Antrodia camphorata Potentiates Neuroprotection against Cerebral Ischemia in Rats via Downregulation of iNOS/HO-1/Bax and Activated Caspase-3 and Inhibition of Hydroxyl Radical Formation

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Antrodia camphorata (A. camphorata) is a fungus generally used in Chinese folk medicine for treatment of viral hepatitis and cancer. Our previous study found A. camphorata has neuroprotective properties and could reduce stroke injury in cerebral ischemia animal models. In this study, we sought to investigate the molecular mechanisms of neuroprotective effects of A. camphorata in middle cerebral artery occlusion (MCAO) rats. A selective occlusion of the middle cerebral artery (MCA) with whole blood clots was used to induce ischemic stroke in rats and they were orally treated with A. camphorata (0.25 and 0.75 g/kg/day) alone or combined with aspirin (5 mg/kg/day). To provide insight into the functions of A. camphorata mediated neuroprotection, the expression of Bax, inducible nitric oxide synthase (iNOS), haem oxygenase-1 (HO-1), and activated caspase-3 was determined by Western blot assay. Treatment of aspirin alone significantly reduced the expressions of HO-1 (P < 0.001), iNOS (P < 0.001), and Bax (P < 0.01) in ischemic regions. The reduction of these expressions was more potentiated when rats treated by aspirin combined with A. camphorata (0.75 g/kg/day). Combination treatment also reduced apoptosis as measured by a significant reduction in active caspase-3 expression in the ischemic brain compared to MCAO group (P < 0.01). Moreover, treatment of A. camphorata significantly (P < 0.05) reduced fenton reaction-induced hydroxyl radical (OH-) formation at a dose of 40 mg/mL. Taken together, A. camphorata has shown neuroprotective effects in embolic rats, and the molecular mechanisms may correlate with the downregulation of Bax, iNOS, HO-1, and activated caspase-3 and the inhibition of OH- signals.

1. Introduction

Stroke denotes to a rapid worldwide neurological impairment that victims may grieve paralysis and speech disorder, as well as loss of cognizance due to either ischemia or hemorrhage. It is considered as one of the leading causes of death and disability worldwide [1]. Currently, intravascular techniques and thrombolytic agents have remarkably decreased functional deficits. Although there are good improvements established in treatment, there is still little that can be done to prevent stroke-related brain damage. Therefore, active prevention and control of stroke are of great clinical value. Aspirin is the most widely used drug for the prevention of secondary stroke. However, the incidence of cerebral haemorrhage and other
bleeding events are major issues, while recurrent stroke is controlled by this treatment [2]. Thus, research has been focused on finding alternative drugs that may act on different pathways that have been used to recover them from the group of inflammation, necrosis, and apoptosis, all of which are associated in ischemic stroke [3]. Natural products are a prolific source of bioactive agents of different structure and varying biological activities. In the search for neuroprotective agents from natural sources, a number of plant extracts and several natural products isolated from them have been reported to provide neuroprotection against ischemic stroke [4].

*Antrodia camphorata* is being used as the complementary and alternative medicines, and it grows only on the inner heartwood wall of the endangered species *Cinnamomum kanehira* Hay (Lauraceae) [5–7]. *A. camphorata* has long been used in Taiwanese folk medicine for abdominal pain, chemical intoxication, diarrhea, hypertension, itchy skin, and hepatitis [8]. Studies have demonstrated that *A. camphorata* induces significant apoptosis of human promyelocytic leukemia (HL-60) cells [9] and its extracts may be used as an adjuvant antitumor agent for human hepatoma cells, which are resistant to most other antitumor agents. Our previous study had showed that *A. camphorata* possesses antioxidant effects against carbon tetrachloride-(CCL4) induced hepatic injury in vivo, via mediating free radical scavenging activities [10]. *A. camphorata* also has shown to reduce H2O2-induced lipid peroxidation and enhance hepatic glutathione-dependent enzymes upon protecting CCL4-induced damage on rat liver [11]. Despite the fact that our very recent study has demonstrated that *A. camphorata* has neuroprotective effect against ischemic stroke in rats through reducing infarct volume and improves neurobehavioral scores and regulating blood perfusion without increasing hemorrhagic transformation [12], the molecular mechanism of action of *A. camphorata* in this effect is remained obscured. Thus, in this study, we investigated the effects and possible mechanisms of action of *A. camphorata* on ischemic stroke in rats.

2. Materials and Methods

2.1 Plant Material. Well Shine Biotechnology Development Co., Pvt. Ltd., Taipei, Taiwan, provided the extracts of *A. camphorata* for this study.

2.2 Animals. Male Wistar rats (250–300 g) were used to determine the effects of *A. camphorata* alone or in combination with aspirin against MCAO induced brain damage. Animal care and the general protocols for animal use were approved by the Institutional Animal Care and Use Committee (IACUC) of Taipei Medical University. All animals were clinically normal, free of apparent infection or inflammation, and showed no neurological deficits while they were checked before undergoing the experimental procedures.

2.3 MCAO-Induced Ischemia. As demonstrated in our previous studies, an autologous blood clot was administered in rats for MCAO-induced ischemia [13–15]. In brief, 0.6 mL of arterial blood was withdrawn from a femoral catheter by using 1-mL syringe and the blood was immediately injected into PE-10 tubes. The tubes were kept at 4°C for 22 h, and the thread-like clots were removed and placed in a saline-filled dish. The clots were then washed to remove blood cells. Washed clots were transferred to fresh dishes, and the washing process was continued until the saline remained clear. The cleared clot sections were cut into 30 mm long fragments and then drawn up with the saline solution into a PE-10 catheter.

At the time of surgical procedure, animals were anesthetized with a mixture of 75% air and 25% O2 gases containing 3% isoflurane. The common carotid artery (CCA) was identified, and approximately 1 cm of the external carotid artery (ECA) was ligated and cut. Consequently, the pterygopalatine artery (PA) was clamped with a 10 mm microaneurysm clamp, and the CCA was similarly clamped before the carotid bifurcation. The internal carotid artery (ICA) was then clamped between the carotid bifurcation and the PA. After that, the PE-50 catheter containing the clot was introduced approximately 5 mm into the previously cut ECA and tied in place with sutures. The ICA clamp was removed, and the clot was flushed into the ICA over a period of approximately 5 s. The PA clamp was removed, and the rat was left in this condition for 1 h.

2.4 Experimental Procedure. Rats were randomly separated into six groups at 1 hr after MCA occlusion: (1) a sham-operated group; (2) a group orally treated with an isovolumetric solvent (distilled water) for 60 days, followed by thromboembolic occlusion; (3) and (4) groups orally treated with *A. camphorata* (0.25 and 0.75 g/kg/day) alone for 60 days, followed by thromboembolic occlusion, respectively; (5) and (6) groups treated with *A. camphorata* (0.25 and 0.75 g/kg/day) and aspirin (5 mg/kg/day), followed by thromboembolic occlusion, respectively. An observer blinded to the identity of the groups assessed the neurological deficits after reperfusion by forelimb akinesia test.

2.5 Immunoblotting Assay. Expressions of HO-1, iNOS, Bax, and active caspase-3 in the ischemic brain at 24 h after thromboembolic occlusion-reperfusion injury were analyzed by immunoblotting as described by our previous study [14]. Thromboembolic occlusion-insulted and sham-operated rats were anesthetized with chloral hydrate (400 mg/kg, i.p.), and then the apex of the heart was penetrated with a profusion cannula inserted through the left ventricle into the ascending aorta. Perfusion with ice-cold PBS was performed, and an incision was made in the right atrium for venous drainage. Brains were freshly removed and sectioned coronally into four sequential parts from the frontal lobe to the occipital lobe. The third of four parts of the right hemisphere was separately collected, snap-frozen in liquid nitrogen, and stored at −70°C. The frozen tissues were placed in homogenate buffer and homogenized and then sonicated for 10 s three times at 4°C. The sonicated samples were subjected to centrifugation (10,000 × g).
The supernatant (50 μg protein) was subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (0.45 μm, Hybond-P, Amersham). After incubation in blocking buffer and being washed three times with TBST buffer (10 mM Tris-base, 100 mM NaCl, and 0.1% Tween 20; pH 7.5), blots were treated with an anti-HO-1 polyclonal antibody (pAb, 1:1000; R&D, Minneapolis, MN), an anti-iNOS monoclonal antibody (mAb; 1:3000, BD Biosciences, San Jose, CA), an anti-Bax pAb (1:1000; Cell Signaling, Beverly, MA), and an anti-active caspase-3 pAb (1:250; Biovision, Mountain View, CA), or an anti-α-tubulin mAb (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA) in TBST buffer overnight. Blots were subsequently washed with TBST and incubated with a secondary horseradish peroxidase- (HRP-) conjugated goat anti-mouse mAb or donkey anti-rabbit immunoglobulin G (IgG)(Amersham) for 1 h. Blots were then washed, and the immunoreactive protein was detected using film exposed to enhanced chemiluminescence (ECL) detection reagents (ECL™ system; Amersham). The bar graph depicts the ratios of semiquantitative results obtained by scanning reactive bands and quantifying the optical density using video densitometry (Bio-1D vers. 99 image software).

2.6. Measurement of Hydroxyl Radical (HO•) Formation by Electron Spin Resonance (ESR) Spectrometry. The ESR method used a Bruker EMX ESR spectrometer (Billerica, MA, USA) as described previously [16]. In brief, a Fenton reaction solution (50 μM FeSO₄ + 2 mM H₂O₂) was pretreated with a solvent control (0.1% DMSO) or Antrodia camphorate (20 and 40 mg/mL) for 10 min. The rate of hydroxyl radical-scavenging activity was defined by the following equation: inhibition rate = 1 – [signal height (A. camphorata)/signal height (solvent control)].

2.7. Data Analysis. Experimental results are expressed as the mean ± S.E.M. and are accompanied by the number of observations. The experiments were assessed by the method of analysis of variance (ANOVA). If this analysis indicated significant differences among the group means, then each group was compared using the Newman-Keuls method. A P value of <0.05 was considered statistically significant.

3. Results

3.1. A. camphorata Inhibits iNOS and HO-1 Expression in Thromboembolic Cerebral Tissues. To examine the effect of A. camphorata in the ischemic brain, we measured the expression of iNOS and HO-1 in thromboembolic occlusion-insulted cerebral tissues. As shown in Figure 1, iNOS was more evidenced in tissues of thromboembolic occlusion-reperfusion injury than the level obtained in the corresponding area of the sham-operated group. Treatment of A. camphorata and aspirin alone at a respective doses of 0.75 g/kg and 5 mg/kg significantly (P < 0.001) diminished iNOS expression compared to the MCAO-untreated rats. Moreover, a combined treatment of A. camphorata with aspirin apparently potentiated A. camphorata mediated suppression of iNOS expression.

A study has revealed that HO-1 is a key player for drugs upon neuroprotection in transient MCAO model [17]. In this study, Western blot was done to investigate whether A. camphorata affects the level of HO-1 expression. The results showed that A. camphorata and aspirin alone significantly (P < 0.001) reduced the expression of HO-1 protein in brain tissues of MCAO-induced rats (Figure 2). However, this protein expression was not changed when A. camphorata was treated with aspirin, since HO-1 expression seemed quite similar as appeared in their individual treatment.

3.2. A. camphorata Reduces Aspirin-Mediated Suppression of Bax-1 and Active Caspase-3 Expressions in Thromboembolic Cerebral Tissues. Bax is the proapoptotic member and caspase-3 is the most abundant cysteine protease in the brain and is acutely cleaved and activated in neurons in the early stages of reperfusion, leading to cell apoptosis. In this study, the expression levels of these apoptotic proteins, which are considered as the most important determining factors for the fate of cell and tissues in response to apoptotic stimulations were determined. We found a significant increase in the
3.3. A. camphorata Reduces In Vitro OH$^\cdot$ Formation. To determine the efficacy of A. camphorata upon inhibiting fenton reaction-induced OH$^\cdot$ formation in vitro, a cell-permeative ROS-sensitive dye, DCFDA (nonfluorescent in a reduced state but fluorescent upon oxidation by ROS) was used [16]. In this study, we found that OH$^\cdot$ was produced during the fenton reaction very obviously. Interestingly, treatment with A. camphorata (40 mg/mL) markedly inhibited the fenton reaction induced OH$^\cdot$ (Figure 4); however no effects were observed when A. camphorata is treated at a concentration of 20 mg/mL.

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Figure 2: Effects of the extracts of A. camphorata combined with aspirin on the expressions of HO-1 in cerebral homogenates 24 h after thromboembolic stroke in rats. Data are presented as the mean ± S.E.M. ***P < 0.001, compared to the sham-operated group, and ###P < 0.001, compared to the MCAO group.

4. Discussion

Our recent study has demonstrated that A. camphorata shows neuroprotective effect against ischemic insults in MCAO model through a mechanism of blood perfusion regulation without increasing hemorrhagic transformation. This treatment also reduced infarct volume in the focal ischemic brain injury and improves neurological outcomes. In this study, we investigated the possible molecular mechanisms of A. camphorata on the observed neuroprotective effect. The results were found that an extract of A. camphorata possesses neuroprotective effect via antiapoptotic and anti-inflammatory effects and reduces OH radical formation in rat thromboembolic stroke.

Recently, researchers have been attracted to notice the hypothesis that secondary brain damages from hemoglobin as well as its byproducts such as ferrous iron released after heme degradation [18]. Heme or hemin released from hemoglobin accumulates in intracerebral hemorrhage (ICH) [19] and the increased hemin induces HO-1, the rate-limiting enzyme in the oxidative degradation of free heme [20]. High levels of heme metabolites such as ferrous iron resulted in neuronal cell death. Although HO-1 serves a cytoprotective function [21], reports of protective effects of HO-1 inhibitors in experimental ICH models support the idea that HO-1 is a mediator of neurotoxicity in ICH [22, 23] and an attractive therapeutic target for ICH.

In this study, we found that A. camphorata exerted neuroprotective effects by reducing the MCAO-induced expression of HO-1. As reported by Chen et al. [24], the induction of HO-1 has been correlated with an experimental model of MCAO and HO-1 knockout mice are reported to be protected from brain injury and functional impairment by ICH [25]. Our results showed that reduced expression of HO-1 by A. camphorata protects the MCAO-induced ischemic brain injury. Several reports proposed that a decrease of HO-1 expression by HO-1 inhibitor may provide a protective effect against stroke in various animal models [26, 27]. Recently, Huang et al. reported that treatment of vitamin C offers neuroprotection via reducing HO-1 activity in methamphetamine-induced neurotoxicity in neuronal cells [28]. Combined with the current data, these reports suggest that modulation of HO-1 might have a potential as a new therapy for stroke.

A study demonstrated that iNOS knock-out mice showing reduced brain damage after ischemia, because of an increased expression of iNOS, may also contribute to enhanced neuronal injury [29] and there is an evidence that iNOS plays a role as a mediator in the reduction of infarct size via late preconditioning [30]. A recent study also suggests that iNOS may be involved in the inflammatory reaction that follows cerebral ischemia and iNOS mRNA and enzymatic activity are expressed in brain after permanent MCA occlusion [31]. Treatment with the selective iNOS inhibitor was reported to be reduced infarct volume, suggesting that iNOS activity contributes to ischemic brain damage [32]. A study reported that bioactive constituents of mycelium of A. camphorata, antroquinonol B, 4-acetyl-antroquinonol B, 2,3-(methylenedioxy)-6-methylbenzene-1,4-diol, and 2,4-dimethoxy-6-methylbenzene-1,3-diol along with antrodin D inhibit iNOS activity in lipopolysaccharide- (LPS-) activated murine macrophages [33]. In the present study, we demonstrated that treatment of A. camphorata in MCAO-induced embolic rats significantly reduced the expression of iNOS, is harmful to the postischemic brain, and may be of worth in the treatment of cerebral ischemia.
Apoptosis is also known as programmed cell death, which is an initiative suicide process after the cells receive a signal or stimulation with some other related gene. The Bcl-2 family proteins are key regulators of apoptosis, which include both antiapoptotic members such as Bcl-2 and the proapoptotic members such as Bax. It has been suggested that a slight change in the dynamic balance of Bcl2/Bax proteins may result either in inhibition or promotion of cell death [34]. Apoptosis has been reported to occur after transient cerebral ischemia and is regulated by the pro- and antiapoptotic proteins and it contributes to ischemic cell damage after stroke [35]. Caspase-3 is an essential protein for brain development, but it also serves as a crucial mediator of neuronal apoptosis [36]. During ischemia, caspase-3 is cleaved and activated whereupon it degrades multiple substrates in the cytoplasm and nucleus leading to cell death [37]. Caspase-3 deficient adult mice reported to be more resistant to ischemic stress both in vivo and in vitro [37]. Therefore, it is of great interest to control the activation of Bax and caspase-3 for the potential therapeutic treatment of neurological diseases. Several studies have demonstrated that treatment of caspase-3 inhibitors reduced ischemic-induced brain damage [38]. A recent study has suggested that inhibition of Bcl2/Bax ratio may be a novel target for the treatment of stroke [39], and these authors have shown that chemokine-like factor 1 (CKLF1), a novel C-C chemokine, with antibodies displays neuroprotective effects against cerebral ischemia via regulation of apoptosis-related protein expression in ischemic hemisphere. In the present study, it has been shown that A. camphorata has neuroprotective effects in MCAO-induced rats via inhibiting Bax and caspase-3 expressions.

Oxidative stress involves the formation of reactive oxygen/nitrogen species (ROS/RNS), which are causal factors in the neuropathology of stroke [40]. Abundant ROS are generated during an acute ischemic stroke through multiple injury mechanisms, such as mitochondrial inhibition, Ca\textsuperscript{2+} overload and reperfusion injury [41]. Brain ischemia generates super oxide radical (O\textsuperscript{2−}), from which H\textsubscript{2}O\textsubscript{2} is formed. H\textsubscript{2}O\textsubscript{2} is the source of hydroxyl radical (OH\textsuperscript{−}). An in vivo study has revealed that a dry matter of fermented filtrate (DMF) from A. camphorata in submerged culture shows antioxidant like effects against H\textsubscript{2}O\textsubscript{2}-induced cytotoxicity in HepG2 and carbon tetrachloride- (CCL\textsubscript{4}) induced hepatotoxicity [11]. They showed that DMF may play a role in preventing oxidative damage in living systems by upregulating hepatic glutathione-dependent enzymes to preserve the normal reduced and oxidized glutathione (GSH/GSSH) ratio and scavenging free radicals formed during CCl\textsubscript{4} metabolism.

A previous study was reported that polysaccharides extracted from fruiting bodies or cultured mycelia of A. camphorata exhibit an antihepatitis B virus effect [42]. In that study, the authors have specified that extracts from cultured mycelia of A. camphorata inhibit N-formyl-methionyl-leucyl-phenylalanine (fMLP) or phorbol 12-myristate 13-acetate- (PMA-) induced ROS production in peripheral human neutrophils (PMN) or mononuclear cells (MNC).
OH• can be produced from O2 under a variety of stress conditions and are involved in numerous cellular disorders such as inflammations, embryo teratogenesis, herbicide effects, cell death, and killing of microorganisms in pathogen-defense reactions. It is generally assumed that OH• is generated in biological systems from H2O2 by the Fenton reaction [43, 44]. Therefore, in the present study, we used fenton reaction to evaluate the hydroxyl radical-scavenging activity of A. camphorata by the ESR experiment. We found that A. camphorata significantly inhibits OH• formation at a higher concentration of 40 mg/mL. These results proposed that neuroprotection by A. camphorata may be involved, at least partly, in the inhibition of free radical formation.

In conclusion, our recent study was demonstrated that A. camphorata provides neuroprotection against MCAO-induced ischemic stroke via improved neurological functional scores and reduced infarct volume without causing hemorrhagic incidence when it is used in conjunction with aspirin therapy; nevertheless, the mechanisms underlying remained intricate. Therefore, we performed this study and found that the neuroprotective effect of A. camphorata is possibly via enhanced inhibition of HO-1, followed by the inhibition of inflammatory responses (i.e., iNOS) and apoptosis (Bax and activated caspase-3) in the ischemic brain. In addition, neuroprotection by A. camphorata may be involved, at least partly, by the inhibition of free radical formation.

Conflict of Interests

The authors declare that they have no conflict of interests to disclose.

Authors’ Contribution

Po-Sheng Yang and Po-Yen Lin contributed equally to this work.

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References

[1] A. Towfighi and J. L. Saver, “Stroke declines from third to fourth leading cause of death in the United States: historical perspective and challenges ahead,” Stroke, vol. 42, no. 8, pp. 2351–2355, 2011.
[2] W. Hacke, M. Kaste, E. Bluhmki et al., “Thrombolysis with alteplase 3 to 4.5 hours after acute ischemic stroke,” The New England Journal of Medicine, vol. 359, no. 13, pp. 1317–1329, 2008.
[3] P. H. Chan, “Reactive oxygen radicals in signaling and damage in the ischemic brain,” Journal of Cerebral Blood Flow & Metabolism, vol. 21, no.1, pp. 2–14, 2001.
[4] Z. A. Malik, M. Singh, and P. L. Sharma, “Neuroprotective effect of Momordica charantia in global cerebral ischemia and reperfusion induced neuronal damage in diabetic mice,” Journal of Ethnopharmacology, vol. 133, no.2, pp. 729–734, 2011.
[5] T. Y. Song, S. L. Hsu, C. T. Yeh, and G. C. Yen, “Mycelia from Antrodia camphorata in submerged culture induce apoptosis of human hepatoma HepG2 cells possibly through regulation of fas pathway,” Journal of Agricultural and Food Chemistry, vol. 53, no.14, pp. 5559–5564, 2005.
[6] Y. L. Hsu, Y. C. Kuo, P. L. Kuo, L. T. Ng, Y. H. Kuo, and C. C. Lin, “Apoptotic effects of extract from Antrodia camphorata fruiting bodies in human hepatocellular carcinoma cell lines,” Cancer Letters, vol. 221, no.1, pp. 77–89, 2005.
[7] P. C. Cheng, C. Y. Hsu, C. C. Chen, and K. M. Lee, “In vivo immunomodulatory effects of Antrodia camphorata polysaccharides in a T1/T2 doubly transgenic mouse model for inhibiting infection of Schistosoma mansoni,” Toxicology and Applied Pharmacology, vol. 227, no.2, pp. 291–298, 2008.
[8] H. Nakano, S. Ikenaga, T. Aizu et al., “Human metallothionein gene expression is upregulated by β-thujaplicin: possible
involvement of protein kinase C and reactive oxygen species,”

Biological & Pharmaceutical Bulletin, vol. 29, no. 1, pp. 55–59, 2006.

Y.-C. Hseu, H.-L. Yang, Y.-C. Lai, J.-G. Lin, G.-W. Chen, and Y.-H. Chang, “Induction of apoptosis by Antrodia camphorata in human promyelocytic leukemia HL-60 cells,” Nutrition and Cancer, vol. 48, no. 2, pp. 189–197, 2004.

G. Hsiao, M.-Y. Shen, K.-H. Lin et al., “Antioxidative and hepatoprotective effects of Antrodia camphorata extract,” Journal of Agricultural and Food Chemistry, vol. 51, no. 11, pp. 3302–3308, 2003.

T. Y. Song and G. C. Yen, “Protective effects of fermented filtrate from Antrodia camphorata in submerged culture against CCl4-induced hepatic toxicity in rats,” Journal of Agricultural and Food Chemistry, vol. 51, no. 6, pp. 1571–1577, 2003.

Y. M. Lee, C. Y. Chang, T. L. Yen et al., “Extract of Antrodia camphorata exerts neuroprotection against embolic stroke in rats without causing the risk of hemorrhagic incidence,” The Scientific World Journal, vol. 2014, Article ID 686109, 8 pages, 2014.

G. Hsiao, K. H. Lin, Y. Chang et al., “Protective mechanisms of inosine in platelet activation and cerebral ischemic damage,” Arteriosclerosis, Thrombosis, and Vascular Biology, vol. 25, no. 9, pp. 1998–2004, 2005.

T. Jayakumar, W.-H. Hsu, T.-L. Yen et al., “Hinokitiol, a natural tropolone derivative, offers neuroprotection from thromboembolic stroke in vivo,” Evidence-based Complementary and Alternative Medicine, vol. 2013, Article ID 840487, 8 pages, 2013.

J. J. Lee, W. H. Hsu, T. L. Yen et al., “Traditional Chinese medicine, Xue-Fu-Zhu-Yu decoction, potentiates tissue plasminogen activator against thromboembolic stroke in rats,” Journal of Ethnopharmacology, vol. 134, no. 3, pp. 824–830, 2011.

D.-S. Chou, G. Hsiao, M.-Y. Shen, Y.-J. Tsai, T.-F. Chen, and J.-R. Sheu, “ESR spin trapping of a carbon-centered free radical from agonist-stimulated human platelets,” Free Radical Biology and Medicine, vol. 39, no. 2, pp. 237–248, 2005.

S. Saleem, H. Zhuang, S. Biswal, Y. Christen, and S. Doré, “Ginkgo biloba extract neuroprotective action is dependent on heme oxygenase-1 in ischemic reperfusion brain injury,” Stroke, vol. 39, no. 12, pp. 3389–3396, 2008.

F.-P. Huang, G. Xi, R. F. Keep, Y. Hua, A. Nemoianu, and J. T. Hoff, “Brain edema after experimental intracerebral hemorrhage: role of hemoglobin degradation products,” Journal of Neurosurgery, vol. 96, no. 2, pp. 287–293, 2002.

A. H. Koeppen, A. C. Dickson, and J. Smith, “Heme oxygenase in experimental intracerebral hemorrhage: the benefit of temesporphyrin,” Journal of Neuropathology & Experimental Neurology, vol. 63, no. 6, pp. 587–597, 2004.

N. G. Abraham and A. Kappas, “Pharmacological and clinical aspects of heme oxygenase,” Pharmacological Reviews, vol. 60, no. 1, pp. 79–127, 2008.

Z.-P. Teng, J. Chen, L.-Y. Chau, N. Galunic, and R. F. Regan, “Adenoviral transfer of the heme oxygenase-1 gene protects striatal astrocytes from heme-mediated oxidative injury,” Neurobiology of Disease, vol. 17, no. 2, pp. 179–187, 2004.

Y. Gong, H. Tian, G. Xi, R. F. Keep, J. T. Hoff, and Y. Hua, “Systemic zinc protoporphyrin administration reduces intracerebral hemorrhage-induced brain injury,” Acta Neurochirurgica, Suplementum, vol. 96, pp. 232–236, 2006.

K. R. Wagner, Y. Hua, G. M. de Courten-Myers et al., “Tinmesoporphyrin, a potent heme oxygenase inhibitor, for treatment of intracerebral hemorrhage: in vivo and in vitro studies,” Cellular and Molecular Biology, vol. 46, no. 3, pp. 597–608, 2000.

P. S. Chen, C.-C. Wang, C. D. Bortner et al., “Valproic acid and other histone deacetylase inhibitors induce microglial apoptosis and attenuate lipopolysaccharide-induced dopaminergic neurotoxicity,” Neuroscience, vol. 149, no. 1, pp. 203–212, 2007.

J. Wang and S. Doré, “Heme oxygenase-1 exacerbates early brain injury after intracerebral haemorrhage,” Brain, vol. 130, no. 6, pp. 1643–1652, 2007.

K. Kawaguchi, F. Lambein, and K. Kusama-Eguchi, “Vascular insult accompanied by overexpressed heme oxygenase-1 as a pathophysiological mechanism in experimental neurolathyrism with hind-leg paraparesis,” Biochemical and Biophysical Research Communications, vol. 428, no. 1, pp. 160–166, 2012.

Y. Guo, Q. Wang, K. Zhang et al., “HO-1 induction in motor cortex and intestinal dysfunction in TDP-43 A315T transgenic mice,” Brain Research, vol. 1460, pp. 88–95, 2012.

Y.-N. Huang, J.-Y. Wang, C.-T. Lee, C.-H. Lin, and C.-C. Lai, “L-Ascorbate attenuates methamphetamine neurotoxicity through enhancing the induction of endogenous heme oxygenase-1,” Toxicology and Applied Pharmacology, vol. 265, no. 2, pp. 241–252, 2012.

C. Iadecola, F. Zhang, R. Casey, M. Nagayama, and M. Elizabeth Ross, “Delayed reduction of ischemic brain injury and neurological deficits in mice lacking the inducible nitric oxide synthase gene,” Journal of Neuroscience, vol. 17, no. 23, pp. 9157–9164, 1997.

J. Imagawa, D. M. Yellon, and G. F. Baxter, “Pharmacological evidence that inducible nitric oxide synthase is a mediator of delayed preconditioning,” British Journal of Pharmacology, vol. 126, no. 3, pp. 701–708, 1999.

C. Iadecola, X. Xu, F. Zhang, E. E. El-Fakahany, and M. E. Ross, “Marked induction of calcium-independent nitric oxide synthase activity after focal cerebral ischemia,” Journal of Cerebral Blood Flow and Metabolism, vol. 15, no. 1, pp. 52–59, 1995.

C. Iadecola, F. Zhang, and X. Xu, “Inhibition of inducible nitric oxide synthase ameliorates cerebral ischemic damage,” American Journal of Physiology—Regulatory Integrative and Comparative Physiology, vol. 268, no. 1, pp. R286–R292, 1995.

S.-S. Yang, G.-J. Wang, S.-Y. Wang, Y.-Y. Lin, Y.-H. Kuo, and T.-H. Lee, “New constituents with iNOS inhibitory activity from mycelium of Antrodia camphorata,” Planta Medica, vol. 75, no. 5, pp. 512–516, 2009.

M. S. Ola, M. Nawaz, and H. Ahsan, “Role of Bcl-2 family proteins and caspases in the regulation of apoptosis,” Molecular and Cellular Biochemistry, vol. 351, no. 1-2, pp. 41–58, 2011.

S. I. Savitz, J. A. Erhardt, J. V. Anthony et al., “The novel β-blocker, carvedilol, provides neuroprotection in transient focal stroke,” Journal of Cerebral Blood Flow and Metabolism, vol. 20, no. 8, pp. 1197–1204, 2000.

A. G. Porter and R. U. Janicke, “Emerging roles of caspase-3 in apoptosis,” Cell Death & Differentiation, vol. 6, no. 2, pp. 99–104, 1999.

D. A. Le, Y. Wu, Z. Huang et al., “Caspase activation and neuroprotection in caspase-3-deficient mice after in vivo cerebral ischemia and in vitro oxygen glucose deprivation,” Proceedings of the National Academy of Sciences of the United States of America, vol. 99, no. 23, pp. 15188–15193, 2002.

M. Sun and C. Xu, “Neuroprotective mechanism of taurine due to up-regulating calpastatin and down-regulating calpain
and caspase-3 during focal cerebral ischemia,” *Cellular and Molecular Neurobiology*, vol. 28, no. 4, pp. 593–611, 2008.

[39] L. L. Kong, Z. Y. Wang, J. Hu et al., “Inhibition of chemokine-like factor 1 protects against focal cerebral ischemia through the promotion of energy metabolism and anti-apoptotic effect,” *Neurochemistry International*, vol. 76, pp. 91–98, 2014.

[40] J. T. Coyle and P. Puttfarcken, “Oxidative stress, glutamate, and neurodegenerative disorders,” *Science*, vol. 262, no. 5134, pp. 689–695, 1993.

[41] S. Cuzzocrea, D. P. Riley, A. P. Caputi, and D. Salvemini, “Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury,” *Pharmacological Reviews*, vol. 53, no. 1, pp. 135–159, 2001.

[42] Y.-C. Shen, C.-J. Chou, Y.-H. Wang, C.-F. Chen, Y.-C. Chou, and M.-K. Lu, “Anti-inflammatory activity of the extracts from mycelia of *Antrodia camphorata* cultured with water-soluble fractions from five different *Cinnamomum* species,” *FEMS Microbiology Letters*, vol. 231, no. 1, pp. 137–143, 2004.

[43] B. Halliwell and J. M. C. Gutteridge, “Biologically relevant metal ion-dependent hydroxyl radical generation. An update,” *FEBS Letters*, vol. 307, no. 1, pp. 108–112, 1992.

[44] E. R. Stadtman, “Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions,” *Annual Review of Biochemistry*, vol. 62, pp. 797–821, 1993.