Neuroprotective Effects of a Novel Antioxidant Mixture Twendee X in Mouse Stroke Model

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Background: Oxidative stress and inflammation are important aggravating factors in acute ischemic stroke. *Methods:* In the present study, the neuroprotective effects of a novel antioxidant mixture Twendee X containing multiple antioxidative ingredients, such as coenzyme Q10, ascorbic acid, and cystine, were evaluated. After the pretreatment of a vehicle or Twendee X (20 mg/kg/d) for 14 days, mice were subjected to transient middle cerebral artery occlusion for 60 minutes and further treated with vehicle or Twendee X for 1 or 5 days. *Results:* Twendee X administration reduced the infarct size, and reduced oxidative stress markers such as 8-hydroxy-2′-deoxyguanosine, 4-hydroxy-2-nonenal, and N^{ε}-(carboxymethyl) lysine (one of advanced glycation end products), as well as inflammatory markers such as ionized calcium binding adapter molecule-1, tumor necrosis factor-α, and monocyte chemotactic protein-1. *Conclusions:* In the present study, the neuroprotective effects of Twendee X were shown on transient middle cerebral artery occlusion mice via antioxidative and anti-inflammatory pathways, providing a potential of Twendee X as one preventive and therapeutic treatment. **Key Words:** Ischemic stroke—middle cerebral artery occlusion—mouse—antioxidative—anti-inflammatory.

Introduction

Ischemic stroke is a leading cause of mortality and neurologic impairments worldwide.[1] Therapeutic strategies against stroke remain limited, and further novel therapies are required in daily clinical practice.[2] Oxidative stress and inflammation are important aggravating factors in acute ischemic stroke.[3,4] Reactive oxygen species (ROS) is gradually generated during cerebral ischemia, then excessively increased after reperfusion notably in the peri-ischemic area with oxidation of cellular DNA, lipids, and proteins,[5-8] and then often focused as a medical treatable target.[9]

We originally showed the strong neuroprotective effects of a free radical scavenger edaravone.[8,10,11] Furthermore, we reported that dietary supplements such as ginkgo extract,[12] platinum nanoparticle species,[13] and antioxidative

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nutrient-rich enteral diet[14] showed neuroprotective effects on the ischemic brains of mice. Dietary supplements did not show the strong effects on diseases as medicinal chemicals. However, they are safe and valuable for the prevention and treatment of various diseases.[15]

Twendee X (TwX) is an anti-aging supplement containing multiple antioxidants and a patented composition.[16] TwX has strong antioxidant effects, and increases superoxide dismutase and cell protection effects.[17] In the present study, we validated whether TwX could be helpful in ameliorating mouse brain damages and oxidative stress following experimental transient middle cerebral artery occlusion (tMCAO).

Materials and Methods

Animals and Focal Cerebral Ischemia

All experimental procedures were approved by the Animal Committee of the Okayama University Graduate School of Medicine (OKU-2015573). Adult male C57BL/6JJcl mice (23-27 g, 8 weeks old) were obtained from CLEA Japan (Tokyo, Japan). The mice were maintained in a temperature-regulated room (23-25°C) on a 12-hour light–dark cycle and allowed free access to food and water. From 9 weeks of age, the mice received vehicle (physiological saline, ip, n = 15) or TwX (20 mg/kg per day, ip, n = 16) for 14 days, and then were subjected to tMCAO for 60 minutes. TwX is a mixture consisting of coenzyme Q10 (3.6 wt%; AQUA Q10 P40-NF, Nissin Pharmaceutical, Tokyo, Japan), niacin amid (.7 wt%), L-cystine (18.2 wt%), ascorbic acid (34.2 wt%), succinic acid (3.6 wt%), fumaric acid (3.6 wt%), L-glutamine (34.6 wt%), and riboflavin (1.5 wt%; Bislase inj; Toa Eiyo, Tokyo, Japan). The TwX mixture was dissolved in saline (Otsuka Pharmaceutical Factory, Tokushima, Japan) and stored at 4°C until use. The TwX solution (20 mg/kg) was intraperitoneally injected to the mice with 500 µL volume.

After 14 days of intraperitoneal administration of the vehicle or TwX (11 weeks old), the mice were anesthetized with a mixture of nitrous oxide : oxygen : isoflurane (69%:30%:1%) during surgery with an inhalation mask, and tMCAO was induced using the intraluminal filament technique.[18] Body temperature was monitored and maintained at 37 ± 0.3°C by placing the animals on a heating pad (BWT-100; Bio Research Center, Aichi, Japan). After the right common carotid artery was exposed, a 7-0 nylon thread with a silicon-coated tip was inserted into the right middle cerebral artery. After 60 minutes of tMCAO, the silicon-coated thread was pulled out to restore blood flow of MCA. After this tMCAO, each mice group (vehicle or TwX) was further treated with the same vehicle or TwX for 1 (n = 7 each) or 5 (n = 8 or 9) days. The mice received the injection of vehicle or TwX once a day over a period of 1 day (V-1d, TwX-1d) or 5 days (V-5d, TwX-5d) (Fig 1).

One day or 5 days after tMCAO, blood was collected from the mice via the retro-orbital puncture 12 hours after the final administration. For histologic analysis, the mice were then deeply anesthetized by intraperitoneal injection of pentobarbital (40 mg/kg), and then transcardially perfused with chilled phosphate-buffered saline (PBS, pH 7.4), followed by 4% paraformaldehyde in PBS. The whole brain was removed and immersed in the same fixative solution overnight at 4°C. Serum samples were separated by centrifugation (1940 *g*, 15 minutes, 4°C) and stored at –80°C. After washing with PBS, the fixed brains were incubated in 20% (wt/vol) sucrose in PBS for 24 hours at 4°C. The tissues were frozen in liquid nitrogen and stored at –80°C. Coronal brain section (10 µm thickness) was cut on a cryostat at –20°C and mounted on silane-coated glass slides.

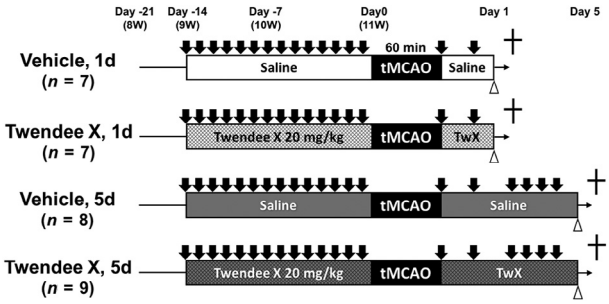

Figure 1. *The 4 experimental mice groups showing V-1d group (saline, sacrificed 1 day after tMCAO), TwX-1d group (TwX, sacrificed 1 day after tMCAO), V-5d group (saline, sacrificed 5 days after tMCAO), and TwX-5d group (TwX, sacrificed 5 days after tMCAO). Filled black arrows indicate intraperitoneal injection of saline or TwX. Open white triangles indicate blood sampling. Abbreviation: tMCAO, transient middle cerebral artery occlusion.*

Serum Measurement of Reactive Oxygen Metabolite Levels and Antioxidant Capacity

The ROS was examined by d-ROMs test (Diacron International, Italy). The reactive oxygen metabolite levels are expressed as arbitrary "Carratelli units" (CARR U), with 1 CARR U corresponding to .08 mg per 100 mL of H_2O_2. The total serum antioxidant capacity was assayed by OXY-Adsorbent test (Diacron International, Grosseto, Italy). The d-ROMs test and OXY-Adsorbent test were performed using a spectrophotometer.[19]

Histology and Immunohistochemistry

For quantification of infarct volume, the brain sections (10 µm) were stained with cresyl violet as Nissl staining and examined by microscopy (SZX-12; Olympus Optical, Tokyo, Japan). The brain sections were prepared at a .8-mm interval each, between .8 mm anterior and 2.4 mm posterior to the bregma. The infarct volumes were measured in the 5 sections by pixel counting using a computer program for Photoshop CC (Adobe, San Jose, CA, USA) and then calculated by summation of the 5 serial infarct areas.

For immunohistochemistry of N^{ε}-(carboxymethyl) lysine (CML), ionized calcium-binding adapter molecule-1 (Iba-