracting acute stress. 5,10 In transgenic mice lacking reactive astrocytes, the demarcation of CNS lesions by astrocytes is inhibited and the infiltration of inflammatory cells is diffusely increased after injury. 11,12 Yet, the failure of reactive astrocytes to return to a resting state can result in neuronal damage through the production of cytotoxic levels of pro-inflammatory and oxidative molecules as well as glial scarring. 10,13 Astrocyte activation has important effects on both innate and adaptive immune responses in the CNS. 14,15 A previous study reported that reactive astrocytes mediated an immune-inflammatory response in the delayed phase of I/R injury, potentially perpetuating neuronal damage at the site of injury. 15 Glial scar formation by reactive astrocytes inhibits axonal regeneration through the secretion of extracellular matrix molecules that prevent axonal regrowth, impeding long-term functional recovery. 9,10,17 Protective astrocytic functions such as glutamate uptake and substrate production are compromised under these conditions. In a rodent model, the suppression of astrocyte proliferation at 7 d after I/R injury was associated with less delayed neuronal death and better neuronal survival in the penumbra. 8 Decreased astrogliosis also promotes functional recovery in rats after cerebral ischemia. Thus, the inhibition of the excessive astrogliosis is an important target in ischemic stroke.

Isosteviol (ent-16-ketobeyeran-19-oic acid) is a tetracyclic diterpenoid derived from stevioside, a widely used sweetener. 18–20 Isosteviol does not appear to induce mutagenesis but retains some of the desirable pharmacological activities of stevial, making it a useful candidate for further clinical development in stroke. Isosteviol sodium (STVNa) is an injectable formulation of isosteviol sodium that was developed to have improved solubility. Previous studies found that STVNa decreased oxidative stress, inflammation, cell apoptosis in the acute phase of I/R injury 21–24 and protected against permanent cerebral ischemia injury in mice 25; however, the neuropro-
tective mechanisms and exact utility of STVNa in ischemic stroke remain unclear. In this study, we investigated different schedules for STVNa treatment and the ability of STVNa to attenuate I/R injury through the regulation of astrogliosis.

MATERIALS AND METHODS

Animals  Adult male Sprague-Dawley rats (250–320 g, 10 weeks old) were purchased from the Experimental Animal Center of Sun Yat-sen University (Guangzhou, China). Animals were housed in a room with controlled temperature (25°C) on a 12-h light/dark cycle with *ad libitum* access to standard rodent food and tap water. The rats were allowed to acclimate for a minimum of 3 d prior to use. All experiments were performed in accordance with an institutionally approved protocol and the guide for the care and use of laboratory animals.

Focal Cerebral Ischemia Model  Focal cerebral ischemia was induced by transient middle cerebral artery occlusion (MCAO) through an intraluminal suture as previously described. Briefly, rats were anesthetized with 5% isoflurane (RuitoaiBio, Beijing, China). During the surgical procedure, body temperatures were maintained constant at 37.0±0.5°C using a thermostatically controlled heating blanket. The right common carotid artery (CCA) and external and internal carotid arteries (ECA, ICA) were exposed through a neck incision under an operating microscope. Then, a silicon-coated nylon filament was introduced into the CCA and advanced into the ICA until its tip reached the origin of the MCA, detected by mild increase in resistance. Two hours after ischemia, the filament was slowly withdrawn to allow reperfusion. Sham-operated rats received the same experimental surgery without filament insertion. The success of I/R was verified by laser-Doppler flowmetry (LDF, Perimed AB, Sweden) using a previously published landmark (2 mm posterior to bregma, 5–6 mm lateral to the midline). Cerebral blood flow (CBF) was measured before MCA occlusion, after MCA occlusion, and after reperfusion. A decrease in ipsilateral regional (r)CBF below 30% relative to baseline was considered to represent sufficient induction of focal cerebral ischemia. After withdrawal of the filament, an increase in ipsilateral CBF up to 70% of baseline was considered to represent successful reperfusion. At the same time, physiological parameters including heart rate (HR), pulse oxygen saturation (SpO2) and breathing rate (BR) were monitored using the MouseOx Plus (Starr Life Sciences, Inc., U.S.A.). Sham animals were subjected to the same surgical procedures without advancing of the suture into the MCA.

STVNa Administration  STVNa was provided by the Chemical Development Laboratories of Key Biological Pharmaceutical Company (DongGuan, China). STVNa was dissolved in a mixture of saline and organic solvent to a final concentration of 2.5 mg/mL.

For multiple treatments, rats were randomized into a vehicle-treated group (n=13), 5 mg/kg STVNa group (n=13), 10 mg/kg STVNa group (n=13), and sham-operated group (n=13). Intraperitoneal treatment with STVNa or vehicle was initiated 2 h after ischemia and continued for 7 consecutive days. For single treatment, a dose of 10 mg/kg STVNa (which was more efficacious than the 5 mg/kg dose in our previous experiment) was administered 2 h after ischemia and followed by multiple administrations of vehicle for 6 consecutive days. To explore the effects of STVNa treatment on reactive astrogliosis in the acute and delayed phases of I/R injury, rats subjected to I/R injury were randomly treated with vehicle (n=10) or 10 mg/kg STVNa (n=10) beginning 2 h after ischemia and then daily until the end of the experiment. Rats were sacrificed at 1 and 7 d after I/R injury (n=5 per group per time point).

Assessment of Neurological Deficits  Neurological assessments were performed at 1, 3, and 7 d after I/R injury. Two neurological grading systems were used. The first grading system was similar to that described by Bederson *et al.* with a minor modification and included the assessment of motor deficits and the observation of circling behavior. A grading scale of 0–4 was used as follows: 0, no observable neurological deficit; 1, failure to extend the left forepaw; 2, decreased resistance to lateral push towards the paretic site (and forelimb flexion) without circling; 3, same behavior as grade 2, with circling; and 4, loss of spontaneous walking and a depressed level of consciousness.

The second grading system used was developed by Garcia *et al.* and allows the assessment of spontaneous movement as well as motor and sensory function. The neurobehavioral evaluation consists of the following 6 tests: (1) spontaneous activity, (2) symmetry in movement of the 4 limbs, (3) forepaw outstretching, (4) climbing, (5) body proprioception, and (6) response to vibrissae touch. Rats with severe impairments in each test were graded as 0 or 1, and those with no observable deficits were graded as 3. The final neurological score given to each rat was the summation of all 6 individual test scores; the minimum score was 3 and the maximum was 18. The investigator administering the behavior tests was not involved in the surgeries and was blinded to the experimental groups.

Measurement of Infarct Size  Rats were sacrificed at 7 d after I/R injury and brains were removed and cut coronally into 6 consecutive 2-mm thick slices with the aid of a brain matrix (JieKai Seiko Electronic Co., Ltd., DongGuan, China). The slices were then stained in 2% 2, 3, 5-triphenyltetrazolium chloride solution (TTC, Sigma-Aldrich, St. Louis, MO, U.S.A.) at 37°C. At the same time, physiological parameters including heart rate (HR), pulse oxygen saturation (SpO2) and breathing rate (BR) were monitored using the MouseOx Plus (Starr Life Sciences, Inc., U.S.A.). Sham animals were subjected to the same surgical procedures without advancing of the suture into the MCA.

Tissue Preparation and Hematoxylin and Eosin (HE) Staining  Twenty-two hours after reperfusion, rats were anesthetized with 5% pentobarbital sodium and perfused transcardially with 100 mL of saline solution followed by 100 mL of freshly prepared 4% (v/v) paraformaldehyde in 0.01 M phosphate buffered saline (PBS, pH 7.4). Brains were then removed and post-fixed in 10% paraformaldehyde for at least 48 h. Standard paraffin blocks were obtained from the center of the lesion, corresponding to 1.0 mm anterior to bregma and 1.0 mm posterior to bregma. A series of 5-μm-thick coronal sections were cut and mounted directly onto glass slides for histochemical staining with HE.

Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate-Digoxigenin Nick End Labeling
(TUNEL) Assay  Cell apoptosis was detected by TUNEL assay as per manufacturer instructions (ApopTag® Plus Peroxidase in Situ Apoptosis Detection Kit S7101, Millipore, Darmstadt, Germany). Briefly, brain sections were treated with proteinase K in 3% H2O2. Then, sections were incubated with terminal deoxyribonucleotidyl transferase (TdT) enzyme at 37°C for 1 h, followed by incubation with peroxidase-conjugated anti-digoxigenin for 30 min at 37°C. 3,3′-Diaminobenzidine (DAB, DAKO, Denmark) was used to stain apoptotic cells. The number of TUNEL-positive cells in the ipsilateral hemisphere were counted in 5 fields from random peri-infarct areas per slice and presented as the number of cells/mm². All positively stained cells (methyl green) were included in the count regardless of their morphology.

Immunohistochemistry Assay  Surviving neurons and reactive astrocytes in brain tissue were detected by immuno-histochemical staining procedures. Sections were placed in boiled citrate buffer (pH 6.0) for 10 min for antigen retrieval after deparaffinization and then allowed to cool. After incubation in 3% H2O2 for 10 min followed by blocking in 5% bovine serum albumin (BSA, Sigma-Aldrich) for 30 min, the sections were stained overnight at 4°C using primary antibodies against neuronal nuclei (anti-NeuN, mouse, 1:5000, Millipore) and glial fibrillary acidic protein (anti-GFAP, mouse, 1:300, Abcam, Cambridge, U.K.). The next day, sections were washed with PBS and incubated in secondary antibody (anti-mouse immunoglobulin G (IgG) antibody EnVision System-horseradish peroxidase (HRP), DAKO) for 40 min at 37°C. After washing in PBS, sections were reacted with DAB as a chromogen and counterstained with hematoxylin. Negative control test was performed using mouse (G3A1) monoclonal antibody (mAb) IgG1 isotype control (Cell Signaling Technology, Danvers, U.S.A.) instead of primary antibody to confirm its specificity. Images were taken at 400× magnification.

Cell Counting  Cell counting was performed using ImageJ1.48 by investigators who were blinded to the experimental treatments. Five non-overlapping visual fields were randomly selected within the regions of interest in each brain section. Numbers of TUNEL-positive and GFAP-positive cells were counted within the ischemic boundary zone which was the transitional zone between the infarcts and normal tissue. Data are presented as the number of positive cells in the high power 400× visual field. Numbers of NeuN-positive cells were counted within the ischemic boundary zone and the contralateral homologous region. Data are presented as the percentage of NeuN-positive cells in the boundary zone to those in contralateral homologous region.

Statistical Analysis  Values are presented as mean±standard error of the mean. Differences between 2 groups were analyzed using 2-sample t-tests. Differences between more than 2 groups were compared by an ANOVA followed by Tukey multiple comparisons tests. The threshold for statistical significance was p<0.05.

RESULTS

CBF and Physiological Variables  LDF was performed to ensure the success of I/R injury. Ipsilateral rCBF was reduced below 30% of baseline after ischemia and was increased sufficiently after reperfusion in all I/R groups (Fig. 1). rCBF values in the vehicle group were not significantly different from those in STVNa-treated groups at any time point. Physiological variables including heart rate (HR), arterial oxygen saturation (SpO2), breathing rate (BR), and body temperature were within normal ranges and also did not differ across study groups (Table 1).

Effects of Multiple STVNa Treatments on I/R Injury  Neurological Behavior and Infarct Volume  In order to determine the effects of multiple STVNa treatments on cerebral I/R injury, we examined neurological behaviors and infarct volumes in response to I/R injury. Rats in the sham-operated group had no neurological deficits or infa...
in vehicle-treated rats. Multiple STVNa treatments significantly reduced infarct volumes in a dose-dependent manner (5 mg/kg: 26.8±0.5%; and 10 mg/kg: 17.1±1.4%), with reductions of 16.3% (p<0.05) and 46.7% (p<0.01), respectively (Fig. 2D).

**Histological Analysis**

HE staining was used to examine morphological changes within the ischemic boundary zone at 7 d after I/R injury (Fig. 3A). In the sham-operated group, the structures of most neurons were clear and showed no notable changes. Conversely, most neurons in the vehicle-treated group showed shrinkage, nuclear pyknosis, vacuolization, disappearance, and enlargement of the intercellular space. Rats that received multiple STVNa treatments showed less extensive damage, with significant improvement noted in the 10 mg/kg dosing group (Fig. 3B).

Cell apoptosis was detected by TUNEL assay (Fig. 3C). TUNEL-positive cells were almost absent in sham-operated animals, but were abundant within the ischemic boundary zone of vehicle-treated rats at 7 d after I/R injury. Multiple STVNa treatments (10 mg/kg) significantly reduced the number of apoptotic cells within the ischemic boundary in a dose-dependent manner (5 mg/kg: 13.5±2.7; and 10 mg/kg: 3.7±0.7) compared to vehicle (25.1±2.6) (Fig. 3E). Neuronal survival was determined by NeuN staining (Fig. 3D) and the quantification of NeuN+ cells (% of contralateral homologous area; Fig. 3F). NeuN+ cells were abundant in brains from the sham-operated group (97.3±1.6%), whereas they were significantly decreased within the ischemic boundary zone in vehicle-treated rats (33.8±3.0%) at 7 d after I/R injury. Compared to vehicle, multiple STVNa treatments significantly increased the percentage of surviving neurons in a dose-dependent manner (5 mg/kg: 45.1±2.3%; and 10 mg/kg: 86.9±3.7%).

**Effects of Single STVNa Treatment on I/R Injury**

**Neurological Behavior and Infarct Volume**

In Bederson's test, single STVNa treatment improved neu-

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**Fig. 2. Effects of Multiple STVNa Treatments on Neurological Behavior and Infarct Volume**

(A) Bederson's test scores at 1, 3, and 7 d after I/R injury. (B) Garcia's test scores at 1, 3, and 7 d after injury. (C) Representative photographs of 2% TTC staining of coronal brain sections. Pale areas represent infarction and dark areas represent non-lesioned regions. (D) Statistic analysis of infarct volumes in each condition. Data are expressed as the mean±S.E.M. (n=8). *p<0.05 vs. vehicle-treated group; **p<0.01 vs. vehicle-treated group; ***p<0.05 vs. 5 mg/kg STVNa-treated group.
rological behaviors at 1 and 3 d after I/R injury compared to vehicle (1 d: 2.5±0.2 vs. 3.1±0.1, p<0.05; and 3 d: 2.1±0.2 vs. 2.9±0.2, p<0.05), but this effect was lost by 7 d post-injury (Fig. 4A). In Garcia’s test, single STVNa treatment improved neurological behaviors at each time point after I/R injury compared to vehicle (1 d: 10.6±0.4 vs. 9.1±0.2; 3 d: 11.0±0.3 vs. 10.0±0.3; and 7 d: 11.6±0.5 vs. 10.0±0.4, all p<0.05; Fig. 4B). Single STVNa treatment significantly reduced infarct volumes compared to vehicle (27.2±1.7 vs. 32.1±1.3%, p<0.05), with a reduction of 15.0% (Fig. 4C).
Cellular Morphology, Cell Apoptosis, Neuronal Survival, and Reactive Astrogliosis

At 7 d after I/R injury, single STVNa treatment significantly improved morphological appearances, decreased the number of apoptotic cells (13.8±1.0% vs. 25.1±2.6% in the vehicle-treated group, *p<0.01; Fig. 5), and increased the percentage of surviving neurons (40.5±2.0% vs. 33.8±3.0% in the vehicle-treated group, *p<0.05) within the ischemic boundary zone. Conversely, reactive astrogliosis was not affected by single treatment (42.08±6.53).

Fig. 4. Effects of Single STVNa Treatment on Neurological Behavior and Infarct Volume

(A) Bederson’s test scores at 1, 3, and 7 d after I/R injury. (B) Garcia’s test scores at 1, 3, and 7 d after injury. (C) Statistical analysis of the infarct volumes in each condition. Data are expressed as the mean±S.E.M. (n=8). *p<0.05, vs. vehicle-treated group.

Fig. 5. Effect of Single STVNa Treatment on Cellular Morphology, Cell Apoptosis, Neuronal Survival, and Reactive Astrogliosis within the Ischemic Boundary Zone at 7 d after I/R Injury

(A) HE staining. (B) Apoptotic cells detected by TUNEL assay. (C) Surviving neurons detected by NeuN staining. (D) Reactive astrogliosis detected by GFAP staining. (E–G) Statistic analysis of TUNEL+, NeuN+, and GFAP+ cells in each condition. Data are expressed as the mean±S.E.M. (n=5). *p<0.05, **p<0.01 vs. vehicle-treated group. Scale bar=50 µm.
Effect of STVNa on Reactive Astrogliosis  To evaluate the effect of STVNa treatment on reactive astrogliosis in the acute and delayed phases of I/R injury, GFAP-positive reactive astrocytes in the ischemic boundary zone were assessed in detail at 1 and 7 d after I/R injury (Figs. 6A, B). In the sham-operated group, most astrocytes did not express detect-
able levels of GFAP. In the vehicle-treated group, reactive astrocytes were common in the ischemic boundary zone at 1 d after I/R injury, but there was no obvious astroglial proliferation at this time point. At 7 d after I/R injury, the number of reactive astrocytes increased to 5.5-fold that on 1 d post-injury (49.5 ± 9.7 vs. 9.2 ± 0.86, respectively, p < 0.01; Fig. 6C). These cells were hypertrophied and formed a glial scar within the border zone of tissue injury. Numbers of reactive astrocytes were not significantly decreased by STVNa treatment at 1 d after I/R injury (8.5 ± 1.2); however, excessive astrogliosis was significantly attenuated on day 7 (16.0 ± 2.5, p < 0.01; Fig. 6D).

**DISCUSSION**

In the current study, we demonstrated that both single and multiple treatments with 10 mg/kg STVNa significantly improved neurological behavior, reduced infarct volume, decreased numbers of apoptotic cells and reactive astrocytes, and increased the percentage of surviving neurons after MCAO I/R injury in rats. Notably, multiple treatments yielded better neuroprotection than single treatment (Fig. 7).

This is the first study to investigate the effects of STVNa treatment on reactive astrogliosis in the area of injury after I/R. In our model, astrogliosis was not fully developed at 1 d post-injury (i.e., in the acute phase of injury), but was profound (approximately 6-fold that on day 1) by day 7. This finding is agreement with other studies describing the appearance of reactive astrocytes at 7 d after I/R injury.\(^8,37\) Additionally, the number of reactive astrocytes at 7 d after I/R injury was significantly reduced by multiple STVNa treatments at a dose of 10 mg/kg, but not by single treatment. Both single and multiple treatments led to significant neuroprotection as shown by reductions in infarct volumes and improvements in neurological behaviors compared to the vehicle-treated group. The ability of single STVNa treatment to produce neuroprotection without significantly inhibiting astrogliosis indicates the possibility that STVNa has no effect on reactive astrocytes in the acute phase of I/R. Although previous *in vivo* studies have shown the benefits of inhibiting astrogliosis after ischemia,\(^37,38\) we were unable to distinguish astrogliosis-dependent and non-astrogliosis-dependent neuroprotective effects in our study. Yet, we were able to conclude that STVNa may regulate the proliferation rather than activation of astrocytes.

In our experiment, we found that infarct volume was significantly reduced by multiple treatments with STVNa. This diverges from a report by Wang *et al.*,\(^8\) in which the inhibition of astrogliosis was not associated with a reduction in infarct volume. Many studies using transgenic animal models have shown that the loss of astrocytes after CNS injury prevents the demarcation of CNS lesions and increases the lesion area,\(^1,12\); however, these transgenic models also omit the beneficial actions of reactive astrocytes in the early stage of CNS injury. This may be an explanation for observed differences between our study and previous research. Additionally,
we found that the ability of STVNa to inhibit astrogliosis at 7 d post-injury was much stronger than that previously reported (70 vs. 40% inhibition). Therefore, it is possible that a higher level of astrocytic inhibition led to a reduction in infarct volume in our study. Scar-forming reactive astrocytes have long been considered to be primary inhibitors of axonal regeneration after brain injury. Thus, it is also possible that STVNa treatment created a more hospitable microenvironment for neuronal repair by moderating astrogliosis in the delayed phase of brain I/R injury.

Previous studies have similarly demonstrated the neuroprotective effects of STVNa, focusing on the suppression of pathological processes in the acute phase of focal cerebral I/R injury such as oxygenation, and apoptosis. Increased B cell receptor signaling is one candidate pathway through which suppressed acute adverse effects against cerebral I/R injury in the acute and delayed phases, most notably accompanied with the inhibition of astrogliosis and glial scarring in the delayed phase of injury. However, the causal relationship needed to be further investigated.

An important limitation of this study was a short treatment period of 7 d after I/R injury. Further studies are needed to elucidate the effects of STVNa on neuronal remodeling and functional recovery in the long term after I/R injury. In summary, STVNa (isosteviol) exerts multiple therapeutic effects by suppressing acute adverse factors through a non-astrogliosis-dependent mechanism, and by attenuating astrogliosis in the delayed phase of I/R injury to promote recovery after neuronal damage and prevent secondary injury.

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Conflict of Interests The authors declare no conflict of interest.

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