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Research Paper

# Amelioration of cerebral infarction and improvement of neurological deficit by a Korean herbal medicine, modified Bo-Yang-Hwan-O-Tang

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## Abstract

**Objectives** Modified Bo-Yang-Hwan-O-Tang (mBHT) is an improved herbal formula of BHT, which has been widely used to treat ischaemic stroke in East Asia, by the addition of five herbs having anti-ischaemic properties. In this study, we investigated whether mBHT would reduce cerebral ischaemic injury in rats.

**Methods** Sprague–Dawley rats were subjected to a 90-min middle cerebral artery occlusion (MCAO) and subsequent 22-h reperfusion. mBHT was administered either intraperitoneally twice 15 min before and 15 min after, or orally once 30 min or 120 min after the onset of MCAO (50 or 200 mg/kg each).

**Key findings** Intraperitoneal administration of mBHT markedly reduced the cerebral infarct size and neurological deficit caused by MCAO/reperfusion. mBHT treatment also significantly improved long-term survival rate after cerebral ischaemic injury. Oral administration of mBHT 30 min after ischaemia also markedly reduced the infarct size after cerebral ischaemia. The anti-ischaemic effect of mBHT was significantly, but not fully, reduced when mBHT-induced hypothermia was abolished. In cultured cortical neurons, we further found that mBHT decreased oxygen-glucose deprivation/re-oxygenation-evoked neuronal injury by inhibiting production of reactive oxygen species, decrease in mitochondrial transmembrane potential, and activation of caspase-3. However, mBHT did not inhibit N-Methyl-D-aspartate (NMDA) receptor-mediated excitotoxicity.

**Conclusions** Taken together, our data suggest that mBHT has multiple anti-ischaemic properties and would be a good therapeutic herbal prescription for the treatment of cerebral ischaemic stroke.

**Keywords** hypothermia; ischaemic injury; mBHT; OGD; ROS

## Introduction

Cerebral ischaemia/hypoxia is one of the most important life-threatening events in brain injury. Cerebral ischaemic injury is evoked by many interrelated causes such as excessive stimulation of excitatory amino acid receptors (i.e. excitotoxicity), intracellular calcium accumulation, energy failure, apoptotic cell death, oxidative injury by free radicals and inflammatory responses.[1] Because of such various cytotoxic causes, anti-ischaemic agents are required to possess multi-functional properties for the efficient treatment of ischaemic injury.[2,3]

In general, herbal medicines possess many components with different cytoprotective properties.[4,5] Thus, traditional herbal medicines have been often used to treat cerebral ischaemic stroke. Bo-Yang-Hwan-O-Tang (BHT), also known as Bu-Yang-Huanwu-Tang in China, is a decoction of seven herbs consisting of Astragali Radix, Angelicae Gigantis Radix, Paeoniae Radix Rubra, Cnidii Rhizoma, Lumbricus, Percicae Semen and Carthami Flos.[6] BHT has been clinically used for the treatment of cerebral or cardiac strokes and vascular dementia.[7–10] BHT also has anti-thrombotic and immune-modulating activity.[11–13] In addition, BHT is reported to protect cultured primary neurons or PC12 cells from hypoxia-induced cell death.[14]

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For the purpose of synergistic enhancement of the anti-ischaemic effect of BHT, we recently improved the classic BHT formula by adding Achyranthis Radix, Salviae Miltiorrhizae Radix, Cinnamomi Ramulus, Polygalae Radix and Acori Graminei Rhizoma to the seven herbs of BHT. Achyranthis Radix shows anti-thrombotic activity[15] and has a potent anti-inflammatory action.[16] Salviae Miltiorrhizae Radix increases dopamine release,[17] protects rat hippocampal neurons[18] and inhibits the production of nitric oxide and superoxide by microglia in ischaemic rat brain.[19,20] Cinnamomi Ramulus possesses an anti-inflammatory property,[21] and the neuroprotective effect of Polygalae Radix has been demonstrated against N-Methyl-D-aspartate (NMDA) receptor-mediated excitotoxicity in cultured rat granule cells.[22] Acori Graminei Rhizoma has been shown to protect rat heart from ischaemia/reperfusion toxicity[23] and to reduce ischaemia-evoked neuronal death and learning/memory impairments.[24] Considering these properties of each herb, thus, the BHT formula was modified in expectation of the synergistic enhancement of the anti-ischaemic effect of BHT.

## Materials and Methods

### Preparation of modified Bo-Yang-Hwan-O-Tang extract

mBHT was composed of twelve different herbs (Table 1), as previously described.[25] In brief, herbal plants were purchased from Medicinal Materials Company (Kwangmyungdang Medicinal Herbs, Ulsan, Korea) and were authenticated by Professor J.-H. Lee, a medical botanist in the Department of Herbology, College of Oriental Medicine, Dongguk University (DUCOM), Seoul, Korea. Voucher specimens were deposited in the Herbarium of DUCOM. The herbal mixture was extracted in boiling water for 3 h, filtered through a two-layer mesh, freeze-dried under vacuum at 700 mmHg for 15 h, and stored at 4°C before use. For in-vivo experiments, the lyophilized powder of mBHT was dissolved in sterile saline before use. For in-vitro studies, the dissolved solution of mBHT was further sterilized by membrane filtration (0.2 μm Minisart; Sartorius, Goettingen, Germany).

**Table 1** Composition of modified Bo-Yang-Hwan-O-Tang

| Botanical name | Herbal name | Combination ratio |
|---|---|---|
| *Astragalus membranaceus* Bunge | Astragali Radix | 10 |
| *Angelica gigas* Nakai | Angelicae gigantis Radix | 2 |
| *Paeonia lactiflora* Pallas | Paeoniae Radix Rubra | 1.5 |
| *Cnidium officinale* Makino | Cnidii Rhizoma | 1 |
| *Pheretima aspergillum* | Lumbricus | 1 |
| *Prunus persica* (L.) Batsch | Percicae Semen | 1 |
| *Carthamus tinctorius* L. | Carthami Flos | 1 |
| *Achyranthes bidentata* Bl. | Achyranthis Radix | 1.5 |
| *Savia miltiorrhiza* Bge. | Salviae Miltiorrhizae Radix | 4 |
| *Cinnamomum cassia* Blume | Cinnamomi Ramulus | 1 |
| *Polygala tenuifolia* Willd. | Polygalae Radix | 1 |
| *Acorus gramineus* Solander | Acori Graminei Rhizoma | 1 |

### Animals

Male Sprague–Dawley rats, 260–270 g, were purchased from Charles River Laboratories (Seoul, Korea) and kept on a 12-h light–dark cycle with free access to food and water. Rats were acclimatised to their environment for five days before use for experiments. All experimental procedures using animals were in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by Korea University Institutional Animal Care & Use Committee.

### Focal cerebral ischaemia and treatment with modified Bo-Yang-Hwan-O-Tang

To induce transient cerebral ischaemia, rats were initially anaesthetized with 3% isoflurane in a 70% $N_2O$–30% $O_2$ (v/v) mixture via a face mask. Anaesthesia was maintained with 2.5% isoflurane. Focal cerebral ischaemia was achieved by right carotid sheath endovascular middle cerebral artery occlusion (MCAO) for 90 min, as previously described.[26] During surgery (i.e. insertion/removal of a filament to induce MCAO/reperfusion in carotid artery), all rats were kept at 37.0–37.9°C by means of an automated heating pad connected to a rectal thermometer, to avoid rapid reduction in body temperature due to anaesthesia. For the rest of experiments, all rats were kept in the same ambient temperature so as not to interfere with the hypothermic effect of mBHT. For treatment with mBHT, rats were randomly divided into groups treated with vehicle, mBHT (50 or 200 mg/kg) or MK-801 (10 mg/kg). mBHT was dissolved in sterile saline and was administered intraperitoneally twice (15 min before starting MCAO and also 15 min after starting reperfusion) or orally once (either 30 min or 120 min after starting MCAO).

In temperature-controlled studies, mBHT was administered intraperitoneally twice (15 min before starting MCAO and also 15 min after starting reperfusion, 200 mg/kg each). Then mBHT-treated rats were placed in a thermo-regulating cage with heating pad/heating lamps and their temperature was kept at 37.0–37.9°C as control animals, until 270 min after the last mBHT injection.

### Measurement of physiological variables

PE-50 polyethylene tubing was inserted into the right femoral artery for continuous monitoring (Harvard Apparatus model 60-3002; Holliston, MA) of physiological parameters, including mean arterial blood pressure (MABP), pH, $PaO_2$ and $PaCO_2$. Physiological parameters were examined in separate sets of rats ($n = 8$) before ischaemia and after reperfusion, respectively. pH, $PaO_2$ and $PaCO_2$ were measured with a blood-gas analyser (i-STAT Analyzer; East Windsor, NJ, USA).

### Measurement of rectal temperature

To avoid traumatic injury to the brain and resultant reduction of brain temperature, we measured rectal temperature instead.[27] In focal brain injury, the changes in rectal temperature well reflects those in both core body and brain temperature when the thermometer probe was inserted into the rectum by at least 6 cm.[28–30] Rats were anaesthetized with isoflurane and received an intraperitoneal injection of 50, 100 or

200 mg/kg mBHT after 30 min of temperature stabilization. The rectal temperature was monitored using a temperature controller system (TR-100; FST, Heidelberg, Germany) at 0 min (immediately before mBHT injection) and every 30 min thereafter up to 270 min after mBHT injection.

## Infarct volume measurements

Rats were anaesthetized with chloral hydrate and decapitated after 22 h of reperfusion. To determine infarct volumes, rat brains were cut into coronal slices of 2 mm in thickness using a rat brain matrix (Ted Pella, Redding, CA, USA). Infarct area was visualized by incubation with a 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC; Sigma, St Louis, MO, USA) at 37°C for 20 min. The cross-sectional area of infarction between the bregma levels of +4 mm (anterior) and −6 mm (posterior) was determined with a computer-assisted image analysis program (OPTIMAS 5.1; BioScan Inc., Edmonds, WA, USA). The total volume of infarction was evaluated by integrating six chosen sections and expressed as percentage of the total brain volume.

## Neurological deficit scores

Neurological deficits were evaluated carefully at 2.5 h and 24 h after starting MCAO by an observer who had no knowledge of the procedures that had been performed. A neurological deficit was graded on a four-point scale: 0, no neurological deficit; 1, forelimb flexion; 2, forelimb flexion and decreased resistance to lateral push without circling; 3, same behaviour as grade 2, with circling, as previously described.[31]

## Cell culture, oxygen-glucose deprivation/re-oxygenation, and excitotoxicity

Primary cortical neuronal cultures were prepared from embryonic 17–18 days old fetal Sprague–Dawley rats, as we described before.[32] Experiments were performed 14–16 days after initial plating.

For in-vitro hypoxic/ischaemic insult, cells were placed in an anaerobic chamber (partial pressure of oxygen < 2 mmHg), and the culture medium was replaced with a glucose-free DMEM bubbled with an anaerobic gas mix (95% $N_2$, 5% $CO_2$) for 30 min to remove residual oxygen. Cells were left in an anaerobic chamber at 37°C for 60 min to produce oxygen deprivation. Control cells, not exposed to oxygen-glucose deprivation (OGD), were maintained in glucose (25 mM)-containing DMEM aerated with an aerobic gas mix (95% air, 5% $CO_2$). OGD was ended by replacing the bathing media with oxygenated DMEM supplemented with 25 mM glucose, and returning the cells to the incubator under normoxic conditions. mBHT or MK-801 was added before starting OGD and maintained during the re-oxygenation process.

The effect of BHT on excitotoxicity-induced cell death was determined in primary cortical neuronal cultures, as previously described.[33] In brief, cortical neurons were treated with NMDA (100 μM) for 10 min and 5 h later lactate dehydrogenase (LDH) release was determined. Cells were continuously treated with mBHT or MK-801 until LDH release was determined.

## Assessment of cell injury or death

Cellular or nuclear morphological changes were observed using either a phase-contrast microscope or a fluorescence microscope after propidium iodide (PI) staining.

Release of LDH into bathing media was measured using a diagnostic kit (Sigma, St Louis, MO, USA). Cell viability was expressed as percentage of total LDH, which was measured in sister cultures frozen and thawed after the experiments.

## Measurement of intracellular level of reactive oxygen species

Cells were loaded with 2,7-dihydrodichlorofluorescein diacetate (DCF-DA, 30 μM, molecular probe) in phosphate-buffered saline (PBS) for 10 min and then rinsed with the same solution. After a 10-min incubation at room temperature, the fluorescence of DCF was measured at an excitation wavelength of 485 nm and emission wavelength of 530 nm using a fluorescence microplate reader (SpectraMax GeminiEM; Molecular Devices, Sunnyvale, CA, USA) or a fluorescence microscope (DM IL HC Fluo; Leica, Wetzlar, Germany) equipped with digital camera (DFC420C; Leica, Wetzlar Germany). DCF-DA diffuses through cell membranes and is subsequently enzymatically deacetylated by intracellular esterases to the nonfluorescent DCF-H. Free radicals such as peroxynitrite and $H_2O_2$ effectively convert DCF-H to the highly fluorescent DCF.[34] Fluorescence intensities were corrected for autofluorescence (i.e. fluorescence of cells not loaded with DCF-DA).

## Measurement of mitochondrial transmembrane potential

The accumulation of tetramethylrhodamine methyl ester (TMRM) in mitochondria has been shown to be driven by their transmembrane potential.[35] In brief, cortical neurons were subject to OGD/re-oxygenation in the presence or absence of mBHT (3 mg/ml). Three hours after re-oxygenation, 10 nM TMRM was loaded on mitochondria at 37°C for 30 min and then replaced with fresh buffer before observation. Mitochondrial uptake of TMRM (at Ex = 552 nm, Em = 570 nm) was measured by a fluorescence microscope (DM IL HC Fluo; Leica, Wetzlar, Germany) equipped with digital camera (DFC420C; Leica, Wetzlar Germany). Imaging analysing program (TOMORO ScopeEye 3.5) was used for quantitative calculation of fluorescence intensity.

## Western blot analysis

The proteins from total cell lysates (20 μg/lane) were separated by electrophoresis on a 10% SDS–polyacrylamide gel (SDS-PAGE) and transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). After brief washing with Tris-buffered saline containing 0.1% Tween 20 for 10 min at room temperature, the blots were blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween 20 at room temperature for 1 h and then incubated overnight at 4°C with primary antibodies. The blots were incubated with horseradish peroxidase-conjugated secondary antibodies (1 : 2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and developed using an enhanced chemiluminescence detection kit (Amersham Biosciences, Little Chalfont, UK). The following

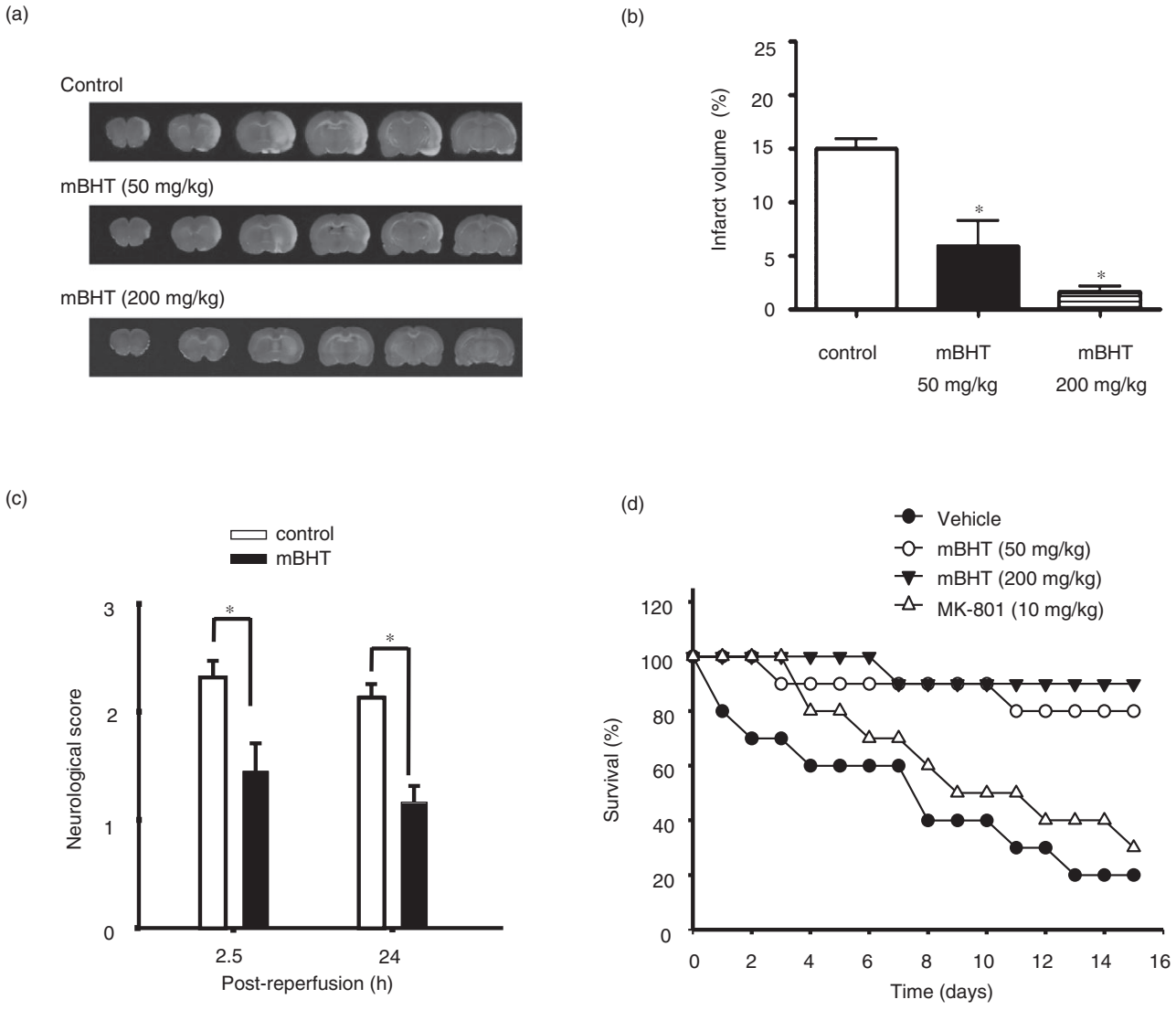


**Figure 1** Effect of modified Bo-Yang-Hwan-O-Tang (mBHT) on cerebral infarction by middle cerebral artery occlusion (MCAO) in rats. mBHT (50 or 200 mg/kg) or MK801 (10 mg/kg) was administered intraperitoneally to rats twice (15 min before starting MCAO and 15 min after starting reperfusion). Infarct areas were determined at 24 h after surgery. (a) Representative 2,3,5-triphenyltetrazolium chloride (TTC)-stained coronal brain sections with six slice (2 mm thick) each 4–16 mm from the frontal pole in each studied group. (b) Percentage changes of infarct volume in TTC-stained brain sections. $n = 15$ for each group. (c) Neurological deficit scores were determined 2.5 h or 24 h after starting MCAO. $n = 15$. (d) Long-term survival rates were determined for 15 days after surgery. $n = 10$. $^*P < 0.05$ compared with vehicle-treated (control) group.

primary antibodies were used: anti-caspase-3 (1 : 1000), anti-$\alpha$-spectrin (1 : 10 000) and anti-$\beta$-actin antibody (1 : 3000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA)

### Measurement of caspase-3 protease activity

The lysates (10 µl) were incubated in a 96-well plate with 90 µl of assay buffer (10 mM HEPES, pH 7.4, 42 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, and 10% sucrose) containing 30 µM caspase-3 substrate (Ac-DEVD-AMC) (Calbiochem, San Diego, CA, USA). AMC from Ac-DEVD-AMC was measured on a microplate fluorescence reader (SpectraMax GeminiEM; Molecular Devices, Sunnyvale, CA, USA) with an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

### Statistical analysis

Data are expressed as mean ± SD or SEM and analysed for statistical significance using unpaired *t*-test or analysis of variance followed by post-hoc Tukeys' test for multiple comparisons, unless otherwise stated. $P < 0.05$ was considered significant.

## Results

First, we examined whether mBHT altered physiological variables, such as mean arterial blood pressure, pH, arterial partial $CO_2$ and $O_2$ pressures and blood glucose concentration, in rats. The physiological variables were determined 15 min

**Table 2** Physiological variables in rats subjected to middle cerebral artery occlusion

| | 15 min before MCAO | | 15 min after reperfusion | |
|---|---|---|---|---|
| | Vehicle | mBHT | Vehicle | mBHT |
| MABP (mmHg) | 106.48 ± 16.54 | 102.68 ± 8.34 | 96.68 ± 11.65 | 98.65 ± 11.30 |
| pH | 7.37 ± 0.04 | 7.36 ± 0.05 | 7.36 ± 0.02 | 7.36 ± 0.05 |
| $PaO_2$ (mmHg) | 130.63 ± 25.82 | 126.38 ± 15.68 | 142.25 ± 17.80 | 135.50 ± 23.34 |
| $PaCO_2$ (mmHg) | 46.86 ± 6.77 | 49.41 ± 7.65 | 46.46 ± 4.05 | 46.16 ± 10.36 |

MCAO, middle cerebral artery occlusion; MABP, mean arterial blood pressure; $PaCO_2$, partial arterial pressure of $CO_2$; $PaO_2$, partial arterial pressure of oxygen. Rats were intraperitoneally administered with 200 mg/kg modified Bo-Yang-Hwan-O-Tang extract. The physiological variables were obtained 15 min before MCAO onset and 15 min after reperfusion. Values are given as means ± SD, $n = 10$.

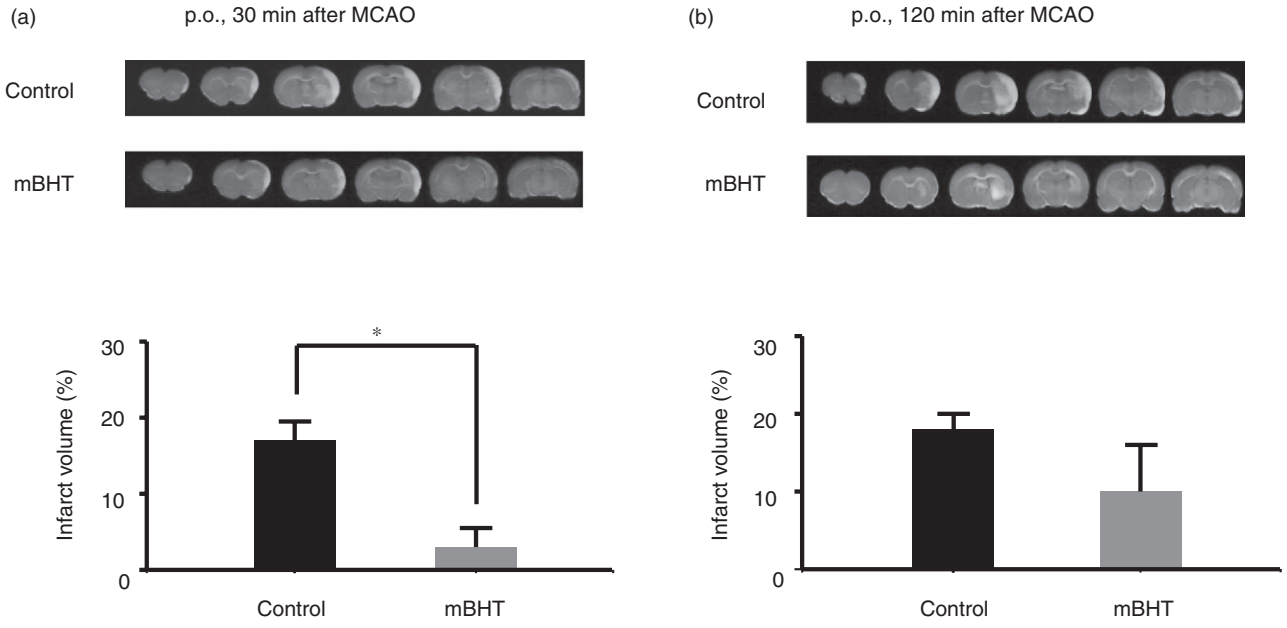


**Figure 2** Reduced infarction by oral administration of modified Bo-Yang-Hwan-O-Tang (mBHT) to rats. mBHT (200 mg/kg) was administered orally to rats 30 min (a) or 120 min (b) after middle cerebral artery occlusion (MCAO) onset. Infarct volumes were calculated 24 h after surgery. $n = 10$. *$P < 0.05$ compared with control group. In (b), mBHT reduced infarct volume, but statistical significance was not obtained ($P = 0.0508$).

before MCAO and 15 min after reperfusion. Intraperitoneal administration of mBHT itself did not change physiological variables in rats (Table 2).

mBHT dose-dependently reduced cerebral ischaemic injury caused by 90 min MCAO/22 h reperfusion. Thus, mBHT reduced cerebral infarct volumes by 89.4% and 60.5% at 200 and 50 mg/kg, respectively (Figure 1a and 1b). Along with the reduced infarct volumes, the sizes of oedema were also reduced by mBHT (data not shown). Ischaemia-induced spontaneous circling behaviour was used to evaluate neurological status after MCAO.[36,37] Neurological deficits were measured twice at 2.5 h and 24 h after starting MCAO. mBHT significantly improved neurological deficit scores after MCAO/reperfusion (Figure 1c). Furthermore, as determined up to 15 days after surgery, the long-term survival rate of mBHT-treated rats was markedly improved compared with that of vehicle-treated or MK-801-treated rats (Figure 1d). Our studies also showed that oral administration of 200 or 500 mg/kg mBHT at 30 min after the onset of MCAO markedly reduced the cerebral infarction (Figure 2, data not shown for 500 mg/kg).

Hypothermia has been well-known to reduce the infarct and recognized as a therapeutic strategy for the clinical treatment of cerebral ischaemic stroke.[38–40] We found that mBHT reduced the rectal temperature, which well reflects body core temperature, in a dose-dependent manner (Figure 3a). Thus, we further examined whether the hypothermic properties of mBHT would contribute to the reduction of infarct. When the core body temperature was maintained at normal level using a thermo-regulating cage with a heating pad and heating lamps, the percentage inhibition of mBHT for cerebral infarct size was significantly, but not completely inhibited (47.2% inhibition in Figure 3b, when compared with 89.4% in Figure 1b), suggesting that the anti-ischaemic effect of mBHT is partly but significantly mediated through its hypothermic properties. Consistently, the neurological deficit scores also showed similar reduction by mBHT (Figure 3c).

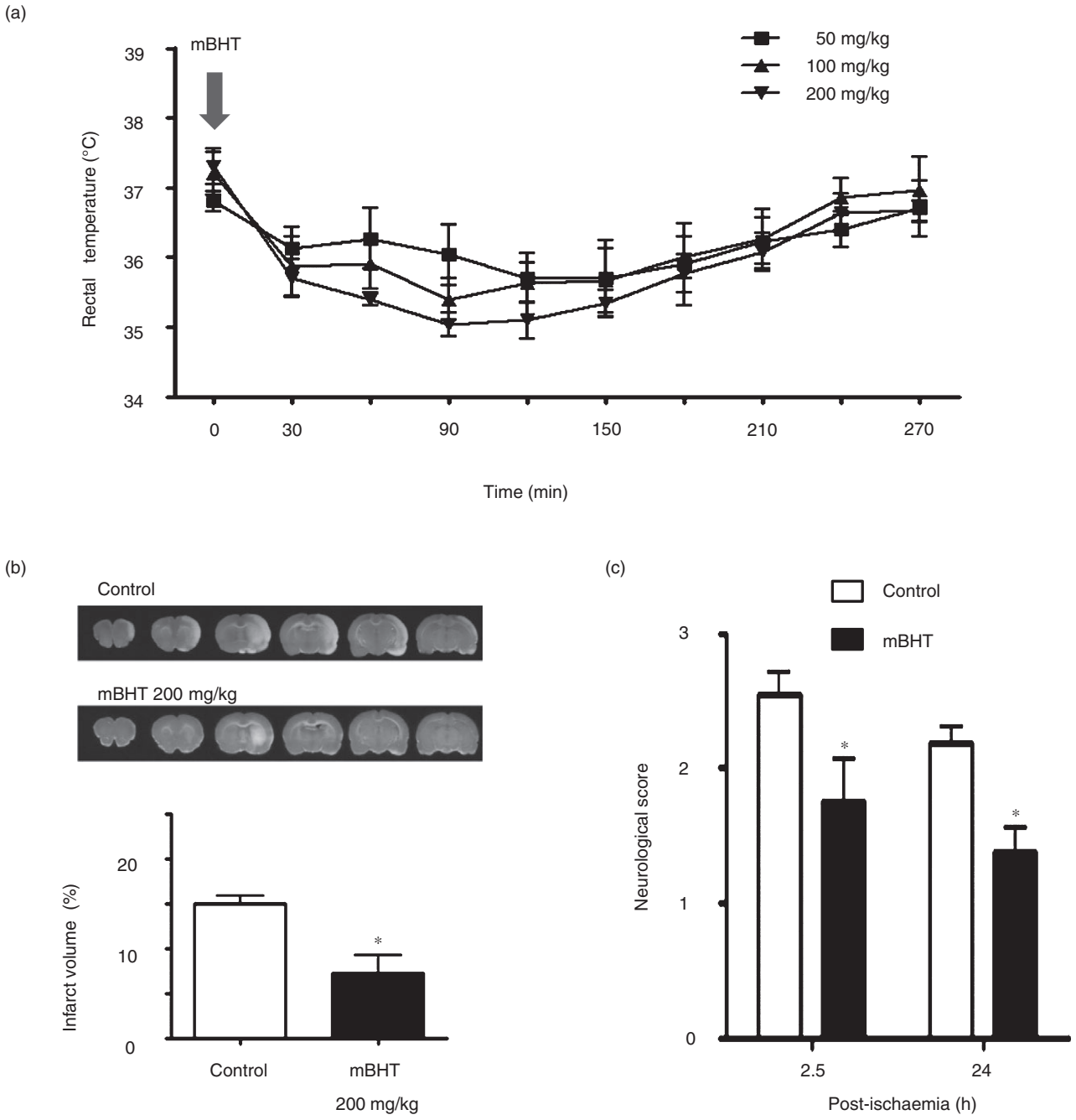


**Figure 3** Dose-dependent effect of modified Bo-Yang-Hwan-O-Tang (mBHT) on rectal temperature of rats. (a) Rats were intraperitoneally administered with 50, 100 or 200 mg/kg of mBHT. The rectal temperature was determined every 30 min using a temperature controller system for 270 min after mBHT treatment. $n = 3$. (b) Effect of mBHT on cerebral infarction under controlled body temperature. Infarct volumes were determined 24 h after surgery in rats whose rectal temperature was maintained within normal range. $n = 8$. (c) Neurological deficit scores in vehicle-treated or mBHT (200 mg/kg)-treated rats were determined 2.5 and 24 h after surgery. $n = 8$. $^*P < 0.05$ compared with control group.

We further examined the cytoprotective effect of mBHT in in-vitro ischaemic models. OGD/re-oxygenation caused morphological deterioration (Figure 4a) and increased the propidium iodide permeability and LDH release (Figure 4a–4c). mBHT markedly reduced the OGD/re-oxygenation-evoked death of neuronal cells. In general, neuronal cell death is caused by OGD/re-oxygenation via NMDA-receptor-mediated excitotoxicity and oxidative stress. We found that mBHT did not suppress NMDA receptor-mediated neuronal cell death (Figure 4d). The intracellular reactive oxygen species (ROS) level was highly increased by OGD/re-oxygenation, and was significantly reduced by mBHT (Figure 5). The mitochondria are very vulnerable to oxidative stress. As expected from its antioxidant activity against ROS,

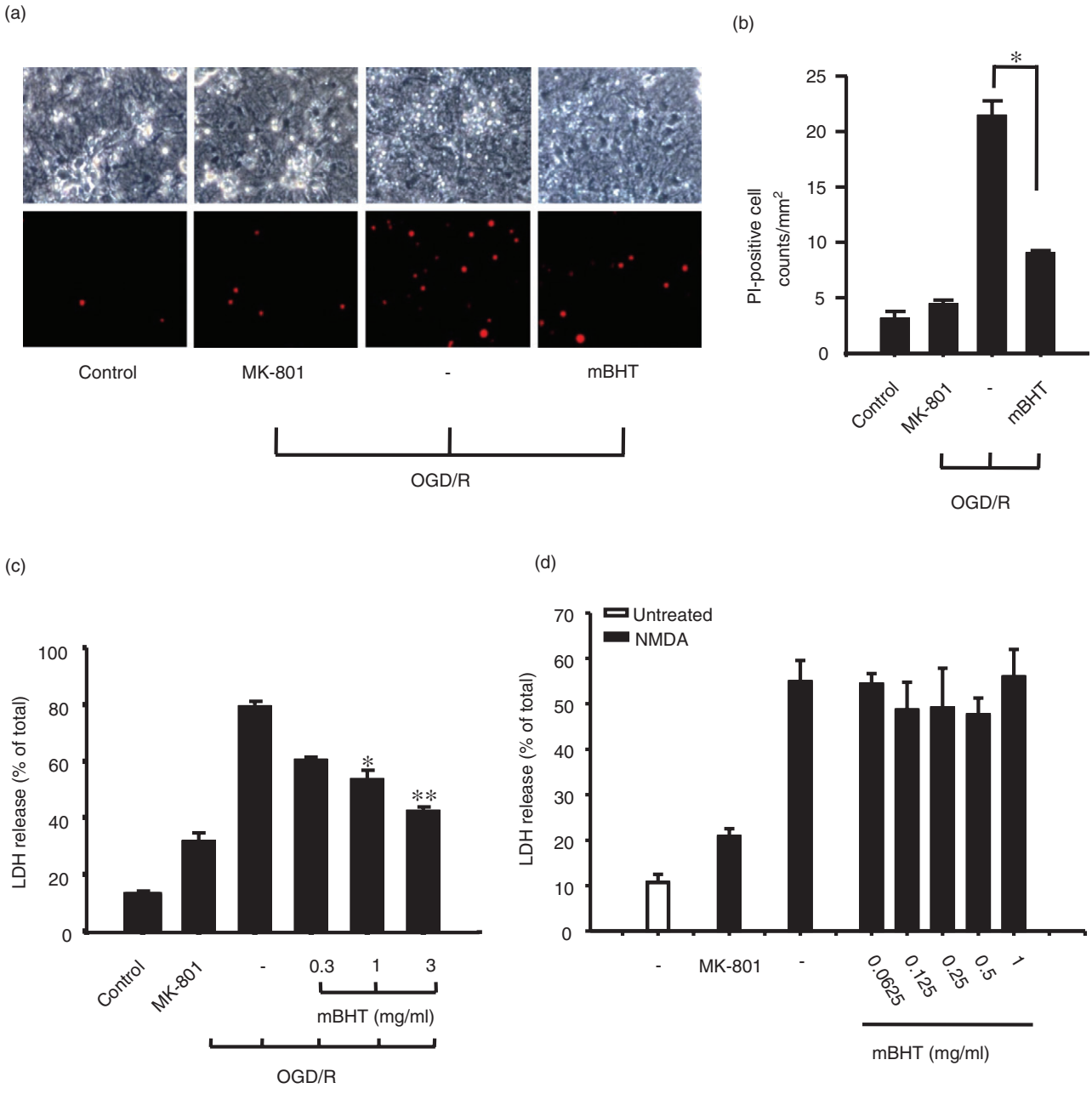


**Figure 4** Modified Bo-Yang-Hwan-O-Tang (mBHT) reduces oxygen-glucose deprivation (OGD)/re-oxygenation-induced cortical neuronal death. (a, b) Rat cortical neuronal cells were exposed to 1-h OGD and 5-h re-oxygenation in the absence or presence of either mBHT (3 mg/ml) or MK-801 (10 μM). Cell injury or death was assessed by propidium iodide (PI) permeability (a) and by counting the PI-positive cells in square millimeters (b). (c) Cortical neurons were treated with mBHT (0.3, 1, or 3 mg/ml) or MK-801 (10 μM), followed by 1-h OGD and 5-h re-oxygenation. Cell death was further quantified by LDH release. Data are expressed as the percentage of total LDH and represent the mean ± SD. (d) Cortical neurons treated with mBHT (0.0625, 0.125, 0.25, 0.5 or 1 mg/ml) or MK-801 (10 μM) were subsequently exposed to NMDA (100 μM) for 10 min and LDH release was determined 5 h later. $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.001$, compared with the untreated group.

mBHT significantly alleviated the depolarization of OGD/re-oxygenation-evoked decrease of mitochondrial transmembrane potential (Figure 6), possibly leading to decreased neuronal cell death.[41]

In general, apoptosis cannot be simply identified by morphological changes. Caspases are key mediators of cell death and, especially, caspase-3 has been recognized as an executioner for the death program in cortical neurons in response to various noxious insults.[42,43] Using a fluorogenic peptide substrate (Ac-DEVD-AMC for caspase-3), we observed that mBHT decreased the activity of caspase-3, which was enhanced by OGD/re-oxygenation (Figure 7a). Western blot analysis also showed that the amount of cleaved fragments of caspase-3 (a 17-kDa band) was increased by OGD/

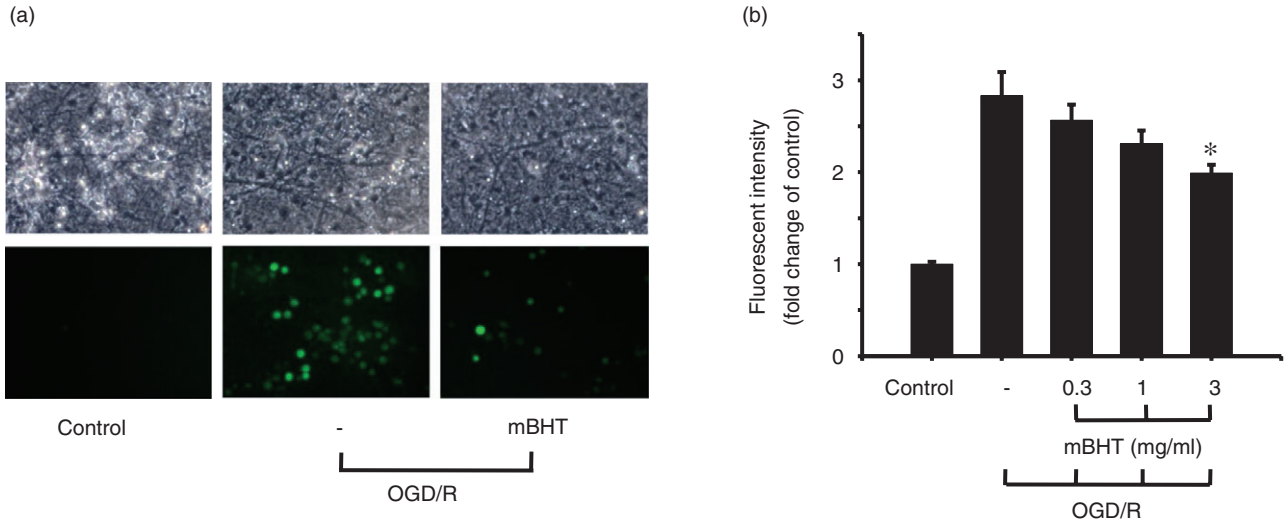


**Figure 5** Modified Bo-Yang-Hwan-O-Tang (mBHT) reduces intracellular reactive oxygen species (ROS) level. (a) Rat cortical neuronal cells exposure to 1-h oxygen-glucose deprivation (OGD) and 3-h re-oxygenation in the absence or presence of mBHT (3 mg/ml). Intracellular levels of free radicals were measured by using DCF-DA. (b) Fluorescence intensities in each experimental condition. Data are expressed as mean ± SD. $n = 3$. $^*P < 0.05$, compared with the fluorescence intensities shown in control group.

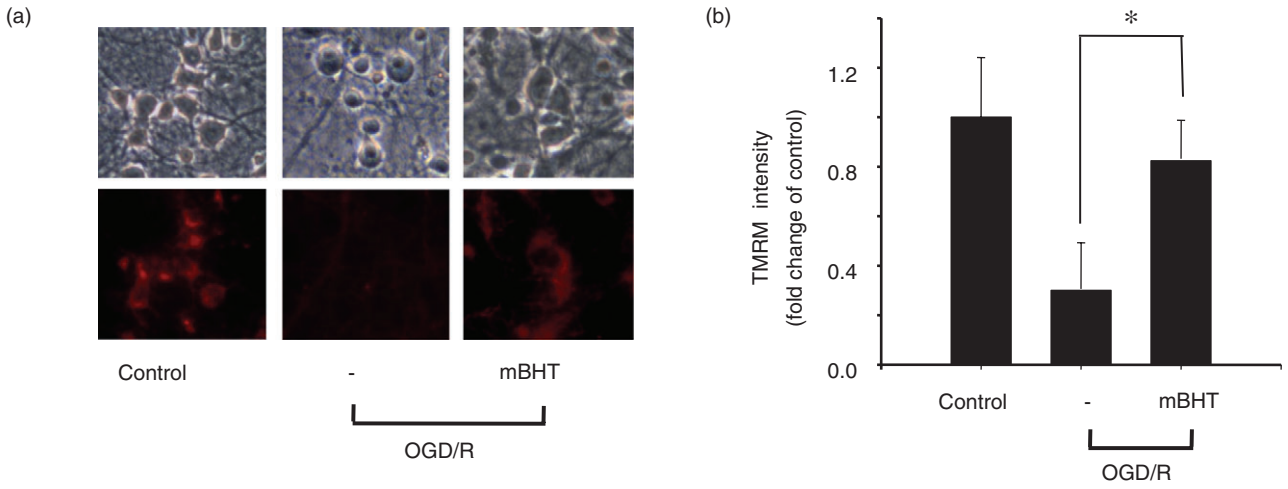


**Figure 6** Inhibition of mitochondrial transmembrane depolarization by mBHT. Rat cortical neurons were subject to 3 h oxygen-glucose deprivation (OGD)/re-oxygenation in the presence or absence of mBHT (3 mg/ml). Data are expressed as mean ± SD. $n = 3$. $^*P < 0.05$, compared with the untreated group.

re-oxygenation, which was reversed by mBHT (Figure 7b). $\alpha$-Spectrin is cleaved by caspase-3 and this event has been regarded as a common and physiologically significant step in the execution phase of apoptosis.[44] mBHT decreased the cleavage of $\alpha$-spectrin, which was increased by OGD/re-oxygenation (Figure 7b).

## Discussion

In this study, we found that mBHT markedly reduced ischaemic injury in in-vivo as well as in-vitro ischaemic models. Furthermore, mBHT markedly improved long-term survival rate in ischaemic rats (Figure 1d), even compared with a relative high dose (10 mg/kg) of MK-801, which caused completely blockage of NMDA-mediated excitotoxicity and maximized neuroprotection.[45] The anti-ischaemic effect of mBHT was observed even at 50 mg/kg, which is a clinically relevant dose in humans, all suggesting the high potential of mBHT as an anti-ischaemic drug.

Many different cell death mechanisms are closely involved in ischaemia/reperfusion-provoked brain injury. mBHT is composed of various herbal constituents, each of which has been shown to have anti-thrombotic,[15] anti-inflammatory,[16] antioxidant[19,20] or anti-excitotoxic activity.[22] This multitude of protective actions may explain why the brain protection by mBHT is so effective and prolonged, compared with that afforded by a single drug or herb therapy.

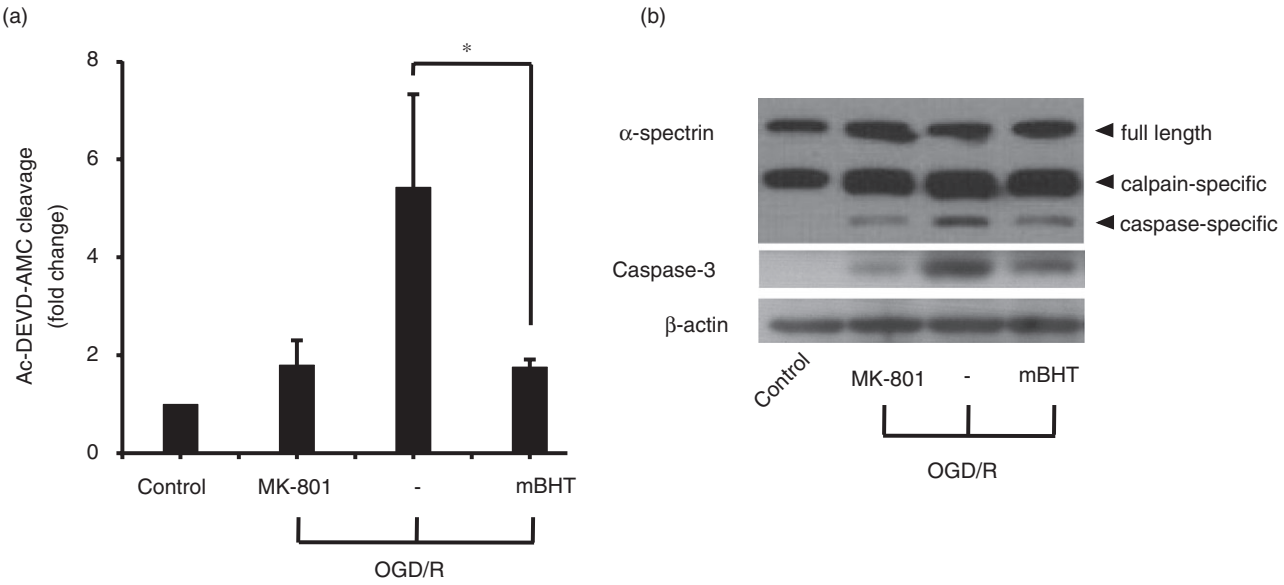


**Figure 7** Inhibition of caspase-3 activity by modified Bo-Yang-Hwan-O-Tang (mBHT). (a) Rat cortical neuronal cells were exposed to 1-h oxygen-glucose deprivation (OGD) and 5-h re-oxygenation in the absence or presence of mBHT (3 mg/ml) or MK-801 (10 μM), Data are expressed as mean ± SEM from three separate experiments. $*P < 0.05$. (b) Western blot. Data are representative of at least three independent experiments.

In general, body temperature increases during ischaemic insult, especially after starting reperfusion. Post-ischaemic hypothermia has been shown to provide prolonged brain protection against cerebral ischaemia in adult rats and gerbils.[46–48] In our experimental conditions, rectal temperature increased 1–1.5°C during ischaemia and reperfusion. However, mBHT treatment reduced the rectal temperature by 2.5–4.0°C, consequently 1.5–3.0°C lower than normal rectal temperature. Previously, this mild hypothermia was shown to protect cerebral ischaemic injury.[38,49] The potential mechanisms underlying the protective actions of mild hypothermia were reviewed by Colbourne *et al*.[50] The underlying mechanisms include a reduction in metabolic rate, a rapid normalization of pH and metabolism, an amelioration of post-ischaemic hypoperfusion, reduced free radicals, reversal of second messenger alterations and reduced glutamate release. These multiple cytoprotective actions may render hypothermia so effective for the treatment of cerebral ischaemia. However, although hypothermia is undoubtedly associated with brain protection by mBHT, our experimental results further indicate that hypothermia is not the only reason for the anti-ischaemic effect. Thus, even when the rectal temperature was maintained within normal range (ca. 37.5°C), mBHT still significantly (but to a lesser extent), reduced cerebral infarct volume.

The mechanisms responsible for the neuronal cell death caused by OGD/reoxygenation are mainly excitotoxicity and radical burst.[51] Our data clearly showed that mBHT did not inhibit NMDA receptor-mediated excitotoxicity (Figure 4d). Thus, the brain protection by mBHT could be at least in part attributed to its antioxidant capacity. The level of ROS in normal cells is tightly regulated by biological antioxidants and antioxidant enzymes.[52] In pathological conditions, the generation of oxidants exceeds the intracellular antioxidant capacity, resulting in oxidative damage to proteins, lipids and DNA. Thus, ROS has been recognized as a key mediator associated with neurodegeneration induced by a variety of insults including ischaemia.[53] In this study, mBHT reduced intracellular ROS level, as assessed by measuring oxidation of DCF during MCAO/reperfusion (Figure 5), thus significantly alleviating the depolarization of mitochondrial transmembrane potential (Figure 6). In our separate paper (Park *et al*., in submission), we delineated the antioxidant activity of mBHT. mBHT possesses a wide range of radical scavenging activity, as assessed by Trolox equivalent antioxidant capacity, oxygen radical absorbance capacity and 1,1-diphenyl-2-picrylhydrazyl radical assays. Furthermore, we also found that mBHT almost completely restored the activity of various antioxidant enzymes, such as superoxide dismutase, catalase and glutathione peroxidase, which had been decreased during ischaemia/reperfusion. Consequently, mBHT increased the level of reduced glutathione in brain tissues, which is one of key reductants for cellular redox homoeostasis.

By HPLC fingerprinting analysis, we previously identified some components of mBHT, including paeoniflorin, cinnamic acid, decursin, calycosin-7-O-glucoside, salvianolic acid B and others.[25] Although bioactive components of mBHT were not fully identified in the present study, the phytochemical screening of mBHT has demonstrated the presence of flavonoids, steroids, triterpenoids and polyphenols, which may act synergistically to exert the anti-ischaemic activity. Previously, many chemicals obtained from each mBHT herbal ingredient have been demonstrated to possess neuroprotective, antioxidant or anti-inflammatory actions. In our preliminary experiments, we also found that some components of mBHT have anti-ischaemic activity: the half maximal inhibitory concentrations (IC50) of some mBHT components against OGD/re-oxygenation toxicity are shown in Table 3. Considering our findings together with other researchers'

**Table 3** Pharmacological activity of modified Bo-Yang-Hwan-O-Tang components

| Botanical name | Major components | IC50 (μM) | Published activity | References |
|---|---|---|---|---|
| *Astragalus membranaceus* Bunge | Daucosterol | ~5 | NP/AO | [54] |
| | Formononetin | – | NP/AI | [55,56] |
| | Astragaloside IV | – | N.A | |
| | Isomucronulatol 7-*O*-glucoside | – | N.A. | |
| | (+)-Pinitol | – | N.A | |
| *Angelica gigas* Nakai | Decursin | ~10 | NP | [57] |
| | Decursinol | ~10 | N.A. | |
| | Nodakenin | – | NP | [58] |
| *Paeonia lactiflora* Pallas | Paeoniflorin | ~10 | NP/AI/AO | [59,60] |
| | Paeonol | ~10 | NP/AI | [61,62] |
| | Benzoylpaeoniflorin | – | NP/AO | [61] |
| | Albiflorin | – | N.A. | |
| *Cnidium officinale* Makino | Senkyunolide A | – | AI | [63] |
| | Z-Ligustilide | – | NP/AI | [64] |
| | Chlorogenic acid | – | N.A. | |
| | Pregnenolone | – | N.A. | |
| *Pheretima aspergillum* (E. Perrier) | Lumbritin | – | N.A. | |
| | Terrestrolumbrilysin | – | N.A. | |
| | Lumbrofebrin | – | N.A. | |
| *Prunus persica* (L.) Batsch | Amygdalin | – | AI | [65] |
| *Carthamus tinctorius* L. | Carthamin | – | NP/AO | [66] |
| | Safflower yellow | – | NP/AO | [66] |
| *Achyranthes bidentata* Bl. | Triterpenoid saponin | – | NP/AI | [67,68] |
| | Oleanol acid | ~5 | N.A. | |
| *Salvia miltiorrhiza* Bge. | Cryptotanshinone | – | NP | [69] |
| | Tanshinone I | – | N.A. | |
| | Tanshinone IIA | – | NP/AO | [70,71] |
| | Salvianolic acid B | – | NP | [72] |
| | Rosmarinic acid | – | NP | [73] |
| | 15,16-Dihydrotanshinone I | – | N.A. | |
| *Cinnamomum cassia* Blume | Cinnamaldehyde | – | N.A. | |
| | 2-Methoxycinnamaldehyde | – | N.A. | |
| | 2-Hydroxycinnamaldehyde | – | N.A. | |
| | Cinnamic acid | ~10 | NP/AO | [74] |
| | Coumarin | – | NP | [58] |
| | Eugenol | ~10 | NP | [75] |
| *Polygala tenuifolia* Willd. | Onjisaponin | – | N.A. | |
| | Prosapogenins | – | N.A. | |
| | Tenuidine | – | N.A. | |
| | Polygalitol | – | N.A. | |
| *Acorus gramineus* Solander | $\alpha$-Asarone | – | NP | [76,77] |
| | $\beta$-Asarone | – | N.A. | |

IC50, half maximal inhibitory concentrations against oxygen-glucose deprivation (OGD)/re-oxygenation toxicity; AI, anti-inflammation; AO, anti-oxidant; N.A., data not available; NP, neuroprotection. Cells were exposed to 1-h OGD and 5-h re-oxygenation in the absence or presence of modified Bo-Yang-Hwan-O-Tang and then cell death was further quantified by lactate dehydrogenase release.

results, we conclude that the anti-ischaemic activity of mBHT may result from the synergistic interactions of the active compounds.

## Conclusions

Taken together, mBHT exerts its anti-ischaemic effect at least in part due to its hypothermic and antioxidant activity. However, before clinical assessment for the treatment of cerebral ischaemic injury, further studies should be carried out to avoid adverse effects that can be induced by hypothermia. Although we did not study anti-inflammatory activity in this study, some constituents of mBHT are known to suppress inflammatory responses. Thus, more studies on interrelated cytoprotective mechanisms of mBHT may provide a good therapeutic prescription of herbal constituents for the treatment of ischaemia/reperfusion-associated brain injury.

## Declarations

### Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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