Research Article

Weisheng-Tang Ameliorates Acute Ischemic Brain Damage in Mice by Maintaining Blood-Brain Barrier Integrity

Min Jae Kim,1,2,3 Ki Hyun Park,2,4 Ji Yun Lee,1,2,3 Ki-Tae Ha,1,2,3,4 Byung Tae Choi1,2,3,4 Jin Ung Baek4, Young Ju Yun4, Seo-Yeon Lee2,3 and Hwa Kyoung Shin1,2,3,4

1Department of Korean Medical Science, School of Korean Medicine, Pusan National University, Yangsan, Gyeongnam 50612, Republic of Korea
2Korean Medical Science Research Center for Healthy-Aging, Pusan National University, Yangsan, Gyeongnam 50612, Republic of Korea
3Graduate Training Program of Korean Medicine for Healthy-Aging, Pusan National University, Yangsan, Gyeongnam 50612, Republic of Korea
4Department of Korean Medicine, School of Korean Medicine, Pusan National University, Yangsan, Gyeongnam 50612, Republic of Korea

Correspondence should be addressed to Seo-Yeon Lee; brainsw@gmail.com and Hwa Kyoung Shin; julie@pusan.ac.kr

Received 6 August 2019; Accepted 11 November 2019; Published 3 December 2019

Academic Editor: Ji C. Bihl

Copyright © 2019 Min Jae Kim et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Stroke is one of the major causes of death and long-term disability worldwide; the associated breakdown of the blood-brain barrier (BBB) aggravates ischemic brain damage. Accordingly, many medicinal herbs and formulas have been used to treat stroke-related symptoms. In this study, we selected two Korean herbal medicine formulas, Weisheng-tang and Tongxuewan, through Dongeuibogam text-mining analysis, and evaluated their protective effect on BBB disruption and brain damage in stroke. Ischemic brain damage was induced in mice by phototrombotic cortical ischemia. The infarct volume, brain edema, neurological deficits, and motor function 24 h after ischemic injury were analyzed. We investigated BBB breakdown by measuring Evans blue extravasation in addition to endothelial cells, tight junction proteins, protease-activated receptor-1 (PAR-1), and matrix metalloproteinase-9 (MMP-9) using immunofluorescence staining and confocal microscopy. Pretreatment with Weisheng-tang significantly reduced infarct volume and edema and improved neurological and motor functions; however, Tongxuewan did not. In addition, Weisheng-tang decreased brain infarction and edema and recovered neurological and motor deficit in a dose-dependent manner (30, 100, and 300 mg/kg). Weisheng-tang pretreatment resulted in significantly less BBB damage and higher brain microvasculature after focal cerebral ischemia. Tight junction proteins, such as zonula occludens-1 (ZO-1) and claudin-5, were preserved in Weisheng-tang-pretreated mice. Moreover, the ischemic brain in these mice showed suppressed PAR-1 and MMP-9 expression. In conclusion, our findings show that Weisheng-tang, which was selected through literature analysis but has not previously been used as a stroke remedy, exerts protective effects against ischemic brain damage and suggest its possible application for potential stroke patients, especially in the elderly.

1. Introduction

Stroke is one of the leading causes of serious and long-term disability worldwide, although stroke mortality has been declining [1]. Treatment of stroke has been traditionally focused on reducing ischemic cell death; however, clinical trials have shown that none of the tested neuroprotective drugs achieve clinical benefit for the treatment of acute stroke [2]. The failure of clinical trials provide evidence that new therapeutic strategies for acute stroke need to be explored; one such strategy might involve the preservation of the integrity of the blood-brain barrier (BBB) [2]. The BBB is a highly
In our previous study, we investigated the literature in the search for novel therapeutic strategies. Indeed, researchers have begun to consult traditional medicines for the development of conventional medicines. Products, which represent a promising source of new ingredients, can contribute to further progression of brain damage [4, 5]. Therefore, BBB disruption is recognized as a hallmark of stroke. We hypothesized that the inhibition of BBB disruption by Tongxuewan or Weisheng-tang could be effective in ameliorating ischemic brain damage. To investigate this hypothesis, we examined the protective effect of Tongxuewan or Weisheng-tang on the parameters of ischemic brain damage and BBB disruption, such as the infarct volume, brain edema, neurological deficits, motor function, and Evans blue extravasation using the focal cerebral ischemia mouse model.

In this study, we evaluated the protective effect of Tongxuewan or Weisheng-tang, which were selected by text-mining analysis, on brain damage in stroke. We hypothesized that the inhibition of BBB disruption by Tongxuewan or Weisheng-tang could be effective in ameliorating ischemic brain damage. To investigate this hypothesis, we examined the protective effect of Tongxuewan or Weisheng-tang on the parameters of ischemic brain damage and BBB disruption, such as the infarct volume, brain edema, neurological deficits, motor function, and Evans blue extravasation using the focal cerebral ischemia mouse model. In addition, we determined the expression of tight junction proteins, protease-activated receptor-1 (PAR-1), and matrix metalloproteinase-9 (MMP-9), to elucidate the mechanisms by which Weisheng-tang regulated BBB disruption.

2. Materials and Methods

2.1. Preparation of Herb Extracts. The components of Tongxuewan and Weisheng-tang (Table 1) were purchased from Hwalim Nature Drug (Busan, Korea). Tongxuewan (88 g) or Weisheng-tang (61.87 g) was boiled in 1.2 L of distilled water for 3 h; subsequently, the samples were filtered twice and concentrated using an evaporator equipped with a decomposition device (EYELA Co., Tokyo, Japan). After freeze drying (Labconco, Kansas City, MO), the extracts of Tongxuewan and Weisheng-tang obtained were 21.45 g and 13.8 g, respectively.

2.2. Animal Experiments. Male C57BL/6 mice were purchased from Hana Biotech (Ansan, Korea). Mice were housed under a 12 h light/dark cycle and allowed ad libitum access to food and water. The animal protocol used in this study was reviewed by the Pusan National University-Institutional Animal Care and Use Committee (PNU-IACUC) as per

---

**Table 1: Composition of Tongxuewan and Weisheng-tang.**

| Family                      | Scientific name          | Amount (g) |
|-----------------------------|--------------------------|------------|
| **Tongxuewan**              |                          |            |
| Cnidium officinale Makino   | Cnidii Rhizoma           | 40         |
| Angelica gigas Nakai        | Angelicae Gigantis Radix | 40         |
| Saposhnikovia divaricata Schiskin | Saposhnikovia Radix     | 40         |
| Schizonepeta tenuifolia Briquet | Schizonepetae Spica    | 40         |
| Rehmannia glutinosa Liborsch. var. purpurea Mak | Rehmanniae Radix     | 20         |
| Paeonia lactiflora Pall.    | Paeoniae Radix Rubra     | 20         |
| Glycyrrhiza glabra Linn.    | Glycyrrhizae Radix       | 20         |
| **Total**                   |                          | 220        |
| **Weisheng-tang**           |                          |            |
| Astragalus mongholicus Bunge| Astragali Radix          | 8          |
| Angelica gigas Nakai        | Angelicae Gigantis Radix | 8          |
| Paeonia lactiflora Pall.    | Paeoniae Radix Alba      | 8          |
| Glycyrrhiza glabra Linn.    | Glycyrrhizae Radix       | 4          |
| **Total**                   |                          | 28         |
their ethical procedures and scientific care, and it has been proven (PNU-2017-1477, PNU-2018-1828). Euthanasia is considered when eating less 40% of food and water, unstable breathing, and continuous for humane endpoint. We assessed and monitored the condition of the feeding and postsurgery daily for any signs of decreased activity and a decrease in food intake. We did not observe any signs of these conditions. Mice were orally administered 0.15 mL of each Tongxuewan or Weisheng-tang extract in 1x PBS at the appropriate concentration once per day for 4 days, as well as 1 h prior to focal cerebral ischemia (a total of five treatments). Experimental drugs, including PAR-1 agonist (TFLLR-NH$_2$: 3 μmol/kg in 40 μL saline, Tocris, Bristol, UK) or control peptide (RLLFT-NH$_2$: 3 μmol/kg in 40 μL saline, Tocris) [9], were injected into the tail vein, 30 min prior to ischemic brain injury.

2.3. Focal Cerebral Ischemia. Focal cerebral ischemia was induced by photothermalism of the cortical microvessels, as previously described [7]. Briefly, mice were anesthetized with 2% isoflurane in 20% O$_2$ and 80% N$_2$O; subsequently, they received an intraperitoneal (i.p.) injection of rose bengal (Sigma-Aldrich, St. Louis, MO) or control peptide (RLLFT-NH$_2$: 3 μmol/kg in 40 μL saline, Tocris) [9], were injected into the tail vein, 30 min prior to ischemic brain injury.

2.4. Infarct Volume and Edema. The brains were removed 24 h after ischemic injury. Subsequently, infarct size was determined using 2,3,5-triphenyltetrazolium chloride (TTC) staining of 2 mm thick brain sections. Infarct size was quantified using i-Solution software (Image & Microscope Technology, Vancouver, Canada). Measurements of the direct infarct volume included areas of the ipsilateral side that had sustained direct damage. The indirect infarct volume was calculated according to the following formula: contralateral hemisphere (mm$^3$)—undamaged ipsilateral hemisphere (mm$^3$). Edema was calculated by subtracting the direct infarct volume from the indirect infarct volume.

2.5. Neurological Score. Neurological deficits were evaluated 24 h after ischemic injury using the following scoring system: 1 = turning in the direction of the ipsilateral (nondamaged) side when held by the tail, 2 = turning in the direction of the contralateral (damaged) side and difficulty bearing weight, 3 = unable to bear weight on the contralateral side, and 4 = no spontaneous movement [7].

2.6. Wire-Grip Test. Vestibular-motor function was evaluated 24 h after the ischemic injury using the wire-grip test. Each mouse was suspended on a metal wire and forced to hang using both forepaws. Wire grip was scored as follows: 1 = not holding onto the wire; 2 = holding onto the wire using both forepaws and hind paws as well as the tail, without movement; 4 = moving on the wire using both forepaws, both hind paws, and tail; and 5 = moving well on the wire [7].

2.7. Determination of Evans Blue Leakage. BBB permeability was determined by the extravasation of Evans blue. Evans blue (2% in saline, 4 mL/kg; Sigma-Aldrich) was injected into the tail vein immediately after induction of focal cerebral ischemia. One hour after injection, mice were euthanized and transcardially perfused with iced PBS. The brains were removed; subsequently, the cortical area of each hemisphere was separated, weighed, and homogenized in 400 μL of N,N-dimethylformamide (Sigma-Aldrich, St. Louis, MO, USA). Following overnight incubation at 55°C, samples were centrifuged at 13,000 rpm for 30 min in 4°C. The absorbance of supernatant was measured at 620 nm via spectrophotometry, whereas Evans blue extravasation (μg/g of brain tissue) was quantified using a standard curve [7].

2.8. Immunofluorescence Staining. Mice were perfused with cold PBS followed by 4% paraformaldehyde 24 h after focal cerebral ischemia. Immediately thereafter, brains were harvested and further fixed for 24 h in 4% paraformaldehyde; subsequently, they were cryoprotected in 30% sucrose for 72 h at 4°C. Each brain was frozen in optical cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA) and stored at -80°C until analysis. The frozen brains were sectioned (thickness: 20 μm) using a CM 3050 cryostat (Leica Microsystems, Wetzlar, Germany). The brain sections were immunostained with anti-CD-31 (1:100, Invitrogen Corporation, Carlsbad, CA), anti-claudin-5 (1:100, Invitrogen Corporation), anti-PAR-1 (1:100, Novus, Centennial, CO), and anti-MMP-9 (1:100, R&D systems, Minneapolis, MN) overnight at 4°C. They were subsequently incubated with Alexa 488 or Alexa 594-conjugated secondary antibodies (1:500, Life Technologies, Carlsbad, CA) for 2 h in total darkness. 4′,6-Diamidino-2-phenylindole (DAPI, Molecular Probes) was used for the staining of the nuclei. Fluorescence images were visualized using a Zeiss LSM 700 laser scanning confocal device (Carl Zeiss). The images were quantified using MetaMorph Microscopy Automation and Image Analysis Software (Molecular Devices, San Jose, CA), i-Solution software (Image & Microscope Technology), and Imagej (NIH).

2.9. Statistical Analysis. Data are presented as the mean ± standard error of the mean (SEM). Statistical comparisons between different groups were performed using Student’s t-tests. Values of p < 0.05 were considered statistically significant.

3. Results

3.1. Pretreatment Effects of Tongxuewan or Weisheng-Tang on Ischemic Brain Damage. We evaluated the pretreatment effects of Tongxuewan or Weisheng-tang on ischemic brain injury. Mice underwent oral administration of Tongxuewan...
Weisheng-tang (500 mg/kg) once daily for 4 days prior to the induction of ischemia as well as 1 h prior to the procedure (Figure 1(a)). The outcome of the acute stroke was assessed on day 1 after focal cerebral ischemia. Direct infarct volume and edema were significantly reduced in mice treated with Weisheng-tang at a dose of 500 mg/kg, whereas Tongxuewan had no significant effect compared with control mice (Figures 1(b)–1(d)). In addition, mice treated with Weisheng-tang showed significant improvement in neurologic (Figure 1(e)) and motor (Figure 1(f)) functions

Figure 1: Effects of Tongxuewan and Weisheng-tang on brain infarction, edema, and behavior following ischemic brain injury. (a) Mice were pretreated via oral administration of 1000 mg/kg of Tongxuewan (n = 9), 500 mg/kg Weisheng-tang (n = 8), or PBS (control group, n = 9) once per day for 4 days prior to ischemic injury as well as 1 h prior to the procedure. Twenty-four hours after ischemic brain injury, the mouse brains were harvested and stained with 2% TTC solution. (b) Representative images of 2,3,5-triphenyltetrazolium chloride-stained brain coronal sections of mice. White region indicates the infarct area. (c, d) Quantification analysis of direct infarct volume (c) and edema (d). *p < 0.05 vs. control group. (e, f) Neurological score (e) and wire-grip tests (f) were performed to evaluate functional outcomes. *p < 0.05 vs. control group. TTC: 2,3,5-triphényltetrazolium chloride.
compared to the control mice, suggesting that the smaller infarct volumes translated into better functional outcomes in the Weisheng-tang-treated group.

3.2. Dose-Dependent Effects of Weisheng-Tang on Ischemic Brain Damage. To determine whether Weisheng-tang exerts dose-dependent effects on ischemic brain injury, mice were pretreated with 30, 100, or 300 mg/kg of Weisheng-tang (Figure 2(a)). Our findings indicated that Weisheng-tang produced dose-dependent reductions in direct infarct and edema volume; moreover, the most significant reductions in infarct volume and edema were observed in mice pretreated with 300 mg/kg of Weisheng-tang (Figures 2(b)–2(d)). Next, we further observed dose-dependent effects of Weisheng-tang pretreatment on neurologic deficits and motor function. Significant improvements in functional outcomes were
Figure 3: Effects of Weisheng-tang on BBB disruption following ischemic brain injury. Mice were pretreated via oral administration of 30, 100, or 300 mg/kg of Weisheng-tang (n = 4–6) or PBS (control group, n = 6) once per day for 4 days prior to ischemic insult, as well as 1 h prior to the procedure. Evans blue (4 mg/kg) was intravenously injected immediately following ischemic insult. (a) Representative photographs of Evans blue leakage in control or Weisheng-tang groups 1 h after ischemic injury and quantification of Evans blue extravasation. * p < 0.01 vs. control group. (b) Endothelial cell staining (CD31, green) shows significantly higher vessel density in the ipsilateral cerebral cortex of the Weisheng-tang-treated group (n = 9 in each group). * p < 0.05 vs. control group. Scale bar = 50 μm. (c–e) Confocal images show costaining of tight junction proteins markers ZO-1 (zonula occludens-1) and claudin-5 (red) with CD31 (green) in the peri-infarct region of control and Weisheng-tang-treated mice. Mice pretreated with Weisheng-tang (300 mg/kg) exhibited increased expression of the tight junction proteins ZO-1 (c) and claudin-5 (d) following focal cerebral ischemia. Representative photographs of ZO-1 (c) and claudin-5 (d). CD31 staining for blood vessels indicated in green. Scale bar = 50 μm. Quantification graphs of ZO-1 and claudin-5 immunofluorescence (e) (n = 9 each, * p < 0.05 vs. control group).
observed in mice pretreated with 300 mg/kg of Weisheng-tang, relative to those observed in the control group (Figures 2(e) and 2(f)).

3.3. Effects of Weisheng-Tang on BBB Disruption following Ischemic Brain Injury. Because Weisheng-tang reduced ischemic brain edema, we evaluated the effect of Weisheng-tang on the BBB breakdown. The BBB permeability was assessed by measuring extravasation of Evans blue. Mice were pretreated with 30, 100, or 300 mg/kg of Weisheng-tang, BBB disruption was induced by photothrombotic ischemia, and Evans blue solution injected into mice extravasates through the damaged BBB (Figure 3(a)). Weisheng-tang produced dose-dependent reductions in BBB disruption, and 300 mg/kg Weisheng-tang significantly reduced BBB disruption compared to that observed in the control group (Figure 3(a)). To examine whether Weisheng-tang also affects the blood vessels in the cerebral cortex after focal cerebral ischemia, we measured the levels of blood vessel with the specific marker CD31 at the peri-infarct region (Figure 3(b)). The numbers of CD31+ cells were significantly greater in the Weisheng-tang-treated group than in the control group, indicating that Weisheng-tang can preserve blood vessel in the ischemic area. Next, we observed the effect of Weisheng-tang on the tight junction proteins for BBB maintenance (Figures 3(c)–3(e)). Immunofluorescence staining revealed increased levels of ZO-1 and claudin-5 following cerebral ischemia in the Weisheng-tang-treated group, suggesting that Weisheng-tang reduces the degree of BBB disruption by increasing the levels of ZO-1 and claudin-5.

3.4. Effects of Weisheng-Tang on PAR-1 Activation following Ischemic Brain Injury. Next, we examined the effects of Weisheng-tang treatment on the expression of a specific thrombin receptor, PAR-1, which has been known to trigger the development of neuronal damage associated with ischemia and BBB breakdown [10]. In line with the results of previous studies, our results showed a high level of PAR-1 expression in the peri-infarct region after focal cerebral ischemia (data not shown), indicating that PAR-1 induction contributes to the expansion of brain damage. Despite an increase in the CD31+ area, Weisheng-tang pretreatment caused significant decreases in PAR-1 expression in the perivascular region (Figures 4(a) and 4(b)), suggesting that Weisheng-tang may protect brain damage via PAR-1 reduction. To confirm this, we further investigated whether PAR-1 mediated the infarct volume and edema reduction, which was functionally improved by Weisheng-tang. As seen in Figure 5, the PAR-1 agonist TFLLR-NH2 [9] combined with Weisheng-tang reversed the protective effect of Weisheng-tang on the infarct and edema volume and neurological and motor function (Figure 5). These findings indicate that pretreatment with Weisheng-tang decreased ischemic brain damage, possibly by PAR-1 suppression.

Next, we examined the effects of Weisheng-tang treatment on the levels of the zinc-containing protease MMP-9. Mice pretreated with Weisheng-tang showed a significant reduction in MMP-9 expression in the perivascular region (Figures 6(a) and 6(b)). These results suggest that MMP-9 reduction by Weisheng-tang may contribute towards the amelioration of the BBB disruption and expansion of brain edema following ischemia.

4. Discussion

We evaluated the protective effects of Tongxuewan and Weisheng-tang selected by text-mining analysis of Dongeui-bogam, the ancient Korean medical literature, on ischemic brain damage. Weisheng-tang significantly reduced ischemic brain damage; however, Tongxuewan did not. In addition,
Weisheng-tang showed less BBB damage via downregulation of tight junction proteins and suppression of PAR-1 and MMP-9 in the ischemic brain. The present study suggests that the protective effect of Weisheng-tang on brain ischemic injury involves its ability to attenuate BBB disruption and expansion of brain edema.

According to the Dongeuibogam, Tongxuewan has long been used to treat the vascular diseases that are caused by blocked blood flow, whereas Weisheng-tang has been used in individuals who suffer from exhaustion and indigestion causing diarrhea [8]. In the present study, our findings indicated that Weisheng-tang treatment significantly reduced direct infarct and edema volume, in addition to dose-dependent increases in neurological and vestibular motor functions (Figures 1 and 2) after focal cerebral ischemia. In contrast, treatment with Tongxuewan produced no significant reductions in the extent or functional impact of ischemic injury (Figure 1). Such findings indicate that Weisheng-tang, which was screened according to certain criteria through literature analysis, exerts protective effects against ischemic brain damage, although it has not previously been used as a stroke remedy.

The blood-brain barrier (BBB) is a specialized barrier consisting of endothelial cells, tight junctions, pericytes, astrocytic end-feet processes, and the basement membrane; it is crucial in the regulation of the passage of ions, proteins, and inflammatory cells between the plasma and brain [3]. Disruption of the BBB in ischemic stroke causes dramatic changes in the chemical and cellular composition of this environment, which can contribute to further progression of brain damage [4, 5]; thus, this makes the BBB an important target to reduce brain damage in stroke. Tight junctions

Weisheng-tang showed less BBB damage via downregulation of tight junction proteins and suppression of PAR-1 and MMP-9 in the ischemic brain. The present study suggests that the protective effect of Weisheng-tang on brain ischemic injury involves its ability to attenuate BBB disruption and expansion of brain edema.

According to the Dongeuibogam, Tongxuewan has long been used to treat the vascular diseases that are caused by blocked blood flow, whereas Weisheng-tang has been used in individuals who suffer from exhaustion and indigestion causing diarrhea [8]. In the present study, our findings indicated that Weisheng-tang treatment significantly reduced direct infarct and edema volume, in addition to dose-dependent increases in neurological and vestibular motor functions (Figures 1 and 2) after focal cerebral ischemia. In contrast, treatment with Tongxuewan produced no significant reductions in the extent or functional impact of ischemic injury (Figure 1). Such findings indicate that Weisheng-tang, which was screened according to certain criteria through literature analysis, exerts protective effects against ischemic brain damage, although it has not previously been used as a stroke remedy.

The blood-brain barrier (BBB) is a specialized barrier consisting of endothelial cells, tight junctions, pericytes, astrocytic end-feet processes, and the basement membrane; it is crucial in the regulation of the passage of ions, proteins, and inflammatory cells between the plasma and brain [3]. Disruption of the BBB in ischemic stroke causes dramatic changes in the chemical and cellular composition of this environment, which can contribute to further progression of brain damage [4, 5]; thus, this makes the BBB an important target to reduce brain damage in stroke. Tight junctions

Figure 5: The protective effect of Weisheng-tang on ischemic brain damage was mediated by PAR-1 reduction. Mice were pretreated via oral administration of 300 mg/kg of Weisheng-tang \((n = 7, \text{ each group})\) or PBS (sham group, \(n = 7\)) once daily for 4 days prior to ischemic insult, as well as 1 h prior to the procedure. PAR-1 agonist (TFLLR-NH₂: 3 μmol/kg in 40 μL saline) or control peptide (RLLFT-NH₂: 3 μmol/kg in 40 μL saline) was injected into the tail vein 30 min prior to ischemic brain injury. Twenty-four hours after focal cerebral ischemia, the mouse brains were harvested and stained with 2% TTC solution. (a) Representative photographs of brain sections stained with TTC. White region indicates the infarct area. (b–e) Quantification graphs of direct infarct volume (b) and edema (c). Neurological score (d) and wire-grip tests (e) were performed to evaluate functional outcomes. \(* p < 0.05\) and \(** p < 0.01\) vs. control group; \(\# p < 0.05\) and \(\## p < 0.01\) vs. control peptide cotreated group with Weisheng-tang. TTC: 2,3,5-triphenyltetrazolium chloride.
in the brain endothelial cells maintain BBB integrity and consist of different proteins, such as claudins and occludins [11]; in acute stroke, there is degradation of tight junctions resulting in the loss of vascular integrity [12]. We found that Weisheng-tang pretreatment significantly reduces BBB leakage and increases endothelial cells (Figures 3(a) and 3(b)). When we examined the expression pattern of ZO-1 and claudin-5 in the capillaries of the peri-infarct region, their expression pattern was clearly disrupted in the control group (Figures 3(c) and 3(d)), whereas relatively homogenous distributions and increased expression were observed in Weisheng-tang-treated mice (Figures 3(c)–3(e)). The results of the present study indicate that Weisheng-tang pretreatment reduces BBB disruption and increases levels of ZO-1 and claudin-5 in the ischemic brain.

Protease-activated receptors (PARs) are G protein-coupled receptors that convert an extracellular proteolytic cleavage event by thrombin into transmembrane signaling [13, 14]. Four members of PARs have been cloned (PAR-1, PAR-2, PAR-3, and PAR-4) in diverse neural cells of the brain [15]; however, PAR-1 is the major thrombin-activated receptor in humans [16]. Many previous studies used an animal model cerebral ischemia to determine whether pharmacological manipulation of PAR-1 signaling could provide an attractive drug discovery target for possible treatments of brain damage associated with ischemic damage and BBB breakdown [10]. PAR-1-mediated neurovascular damage during cerebral ischemia was demonstrated using in vivo and in vitro knockdown of PAR-1 [17] and an animal model of PAR-1 deficiency [18]. In addition, suppression of PAR-1 activity plays a role in the maintenance of microvascular integrity in rats undergoing subarachnoid hemorrhage [19]. Injury-induced BBB breakdown sufficient to allow extravasation of PAR-1 activators, such as thrombin, may be a result of the PAR-1-mediated mechanism underlying the pathogenesis of brain injury [20]. In the present study, we found that Weisheng-tang pretreatment significantly decreases PAR-1 expression in the perivascular region (Figures 4(a) and 4(b)); moreover, a PAR-1 agonist TFLLR-NH₂ [9] reversed the protective effect of Weisheng-tang on ischemic brain damage (Figure 5). Collectively, these findings indicate that Weisheng-tang pretreatment may attenuate BBB dysfunction and edema following ischemic brain injury by promoting the increased expression of ZO-1 and claudin-5 and inhibition of PAR-1. However, further studies are required to investigate the mechanisms underlying the effects of Weisheng-tang on the expression of tight junction proteins and PAR-1.

Matrix metalloproteinase-9 (MMP-9) plays a key role in protease-mediated physiological and pathological changes in BBB breakdown [21, 22]. In ischemic stroke, increased MMP-9 in the damaged brain is one of the significant causes of BBB breakdown [22]; moreover, PAR-1 is a main receptor for thrombin-induced expression of MMP-9 and pathological changes in BBB breakdown [21]. BBB-constituting cells, including the brain microvascular endothelial cells, astrocytes, and brain pericytes, can release MMP-9 upon thrombin stimulation [21, 23, 24]. Lastly, we reported that treatment of PAR-1 antagonists inhibited breakdown of BBB via the downregulation of MMP-9 expression and preservation of the expression of tight junction proteins in the brain [25]. In this study, we showed Weisheng-tang pretreatment significantly increases MMP-9 expression in the perivascular region (Figures 6(a) and 6(b)), suggesting that
Weisheng-tang can prevent ischemic brain injury by stabilizing the disrupted BBB via downregulation of PAR-1 and MMP-9 and upregulation of tight junction proteins.

5. Conclusion
In the present study, we selected two Korean herbal medicine formulas, Weisheng-tang and Tongxuewan, through Dongguibogam text-mining analysis, and evaluated their protective effects on BBB disruption and brain damage in stroke. Weisheng-tang exerts protective effects against ischemic brain damage and promotes the recovery of neurological and motor function after focal cerebral ischemia, although it has not previously been used as a stroke remedy. Further experimental and clinical investigations of Weisheng-tang may aid in the development of novel therapeutic strategies for ischemic stroke.

Data Availability
The data used to support the findings of this study are available from the corresponding authors upon request.

Conflicts of Interest
The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors’ Contributions
Min Jae Kim, Seo-Yeon Lee, Byung Tae Choi, Jin Ung Baek, and Hwa Kyoung Shin participated in research design. Min Jae Kim, Ki Hyun Park, and Ji Yun Lee conducted experiments. Young Ju Yun, Ki-Tae Ha, and Jin Ung Baek contributed new reagents or analytic tools. Min Jae Kim, Seo-Yeon Lee, and Hwa Kyoung Shin performed data analysis. Ki Hyun Park, Seo-Yeon Lee, Byung Tae Choi, Jin Ung Baek, and Hwa Kyoung Shin wrote or contributed to the writing of the manuscript. Min Jae Kim and Ki Hyun Park contributed equally to this work.

Acknowledgments
This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIP) (2014R1A5A2009936; 2019R1A2C1087515).

References
[1] D. T. Lackland, E. J. Roccella, A. F. Deutsch et al., "Factors influencing the decline in stroke mortality: a statement from the American Heart Association/American Stroke Association," Stroke, vol. 45, no. 1, pp. 315–353, 2014.
[2] M. A. Moskowitz, E. H. Lo, and C. Iadecola, "The science of stroke: mechanisms in search of treatments," Neuron, vol. 67, no. 2, pp. 181–198, 2010.
[3] P. Ballabh, A. Braun, and M. Nedergaard, "The blood-brain barrier: an overview: structure, regulation, and clinical implications," Neurobiology of Disease, vol. 16, no. 1, pp. 1–13, 2004.
[4] U. Dirnagl, "Pathobiology of injury after stroke: the neurovascular unit and beyond," Annals of the New York Academy of Sciences, vol. 1268, no. 1, pp. 21–25, 2012.
[5] E. H. Lo, "A new penumbra: transitioning from injury into repair after stroke," Nature Medicine, vol. 14, no. 5, pp. 497–500, 2008.
[6] F. E. Koehn and G. T. Carter, "The evolving role of natural products in drug discovery," Nature Reviews Drug Discovery, vol. 4, no. 3, pp. 206–220, 2005.
[7] M. J. Kim, S. Y. Lee, J. Y. Hwang et al., "Pretreatment with Shuanghe-tang extract attenuates postischemic brain injury and edema in a mouse model of stroke: an analysis of medicinal herbs listed in Dongui Bogam," Oxidative Medicine and Cellular Longevity, vol. 2018, Article ID 2479602, 14 pages, 2018.
[8] J. Heo, A New Enlarged Edition, A Translation Printed Side by Side With Original Dongguibogam, Bubin Publishers Co, Seoul, 2012.
[9] L. de Garavilla, N. Vergnolle, S. H. Young et al., “Agonists of proteinase-activated receptor 1 induce plasma extravasation by a neurogenic mechanism,” British Journal of Pharmacology, vol. 133, no. 7, pp. 975–987, 2001.
[10] C. E. Junge, T. Sugawara, G. Mannaioni et al., “The contribution of protease-activated receptor 1 to neuronal damage caused by transient focal cerebral ischemia,” Proceedings of the National Academy of Sciences of the United States of America, vol. 100, no. 22, pp. 13019–13024, 2003.
[11] H. Wolburg and A. Lippoldt, “Tight junctions of the blood-brain barrier: development, composition and regulation,” Vascular Pharmacology, vol. 38, no. 6, pp. 323–337, 2002.
[12] M. Nour, F. Scalzo, and D. S. Liebeskind, "Ischemia-reperfusion injury in stroke," Interventional Neurology, vol. 1, no. 3-4, pp. 185–199, 2013.
[13] S. R. Coughlin, "How the protease thrombin talks to cells," Proceedings of the National Academy of Sciences of the United States of America, vol. 96, no. 20, pp. 11023–11027, 1999.
[14] S. R. Coughlin, "Thrombin signalling and protease-activated receptors," Nature, vol. 407, no. 6801, pp. 258–264, 2000.
[15] W. Luo, Y. Wang, and G. Reiser, "Protease-activated receptors in the brain: receptor expression, activation, and functions in neurodegeneration and neuroprotection," Brain Research Reviews, vol. 56, no. 2, pp. 331–345, 2007.
[16] G. Cirino and B. Severino, "Thrombin receptors and their antagonists: an update on the patent literature," Expert Opinion on Therapeutic Patents, vol. 20, no. 7, pp. 857–884, 2010.
[17] P. S. Rajput, P. D. Lyden, B. Chen et al., "Protease activated receptor-1 mediates cytotoxicity during ischemia using in vivo and in vitro models," Neuroscience, vol. 281, pp. 229–240, 2014.
[18] J. Wang, H. Jin, Y. Hua, R. F. Keep, and G. Xi, "Role of protease-activated receptor-1 in brain injury after experimental global cerebral ischemia," Stroke, vol. 43, no. 9, pp. 2476–2482, 2012.
[19] J. Yan, A. Manaenko, S. Chen et al., "Role of SCH779797 in maintaining vascular integrity in rat model of subarachnoid hemorrhage," Stroke, vol. 44, no. 5, pp. 1410–1417, 2013.
[20] O. Nicole, A. Goldshmidt, C. E. Hamill et al., "Activation of protease-activated receptor-1 triggers astrogliosis after brain injury," The Journal of Neuroscience, vol. 25, no. 17, pp. 4319–4329, 2005.
[21] M. S. Choi, Y. E. Kim, W. J. Lee et al., “Activation of protease-activated receptor1 mediates induction of matrix metalloproteinase-9 by thrombin in rat primary astrocytes,” *Brain Research Bulletin*, vol. 76, no. 4, pp. 368–375, 2008.

[22] A. Rosell and E. H. Lo, “Multiphasic roles for matrix metalloproteinases after stroke,” *Current Opinion in Pharmacology*, vol. 8, no. 1, pp. 82–89, 2008.

[23] K. Kolev, J. Skopal, L. Simon, E. Csonka, R. Machovich, and Z. Nagy, “Matrix metalloproteinase-9 expression in post-hypoxic human brain capillary endothelial cells: H$_2$O$_2$ as a trigger and NF-$\kappa$B as a signal transducer,” *Thrombosis and Haemostasis*, vol. 90, no. 3, pp. 528–537, 2003.

[24] T. Machida, F. Takata, J. Matsumoto et al., “Brain pericytes are the most thrombin-sensitive matrix metalloproteinase-9-releasing cell type constituting the blood-brain barrier in vitro,” *Neuroscience Letters*, vol. 599, pp. 109–114, 2015.

[25] H. N. Kim, Y. R. Kim, S. M. Ahn, S. K. Lee, H. K. Shin, and B. T. Choi, “Protease activated receptor-1 antagonist ameliorates the clinical symptoms of experimental autoimmune encephalomyelitis via inhibiting breakdown of blood-brain barrier,” *Journal of Neurochemistry*, vol. 135, no. 3, pp. 577–588, 2015.