

## Neuroprotective Effects of a Butanol Fraction of *Rosa hybrida* Petals in a Middle Cerebral Artery Occlusion Model

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### Abstract

The neuroprotective effects of a butanol fraction of white rose petal extract (WRPE-BF) were investigated in a middle cerebral artery occlusion (MCAO) model. Seven week-old male rats were orally administered WRPE-BF for 2 weeks and subjected to MCAO for 2 h, followed by reperfusion. Twenty-four h later, MCAO-induced behavioral dysfunctions were markedly improved in a dose-dependent manner by pretreatment with WRPE-BF. Moreover, higher dose of WRPE-BF not only decreased infarction area but also effectively reduced astrogliosis. The expression of inducible nitric oxide synthase, cyclooxygenase-2, and glial fibrillary acidic protein in MCAO model were markedly inhibited by WRPE-BF treatment. Notably, WRPE-BF decreased nitric oxide and malondialdehyde levels in the striatum and subventricular zone of stroke-challenged brains. These data suggested that WRPE-BF may exert its neuroprotective effects via anti-oxidative and anti-inflammatory activities against ischemia-reperfusion brain injury and could be a good candidate as a therapeutic target for ischemic stroke.

**Key Words:** Ischemic brain injury, Middle cerebral artery occlusion, Glial fibrillary acidic protein, *Rosa hybrida*, Antioxidation, Anti-inflammation

### INTRODUCTION

Stroke is generally characterized by the sudden loss of blood circulation in the brain, resulting in a corresponding loss of neurologic function, complications, and death. This can be due to a lack of blood flow caused by blockage or a hemorrhage (Sims and Muyderman, 2010). Astrocytes, the principal housekeeping cells of the nervous system, are not merely supportive of neurons but also perform crucial brain functions, including maintenance of ionic homeostasis, neovascularization, synaptogenesis, neurogenesis, prevention of excitotoxicity, and free radical scavenging (Raghubir, 2008). Thus, protection and maintenance of astrocyte function during focal stroke may be more important than that of neurons. However, astrocytes may negatively affect the balance of life and death

in certain situations. After some ischemic insults, such as permanent MCAO and transient ischemia, reactive astrocytes express inflammatory mediators that may contribute to growth of a stroke (Asano *et al.*, 2005)

Several studies have reported that inhibiting inflammatory processes such as production of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) reduces delayed increases in stroke volume in animal models. Thus, inflammatory signaling cascades are a target of translational research on stroke (Iadecola *et al.*, 1997; Iadecola and Alexander, 2001; Lakhan *et al.*, 2009). Many pro-inflammation enzymes such as iNOS, COXs and xanthine oxidase participate in oxidative injury during cerebral ischemia (Chan, 2001). iNOS is found in astrocytes, microglia, and macrophages where it is a major source of reactive oxygen species (ROS) generation

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(Vaughan and Delanty, 1999).

The rose (genus *Rosa*), allied to its use as a source of aromatic oils for the perfume industry, has been an alternative model for pharmaceutical candidates to treat diabetes mellitus, pain, and inflammatory diseases (Cho *et al.*, 2003; Ng *et al.*, 2005; Jeon *et al.*, 2009). Previously, we reported that the hexane fraction from white rose flowers, which may contain high amounts of gallic acid and volatiles, displayed a broad range of anti-oxidant, anti-microbial, anti-allergic and anti-inflammatory effects (Park *et al.*, 2009; Joo *et al.*, 2010; Lee *et al.*, 2011). In the present study, the neuroprotective effects of a butanol fraction of a white rose petal extract (WRPE-BF) were evaluated using the MCAO technique, as an animal model of human ischemic stroke, by analyzing anti-oxidative and anti-inflammatory profiles as well as morphological and neurobehavioral changes in rats.

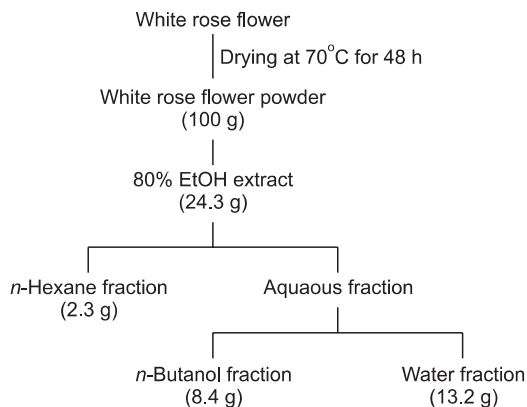
## MATERIALS AND METHODS

### Fractionation of the white rose petal extract

Fresh white rose petals (*R. hybrida*) were collected in Jincheon, Republic of Korea in May 2010 and freeze-dried. A voucher specimen (KWNU80941) was authenticated by Dr. Jang (Gangneung-Wonju National University, Republic of Korea) and deposited at the herbarium of Kangwon National University, Republic of Korea. The dried petals were ground in a rotor mill, and the pulverized petals were sterilized using a 70% ethanol spray, followed by drying at 70°C for 48 h and storage at 4°C before use. The finely-ground powder was extracted with 80% ethanol at room temperature for 24 h. The ratio of powder to solvent was 1:20 (w/v). Total ethanol extracts were filtered through Watmann No. 1 filter paper (Watmann International Ltd., Maidstone, UK). These filtrates were collected and evaporated at 50°C and freeze-dried. Then, fractionation was sequentially performed using *n*-hexane, *n*-butanol and water (Fig. 1). Among three resulting fractions, the butanol fraction was concentrated under vacuum at 50°C and completely dried for further study.

### Animals

Six-week-old male Sprague-Dawley rats, weighing 180-220 g, were purchased from Daehan Biolink (Chungbuk, Republic



**Fig. 1.** Schematic of the fractionation from white rose petal (*R. hybrida*).

of Korea). They were housed in an environmentally-controlled room at constant temperature (23 ± 3°C) and relative humidity (50 ± 10%) and exposed to a 12 h light/dark cycle. The animals were fed a standard rodent chow and purified water *ad libitum*. All experimental procedures were carried out in accordance with the Standard Operating Procedures of the Laboratory Animal Center, Chungbuk National University, Korea, and this protocol was approved by the Institutional Animal Care and Use Committee (Approval #: CBNUA-341-11-01).

### Gas chromatography-mass spectrometry (GC-MS)

Analysis of the WRPE-BF was performed on an Agilent Technologies 5975C GC/MS instrument equipped with a CTC CombiPAL autosampler system (Palo Alto, CA, USA). Chromatographic separation of the WRPE-BF was carried out in helium carrier gas on an HP-5 column (Agilent Technologies, 250 μm×0.25 μm×30 m). A 10 μl volume of sample was injected into a split injector operated in split mode using a 5:1 split ratio with a 5 ml/min split flow and 1 ml/min column flow. The injector temperature was held at 250°C, and the transfer line was held at 250°C. The GC oven was held at 50°C for 5 min, ramped at 3°C/min to 280°C, with a hold for 5 min. The ion source temperature was 250°C with an electron impact ionization energy of -70 V. Following a 4 min delay, data were collected from 35-250 *m/z* using a detector voltage of 1059 V. Components were identified based on a comparison of their relative retention times and mass spectra with those of standards from the Wiley7N library data of the GC-MS system.

### Treatment

After a 1-week acclimation to the laboratory environment, the rats were divided into four groups: sham operation, vehicle-treated MCAO, 10 mg/kg WRPE-BF-treated MCAO and 32 mg/kg WRPE-BF-treated MCAO (n=15/group). The animals were orally administered the WRPE-BF at dose levels of 10 or 32 mg/kg/day or its vehicle (purified water) for 2 weeks and then subjected to MCAO 30 min after the last treatment.

### MCAO surgery

Silicone-coated thread was prepared based on the method described previously (Schmid-Elsaesser *et al.*, 1998). Briefly, polyethylene tubing (Intramedic, Batavia, IL, USA), with a nominal internal diameter of 0.38 mm, was filled with silicone (Koreseal, Busan, Republic of Korea). A 3/0 monofilament nylon suture (Ailee, Busan, Republic of Korea) was inserted 5 mm into one end of the polyethylene tubing (Choi *et al.*, 2012). The tubing and encased silicone were cut with a razor blade at a point 0.5 mm beyond the tip of the suture after 24 h. Immediately prior to surgery, the suture was removed from the tubing, leaving a uniform coating of silicone bonded to the distal 5 mm of the artery. Focal cerebral ischemia was produced as described previously with a slight modification (Longa *et al.*, 1989; Choi *et al.*, 2012; Park *et al.*, 2012). Briefly, rats were anesthetized with 5% isoflurane in 25% O<sub>2</sub>/75% N<sub>2</sub> for induction and maintained with 1.5-2% isoflurane. A midline incision was made on the ventral side of the neck, exposing the left common carotid artery, which was freed from surrounding tissues. The external carotid artery was ligated and dissected. The silicone-coated thread was introduced through the external carotid artery and advanced 18 mm via the internal carotid artery up to the origin of the middle cerebral artery. After occlusion was achieved, the silicone-coated thread was secured

in place by ligature, and the incision was sutured. Only the filament was introduced into the external carotid artery but was not advanced in the sham-operated group. The rats were anaesthetized with isoflurane via a nose cone during the operation, and rectal body temperature was maintained at 37 °C using a temperature-regulated heating pad. The silicone thread was removed for reperfusion after a 2 h occlusion.

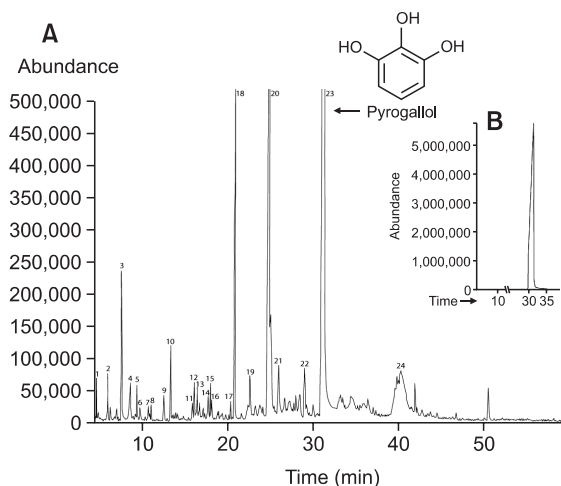
**2,3,5-Triphenyltetrazolium chloride (TTC) staining**

Rat brains were carefully dissected 24 h after MACO, and 2-mm coronal sections were cut in a pre-cooled stainless matrix. Individual sections were placed in 24-well plates and incubated in 2% TTC stain in saline for 15 min at 37°C. Gentle stirring of the plates ensured even staining exposure. Excess TTC was drained off, and slices were fixed in 10% neutral buffered formalin. The area of infarction of each section was determined using a computerized image analysis system. Total lesion volume was calculated by summing up the infarction areas in each section and multiplying by slice thickness.

**Measurement of lipid peroxidation**

Lipid peroxidation was measured by determining the formation of malondialdehyde (MDA)-based ions in the presence of thiobarbituric acid-reactive substances (TBARS) in the brain (Ohkawa *et al.*, 1979; Callaway *et al.*, 1998). Briefly, rat brains were dissected immediately after intracardial perfusion with cold saline. Brain tissue was homogenized in 10 volumes of cold phosphate-buffered saline (PBS) and centrifuged at 4,000 rpm for 5 min to obtain the supernatant. Sodium dodecyl

sulfate (SDS; 500 µl of 8.1% w/v solution) and 1 ml of 20% acetic acid (pH 3.5) were added to the brain homogenate and centrifuged. Aliquots of the clear supernatant were mixed with an equal volume of thiobarbituric acid solution (0.8% w/v) and



**Fig. 2.** Gas chromatography-mass spectrometry (GC-MS) chromatogram (A). Peaks were analyzed based on the GC-MS library listed in Table 1. The presence of pyrogallol in WRPE-BF was identified by the same retention time (31 min) and a single pyrogallol peak was referenced as shown in B.

**Table 1.** Comparison of the gas chromatography-mass spectrometry (GC-MS) library of WRPE-BF<sup>a</sup>

Peak	Retention time (min)	Area (%)	Library
1	4.617	0.21	1-hydroxy-2-propanone
2	5.957	0.32	N-methyl-2-propenamide
3	7.556	1.82	Furfural
4	8.617	0.68	2-furanmethanol
5	8.638	0.57	Methyl isobutyrate
6	9.753	0.19	1,3-dihydroxy-2-propanone
7	10.763	0.22	Hexene-2
8	11.074	0.12	2-hydroxy-2-Cyclopenten-1-one
9	12.538	0.43	5-methyl-2-Furancarboxaldehyde
10	13.386	0.65	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one
11	15.930	0.42	N,N'-Dimethylpiperazine
12	16.133	0.5	Benzeneacetaldehyde
13	16.769	0.18	2,5-Dimethyl-4-hydroxy-3(2H)-furanone
14	17.742	0.23	m-Fluoroanisole
15	18.012	0.5	Methyl2-furoate
16	18.137	0.23	2,5-Dimethyl-4-hydroxy-3(2H)-furanone
17	20.363	0.08	2-acetyl-2-hydroxy-gamma-butyrolactone
18	20.834	4.95	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one
19	22.603	1.06	3,5-Dihydroxy-6-methyl-4H-pyran-4-one
20	24.844	23.73	5-(hydroxymethyl)-2-Furancarboxaldehyde
21	25.979	1.44	1,2,3-propanetriol, monoacetate
22	29.009	1.05	2-Naphthalenol
23	31.176	43.6	1,2,3-Benzenetriol (Pyrogallol)
24	40.321	1.56	Methyl-beta-D-ribosepyranoside

<sup>a</sup>Components were identified based on comparisons of their relative retention time and mass spectra using the Wiley 7N library data of the GC-MS system.

heated in a glass tube capped with aluminum foil at 95°C for 30 min. Samples were cooled on ice, 100 µl of each sample was pipetted into 96 well plates, and absorbance was read at 532 nm.

#### Determination of NO in cerebrospinal fluid

Cerebrospinal fluid (CSF) was collected carefully so as not to be contaminated with blood by puncturing the cervical cisterna membrane after sacrificing the rats with ether anesthesia. Nitrite in CSF was measured as a final product of NO generation using a Griess reagent system at 540 nm (Promega, Madison, WI, USA).

#### Glial fibrillary acidic protein (GFAP) immunohistochemistry

For immunohistochemical staining for GFAP, formalin-fixed paraffin-embedded brain sections (4 µm in thickness) were rinsed in PBS and treated with 3% hydrogen peroxide for 15 min to block endogenous peroxidase activity. After washing with PBS and blocking with 3% bovine serum albumin (BSA) for 10 min, the sections were incubated overnight at 4°C with antibodies specific to GFAP (Dako, Glostrup, Denmark) and then with biotinylated goat anti-rabbit IgG (Vector Laboratories Inc, Burlingame, CA, USA) for 1 h at room temperature, followed by avidin-conjugated peroxidase complex for 30 min at room temperature. The peroxidase reaction was visualized using 3,3'-diaminobenzidine tetrahydrochloride (0.02%) as a chromogen and counterstained with hematoxylin for 4 min.

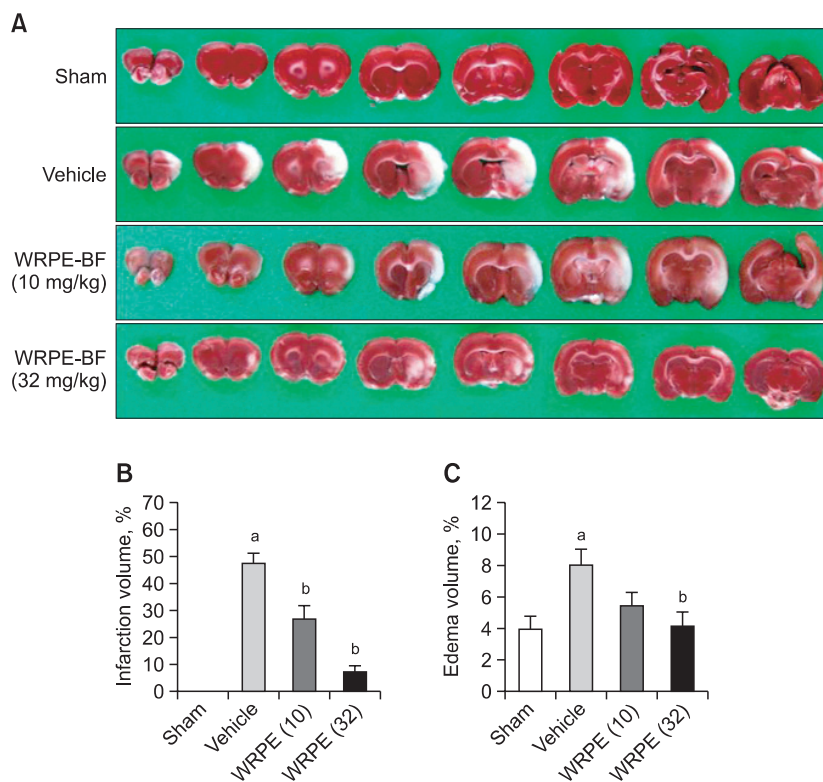
The sections were rinsed, mounted on poly-L-lysine-coated slides, dehydrated, and cover-slipped for light microscopy and photography.

#### Western blot analysis

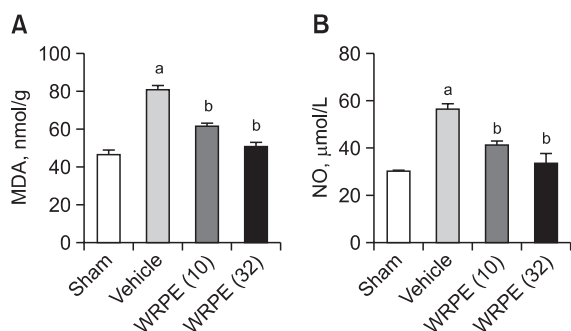
Brain tissues were homogenized in 10 volumes of RIPA buffer containing protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany), and the homogenates were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Then, proteins were transferred onto polyvinylidene fluoride membranes and the membranes were blocked with 5% skim milk in Tris-buffered saline solution containing 0.1% Tween-20. The membranes were then immunoblotted with primary antibodies, anti-COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-iNOS (Santa Cruz Biotechnology), anti-GFAP and anti-β-actin (Santa Cruz Biotechnology), followed by incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (Stressgen Biotechnologies Inc, San Diego, CA, USA). Blots were developed using an ECL solution (Thermo Scientific, Rockford, IL, USA).

#### Measurement of neurobehavioral functions

Motor coordination and balance were evaluated using a rota-rod test system (Panlab Technology, Barcelona, Spain). Rats were placed on the rotating rod at an accelerating speed from 4 to 40 rpm over a period of 5 min, and the time of falling off the rod was recorded.



**Fig. 3.** Effects of white rose petal extract-butanol fraction (WRPE-BF) on the brain infarction and edema induced by middle cerebral artery occlusion (MCAO). Brain slices from rats subjected to 2-h MCAO followed by 22-h reperfusion were stained with 2,3,5-triphenyltetrazolium chloride (TTC). A, representative images of brain sections stained with 2% TTC; B, total infarction area summed up from all slices; C, edema volume. <sup>a</sup>Significantly different from sham control ( $p < 0.05$ ). <sup>b</sup>Significantly different from vehicle control ( $p < 0.05$ ).



**Fig. 4.** Effects of white rose petal extract-butanol fraction (WRPE-BF) on the levels of malondialdehyde (MDA) and nitric oxide (NO) increased by middle cerebral artery occlusion (MCAO). MDA in brain homogenate (A) and NO in CSF (B) from rats subjected to 2-h MCAO followed by 22-h reperfusion were analyzed. <sup>a</sup>Significantly different from sham control ( $p < 0.05$ ). <sup>b</sup>Significantly different from vehicle control ( $p < 0.05$ ).

Spontaneous locomotor activity was evaluated using a video tracking system (Smart v2.5; Panlab Technology), connected to a CCTV monitor. Rats were placed in a quiet chamber (50×50×30 cm), and each time of the movement types; i.e., resting, slow-moving, and fast-moving, was recorded for 2 min following 15-sec adaptation time. Animals were tested three times during one session and the ratio of movement types was analyzed.

**Statistical analysis**

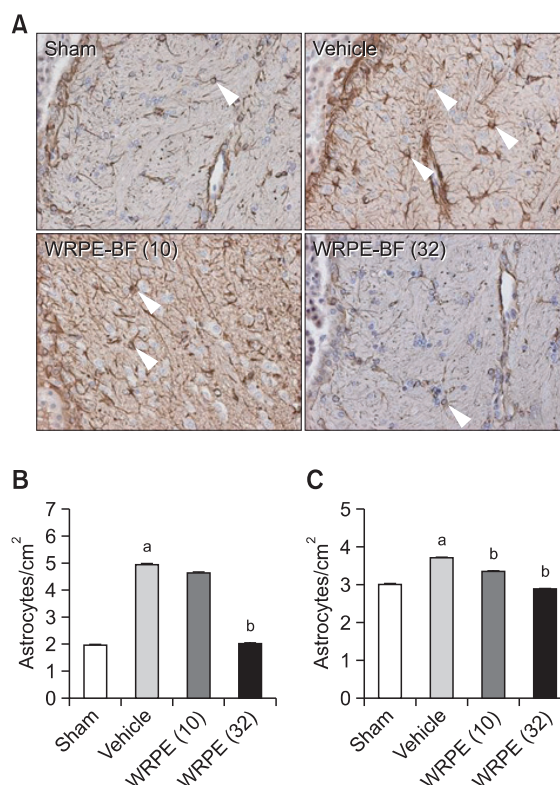
Statistical comparisons between the groups were performed using one-way analysis of variance followed by Dunnett's *post-hoc* test, which were performed using SPSS software v.13 (SPSS Inc., Chicago, IL, USA). Statistical significance was set *a priori* at  $p < 0.05$ .

**RESULTS**

The GC-MS analysis showed that the WRPE-BF contained various components with different retention times (Fig. 2A). GC-MS identified three major components (Table 1), including 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (4.95%), 5-(hydroxymethyl)-2-furancarboxaldehyde (23.73%) and 1,2,3-benzenetriol (pyrogallol) (43.60%) which constituted 72.30% of the total peak area. Pyrogallol, a major substance, was confirmed by analyzing the results with commercial pyrogallol (cat. no. P-0381, Sigma-Aldrich Co., St. Louis, MO, USA) (Fig. 2B).

Brain injury was evaluated by quantifying the total infarction area in rat brain tissues 22 h after reperfusion following a 2 h occlusion. Representative TTC staining patterns of the rat brain tissues are illustrated in Fig. 3A. When pretreated with WRPE-BF, infarction volume was significantly reduced in a dose-dependent manner to 26.6% and 7.3% with 10 and 32 mg/kg WRPE-BF, respectively, compared to the control group (vehicle) (Fig. 3B). WRPE-BF also attenuated brain edema, particularly to the near-normal level at 32 mg/kg (Fig. 3C).

The elevated level of MDA in the MCAO brains (vehicle, 81.0 nmol/g) was remarkably decreased by pretreatment with WRPE-BF in a dose-dependent manner to 61.4 and 50.6 nmol/g at 10 and 32 mg/kg, respectively (Fig. 4A). Coinciden-



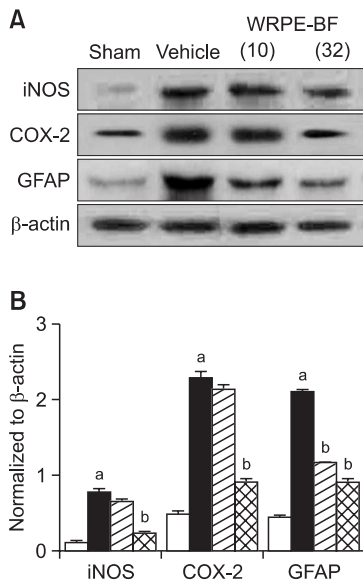
**Fig. 5.** Effects of white rose petal extract-butanol fraction (WRPE-BF) on the number of activated astrocytes increased by middle cerebral artery occlusion (MCAO). Activated astrocytes (A, arrow heads) were detected by immunohistochemistry to glial fibrillary acidic protein (GFAP) in subventricular zone (B) and striatum (C). <sup>a</sup>Significantly different from sham control ( $p < 0.05$ ). <sup>b</sup>Significantly different from vehicle control ( $p < 0.05$ ).

tally, NO production in CSF in the MCAO brains (vehicle, 56.7 µmol/L) was significantly suppressed to 41.5 and 32.5 µmol/L when pretreated with 10 and 32 mg/kg of WRPE-BF, respectively.

The number of activated astrocytes increased greatly in the subventricular zone and striatum of MCAO rats, although the response was much greater in the subventricular zone than that in striatum (Fig. 5A). However, astrocytic activation was inhibited significantly by pretreatment with WRPE-BF (32 mg/kg) to the normal level in both the subventricular zone (Fig. 5B) and striatum (Fig. 5C).

Moreover, the production of inflammatory mediators, iNOS and COX-2 was greatly increased in MCAO rats. Moreover, GFAP was also increased similar to the level of iNOS and COX-2. However, Western blot profiles represented the elevated inflammatory mediators were remarkably attenuated by pretreatment with WRPE-BF in a dose-dependent manner, although GFAP expression was more responsive to the low dose (Fig. 6).

Interestingly, as shown in Fig. 7A, the decreased latency time in rota-rod performance of MCAO rats was significantly recovered by pretreatment with WRPE-BF in a dose-dependent manner. Moreover, locomotor activity tests for the assessment of global activity (resting, slow-moving and fast-moving times) revealed that the decreased physical activity of



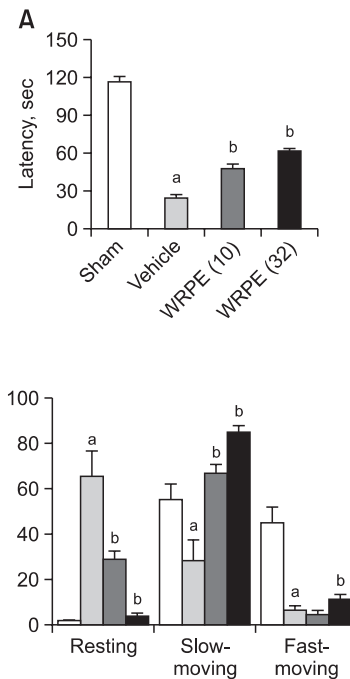
**Fig. 6.** Effects of white rose petal extract-butanol fraction (WRPE-BF) on the expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and glial fibrillary acidic protein (GFAP) increased by middle cerebral artery occlusion (MCAO). The production of enzymes and GFAP proteins was analyzed by Western blotting (A), and quantified relative to the amount of β-actin (B). <sup>a</sup>Significantly different from sham control ( $p < 0.05$ ). <sup>b</sup>Significantly different from vehicle control ( $p < 0.05$ ). White, sham; black, vehicle; shaded, 10 mg/kg; checked, 32 mg/kg.

the MCAO rats was remarkably restored by pretreatment with WRPE-BF in a dose-dependent manner, exhibiting reduced resting times and increased moving times (Fig. 7B).

## DISCUSSION

Many efforts to screen neuroprotective materials using the MCAO animal model have been made, because MCAO recapitulates the biochemical and pathological features of human stroke, such as oxidative stressors and inflammatory responses (Pan *et al.*, 2009). Functional behavioral deficits are common neurological sequelae in patients with brain injuries and in animal models of cerebral ischemia. Thus, behavioral parameters are useful measures of functional deficits following experimental focal cerebral ischemia, and the degree of sensorimotor dysfunction is an important indicator of injury severity (Rogers and Hunter, 1997).

Previously, we reported that neurobehavioral deficits correlated with cerebral infarction volume in MCAO rats and that reperfusion facilitated the brain injury triggered by ischemia (Choi *et al.*, 2012; Park *et al.*, 2012). The WRPE-BF treatment not only reduced infarct size and edema but also improved behavioral deficits, indicating a good relationship between neuroprotection and functional recovery. ROS can be used to assess cellular damage and death during cerebral ischemia and reperfusion as a biochemical mechanism of ischemic brain injury (Chan, 2001; Clark *et al.*, 2001; Margail *et al.*, 2005), because generation and release of excessive ROS during reperfusion plays a major role in brain injury (Chan, 1996; Choi *et al.*, 2012). The elevated free radicals that occur



**Fig. 7.** Effects of white rose petal extract-butanol fraction (WRPE-BF) on the neurobehavioral dysfunctions induced by middle cerebral artery occlusion (MCAO). Latency time in accelerating (from 4 to 40 rpm over a period of 5 min) rota-rod performances (A) and resting, slow-moving and fast-moving times were analyzed in a locomotor activity system (B). <sup>a</sup>Significantly different from sham control ( $p < 0.05$ ). <sup>b</sup>Significantly different from vehicle control ( $p < 0.05$ ).

during ischemic stroke could increase the levels of lipid peroxidation products including TBARS (MDA) and lipid peroxides in the plasma, blood, and brain tissues (Pan *et al.*, 2009; Thakur and Sravanthi, 2010). In the present study, MDA levels increased by 75% in MCAO brains, indicating severe tissue injuries was markedly decreased when pretreated with WRPE-BF.

NO is involved in brain injury development during stroke as well as neurogenesis in the adult hippocampus (Mohammadi *et al.*, 2012). Notably, iNOS is implicated as an important mediator of oxidative and inflammatory responses during ischemia-reperfusion. Astrocytes are the most abundant cells in the brain and serve as an important source of inflammatory mediators during the course of neuroinflammation (Mojsilovic-Petrovic *et al.*, 2007). Activation of the iNOS-NO pathway occurred in parallel with the increased number of activated astrocytes, which were confirmed by up-regulation of GFAP production, while activation of astrocytes was effectively suppressed by WRPE-BF.

COX-2 is constitutively expressed in excitatory neurons, upregulated in many organs by a wide variety of stimuli, and plays an important role in the COX-2-PG inflammatory stream besides the iNOS-NO pathway (Collaço-Moraes *et al.*, 1996; Nogawa *et al.*, 1997). Moreover, COX-2 is upregulated after cerebral ischemia in 12-24 h, and induces a deleterious role during cerebral ischemia in collaboration with NO (Nogawa *et al.*, 1998). Activated astrocytes are also an important source of inflammatory COX-2 in addition to iNOS (Mojsilovic-Petrovic *et al.*, 2007). Our study revealed that iNOS and COX-2

expression were downregulated by WRPE-BF, suggesting its pivotal role for anti-inflammatory activity.

It is well known that activation of astrocytes is a result of brain injury. Thus, the increased expression of GFAP, an astrocyte cytoskeletal protein, is a quantitative marker of neural tissue injury (Park *et al.*, 2011). As predicted, WRPE-BF decreased the number of activated astrocytes in the subventricular zone and striatum in parallel with decreased GFAP expression and infarction area. Such a neuroprotective effect of WRPE-BF is inferred from the anti-oxidant and anti-inflammatory activities of pyrogallol, a major component, which belongs to reducing species having known to strongly scavenge free radicals and inhibit inflammatory mediators (Kondo *et al.*, 1999; Nicolis *et al.*, 2008). Taken together, the WRPE-BF exerted neuroprotective effects in a MCAO model of stroke by suppressing astrocyte activation and inhibiting inflammatory and oxidative indices. Although the active ingredients in WRPE-BF remain to be clarified, WRPE-BF improved neurobehavioral functions via neuroprotective activity. Therefore, we concluded that WRPE-BF may be a good candidate for neuroprotective agents, which is advantageous for pharmaceutical applications for ischemic stroke.

## ACKNOWLEDGMENTS

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