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A Water-Ethanol Extract from the Willow Bracket Mushroom, *Phellinus igniarius* (Higher Basidiomycetes), Reduces Transient Cerebral Ischemia-Induced Neuronal Death

Jin Hee Kim,[1] Bo Young Choi,[1] Hyun Jung Kim,[1] In Yeol Kim,[2] Bo Eun Lee,[1] Min Sohn,[3] Hyoung Jin Park,[4] & Sang Won Suh[1,*]

[1]Institute of Medical Science, Hallym University, Chuncheon, Republic of Korea; [2]Department of Physiology, College of Medicine, Hallym University, Chuncheon, Republic of Korea; [3]Department of Nursing, Inha University, Incheon, Republic of Korea; [4]MushMed Co., Ltd, Chuncheon, Gangwon-do, Republic of Korea

*Address all correspondence to: Sang Won Suh, Department of Physiology, 1-Okcheon Dong, Hallym University College of Medicine, 39 Hallymdaehak-gil, Chuncheon, Republic of Korea 200-702; Tel.: (82-10) 8573-6364 ; Fax : (82-33) 256-3426 ; swsuh@hallym.ac.kr.

ABSTRACT: This study investigated the potential neuroprotective effect of a mushroom extract from *Phellinus igniarius* (Piwep) after transient cerebral ischemia. *Ph. igniarius*, which has a history of traditional medicinal use, contains immunomodulatory compounds that have been described to have effects on the human immune system. Using a model of transient cerebral ischemia induced by both common carotid artery occlusion and hypovolemia, a water–ethanol extract precipitate of *Ph. igniarius* (Piwep) was delivered intraperitoneally immediately after the insult and was injected subsequently every other day for the experimental course. Neuronal death was examined by Fluoro-Jade B staining 1 week after the insult. Piwep injection lead to decreased hippocampal neuronal death, suppression of oxidative injury, activation of microglia, and disruption of the blood–brain barrier. We conclude that Piwep potently inhibits hippocampal neuronal death following ischemia and may have a high therapeutic potential for ameliorating stroke-induced neuron death in the clinical setting.

KEY WORDS: medicinal mushrooms, ischemia, *Phellinus igniarius*, oxidative injury, microglia activation, blood–brain barrier disruption, hippocampus

ABBREVIATIONS: 4HNE, 4-hydroxy-2-nonenal; **BBB**, blood-brain barrier; **CA**, cornu ammonis; **CCA**, common carotid artery; **IgG**, immunoglobulin G; **PBS**; phosphate-buffered saline; **PFA**, paraformaldehyde; **Piwep**, *Phellinus igniarius* water-ethanol extract precipitate.

I. INTRODUCTION

Multiple pharmacological agents have been shown to provide neuroprotection when administered within 3 hours after the onset of ischemia, including glutamate receptor antagonists, free-radical scavengers, and the thrombolytic agent tissue-plasminogen activator.[1–4] However, these promising findings may not translate well into clinical improvements because the majority of patients experiencing stroke do not seek treatment until after this short time window. Therefore the need to identify clinically useful agents that provide neuroprotection with longer latencies and that have minimal side effects is paramount.

Mushrooms belonging of the genus *Phellinus* Quél. (Hymenochaetaceae, higher Basidiomycetes) have been traditionally used as a medicine in Asia to treat inflammation and cancer, and their medicinal functions are currently being investigated.[5] A promising finding regarding these medicinal properties is that polysaccharides extracted from *Ph. linteus* have strong immune-modulating properties. This effect likely underlies the anticancer action of this extract, and further research to understand the mechanisms of its anticancer activities is ongoing.[6–8] So far, 9 compounds were isolated from the aqueous fraction of the *Ph. linteus*, including protocatechuic acid,

caffeic acid, ellagic acid, protocatechualdehyde, hispidin, davallialactone, hypholomine B, interfungins A, and inoscavin A.[9]

The willow bracket mushroom, *Ph. igniarius* (L.) Quél., is another mushroom belonging to the genus *Phellinus* that has a history of use as a naturally occurring medicine in Asia.[5] This mushroom's immunoregulatory properties have been previously described.[10] Polysaccharides, especially β-glucan, are believed to responsible for the observed biological activity of this mushroom.[11] Therefore, we sought to identify the potential therapeutic effects of an extract of *Ph. igniarius* in an animal model of transient cerebral ischemia.

Bilateral common carotid artery occlusion combined with hypovolemia is a commonly used animal model of ischemia. Using this model, we investigated the potential for systemic administration of mushroom extract—a *Ph. igniarius* water-ethanol extract precipitate (Piwep)—to provide neuroprotection against hippocampal neuronal death. Piwep administration reduced hippocampal neuronal death and showed a marked reduction in microglial activation and blood–brain barrier (BBB) disruption. These results suggest that the Piwep has potential therapeutic effects in the treatment of stroke.

II. MATERIALS AND METHODS

The care and handling of animals were in accordance with institutional guidelines and were approved by the Institutional Animal Studies Committee of Hallym University in Chuncheon, Korea (protocol no. Hallym 2012–28). Animal surgery was performed under isoflurane anesthesia, and all efforts were made to minimize suffering. This article was written in accordance with the "Animal Research: Reporting *In Vivo* Experiments" guidelines.[12]

A. Preparation of Piwep

Piwep was prepared as previously described.[13,14] The dried fruit bodies of *Ph. igniarius* (Amazing Grace Health Products, Bangkok, Thailand) was first ground to a crude powder. This powder was boiled at 103°C in distilled water for 3 hours. The aqueous extract then was mixed with 2 volumes of cold (–20°C) 95% ethanol and stored in a refrigerator for 24 hours. Dark brown precipitate was collected after centrifugation and freeze-dried. The extract was loaded on an open chromatography column (8 × 45 cm) of HP-20 (Supelco) and sequentially fractioned with water, 25% ethanol, and 50% ethanol. Each fraction was freeze-dried.

B. Transient Forebrain Ischemia

Transient forebrain ischemia was produced with bilateral common carotid artery (CCA) occlusion as described by Smith et al.[15] Rats were anesthetized with 1% isoflurane in a closed chamber and maintained with 30% O_2/70% N_2O delivered via a facemask. Core temperature was monitored and maintained at 37 ± 0.5°C throughout the surgical procedure using a homoeothermic blanket (Harvard Apparatus, Holliston, MA, USA). The CCAs were exposed through a midline neck skin incision and carefully separated from the adjacent vagus nerves. The exposed CCAs were occluded by microvascular clips, and systemic mean arterial pressure was lowered to 40 ± 5 mmHg by withdrawing blood (7–10 mL) from the femoral artery into a heparinized syringe maintained at 37°C. The clips were removed from the CCAs 10 min later and the blood was reintroduced into the femoral artery. The incisions were closed, the femoral artery catheter was removed, and anesthesia was discontinued. Animals exhibiting seizures following reperfusion were euthanized and not assigned to control or drug-treatment groups.

C. Treatment Schedule

Piwep was administered by intraperitoneal injection at a dose of 100 mg/kg in a volume of 1.0 mL of 0.9% saline. Treatment was initiated immediately after ischemia, and subsequent injections were given at 24-hour intervals for 7 days. The control group received injections of saline vehicle alone. Rats were euthanatized at the designated time

points, and brains were perfusion-fixed with 4% formaldehyde.

D. Assessment of Neuronal Death

To identify degenerating neurons after ischemia, animals were deeply anesthetized with isoflurane (3%) 7 days after transient cerebral ischemia and intracardially perfused with 200 mL of 0.9% saline followed by 4% paraformaldehyde (PFA) for 5 min. The brains were post-fixed for 1 hour in the same fixative solution and immersed in 20% sucrose until they sank to bottom. Cryostat sections (25 μm) were mounted on superfrosted coated slides (Fisher Scientific, Pittsburgh, PA, USA). Fluoro-Jade B staining was performed as described by Schmued and Hopkins[16] and Suh et al.[17] In brief, the slides were immersed in a basic alcohol solution followed by 0.06% potassium permanganate. The slides then were immersed in 0.0004% Fluoro-Jade B (Histo-Chem Inc., Jefferson, AR, USA) for 20 min and washed in distilled water. Sections were photographed with a Leica confocal laser-scanning microscope with blue (450–490 nm) excitation light and a barrier filter wavelength of 515 nm. Five coronal sections were collected from each animal by starting 4.0 mm posterior to the bregma and collecting every fourth section (75-μm intervals) until 5 sections were in hand. These sections then were coded and given to a second, blinded experimenter who counted the number of degenerating neurons in the hippocampal cornu ammonis (CA) 1, CA3, subiculum and hilus regions of both hemispheres. The total numbers of degenerating neurons in a region in each of the 5 sections from each brain were averaged.

E. Immunohistochemistry

The formaldehyde-fixed brains were cut in 25-μm cryostat sections through the rostral-caudal extent of the hippocampus, and 5–6 evenly spaced sections from each brain were evaluated and analyzed for each histological variable. Immunohistochemistry was performed in floating sections as described previously.[18] The primary antibodies used were mouse monoclonal antirat CD11b (1:100 dilution) (Serotec, Oxford, UK). Secondary antibodies were mouse, rabbit, or sheep anti–immunoglobulin G (IgG) conjugated with Alexa Fluor 594 or 488 (1:500 dilution each) (Molecular Probes Inc., Eugene, OR, USA). Fluorescence signals were visualized by confocal microscopy with appropriate filter sets. Immunostaining was evaluated using the Zeiss LSM 510 confocal image system with a sequential scanning module by an observer who was blinded to the experimental conditions. Controls prepared with primary or secondary antibodies omitted showed no detectable fluorescence under the conditions used for confocal photography.

F. Evaluation of Brain Microglial Activation

Immunostaining was performed on PFA-fixed, tissue and coronal cryosections were cut at a thickness of 25 μm. After rinsing with 10 mmol/L phosphate-buffered saline (PBS), nonspecific protein binding of the brain tissue was blocked by a 1-hour incubation in blocking buffer (10% goat serum and 0.1% Triton X-100 in 100 mmol/L PBS) at room temperature. The sections then were immunostained with a mouse antibody to rat CD11b (Serotec) at a 1:200 dilution. After washing, the sections were incubated with Alexa Fluor 488–conjugated goat anti-mouse IgG secondary antibody (Molecular Probes Inc.) at a dilution of 1:500 for 2 hours at room temperature. Negative controls were treated with secondary antibody alone and no staining.

G. Detection of Oxidative Injury

Oxidative injury was estimated by evaluating levels of the lipid peroxidation product 4-hydroxy-2-nonenal (4HNE). Immunostaining with 4HNE antibodies (Alpha Diagnostic Int. Inc., San Antonio, TX, USA) was performed as described previously.[19] Tissues were incubated in a mixture of polyclonal rabbit anti-hydroxy-2-nonenal antiserum (diluted at 1:500; Alpha Diagnostic Int. Inc.) in PBS containing 0.3% Triton X-100 overnight at 4°C. After washing 3 times for 10 min each with PBS, sections also were incubated in a mix-

ture of Alexa Fluor 594–conjugated goat antirabbit IgG secondary antibody (Invitrogen, Grand Island, NY, USA) at a dilution of 1:250 for 2 hours at room temperature. The sections were washed with PBS 3 times for 10 min each and mounted on gelatin-coated slides.

H. Detection of BBB Disruption by IgG Immunostaining

Rats were examined for the extravasation of presumed endogenous serum IgG after ischemia. Animals treated with Piwep were killed 7 days after the ischemia. The avidin-biotin peroxidase complex immunoperoxidase method was used to detect IgG-like immunoreactivity.[20] Brains were fixed by perfusion (4.0% PFA). Coronal sections (30 μm thick) were incubated with rabbit serum, followed by purified biotinylated rabbit anti-rat IgG (Vector Laboratories, Burlingame, CA, USA) at a dilution of 1:250.

I. Statistical Analysis

Results are presented as a mean ± standard error. Immunostaining data were assessed by the nonparametric Kruskal-Wallis one-way analysis of variance test, followed by Dunn's test for multiple group comparison. Cell death was assessed by one-way analysis of variance followed by the Tukey-Kramer test for multiple comparisons between the treatment groups.

III. RESULTS

A. Piwep Reduces Neuronal Death after Transient Cerebral Ischemia

Transient cerebral ischemia induces a reproducible pattern of neuronal death when evaluated at 7 days after the insult. Fluoro-Jade B staining provides a selective, unambiguous marker of neuronal degeneration, and staining showed widespread neuronal death in the hippocampal CA1, CA3, subiculum, and hilus areas (Fig. 1). Rats that received blood reperfusion plus Piwep (100 mg/kg intraperitoneally) at the beginning of reperfusion showed a marked reduction in neuronal death (Fig. 1A) compared with those receiving blood reperfusion alone. As quantified in Fig. 1B, rats receiving Piwep had significantly fewer degenerating neurons than the rats given vehicle.

B. Piwep Reduces Ischemia-Induced Oxidative Injury

Rat brain sections were immunohistochemically stained with 4HNE at 7 days after the insult to determine whether oxidative injury had occurred in hippocampal neurons. Compared with vehicle-treated animals, Piwep-treated animals showed a significant reduction in oxidative injury to hippocampal neurons. 4HNE fluorescence intensity was increased adjacent to the area occupied by neuronal cell bodies of the hippocampus of vehicle-treated rats. However, 4HNE fluorescence intensity was reduced in Piwep-treated rats (Fig. 2A and B).

C. Piwep Prevents Ischemia-Induced Microglial Activation

After ischemia, neuronal deprivation of oxygen and glucose and its subsequent deleterious signaling cascade may not only impose neuronal damage via neuron-specific effects but also may promote indirect damage via microglial activation. Hallmarks of microglial activation include a gradual change in morphology (from a highly ramified to an amoeboid shape); proliferation; migration to the site of injury; increased expression of surface molecules; increased secretion of cytokines, chemokines, free radicals, and proteases; and phagocytotic activity.[21] We investigated the degree of microglial activation after ischemia with or without Piwep administration. Compared with saline-treated rats, microglial activation in the hippocampus was significantly reduced by Piwep administration (Fig. 3A and B).

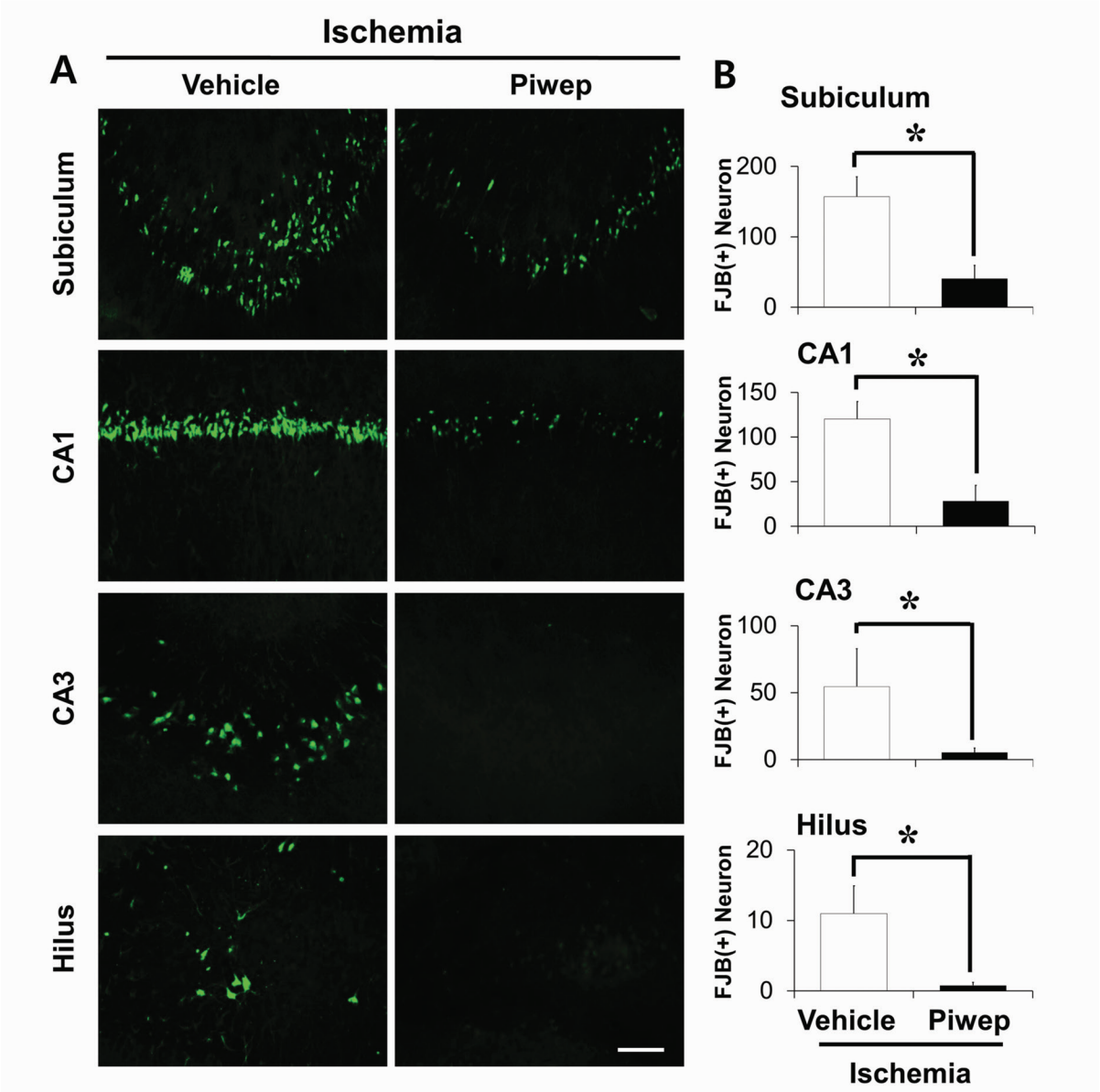

FIG. 1: Ischemia-induced neuron death is prevented by *Phellinus igniarius* water-ethanol extract precipitate (Piwep) administration. **A:** Fluorescence images of Fluoro-Jade B (FJB)–stained brain sections 7 days after ischemia. FJB staining shows numerous degenerating neurons (green fluorescence) in the hippocampal cornu ammonis (CA) 1, CA3, subiculum and hilus. Rats given 100 mg/kg Piwep (intraperitoneal) with blood glucose reperfusion at the end of the ischemia showed fewer degenerating neurons. Photos are representative of 7–9 rats in each treatment condition. Scale bar = 100 μm. **B:** Graphs show quantitated neuronal degeneration after ischemia. Compared with the saline-treated group, the Piwep-treated group showed reduced FJB(+) neurons in the hippocampal CA1, CA3, subiculum, and hilus area. Data are mean ± standard error of the mean (n = 7–9). **P* < 0.05 compared with the saline-treated group.

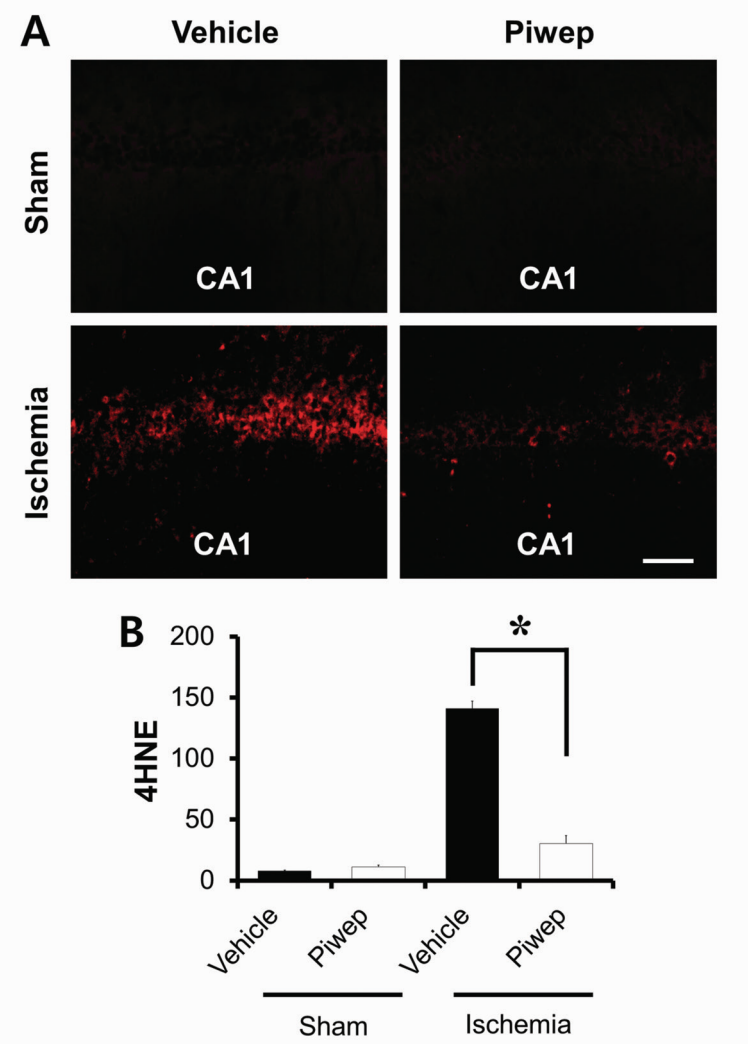

FIG. 2: Ischemia-induced oxidative injury is reduced by *Phellinus igniarius* water-ethanol extract precipitate (Piwep) administration. **A:** Neuronal oxidative injury was detected by immunostaining with 4-hydroxy-2-nonenal (4HNE) in the hippocampal cornu ammonis (CA) 1 area 7 days after ischemia. Sham-operated brain section shows almost no 4HNE-stained neurons in the hippocampus. Fluorescence images show several 4HNE(+) neurons in the hippocampal area after ischemia. Intraperitoneal treatment with Piwep immediately after ischemia attenuated 4HNE fluorescent intensity in the hippocampal area compared with the vehicle-treated group. Scale bar = 20 μm. **B:** Bar graph shows the quantification of 4HNE fluorescence intensity in the hippocampus. The fluorescence intensity in the hippocampal CA1 area is significantly different between the vehicle- and Piwep-treated groups. Data are mean ± standard error of the mean (n = 10 from each group). *Significantly different from the vehicle-treated group ($P < 0.05$).

D. Piwep Reduces Ischemia-Induced BBB Disruption

We evaluated BBB disruption by assaying leakage of serum IgG using immunohistochemistry, as previously reported.[22,23] In normal animals, IgG staining was restricted to the vasculature, whereas in ischemia subjects we observed extravascular IgG staining in the hippocampus. IgG staining formed halos with a concentration gradient around vessels in the dentate granular layer and the hilus. In the CA1/CA3 areas, IgG staining was widespread in the parenchyma. The cytoplasm and dendrites of numerous pyramidal neurons also were immunore-

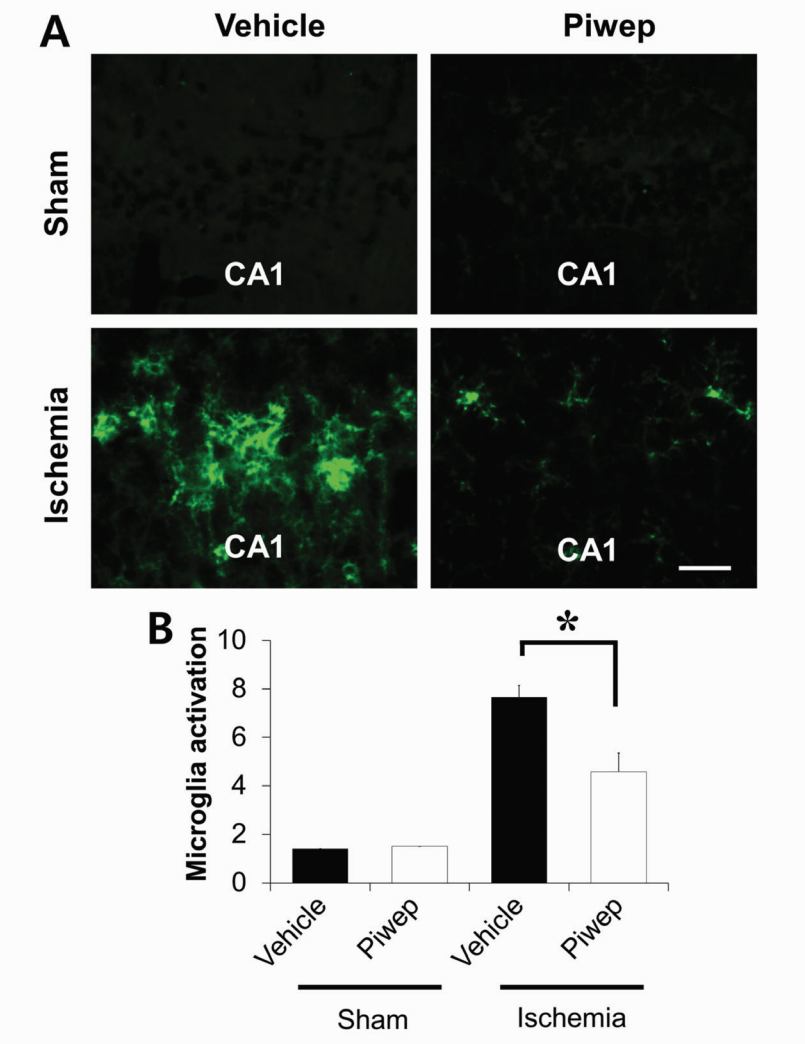

FIG. 3: Ischemia-induced microglial activation is prevented by *Phellinus igniarius* water-ethanol extract precipitate (Piwep) administration. **A:** Fluorescent microscopic images show morphological changes and an increase in the intensity of microglial immunostaining after ischemia. Piwep substantially decreased microglial activation in the hippocampal cornu ammonis (CA) 1 compared with saline-treated animals. Scale bar = 100 μm. **B:** Microglial activation in the hippocampal CA1 area was quantified. As shown in the graph, ischemia-induced microglial activation is strongly reduced by Piwep administration. Data are mean ± standard error of the mean (n = 6–8). **P* < 0.05 compared with the saline-injected group.

active for IgG. However, treatment with Piwep after ischemia reduced immunoglobulin leakage by BBB disruption in the hippocampus (Fig. 4A and B).

IV. DISCUSSION

Here we show that administration of a Piwep, a mushroom extract from *Ph. igniarius*, following transient ischemia-reperfusion can prevent neuronal death and suppress oxidative injury, microglial activation, and BBB disruption. These results clearly demonstrate that Piwep inhibits the neuropathological changes of stroke and, importantly, Piwep is effective at delayed latencies after an insult.

To date, many of the beneficial effects attribut-

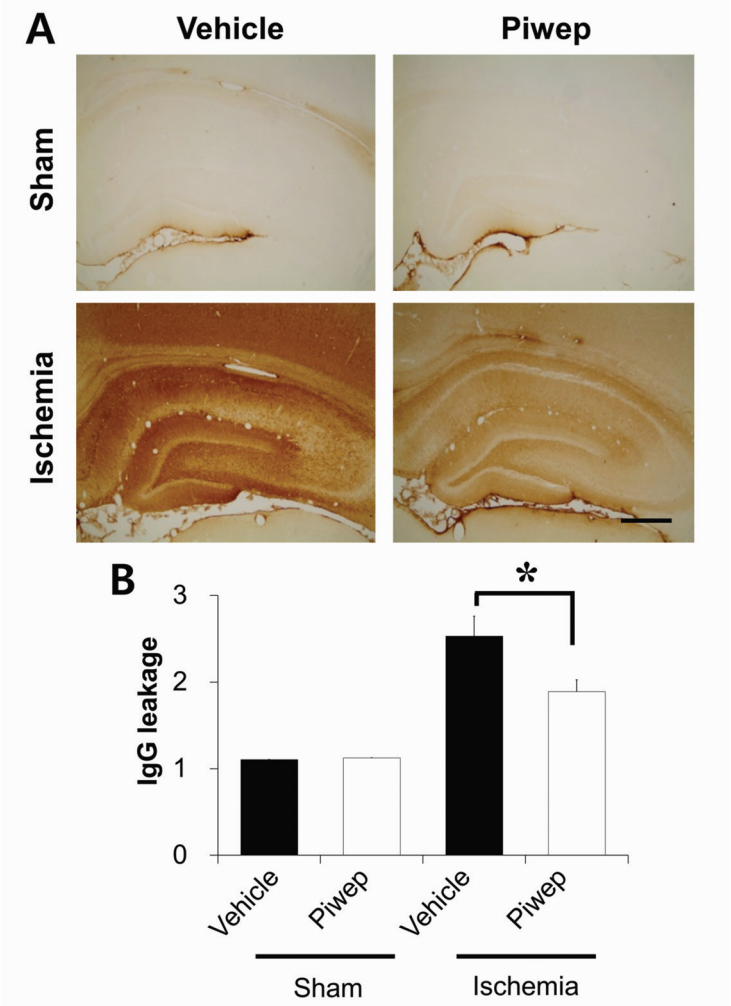

FIG. 4: Ischemia-induced blood–brain barrier (BBB) damage is reduced by *Phellinus igniarius* water-ethanol extract precipitate (Piwep) administration. This figure displays BBB damage in the hippocampus after ischemia. **A:** Low-magnification photomicrographs show immunoglobulin G (IgG)–stained coronal hippocampal sections. Sham-operated animals showed sparse IgG staining in the hippocampus. At 7 days after ischemia, the entire hippocampus is intensely stained and shows IgG immunoreactivity, indicating that substantial BBB damage has occurred in vehicle-treated animals. Injection of Piwep after ischemia reduced the intensity of IgG staining in the hippocampus compared with the vehicle-treated group. Scale bar = 500 μm. **B:** Bar graph shows the quantification of IgG intensity in the hippocampus. The intensity in the hippocampus is significantly different between the vehicle- and Piwep-treated group. Data are mean + standard error of the mean (n = 10 from each group). *Significantly different from the vehicle-treated group ($P < 0.05$).

ed to medicinal mushrooms have focused on their anticancer properties. However, medicinal mushrooms also have been shown to modulate diverse systemic responses to help restore a healthy immune response.[6,24–26] We previously demonstrated that Piwep inhibits the clinical features and neuropathological changes associated with experimental autoimmune encephalomyelitis. Those results suggested that a mushroom extract, Piwep, might possess high therapeutic potential for ameliorating progression of multiple sclerosis.[14]

The mechanism of the neuroprotective effects of Piwep after ischemia is not clear in this study. However, one hypothesis is that Piwep may affect

inflammatory response and BBB integrity after transient cerebral ischemia. Several studies have shown that inflammatory processes are involved in the progression of neuronal death after brain ischemia.[27] Thus, this study investigates whether microglial activation is modulated by Piwep after ischemia. Activation of microglia is characterized by a morphological change into an amoeboid shape. Activated microglia release reactive oxygen species, including superoxide and nitric oxide.[28] Here we found that Piwep treatment significantly reduced microglial activation and oxidative injury after ischemia. This study also demonstrates that ischemia increased diffuse IgG immunoreactivity throughout the hippocampus, which indicates immunoglobulin leakage by BBB breakdown after ischemia. Treatment with Piwep after ischemia diminished immunoglobulin leakage. Therefore, these results suggest that the neuroprotective effects of Piwep after ischemia may be accomplished by reducing microglia activation and BBB disruption.

Several medicinal mushrooms also have been tested for prevention of ischemia-induced neuron death using global ischemia and focal ischemia animal models. *Ophiocordyceps sinensis* (=*Cordyceps sinensis*) has been used to protect against ischemia-induced brain infarction. Liu et al.[29] reported that an extract from *O. sinensis* reduced middle cerebral artery occlusion–induced brain infarction by increasing antioxidant activity. Lee et al.[30] also reported that an extract from Hericium erinaceus showed neuroprotective effects after global ischemia through modulation of inducible nitric oxide synthase and p38 mitogen-activated protein kinase. Use of medicinal mushrooms to restore antioxidant homeostasis in the brain after ischemia may have helped facilitate brain recovery following ischemic injury.

Currently, it remains unknown which substance(s) in Piwep possesses neuroprotective qualities or how it relates to postischemic neuronal injury. Various bioactive substances—such as polysaccharides, cyclophellitol, furan derivatives, hispidin, and hispolon—have been identified from *Ph. linteus*, another mushroom belonging to the genus *Phellinus*.[6] Several bioactive polysaccharides, such as hetero-β-glucans and their protein complexes, have been isolated from *Phellinus* mushrooms.[11] Polysaccharides isolated from the fruiting bodies of *Ph. igniarius* by hot-water extraction have been shown to include fructose, galactose, mannose, and 3-O-Me-galactose.[31,32] These polysaccharides from *Ph. igniarius* have been shown to mediate many biologically relevant actions, such as antitumor and immunomodulatory effects, and to have low toxicity.[33] Lanostanes from *Ph. igniarius* significantly inhibited lipopolysaccharide-induced nitric oxide production in a dose-dependent manner without affecting cellular viability.[34] Future studies are needed to identify the active component(s) of Piwep.

V. CONCLUSION

A *Ph. igniarius* extract, Piwep, ameliorates ischemia-induced neuronal death in rats when administered after insult. The neuroprotective effects were accompanied by inhibition of ischemia-associated oxidative injury, BBB disruption, and microglial activation in the hippocampus. Taken together, our results suggest that a very potent and safe therapeutic agent for stroke patients could be developed from the polysaccharide-enriched fraction obtained from the medicinal mushroom *Ph. igniarius.*

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REFERENCES

1. Lipton SA. Failures and successes of NMDA receptor antagonists: molecular basis for the use of open-channel

blockers like memantine in the treatment of acute and chronic neurologic insults. NeuroRx. 2004;1(1):101–10.
2. Sugawara T, Chan PH. Reactive oxygen radicals and pathogenesis of neuronal death after cerebral ischemia. Antioxid Redox Signal. 2003;5:597–607.
3. Jian Liu K, Rosenberg GA. Matrix metalloproteinases and free radicals in cerebral ischemia. Free Radic Biol Med. 2005;39:71–80.
4. del Zoppo GJ. Thrombolysis: from the experimental findings to the clinical practice. Cerebrovasc Dis. 2004;17(Suppl 1):144–52.
5. Dai HY, Wang P, Feng LY, Liu LM, Meng ZQ, Zhu XY, Wang K, Hua YQ, Mao YX, Chen LY, Chen Z. The molecular mechanisms of traditional Chinese medicine ZHENG syndromes on pancreatic tumor growth. Integr Cancer Ther. 2010;9(3):291–97.
6. Zhu T, Kim SH, Chen CY. A medicinal mushroom: Phellinus linteus. Curr Med Chem. 2008;15:1330–35.
7. Lu TL, Huang GJ, Lu TJ, Wu JB, Wu CH, Yang TC, Iizuka A, Chen YF. Hispolon from Phellinus linteus has antiproliferative effects via MDM2-recruited ERK1/2 activity in breast and bladder cancer cells. Food Chem Toxicol. 2009;47:2013–21.
8. Song KS, Li G, Kim JS, Jing K, Kim TD, Kim JP, Seo SB, Yoo JK, Park HD, Hwang BD, Lim K, Yoon WH. Protein-bound polysaccharide from Phellinus linteus inhibits tumor growth, invasion, and angiogenesis and alters Wnt/beta-catenin in SW480 human colon cancer cells. BMC Cancer. 2011;11:307.
9. Lee YS, Kang YH, Jung JY, Lee S, Ohuchi K, Shin KH, Kang IJ, Park JH, Shin HK, Lim SS. Protein glycation inhibitors from the fruiting body of Phellinus linteus. Biol Pharm Bull. 2008;31:1968–72.
10. Yang Q, Hu XG, Wan Q, Wang WQ. [Study on antitumor effect of medicinal fungi Phellinus igniarius extracts]. Zhongguo Zhong Yao Za Zhi. 2006;31:1713–15.
11. Lee IK, Yun BS. Highly oxygenated and unsaturated metabolites providing a diversity of hispidin class antioxidants in the medicinal mushrooms Inonotus and Phellinus. Bioorg Med Chem. 2007;15:3309–14.
12. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS Biol. 2012;8:e1000412.
13. Lee S, Kim JI, Heo J, Lee I, Park S, Hwang MW, Bae JY, Park MS, Park HJ. The anti-influenza virus effect of Phellinus igniarius extract. J Microbiol. 2013;51:676–81.
14. Li L, Wu G, Choi BY, Jang BG, Kim JH, Sung GH, Cho JY, Suh SW, Park HJ. A mushroom extract Piwep from Phellinus igniarius ameliorates experimental autoimmune encephalomyelitis by inhibiting immune cell infiltration in the spinal cord. BioMed Res Int. 2014;2014:218274.
15. Smith ML, Auer RN, Siesjo BK. The density and distribution of ischemic brain injury in the rat following 2–10 min of forebrain ischemia. Acta Neuropathol. 1984;64:319–32.
16. Schmued LC, Hopkins KJ. Fluoro-Jade B: a high affinity fluorescent marker for the localization of neuronal degeneration. Brain Res. 2000;874:123–30.
17. Suh SW, Aoyama K, Chen Y, Garnier P, Matsumori Y, Gum E, Liu J, Swanson RA. Hypoglycemic neuronal death and cognitive impairment are prevented by poly(ADP-ribose) polymerase inhibitors administered after hypoglycemia. J Neurosci. 2003;23:10681–90.
18. Kim JH, Jang BG, Choi BY, Kim HS, Sohn M, Chung TN, Choi HC, Song HK, Suh SW. Post-treatment of an NADPH oxidase inhibitor prevents seizure-induced neuronal death. Brain Res. 2013;1499:163–72.
19. Suh SW, Gum ET, Hamby AM, Chan PH, Swanson RA. Hypoglycemic neuronal death is triggered by glucose reperfusion and activation of neuronal NADPH oxidase. J Clin Invest. 2007;117:910–18.
20. Hsu SM, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem. 1981;29:577–80.
21. Kreutzberg GW. Microglia: a sensor for pathological events in the CNS. Trends Neurosci. 1996;19:312–18.
22. Ruth RE, Feinerman GS. Foreign and endogenous serum protein extravasation during harmaline tremors or kainic acid seizures in the rat: a comparison. Acta Neuropathol. 1988;76:380–87.
23. Rigau V, Morin M, Rousset MC, de Bock F, Lebrun A, Coubes P, Picot MC, Baldy-Moulinier M, Bockaert J, Crespel A, Lerner-Natoli M. Angiogenesis is associated with blood-brain barrier permeability in temporal lobe epilepsy. Brain. 2007;130:1942–56.
24. Park SK, Kim GY, Lim JY, Kwak JY, Bae YS, Lee JD, Oh YH, Ahn SC, Park YM. Acidic polysaccharides isolated from Phellinus linteus induce phenotypic and functional maturation of murine dendritic cells. Biochem Biophys Res Commun. 2003;312:449–58.
25. Kim GY, Oh WK, Shin BC, Shin YI, Park YC, Ahn SC, Lee JD, Bae YS, Kwak JY, Park YM. Proteoglycan isolated from Phellinus linteus inhibits tumor growth through mechanisms leading to an activation of CD11c+CD8+ DC and type I helper T cell-dominant immune state. FEBS Lett. 2004;576:391–400.
26. Inagaki N, Shibata T, Itoh T, Suzuki T, Tanaka H, Nakamura T, Akiyama Y, Kawagishi H, Nagai H. Inhibition of IgE-dependent mouse triphasic cutaneous reaction by a boiling water fraction separated from mycelium of Phellinus linteus. Evid Based Complement Alternat Med. 2005;2(3):369–74.
27. Inamasu J, Suga S, Sato S, Horiguchi T, Akaji K, Mayanagi K, Kawase T. Post-ischemic hypothermia delayed neutrophil accumulation and microglial activation following transient focal ischemia in rats. J Neuroimmunol. 2000;109:66–74.
28. Vilhardt F. Microglia: phagocyte and glia cell. Int J Bio-

chem Cell Biol. 2005;37:17–21.
29. Liu Z, Li P, Zhao D, Tang H, Guo J. Protective effect of extract of Cordyceps sinensis in middle cerebral artery occlusion-induced focal cerebral ischemia in rats. Behav Brain Funct. 2010;6:61.
30. Lee KF, Chen JH, Teng CC, Shen CH, Hsieh MC, Lu CC, Lee KC, Lee LY, Chen WP, Chen CC, Huang WS, Kuo HC. Protective effects of Hericium erinaceus mycelium and its isolated erinacine A against ischemia-injury-induced neuronal cell death via the inhibition of iNOS/p38 MAPK and nitrotyrosine. Int J Mol Sci. 2014;15:15073–89.
31. Yang Y, Zhang J, Liu Y, Tang Q, Zhao Z, Xia W. Structural elucidation of a 3-O-methyl-D-galactose-containing neutral polysaccharide from the fruiting bodies of Phellinus igniarius. Carbohydr Res. 2007;342:1063–70.
32. Yang Y, Ye L, Zhang J, Liu Y, Tang Q. Structural analysis of a bioactive polysaccharide, PISP1, from the medicinal mushroom Phellinus igniarius. Biosci Biotechnol Biochem. 2009;73:134–39.
33. Chen L, Pan J, Li X, Zhou Y, Meng Q, Wang Q. Endopolysaccharide of Phellinus igniarius exhibited anti-tumor effect through enhancement of cell mediated immunity. Int Immunopharmacol. 2011;11:255–59.
34. Wang GJ, Tsai TH, Chang TT, Chou CJ, Lin LC. Lanostanes from Phellinus igniarius and their iNOS inhibitory activities. Planta Med. 2009;75:1602–7.