Agomelatine prevents macrophage infiltration and brain endothelial cell damage in a stroke mouse model

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ABSTRACT

Background and purpose: Ischemic/reperfusions are regarded as the clinical consensus for stroke treatment, which results in secondary injury of brain tissues. Increased blood-brain barrier (BBB) permeability and infiltration of inflammatory cells are responsible for the ischemic/reperfusion injury. In the present study, we aimed to investigate the effects of Agomelatine on brain ischemic/reperfusions injury and the underlying mechanism.

Methods: MCAO model was established in mice. The expressions of CD68 and claudin-5 in the cerebral cortex were determined using an immunofluorescence assay. Brain permeability was evaluated using Evans blue staining assay. A two-chamber and two-cell trans-well assay was used to detect the migration ability of macrophages through endothelial cells. The expression levels of claudin-5 and MCP-1 in the endothelial cells were determined using qRT-PCR and ELISA.

Results: CD68 was found to be up-regulated in the cerebral cortex of MCAO mice but was down-regulated by treatment with Agomelatine. The expression level of down-regulated claudin-5 in the cerebral cortex of MCAO mice was significantly suppressed by Agomelatine. Deeper staining of Evans blue was found in the MCAO group, which was however faded significantly in the Agomelatine treated MCAO mice. The migrated macrophages were significantly increased by hypoxia incubation but were greatly suppressed by the introduction of Agomelatine. The down-regulated claudin-5 by hypoxic incubation in endothelial cells was up-regulated by treatment with Agomelatine. Furthermore, the increased expression of MCP-1 in endothelial cells under hypoxic conditions was significantly inhibited by Agomelatine.

Conclusion: Agomelatine prevents macrophage infiltration and brain endothelial cell damage in a stroke mouse model.

INTRODUCTION

Stroke is defined as a syndrome of neurological deficits induced by local or holistic ischemia and hypoxia of brain tissue resulting from an obstruction in cerebrovascular circulation [1, 2]. Focal neurological deficits are reported as the main characteristics of stroke, the clinical symptoms of which include sudden inactiveness on one side of the body, language disorder, and tactus disorders, such as impaired vision and hearing, dizziness, ear irritation, and swallowing difficulty [3]. Reports state that stroke is the secondary inducer of dementia-related brain injury and death amongst the elderly population with more than 60 years of age. The cost of treating stroke takes 3–7% of the overall medical expenditure in developed countries [4]. In China, the mortality of cardiovascular disease has remained in the top three for many years and increases with aging [5]. Stroke adversely affects the quality of life of patients and is also burdensome to the patients'

families and society at large. It is of great significance to investigate the pathological mechanism of stroke and explore the potential therapeutic treatments. Ischemic/reperfusion was found to be harmful to the recovery of the tissues when people noticed that a new type of tissue injury could arise from the sudden oxygen supply post-long-term hypoxia, which is also verified in the aggravation of brain dysfunction after ischemic/reperfusion on brain tissues [6, 7]. However, currently, quick recovery of the blood supply has been regarded as the clinical consensus for the treatment of stroke, in which way, ischemic/reperfusion is inevitable. Therefore, the injury to brain tissues resulting from ischemic/reperfusion has been taken as a tough task post clinical stroke treatment.

It is reported that the permeability of the BBB is promoted greatly after the ischemic/reperfusion treatments for stroke, the mechanism of which is currently unknown [8]. With the increase in BBB permeability, inflammation is induced by chemical mediators and infiltration of inflammatory cells, such as macrophages, which further enhances the permeability of the BBB [9]. The BBB is a tight structure that mainly consists of blood endothelial cells, astrocytes, and extracellular matrix [10]. The BBB is connected by tight junction proteins on the endothelial cell surface. There are three types of transmembrane tight junction proteins: claudins, occludin, and junctional adhesion molecules. At the BBB, claudin-5 is the most highly expressed tight junction protein and its dysfunction has been implicated in stroke and brain injury [11]. In the early stage of permeability increase, a widened endothelial gap could be observed instead of any obvious injury. However, as the permeation of the BBB proceeded, inflammatory and chemotactic factors were produced in quantity by astrocytes or infiltrated macrophages around endothelial cells and vessels [12]. One of the key chemotactic factors is MCP-1, and the high levels of MCP-1 have been found in the brain several hours after cerebral ischemia [13]. The expressions of adhesion molecules in endothelial cells are up-regulated by chemical mediators, such as interleukins and tumor necrosis factors. To recruit the leukocytes to the injured location, the metalloprotease (MMP) is activated, by which the ligandin and extracellular matrix are degraded in endothelial cells [14, 15]. The pathological state of brain tissues is further aggravated by up-regulated inflammatory factors, chemotactic factors, MMP, and adhesion molecules [16, 17]. Preventing the infiltration of inflammatory cells, such as macrophages, down-regulating the expressions of chemotactic factors may be an effective way to decrease the permeability of the BBB and protect the

brain from the injury by ischemic/reperfusion treatments.

Agomelatine is the first line medication for the treatment of depression. Agomelatine's antidepressant actions attribute to its sleep-promoting and modulation of circadian rhythm by activating melatonin MT1 and MT2 receptors in the suprachiasmatic nucleus and its blockage of 5-HT2c receptors in the hippocampus, amygdale, and prefrontal cortex [18]. Agomelatine treatment inhibits melatonin synthesis, promotes dopamine production in the prefrontal lobe, accelerates epinephrine release in the nucleus ceruleus, and promotes neuron growth [19, 20]. Besides its action on the CNS, Agomelatine exhibits broad effects in other tissues. It has been proposed that the melatonin receptor agonists could possess cardiovascular benefit [21, 22]. Recent studies further reveal the potential effects of their modulation in the cardiovascular system. The experiment *in vitro* shows that Agomelatine alleviates oxidative stress by inhibiting ROS production and promoting antioxidant levels [23]. In a preclinical animal experiment, Agomelatine administration protected animals from lipopolysaccharide-induced cardiovascular toxicity by suppressing the NF-kβ pathway [24]. An anti-depression study found that Agomelatine inhibits the infiltration and polarization of macrophages [25]. Agomelatine therapy increases circulated brain-derived neurotrophic factor BDNF levels but reduces circulating CRP levels in depressive patients [26, 27]. The evidence indicates that the anti-depression drug Agomelatine possesses anti-ROS and anti-inflammatory properties, and it is a highly interesting topic to evaluate the potential therapeutic effect in the cardiovascular disease model. In the present study, the anti-inflammatory effects of Agomelatine on endothelial cells were investigated to explore its potential therapeutic effects on injury induced by ischemic/reperfusion treatments for stroke.

RESULTS

Agomelatine inhibited macrophage infiltration in the cortex of the MCAO mouse model

To evaluate the effects of Agomelatine on the infiltration of macrophages in MCAO mice, the MCAO mice were treated with Agomelatine and the cortex was isolated to determine the expression of CD68 by immunofluorescence, which is a representative marker of M2 macrophages. The structure of Agomelatine is shown in Figure 1. As shown in Figure 2, CD68 was found to be up-regulated in the cortex isolated from MCAO mice, compared to the control, which was

significantly reversed by the treatment with Agomelatine. These data indicate that the infiltration of macrophages into the cortex was greatly increased by establishing the MCAO model in mice and was alleviated by Agomelatine.

Agomelatine promoted the expression of the down-regulated tight junction protein in the MCAO mouse model

To explore the effects of Agomelatine on the tight junctions between endothelial cells in MCAO mice, the expression level of claudin-5 was evaluated. As shown in Figure 3, claudin-5 was significantly down-regulated in the cortex by MCAO modeling in mice but was greatly up-regulated by the introduction of Agomelatine, indicating that the damaged tight junction by MCAO modeling was repaired by Agomelatine.

BBB permeability in MCAO mice was diminished by Agomelatine

The permeability of the BBB is of great importance in preventing damage to brain tissues by chemical materials and infiltrated inflammatory cells. Following MCAO modeling and Agomelatine administration, the permeability of the BBB of each animal was evaluated by the Evans blue staining assay. As shown in Figure 4, deeper staining of Evans blue was found in the MCAO group which was however faded significantly in Agomelatine treated MCAO animals. These data indicate that the increased BBB permeability by MCAO modeling was reversed by Agomelatine.

The migration of macrophages was suppressed by Agomelatine in hypoxic endothelial cells

Following hypoxic incubation, bEnd.3 cells were incubated with 10 μM Agomelatine. A two-chamber two-cell trans-well assay was performed to test the migration ability of macrophages through bEnd.3 cells. Figure 5A shows the representative schematic of the assay. BEnd.3 endothelial cells were plated in the bottom chamber and macrophages (IC21 cells) were suspended in the top chamber and allowed to migrate through the endothelial cells layer. As shown in Figure 5B and 5C, compared to the control, the number of migrated macrophages was significantly increased by hypoxia incubation, but was greatly suppressed by the introduction of Agomelatine, indicating that hypoxia incubation-induced macrophage migration was inhibited by Agomelatine.
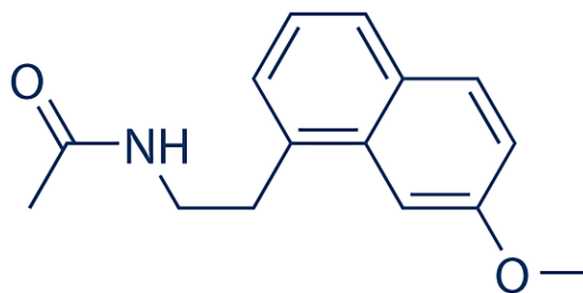

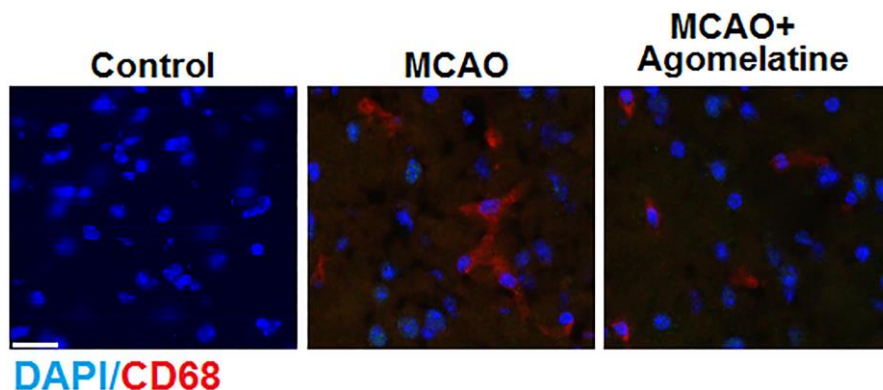**Figure 1. Molecular structure of Agomelatine.**

Figure 2. **Agomelatine prevents macrophage infiltration in the cortex of the MCAO mouse model.** Representative images of staining for macrophages using CD68 in the cerebral cortex 3 days post-tMCAO. Scale bar, 100 μM.
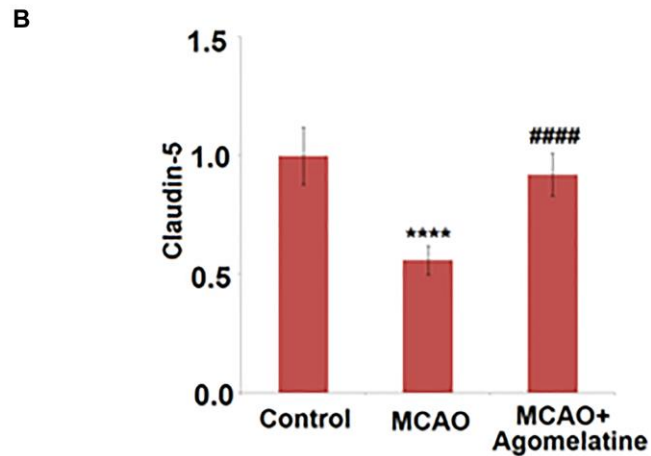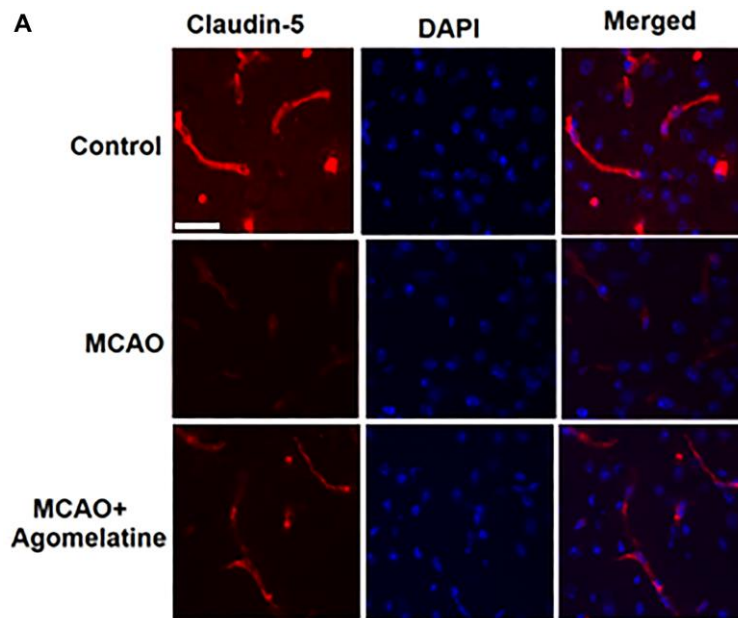
Figure 3. Agomelatine restored the reduction of tight junction protein expressions in the MCAO mouse model. (A). Representative images of staining for claudin-5 in the cerebral cortex 3 days post-tMCAO; (B). Quantification of claudin-5 staining. Scale bar, 100 µM (****$P < 0.0001$ vs. vehicle group; ####$P < 0.0001$ vs. MCAO group).
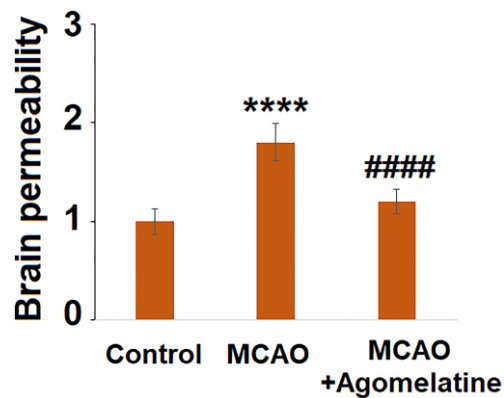

Figure 4. Agomelatine protects against impairment of brain permeability in MCAO mice. Brain permeability of MCAO mice was measured by Evans blue staining assay (****$P < 0.0001$ vs. vehicle group; ####$P < 0.0001$ vs. MCAO group).

The expression level of claudin-5 was up-regulated by Agomelatine in hypoxic endothelial cells

To evaluate the effects of Agomelatine on the tight junctions between hypoxic endothelial cells, the expression level of claudin-5 was evaluated by qRT-PCR and western blot analysis. As shown in Figure 6, claudin-5 was significantly down-regulated by hypoxic incubation, the expression of which was greatly restored by Agomelatine. These data indicate that the injured tight junctions between endothelial cells by hypoxic incubation were significantly repaired by Agomelatine.

MCP-1 was down-regulated by Agomelatine in hypoxic endothelial cells

To explore the effects of Agomelatine on the production of chemotactic factors, the expression of MCP-1, a representative chemotactic factor, was determined in the hypoxic endothelial cells in the presence or absence of Agomelatine. As shown in Figure 7, MCP-1 was greatly up-regulated by hypoxic incubation, the expression of which was significantly decreased by Agomelatine,

indicating that the chemotactic function of endothelial cells to inflammatory cells, such as macrophages, was suppressed by Agomelatine.

DISCUSSION

The brain is the most sensitive organ to hypoxia in mammals and vital activity within the brain is mainly dependent on the energy provided by the aerobic oxidation of glucose. Therefore, irreversible injury is induced under a long-time hypoxic environment [28, 29]. Thrombolytic therapy and mechanical recanalization are effective methods for blood re-supply after ischemia. However, for some specific patients, the injury induced by ischemia can be aggravated by ischemic/reperfusion, which is termed as "brain ischemic/reperfusion injury" [30].

Multiple types of pathological processes are involved in the injury, including changes in energy and amino acid metabolism, the cellular overload of calcium, activation of reactive oxygen species, inflammatory reactions, and damage of the BBB, which culminates in the apoptosis
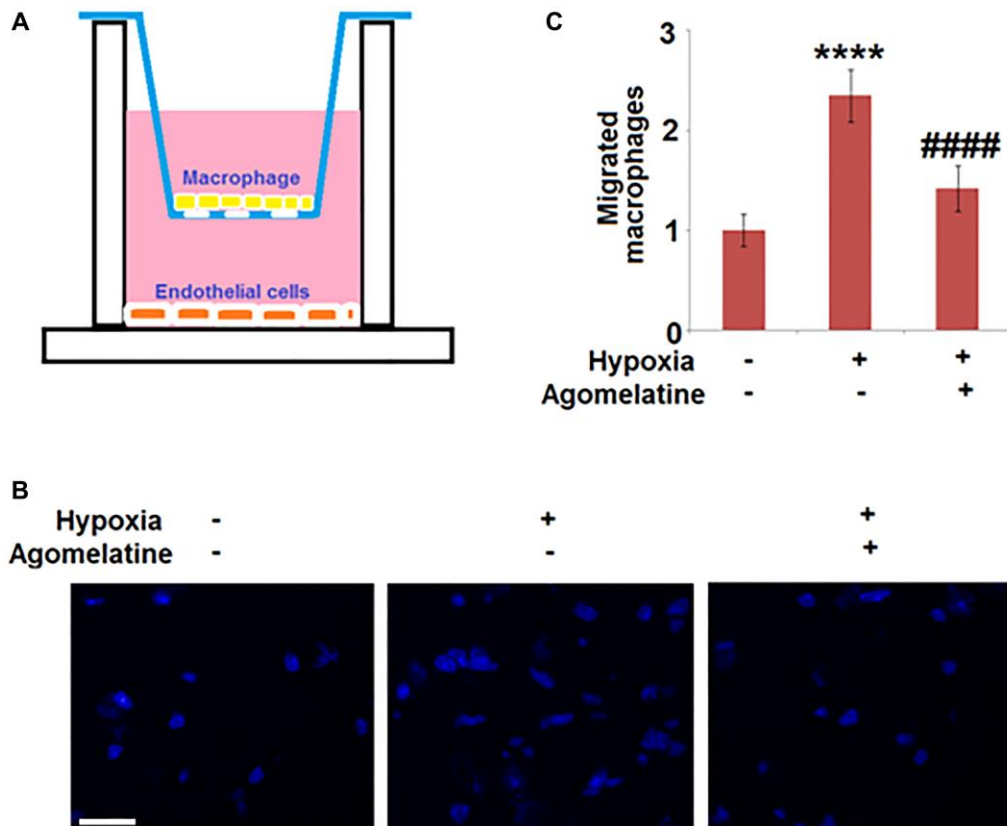

Figure 5. **Agomelatine treated hypoxic endothelial cells inhibit macrophage migration.** Brain bEnd.3 endothelial cells were incubated with 10 μM Agomelatine in the process of hypoxia/ reperfusion. (**A**). A representative schematic of the two-chamber two-cell trans-well assay. Brain bEnd.3 endothelial cells were subjected to hypoxia/reoxygenation (H/R) are plated in the bottom chamber and macrophages (IC21) are suspended in the top chamber and allowed to migrate. (**B**). Migrated IC21 macrophages were stained with DAPI; (**C**). Quantification of migrated macrophages. Scale bar, 100 μM (****$P < 0.0001$ vs. vehicle group; ####$P < 0.0001$ vs. H/R group).

of neurons [31, 32]. Ischemic/reperfusion is usually accompanied by the overexpression of inflammatory factors and adhesion molecules, as well as the infiltration of leukocytes. Direct damage is induced on the BBB by proteolytic enzymes produced by infiltrated leukocytes, including MMP, reactive oxygen species, and the metabolites of arachidonic acid [33, 34]. As verified by the clinical trials and animal experiments, brain ischemic/reperfusion injury can be induced by vasogenic cerebral edema, hemorrhagic transformation, and infarction resulting from the destruction of the BBB [35]. In the present study, a middle cerebral artery occlusion (MCAO) model was established in mice to evaluate the therapeutic effects of Agomelatine on brain ischemic/reperfusion. We found that the permeability of the BBB was significantly increased by MCAO modeling, which was confirmed by the results of Evans blue staining and repaired by the treatment with Agomelatine, indicating recovery effects of Agomelatine on by MCAO modeling-induced BBB
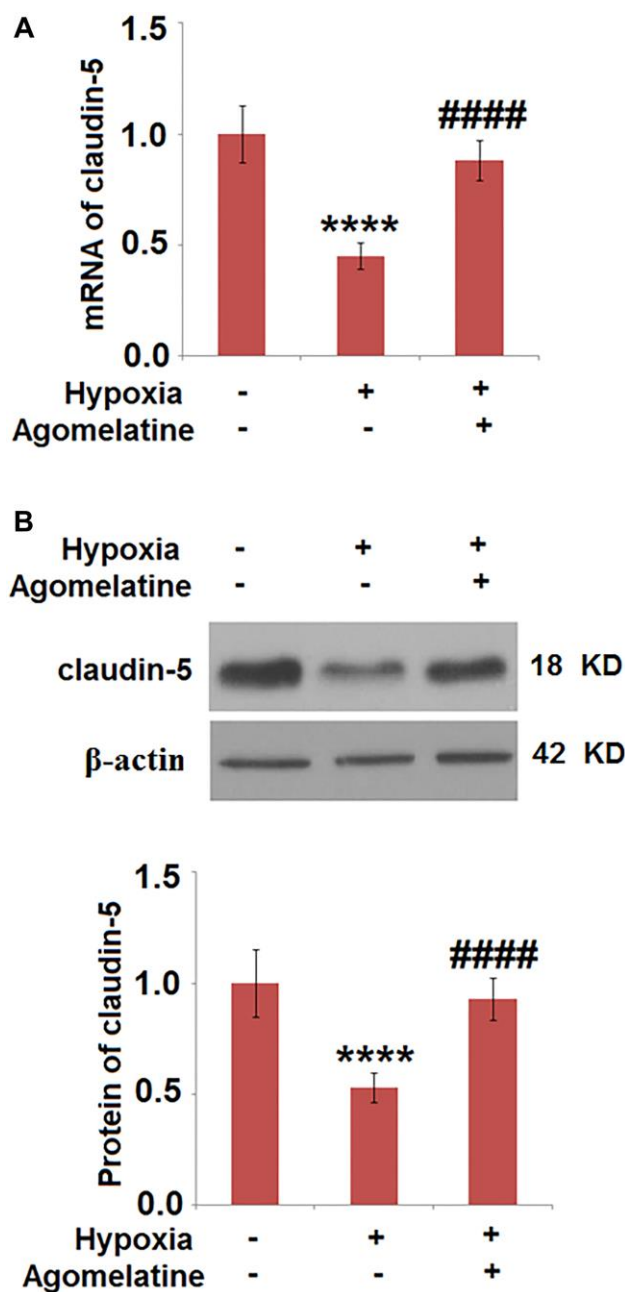

Figure 6. Agomelatine restored the expression of claudin-5 in hypoxic endothelial cells. Brain bEnd.3 endothelial cells were incubated with 10 μM Agomelatine in the process of hypoxia/ reperfusion (H/R). (**A**). mRNA of claudin-5 as measured by real-time PCR; (**B**). Protein expression of claudin-5 as measured by western blot analysis (****$P < 0.0001$ vs. vehicle group; ####$P < 0.0001$ vs. H/R group).

injury. The repair function of Agomelatine on the BBB was indirectly claimed by its promising inhibitory effects on the infiltration of macrophages into the brain tissue. We found that the increased infiltrated macrophages induced by MCAO modeling, especially M2 macrophages, were significantly suppressed by the introduction of Agomelatine. In *in vitro* macrophage migration assay, macrophages were found to easily migrate through endothelial cells under hypoxic conditions compared to endothelial cells under regular conditions or cells under hypoxic conditions but incubated with Agomelatine, indicating the promising inhibitory effects of Agomelatine on macrophage migration. We suspect that the inhibitory effects of Agomelatine on macrophage migration or infiltration are related to its repair function on the tight junctions between endothelial cells, which are the main components of the BBB. To verify our hypothesis, the expression level of a representative protein of the tight junctions between endothelial cells, claudin-5, was evaluated both *in-vivo* and *in vitro*. We found that in both the cerebral cortex of MCAO mice and the endothelial cells under hypoxic conditions, claudin-5 was significantly down-regulated, indicating destructed tight junctions. As predicted, the decreased expression of claudin-5 was promoted by Agomelatine, claiming a potential mechanism underlying the effects of Agomelatine on macrophage infiltration and BBB permeability by enhancing the tight junctions between endothelial cells.

Integrated inflammatory reactions are initiated by the central nervous system to prevent the brain from external injuries and acute inflammatory reactions play an important role in the secondary injury induced by brain ischemic/reperfusion. Following ischemic/reperfusion, the production of adhesion molecules and chemotactic factors are promoted to recruit more and more leukocytes, macrophages, and neutrophil granulocytes [36]. The activated and recruited macrophages and neutrophil granulocytes migrate and adhere to the endothelial cells, which is reported to be initiated 1-hour post-ischemic/reperfusion and achieves the peak at approximately 24–48 hours post-ischemic/reperfusion. Multiple types of pro-inflammatory mediators, such as reactive oxygen species, proteases, and lysosomal enzymes, are released by the infiltrated macrophages and neutrophil granulocytes, which induce the secondary injury on brain tissue post- ischemic/reperfusion. Leukocytes trigger injury to capillaries by changing the hemorheology, diameter. and permeability of capillaries [37, 38]. In the present study, the expression level of MCP-1, a representative chemotactic factor, was evaluated to claim the effects of Agomelatine on the chemotactic function of injured endothelial cells to inflammatory cells. We found that MCP-1 was significantly up-regulated by hypoxia and down-regulated by Agomelatine, indicating a potential chemotactic inhibitory effect of Agomelatine under the secondary injury of ischemic/reperfusion.

There were limitations to the study, one of which is that we still do not fully understand the protective mechanism of Agomelatine acting on brain endothelial cells and macrophages in stroke animals. The effect of Agomelatine could be dependent on its suppression of 5-HT2C or its activation of melatonin receptors. The role of 5-HT2c in ischemic injury has not been well confirmed [39]. Melatonin MT1 and MT2 receptors have been shown to be involved in ischemic diseases. Activation of the MT1 receptor inhibits neuronal mitochondrial cell death pathways, while the action of the MT2 receptor protects against ischemia/reperfusion injury [40]. In isolated brain endothelial cells, MT1 and MT2 are required for
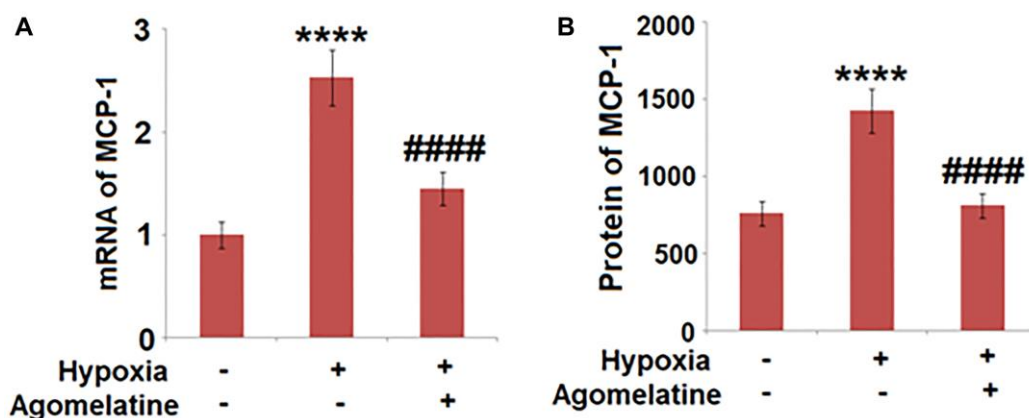

Figure 7. Agomelatine reduced the expression and secretion of MCP-1 in hypoxic endothelial cells. Brain bEnd.3 endothelial cells were incubated with 10 µM Agomelatine in the process of hypoxia/ reperfusion (H/R). (**A**). mRNA of MCP-1 was measured; (**B**). Secretions of MCP-1 were measured (****$P < 0.0001$ vs. vehicle group; ####$P < 0.0001$ vs. H/R group).

protecting the BBB from neurotoxin-induced impairment [41]. Macrophages expressing MT1 and MT2 are reported to regulate macrophage polarization involving multiple pathways including NF-κB [42] Therefore, the MT receptor-mediated pathway could be an important mechanism that Agomelatine protects against brain injury following stroke associated with ischemia/reperfusion. In addition, since Agomelatine has been shown to exhibit pleiotropic effects, some of the mechanism acting on neuroprotection could be independent of its binding to melatonin receptors or 5-HT2c. A recent study shows a short time injection of Agomelatine inhibits myocardial mitochondrial permeability transition pore opening and improves myocardial ischemia injury in an isolated rat heart model [43]. It is likely that Agomelatine could also protect the neuronal mitochondria from ischemia conditioning. Further investigation should be performed on the underlying molecular mechanism to illustrate more clearly how Agomelatine exerts neuroprotection in our future study.

MATERIALS AND METHODS

Collectively, we found that Agomelatine might prevent macrophage infiltration and brain endothelial cell damage in a stroke mouse model.

Middle cerebral artery occlusion (MCAO) model and drug administration

Twelve CD-1 mice were housed in a 12 hour light/dark cycle, temperature-controlled room, and divided into 3 groups randomly. Agomelatine (10 mg/kg/day, Servier Laboratories, Suresnes, France) or its vehicle HEC (hydroxyethyl cellulose 1%, Servier Laboratories) were administered intraperitoneally (i.p.) on a daily basis for 15 days [44, 45]. Animals were used to establish the MCAO model according to the procedure reported by Kusaka [46]. Briefly, mice were anesthetized intraperitoneally with a mixture of ketamine (80 mg/kg) and xylazine (20 mg/kg). Body temperatures and respiration rates were recorded perioperatively. A vertical midline cervical incision was conducted, and the animals' right common carotid artery was exposed. The external carotid artery was isolated, coagulated, transected, and divided (leaving 3–4 mm). The internal carotid artery was isolated, and the pterygopalatine artery was ligated close to its origin. A 5 mm aneurysm clip was utilized to fix the common carotid artery. The external carotid artery stump was reopened, and a 4.0 monofilament nylon suture was inserted through the internal carotid artery. The insertion was stopped when resistance was felt, occluding the origin of the right MCA. Post 2 hours of occlusion, the suture was withdrawn to allow for reperfusion. The external carotid artery was ligated, and the aneurysm clip was removed. The skin was sutured (1% lidocaine was applied), and the animal was left to recover. 98% purity of Agomelatine was purchased from Sigma (St. Louis, MO).

Immunofluorescence

The forebrain tissues were collected and sectioned using a Cryostats. The cortex tissue was then fixed using 100% cold methanol for 10 minutes and permeabilized with 0.1% Triton-X in PBS. The slides were then blocked and incubated with primary rabbit anti-CD68 or anti-claudin-5 (1:1000, Abcam, Cambridge, MA) antibody overnight at 4°C. Following three washes with PBS, cells were incubated with secondary Cy3-conjugated anti-rabbit IgG (1:200, Abcam, Cambridge, MA) for an additional 30 min at room temperature. DAPI was added to dye the nucleus for 5 minutes and 50% glycerinum was used to block the medium. Stained cells were photographed under a fluorescence microscope (Olympus, Tokyo, Japan).

Evans Blue (EB) extravasation assay

To examine BBB integrity, EB extravasation assays were performed. Following drug treatment and the MCAO experiment, mice were injected with 0.25 mL EB dye (2%) (Sigma, St. Louis, MO) via the caudal vein. Two hours later, the animals were anaesthetized and killed by saline infusion. The left hemisphere of each brain was weighed, added into 500 μL N, N-dimethylformamide, and incubated at 72°C for 3 days. Samples were centrifuged twice at 1516 g for 20 minutes. The supernatant was collected and aliquoted (200 μL) into a 96-well glass plate. Fluorescence was quantified using a spectrophotometer at an excitation wavelength of 620 nm and an emission wavelength of 680 nm.

Cell culture and hypoxic injury model

The mouse microvascular endothelial cell line, bEnd.3 cells, were purchased from American Type Culture Collection (ATCC, Rockville, MD USA). DMEM medium with 10% fetal bovine serum was used to culture the cells at 37°C with 5% CO_2. The hypoxia model was established according to a previously reported protocol [47]. Cells were placed in a hypoxia incubator (1% O_2, 5% CO_2, 94% N_2) for 8 hours. An automated regulator with a built-in flow meter and oxygen sensor was used to ensure and maintain the proper composition of gas mixture within the incubator. After hypoxia treatment, cells were removed from the chamber and utilized for the subsequent experiments.

Trans-well migration assay

An approximate 5×10^5 macrophages were suspended in serum-free DMEM and plated into the upper insert of a six-well trans-well plate (BD, Minneapolis, MN) and 5×10^5 bEnd.3 cells were plated into the bottom of the lower chamber. The cells were incubated at 37°C for 8 hours. The non-migratory cells in the upper layer were removed and the migratory cells were fixed with 4% paraformaldehyde at room temperature for 10 minutes, followed by staining with DAPI. Images were photographed and quantified by counting cell numbers of five randomly picked fields of view for each well.

Reverse transcriptase-polymerase chain reaction (qRT-PCR)

Total RNA was collected from the cells using an RNA Extraction Kit (Thermo Fisher Scientific, Waltham, USA) in terms of the instructions of the manufacture. Extracted RNA was quantified with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, USA). A specific RT primer was used to reverse-transcribe the complementary DNA. SYBR Premix Ex Taq TM (Thermo Fisher Scientific, Waltham, USA) with an Applied Bio-Rad CFX96 Sequence Detection system (Genscript, NanJing, China) was used in the real-time PCR procedure. The following primers were used in this study: claudin-5 (Forward: 5′-ACGGGAGGAGCGCTTTAC -3′, Reverse: 5′-GTTGGCGAACCAGCAGAG -3′); MCP-1: (Forward: 5′-CCCAGGAGTGCCTTGATTC -3′, Reverse: 5′-CGCCCCATAATTCTGACATC -3′); GAPDH (Forward: 5′-AAGAGGGATGCTGCCCTTAC -3′, Reverse: 5′-CCATTTTGTCTACGGGACGA-3′). The expression levels of claudin-5 and MCP-1 were determined by the threshold cycle (Ct), and relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

The expression level of GAPDH in the tissue was taken as a negative control.

Western blot assay

Total proteins were isolated from cells using the nuclear and cytoplasmic protein extraction kit (Thermo Fisher Scientific, Waltham, USA). Approximately 40 μg of the protein were separated on 12% SDS-polyacrylamide gel (SDS-PAGE) and the gel was transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, MIT, USA). The membrane was blocked with 5% non-fat dry milk in TBST (Trisbuffered saline/0.1% Tween-20, pH 7.4) for 1 hour at room temperature and incubated overnight with primary antibodies against claudin-5

(1:1000), MCP-1 (1:1000) and GAPDH (1:1000) (Abcam, Cambridge, MA). A horseradish peroxidase (HRP)-conjugated antibody against rabbit IgG (1:5000, Abcam, Cambridge, MA) was used as a secondary antibody. Blots were incubated with the ECL reagents (Amersham Pharmacia Biotech, Inc, USA) and exposed to Tanon 5200-multi to detect protein expression. The intensity of bands was quantified using Image Lab software (Bio-Rad, Herculus, USA). The relative expression of the protein was normalized to the control sample and presented as fold change to the initial value.

Statistical analysis

Mean ± standard deviation (SD) was displayed to show the data. GraphPad Prism 6.0 (GraphPad, China) was used to analyze the data. Analysis of variance (ANOVA) followed by Tukey's HSD post-hoc test was utilized for the contrast among different groups. $P < 0.05$ was regarded as a statistically significant difference between the groups.

AUTHOR CONTRIBUTIONS

ACKNOWLEDGMENTS

CONFLICTS OF INTEREST

There is no conflicts of interest for all authors.

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