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Neuroprotective Effects of Quercetin 3-*O*-Methyl Ether, Quercetin and (±)-Dihydroquercetin in a Rat Model of Transient Focal Cerebral Ischemia

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Stroke is the third leading cause of death in most industrialized countries, and the most important source of chronic disability, among which cerebral ischemic stroke represents about 85% of all.[1] Ischemic stroke occurs by thrombotic or embolic blockade of an artery to the brain resulting in a deficiency in blood flow and causing a brain infarction. During ischemic stroke, diminished blood flow initiates a series of events such as inducing excessive release of excitatory amino acids and subsequent several receptor activations leading to elevated calcium influx that may result in damage to brain cells.[2]

Oxidative stress is one of the primary factors that exacerbate damage in cerebral ischemia.[3,4] Moreover, the reactive oxygen species can create a secondary source of extensive cell injury and death since re-supply of oxygen to the brain through the reperfusion is relevant during the ischemic phase.[5] Therefore, antioxidants that can scavenge oxygen free radicals have the therapeutic potential for the treatment of neuronal injury following ischemia and reperfusion.[6] Many natural antioxidants are reported to reduce reactive oxygen species and protect neuronal cells in animal models of cerebral ischemia.[7-9]

Previously, we reported protective effects of quercetin 3-*O*-methyl ether (**1**) and its related compounds (**2**, **3**) isolated from *Opuntia ficus-indica* var. *saboten* against oxidative neuronal injuries induced in primary cultured rat cortical cells (Fig. 1).[10] Quercetin (**2**) was found to inhibit H_2O_2- or xanthine (X)/xanthine oxidase (XO)-induced oxidative neuronal cell injury with an estimated IC_{50} value of 4-5 μg/mL. (+)-Dihydroquercetin (**3**) inhibited oxidative neuronal injuries concentration-dependently, but it was 2-3 fold less potent than **2**. On the other hand, quercetin 3-*O*-methyl ether (**1**) potently inhibited H_2O_2- and X/XO-induced neuronal injuries with IC_{50} values of 0.6 and 0.7 μg/mL, respectively, indicating that **1** appeared to be the most potent neuroprotectant among three flavonoids isolated from this plant. Recently, antioxidative activities in cell-free systems of **1** have also been reported.[11,12] However, neuroprotective effects of **1** in ischemic animal models have not yet been reported. The present study, therefore, examined neuroprotective effects of **1** in a transient focal cerebral ischemic rat model and compared with those of related flavonoids **2** and (±)-**3**.

The middle cerebral artery occlusion (MCAO) model has usually been used for assessing neuroprotective effects since most ischemic strokes occur in the territory of the middle cerebral artery. Transient focal cerebral ischemia was induced by occlusion of the right middle cerebral artery for 2 h with a silicone-coated 4-0 nylon monofilament in male Sprague-Dawley rats.[13] The antioxidative flavonoids dissolved in 0.9% saline vehicle were administered intraperitoneally at a dose of 10 mg/kg 30 min after onset of ischemia. Infarct size and % edema were measured 24 h after onset of ischemia using a 2,3,5-triphenyltetrazolium chloride (TTC) staining method.[14] Neurological scoring was performed 30 min and 24 h after MCAO. The neurobehavioral tests consisted of scoring the degree of left forelimb flexion (0 to 3), the duration of left forelimb flexion (0 to 4) and symmetry of movement/forepaw outstretching (0 to 3). Rats were scored on a ranking scale of 0 to 10, which reflects the cumulative score of the individual tests with a score of 10 reflecting normal behavior.[15] Representative TTC staining is shown in Figure 2 to illustrate neuroprotective effects of **1**, **2** and (±)-**3**. Area stained red with TTC was considered normal, while area not stained red with TTC was considered infarcted.

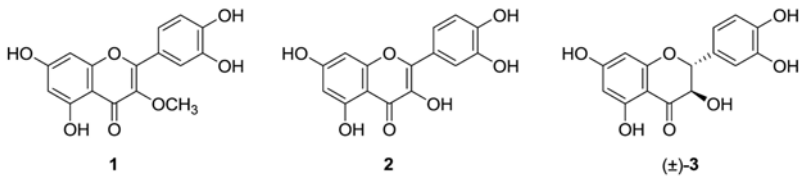

Figure 1. Structures of tested compounds.

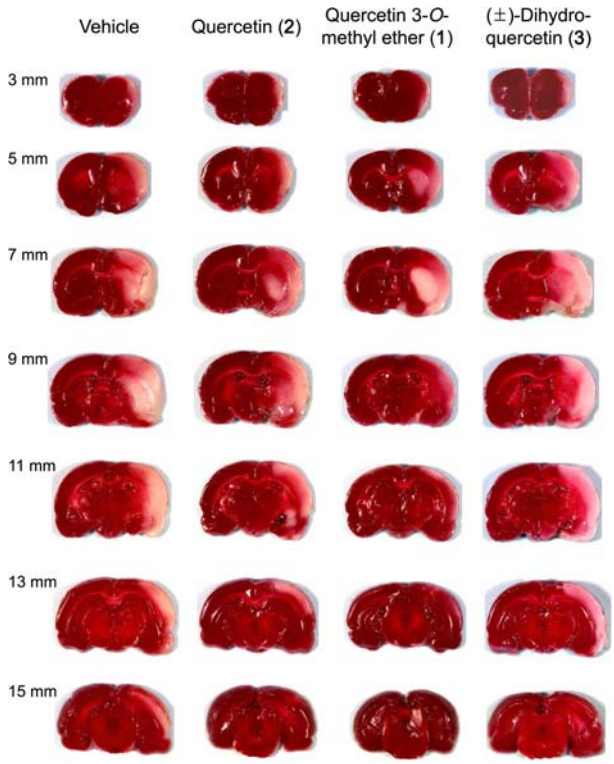

Figure 2. Representative TTC staining illustrating effects of **1**, **2** and (±)-**3** administered at a dose of 10 mg/kg (i.p.) to the rats subjected to middle cerebral artery occlusion (MCAO) for 2 h. Each brain was cut into seven serial slices with thickness of 2 mm starting at 1 mm from the frontal pole 24 h after MCAO. Posterior surface of each slice was imaged. TTC stained normal areas of brain deep red but did not stain infarcted tissue. Values in the left indicate distance from the frontal pole.

As shown in Figure 3, treatment with **1** significantly reduced absolute total and cortical infarct volume by 49.6% (110.1 *vs* 218.6 mm^3) and 55.3% (81.2 *vs* 181.8 mm^3), respectively, compared with a vehicle (0.9% saline)-treated control group. In addition, corrected total infarct volume and edema were significantly reduced by 45.6% (81.6 *vs* 150.0 mm^3) and 54.7% (5.8 *vs* 12.8% edema), respectively. It also produced significant behavioral recovery effect when measured at 24 h after MCAO, increasing neurological score to 4.81 ± 0.44 compared with 2.50 ± 0.41 of the control group. Treatment with **2** significantly reduced only edema by 28.9% (9.1 *vs* 12.8% edema), producing significant behavioral recovery effect comparable to that of **1**. However, treatment with (±)-**3** did not produce any significant neuroprotective effects. The *in vivo* neuroprotective potencies of three antioxidative flavonoids shown in the present study are well correlated with those reported *in vitro* against oxidative injuries in cortical cultures.[10]

Moreover, when **1** was administered intravenously at the same dose, it also produced a similar degree of neuroprotection to the intraperitoneal administration, which was comparable to that of edaravone, a well-known antioxidative neuroprotectant clinically approved only in Japan,[16] administered at a dose of 3 mg/kg (Fig. 4).

In summary, the present study was the first to show that quercetin 3-*O*-methyl ether (**1**) had neuroprotective effect against transient focal ischemic neuronal injury in a MCAO rat model. Compound **1** significantly reduced corrected total infarct volume and edema by 45.6% and 54.7%, respectively, with significant behavioral recovery effect at a dose of 10 mg/kg. Its *in vivo* neuroprotective activity was greater than those of quercetin and dihydroquercetin. Since **1** possesses the most potent *in vivo* neuroprotective activity among the tested antioxidative flavonoids, it is suggested that this compound can serve as a lead chemical for the development of neuroprotective agents by providing neuroprotection against focal ischemic neuronal injury.

Experimental Section

Materials and Methods. Compound **1** was synthesized according to the literature procedure.[17] Compound **2** was obtained from the EtOAc fraction of *Opuntia ficus-indica* var. *saboten*[10] and compound (±)-**3** was prepared according to the literature procedures.[18,19]

Preparation of a Rat Model of Transient Focal Cerebral Ischemia. Under anesthesia of male Sprague-Dawley rats (250 to 300 g) with a gas mixture of 70% N_2O and 2% isoflurane (the balance being O_2), the right MCA was occluded with a silicone rubber cylinder introduced from the bifurcation of the internal carotid artery (ICA) immediately after ligation of the ipsilateral common and external carotid artery. The cylinder was made of 4-0 nylon surgical thread 17 mm long coated with silicone mixed with a hardener to increase the thickness of the distal 5 mm to 0.3-0.4 mm. The proximal tip of the thread was heated, creating a globular stopper for embolization and for easy removal of the cylinder. After introducing the embolus, the ICA was ligated just distal to the point of insertion. The embolus extended from the bifurcation of the ICA to the proximal portion of the anterior cerebral artery. The origins of the right MCA and posterior communicating artery were occluded by the silicone rubber cylinder. Surgery was performed within 15 min and body temperature was kept within normal limits with a heating pad. Following surgery, anesthesia was discontinued and only rats showing neurological deficits characterized by left hemiparesis with upper extremity dominant and/or circling to the left were included in cerebral ischemic groups. After 2-h transient MCAO, reperfusion was achieved by pulling the thread out of the ICA about 10 mm under the same anesthetic conditions as during surgery.

Histological Evaluation of Brain Injury. To examine cerebral infarction caused by transient MCAO, rats were sacrificed by decapitation 24 h after onset of ischemia. After removing brains, seven serial coronal slices of 2-mm thickness were made starting at 1 mm from the frontal pole, incubated in a 2% solution of TTC in normal saline at 37 °C for 60 min for vital staining, and fixed in 10% phosphate-buffered formalin for photography. The areas of the absolute infarct area in the cortex and striatum, and the total areas of both hemispheres were measured for each slice using a com-

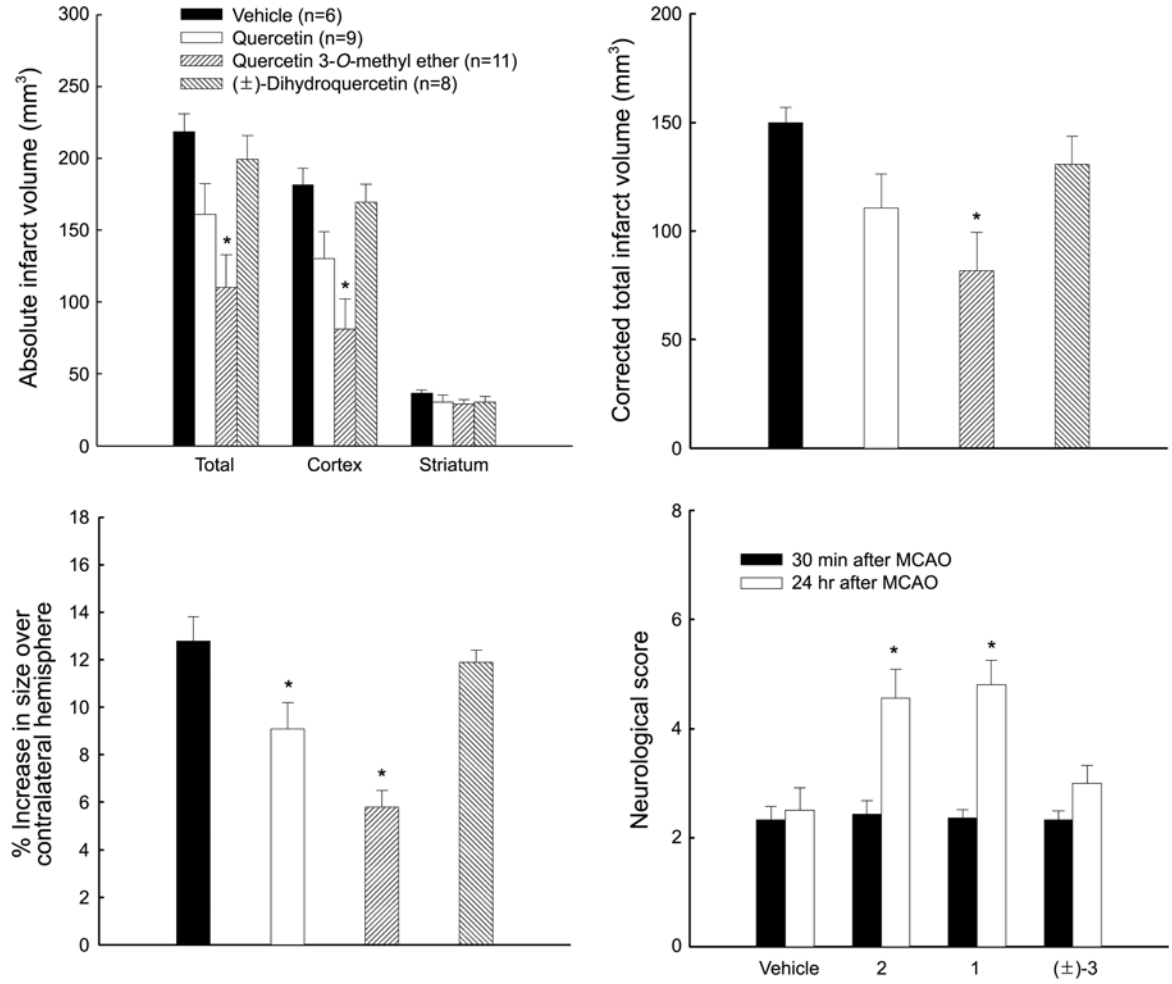

Figure 3. Effects of **1**, **2** and (±)-**3** administered at a dose of 10 mg/kg (i.p.) to the rats subjected to middle cerebral artery occlusion (MCAO) for 2 h on absolute and corrected infarct volume, % edema and neurological score. Data are presented as mean ± S.E.M. *Significantly different from the vehicle-treated control group by Duncan's multiple range test ($p < 0.05$).

puterized image analysis system (Optimas). The absolute infarct volume was calculated by multiplying the area by the slice thickness and summing the volumes. The corrected infarct area in a slice was calculated to compensate for the effect of brain edema by subtracting the area of normal tissue in the ipsilateral hemisphere from the total area of the contralateral hemisphere. Corrected total infarct volume was then calculated by multiplying the area by the slice thickness and summing the volumes. Hemispheric swelling representing tissue edema was expressed as the percent increase in the size of the ipsilateral hemisphere compared with the contralateral hemisphere (% edema).

Evaluation of Neurological Deficits. Neurological deficits in the rats were measured 30 min and 24 h after onset of ischemia. The neurobehavioral tests consisted of the following. (1) In the forelimb flexion test, the rat was held in the air by the tail, and the degree of left forelimb flexion was scored between 0 and 3 (0, no movement on left side; 1, limited movement on left side; 2, less extended or slower movement on left side; 3, symmetrical movement). (2) In the duration of forelimb flexion test, the rat was held in the air by the tail, and the score (0 to 4) was determined by the duration of left forelimb flexion during a 10-second period (0, 8-10 s; 1, 6-8 s; 2, 4-6 s; 3, 2-4 s; 4, 0-2 s). (3) In the symmetry of movement/forepaw outstretching test, the rat was made to walk along the bench on its forelimbs while being held by the tail to keep the hindlimbs in the air. Symmetry in forepaw outstretching was observed and scored between 0 and 3 (0, left forelimb does not move; 1, left forelimb moves minimally and rat circles; 2, left forelimb outstretches less than right; 3, forelimbs outstretched and rat walks normally). Rats were scored on a ranking scale of 0 to 10, which reflects the cumulative score of the individual tests, with a score of 10 reflecting normal behavior.

Statistical Analysis. Data were expressed as mean ± S.E.M. All statistical analyses were performed using SPSS and SigmaPlot software. Statistical significance was assessed by the one-way analysis of variance test followed by Duncan's multiple range test. A value of $p < 0.05$ was considered statistically significant.

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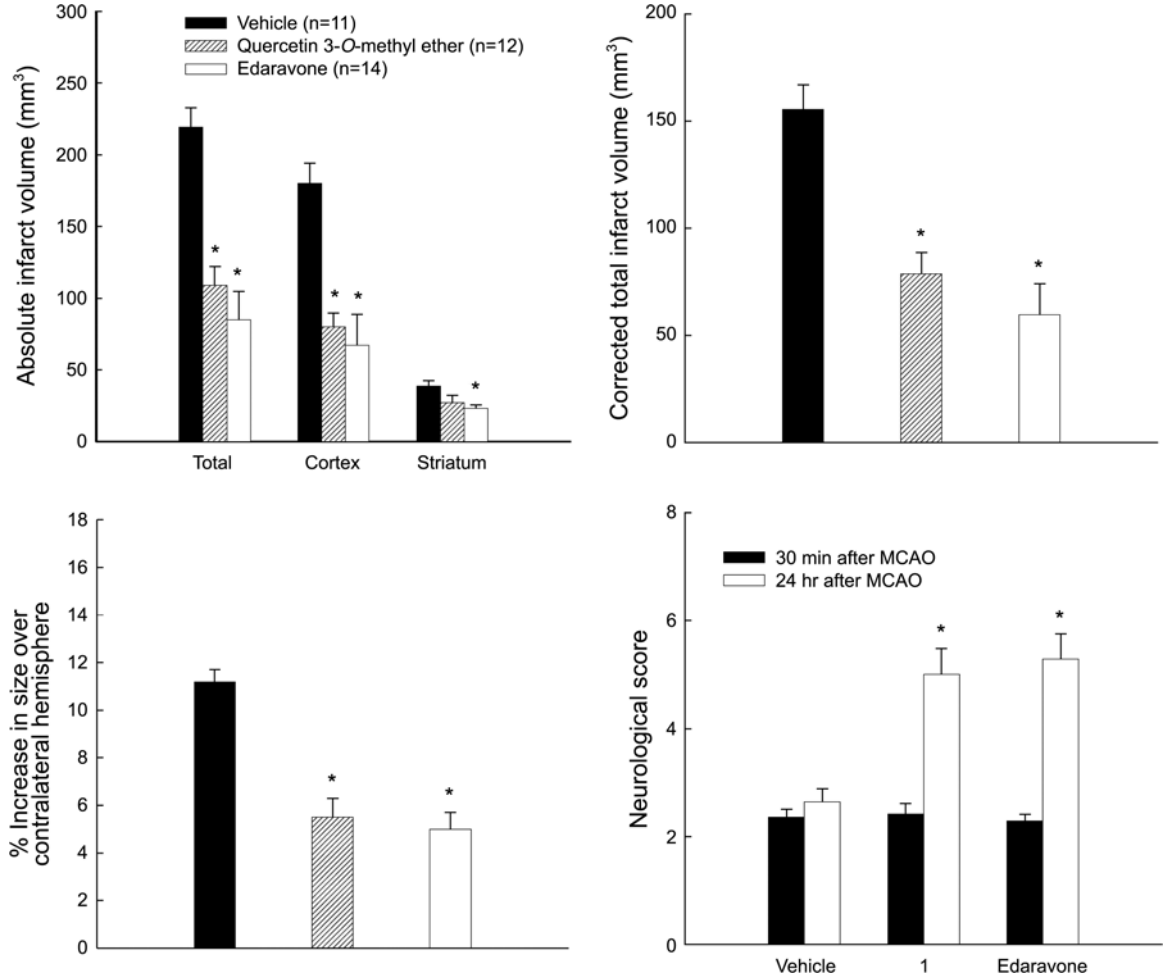

Figure 4. Effects of **1** (10 mg/kg) and edaravone (3 mg/kg) administered intravenously to the rats subjected to middle cerebral artery occlusion (MCAO) for 2 h on absolute and corrected infarct volume, % edema and neurological score. Data are presented as mean ± S.E.M. *Significantly different from the vehicle-treated control group by Duncan's multiple range test ($p < 0.05$).

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