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Abstract

Background: Blood–brain barrier (BBB) disruption mediated by proteases plays a pivotal role in neural tissue damage after acute ischemic stroke. In an animal stroke model, the activation of matrix metalloproteinases (MMPs), especially MMP-9, was significantly increased and it showed potential association with blood–brain barrier (BBB) disruption and cerebral edema. Theoretically, it is expected that early blockade of expression and activation of MMP-9 after ischemic stroke provides neuroprotective effects from secondary neural tissue damage. This study was aimed to determine the ability of rutin to influence MMP-9 expression, activity and BBB disruption using a photothrombotic focal ischemic model in rats.

Methods: Adult male Sprague–Dawley rats, weighing between 250 and 300 g (aged 8 weeks) received focal cerebral ischemia by photothrombosis using Rose Bengal (RB) and cold light. Injured animals were divided into two groups; one group received 50 mg/kg of rutin intraperitoneally, starting 1 h after injury and at 12 h intervals for 3 days, while animals in the control group received weight-adjusted doses of saline vehicle over the same period. In each group, the expressions and activities of MMP-9 were assessed by Western blot and gelatin zymography at 6, 24, 48, and 72 h after photothrombotic insult. The effects of rutin on BBB disruption and functional outcomes were also determined.

Results: Western blot and zymographic analysis showed up-regulated MMP-9 expression and activity in the ischemic cortex. The expression and activity of MMP-9 were significantly elevated at 6 h after photothrombotic insult, which remained up-regulated for at least until 72 h after injury. In the rutin-treated group, MMP-9 expression and activity were significantly attenuated at 6, 24, and 48 h compared to the control group. Relative to the control group, BBB permeability was significantly reduced in the rutin-treated group. The results of the rotarod test revealed that rutin treatment significantly improved functional outcomes.

Conclusions: Rutin treatment starting 1 h after injury attenuated BBB disruption during photothrombotic focal ischemia, which was partly, at least, achieved through inhibitory effects on MMP-9 expression and activity. The results of this study suggest that rutin might be useful in clinical trials aimed to improve the outcome of patients suffering from acute ischemic stroke.

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1. Introduction

Acute ischemic stroke is the major cause of death worldwide among cerebral disorders and is a devastating clinical condition that may result in permanent neurologic deficits. Cerebral ischemia results from a transient or permanent reduction of cerebral blood flow which in turn restricts a territory of a major brain artery. Interruption of blood supply to the brain quickly results in a severe failure of metabolic energy support, which consequently initiates several disturbances of cellular mechanisms essential for neuronal functioning. The major important pathophysiological mechanisms involved during cerebral ischemia include neuronal excitotoxicity, inflammation, oxidative stress, and apoptosis [1,2]. The brain is very sensitive to damage caused by oxidative stress, due to its rapid oxidative metabolic activity, low antioxidative capacity, and poor neural cell recovery ability, therefore, it is safe to state that oxidative stress is an important factor in acute ischemic stroke [3]. Reactive oxygen species and other toxic free radicals, released by inflammatory cells, are known to contribute to functional disruption and neuronal death. Blood–brain barrier (BBB)
provides a structural barrier that selectively filters blood elements. BBB is formed by the basal lamina which surrounds and anchors endothelial cells and astrocytes [4]. Accumulation of toxic free radicals plays a pivotal role in the early molecular cascades of BBB disruption, and these processes have been known to be activated via proteases such as matrix metalloproteinases (MMPs) in ischemic stroke [5].

MMPs are a family of zinc-binding proteolytic and matrix degrading enzymes of the basement membrane that play multifactorial actions in the pathology and physiology of the nervous system [6,7]. MMPs are essential for remodeling of the extracellular matrix (ECM), tissue morphogenesis, and wound healing. However, overexpressed proteolytic activity of MMPs after tissue injury induces endothelial damage and can further cleave protein components of the ECM including proteoglycans, collagens, and basal laminin [7,8]. Eventually, it leads to transmigration of inflammatory cells and toxic molecules into brain parenchyma and aggravates numerous pathologic conditions, including neural tissue injury. Among the MMP family, MMP-9 has been implicated in abnormal vascular permeability associated with either hemorrhagic injury or inflammation. Some reports demonstrated dramatically increased MMP-9 activity after ischemic cerebral injury and showed its possible temporal and partial associations with BBB disruption [5,9]. Abnormal increases of MMP-9 in both inflammatory cells and endothelial cells may collectively impair endothelial barrier function by degrading the vascular basement membrane [9,10]. Therefore, it has been suggested that pharmacological blockade of MMP-9 expression will attenuate disruption of the BBB and improve functional neurologic outcomes after ischemic cerebral stroke.

Rutin (quercetin-3-rhamnosyl glucoside) is a flavonoid glycoside found in buckwheat which is abundantly present in vegetables, fruits, tea, wine and herbs [11,12]. It has several physiological abilities as a high radical scavenger along with antioxidant, anticancer, and anti-inflammatory properties [13]. Moreover, cell protective effects of rutin after ischemic injury of organs, including the heart, brain, and kidney have been reported [14-16]. In particular, administration of rutin before transient cerebral ischemia or at the onset of reperfusion has shown a reduction of ischemic neural apoptosis by increasing endogenous antioxidant enzymatic activities in experimental animals [14]. However, the neuroprotective effects of rutin are complex and are not fully discovered. This study was conducted to determine the effects of rutin on MMP-9 activity and expression, BBB disruption, and functional neurologic outcomes in a rat phototothrombotic stroke model.

2. Materials and methods

2.1. Phototothrombotic ischemic stroke model

All surgical procedures and postoperative care were performed in accordance with the guidelines of the Chonnam National University Animal Care and Use Committee. Male Sprague–Dawley rats, weighing between 250 and 300 g were used in this study. These animals were maintained on a 12 h light/dark cycle and were allowed free access to food and water.

Focal cerebral cortical lesion was induced by phototothrombosis of cortical microvessels using Rose Bengal (RB, Sigma Chemical Co., St. Louis, MO, USA) with cold light (Zeiss KL1500 LCD, Germany). Each rat was anesthetized with 5% isoflurane and maintained with 2% isoflurane in an oxygen/air mixture using a gas anesthesia mask in a stereotaxic frame (Stoelting, Wood Dale, IL, USA). After obtaining a deep level of anesthesia, rats were placed in the prone position with a warmed surgical pad controlled by a rectal probe to preserve body temperature at 37 ± 0.5 °C throughout the procedure. For illumination, a 4.5 mm fiber optic bundle, from a cold light source, was positioned onto the exposed skull 0.5 mm anterior to the bregma and 3.7 mm lateral to the midline over the left sensorimotor cortex. The brain was illuminated for 10 min after infusion of 50 μg/kg of RB in normal saline into the right femoral vein via a microinjection pump within 1 min. The scalp was sutured, and the rats were then returned to their individual cages and kept heated under an infrared lamp until full recovery from anesthesia. Animals for the sham model (sham group, n = 5) received only illumination after infusion of normal saline instead of RB.

2.2. Drug administration

Rutin (Sigma-Aldrich) was dissolved in saline. All experimental animals were randomly allocated into two groups: one group received 50 mg/kg of rutin intraperitoneally (PFI + RU group: phototothrombotic focal ischemic insult and rutin administration group, n = 5), starting 1 h after injury at 12 h intervals for 3 days, while the other group received weight-adjusted doses of saline vehicle according to the same schedule (PFI group: phototothrombotic focal ischemic insult and saline administration group, n = 5).

2.3. Preparation of tissue extracts for protein analysis

To analyze protein expression patterns in the ipsilateral injured ischemic hemisphere by phototothrombotic insult, protein extracts of the tissues were prepared. After the animals were deeply anesthetized with isoflurane, injured ischemic hemispheres were removed at 6, 24, 48, and 72 h after surgery for Western blot and gelatin zymography. Following this, ischemic brain tissues were homogenized in a lysis buffer with protease inhibitors. After centrifugation, the supernatants were collected. The total protein concentrations were determined using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). This process was performed in all experimental groups including sham, PFI, and PFI + RU groups, respectively.

2.4. Electrophoresis and western blot analysis

To investigate the protein expression of MMP-9 in brain tissue extracts using Western blot analysis, samples containing 50 μg of protein were boiled for 5 min at 100 °C with an appropriate volume of 4× sample buffer (NuPAGE® LDS Sample Buffer (4×) NP0007; Invitrogen). The samples were then placed on ice to cool before loading onto a 8% sodium dodecyl sulfate-polyacrylamide (SDS) gel and were separated at 120 V for 3 h. The gels were then transferred onto a polyvinylidene difluoride membrane at 300 mA for 90 min at 4 °C. The membranes were reversibly stained with Ponceau S to confirm the transfer of proteins and destained in water. The membranes were then incubated for 1 h at room temperature in a blocking buffer (5% non-fat dry milk in Tris-buffered saline with 0.2% Tween 20), then probed overnight at 4 °C with primary anti-MMP-9 (rabbit anti-Rat MMP-9 polyclonal, dilution = 1:2000; Chemicon International, Temecula, CA, USA), and finally incubated for 1 h at room temperature with horseradish peroxidase-conjugated immunoglobulin (polyclonal Goat anti-rabbit IgG, dilution = 1:5000; Dako Cytomation, Glostrup, Denmark and Goat anti-mouse IgG, dilution = 1:5000; Zymed, California, USA respectively). β-Actin (monoclonal anti-β-actin, dilution = 1:40,000; Sigma Aldrich Co., St. Louis, Mo, USA) was used as a control for loading. The reaction was developed with a chemiluminescence reagent containing luminal reagent and peroxide solution (Millipore). Optical densities were measured by a Luminescent Image Analyzer (Fujifilm LAS-3000; Fuji Photo Film Co., Tokyo, Japan).

2.5. Gelatin zymography

To determine the effect of rutin on the activity of MMP-9, gelatin zymography was performed. Equal amounts of protein samples (50 μg/lane) were loaded and separated by electrophoresis on an 8% SDS-polyacrylamide gel containing 1 mg/ml gelatin at 4 °C. After
separation by electrophoresis, the gel was briefly rinsed with distilled water and washed three times in 2.5% Triton X-100 solution for 30 min. After washing, the gel was incubated with a developing buffer at 37 °C overnight. Following this, the gel was stained with 0.5% Coomassie Brilliant Blue R-250 (Sigma Chemical) in a mixture of methanol:acetic acid:water (2:1:7) for 2 h and then destained. To quantify the relative levels of MMP-9 expression as detected by gelatin zymography, the gels were digitized, and the area of lysis for each detected band was quantified using the J-Image program (NIH, Bethesda, USA) of the lytic zone area in square millimeters.

2.6. Determination of BBB disruption

Piao et al. [17] reported that Evans blue extravasation was increased within 2 h after photothermotic insult and reached a peak level at 24 h after the insult. Therefore, disruption of the BBB was assessed 24 h after ischemia using Evans blue dye in the present study (n = 5 for each group). Evans blue dye (2%, 4 ml/kg) was injected over 2 min into the left femoral vein; it was allowed to circulate for 60 min. The rats were deeply anesthetized and transcardially perfused with phosphate-buffered saline (PBS) until colorless perfusion fluid was obtained from the right atrium. After decapitation, the brain was removed and coronal sections were divided into the right and left hemispheres. The amount of extravasated Evans blue dye in the brain was determined by spectrophotometry and quantified as micrograms per gram of brain tissue.

2.7. Immunohistochemistry

Forebrains were removed from the animals that had undergone focal cerebral ischemia after 24 h with rutin or without rutin administration. The sham-operated rats, euthanized after 24 h, were used as controls. After standard histological processing, the author reviewed the hematoxylin and eosin stained slides of the brain specimens and then selected tissue blocks from the infarct area to make formalin-fixed, paraffin-embedded samples of brain tissue. Immunohistochemical analysis included antibodies for MMP-9 (Abcam, MA, USA; dilution 1:200). Immunostains were performed by the routine avidin–biotin complex (ABC) method. Briefly, representative paraffin blocks were consecutively cut into 4 micron thick samples, and immunohistochemical staining was carried out using the Microprobe Immuno/DNA stainer (Fisher Scientific, CA, USA). The sections were deparaffinized in xylene and treated with 0.3% hydrogen peroxide in methanol for 20 min to block endogenous peroxidase activity. For MMP-9, the sections were subjected to pressure cooker heat in 10 mM citrate buffer (pH 6.0), then incubated with the antibodies at 4 °C overnight. Anti-mouse immunoglobulin G (Sigma, St. Louis, MO, USA) labeled with biotin was used as a secondary antibody for the detection of primary antibodies and was incubated for 7 min at 45 °C. The streptavidin–horseradish peroxidase (Research Genetics, USA) detection system was applied to capillary channels, followed by 10 min of incubation at 45 °C. After drainage, the tissue sections were ready for the chromogen reaction with 0.02% diaminobenzidine. The sections were counterstained with hematoxylin and mounted in Universal Mount (Research Genetics, USA). Negative controls were treated similarly except for the primary antibodies.

The total numbers of MMP-9 immunopositive neurons, within a defined grid area, were counted, based on the methods modified from Magnoni et al. [18]. Each image was captured by a JVC Digital Camera system (JVC Digital Camera KY-F70B, JVC Corporation, Tokyo, Japan) mounted on a Nikon microscope (Nikon E600, Tokyo, Japan) at ×200 magnification. Each ×200 magnification area was 0.48 mm².

2.8. Assessment of functional neurologic outcome

Functional motor impairment was evaluated by the accelerating rotarod test. A 5-min activity test was performed for each rat model. Prior to induction of ischemia, the rats were trained on the rotarod for three consecutive days, for a total of nine sessions. The rotating drum was accelerated from 4 to 40 rpm over 5 min, and the latency (in seconds) for the animal to fall off the drum was recorded. Each session included three consecutive trials, with a maximum time of 300 s; the mean fall latency was calculated from three trials. Animals that did not stay on the rod for an average of at least 1 min at the end of training were excluded from stroke surgery. The functional state of rats after photothermal insults was the lowest value at 3 days after surgery, and the initial significant improvement of functional outcome showed at 7 days after surgery in preliminary results. Therefore, behavioral testing was conducted and compared at 3, 7, 14, 21, 28, 35, 42, 49, and 56 days after surgery.

2.9. Statistical analysis

Statistical analysis was performed using the SPSS software program (version 14.0 for Windows; SPSS INC, Chicago, IL, USA). Data are expressed as mean ± standard deviation (SD). Statistical analysis was performed by Student’s t test (for comparisons between two groups) and ANOVA followed by Bonferroni post hoc test (for comparisons among multiple groups). The criterion for statistical significance was p < 0.05. All measurements were analyzed by observers who were blinded to individual treatments.

3. Results

3.1. Expression and activity of MMP-9

In Western blot analysis, MMP-9 protein expression was increased 6 h after the focal ischemic insult and reached a peak level at 24 h after the ischemic insult in the PFI group, and it gradually decreased 72 h after the ischemic insult. Rutin treatment substantially decreased the expression of MMP-9 at 6, 24, and 48 h after injury compared to the PFI group (p < 0.05) (Fig. 1). Zymogram revealed significantly elevated MMP-9 activity at 6 h after the ischemic insult and reached a peak level at 24 h after injury, which remained upregulated at least until 72 h after injury. Similar to the Western blot findings, rutin administration significantly attenuated the activity of MMP-9 at 6, 24, 48 and 72 h after the onset of photothermal ischemic injury (p < 0.05) (Fig. 2). MMP-9 protein expression and activity were very low in sham-operated brains.

3.2. BBB permeability

To determine the effect of rutin on BBB permeability, the author chose the 24 h-time point after ischemia, at which the permeability of

![Fig. 1. Western blots of MMP-9 demonstrating that rutin treatment significantly decreased the expression of MMP-9 at 6, 24, and 48 h after the onset of photothermal ischemic injury (*p < 0.05).](image-url)
the BBB for Evans blue reached a peak. At 24 h, Evans blue extravasation was 11.5 ± 0.3 μg/g tissue. Rutin treatment significantly decreased the Evans blue extravasation to 6.7 ± 0.4 μg/g tissue in the ischemic brain compared with that of the PFI group (p < 0.05) (Fig. 3).

3.3. Immunohistochemistry for MMP-9

A few neurons with weak MMP-9 immunoreactivity were observed in the sham (Fig. 4A). In the PFI and PFI + RU group, positive MMP-9 immunoreactivity was easily identified in the cytoplasm of the neurons with a dot-like pattern; they were located in the peri-infarct area and infarct core regions of the ipsilateral hemisphere (Fig. 4B). However, the MMP-9 immunoreactivity neurons were relatively decreased by rutin treatment compared with those in the PFI group (Fig. 4C).

3.4. Functional recovery

Before surgery, there was no significant difference in the time spent on the accelerating rotarod between the PFI group and the PFI + RU group. The time spent on the rotarod was expressed as a percent of presurgery control values. Focal cortical photothrombosis caused severe impairment in the rotarod performance. Significantly greater recovery during the postoperative period was observed in the PFI + RU group relative to the PFI group (Fig. 5).

4. Discussion

The present study shows that administration of rutin effectively attenuates MMP-9 protein expression and enzyme activity after photothrombotic ischemic insult in rats. These rutin-induced attenuations of MMP-9 expression and activity were correlated with the decreases of BBB disruption assessed by Evans blue. Moreover, rutin treatment improved functional outcomes assessed by the rotarod test after photothrombotic ischemic insult.

For the purpose of neuroprotection after an incidence of cerebral ischemia, the damage of the BBB should be minimized for the maintenance of homeostasis and functioning of the brain. The basement membrane which is composed of ECM plays a critical role in maintaining the integrity of the BBB by providing structural support to the endothelial
cell wall [19]. After cerebral ischemia, formation of free radicals by oxidative stress develops and subsequently increases. Accumulation of free radicals during cerebral ischemia triggers molecular cascades sensitive to ischemic injury, leading to activation of various proteolytic enzymes. Protein and polysaccharides, which compose the basal lamina of the BBB, are degraded by activated extracellular proteases, eventually resulting in increased BBB permeability [20]. Transmigration of inflammatory cells and toxic molecules into the brain occurs due to BBB disruption and results in cerebral edema, hemorrhage and neural cell apoptosis [5,6]. Free radicals influence the action of proteases at multiple levels including transcription, mRNA processing and activation of latent proteases. Jian Liu and Rosenberg [6] demonstrated that oxygen free radicals involve nitrosylation or oxidation of MMP directly, resulting in an increase in the protein expression and enzymatic activities of MMP. Theoretically, it is possible to construct novel strategies for stroke therapy from pharmacological inhibition of MMP and free radical activation in the ischemic brain.

Many recent studies have been focused on MMP expression and activation in pathogenic mechanisms after cerebral ischemia [5,9,21]. MMPs are zinc- and calcium-dependent endopeptidases and can cleave most components of the ECM including fibronectin, laminin, proteoglycans and type IV collagen [22,23]. After the onset of cerebral ischemia, MMPs are overexpressed and activated, and excessively expressed and activated MMPs cause damage to the basal lamina of the BBB by degrading the ECM [19,24,25]. Among the MMP family, MMP-2 and MMP-9 have been regarded as the key enzymes related with secondary damage after cerebral ischemia or traumatic accidents [10]. However, some studies have shown that MMP-2 is elevated at several days or weeks after the attack, whereas MMP-9 is elevated within several hours after cerebral infarction [8,10,26]. This indicates that the delayed expression of MMP-2 after cerebral ischemia or traumatic accidents may be associated with tissue healing processes and is correlated with better clinical outcomes [26]. Therefore, recent studies about proteases related to BBB disruption after cerebral ischemia have highlighted the involvement of MMP-9 [9,17,27]. The presence of high MMP-9 levels, not only in infarcted tissues but also in peri-infarct areas, has suggested a role for MMPs in the process of infarct expansion. Emerging clinical data support the finding of significantly increased MMP-9 levels after cerebral ischemia, with the levels correlating with infarct size, stroke severity, hemorrhagic transformation, and functional outcomes [7]. In the present study, MMP-9 expression and activity were increased as early as 6 h after photothrombosis cerebral ischemia and rapidly increased thereafter reaching a peak level at 24 h. Evans blue extravasation was increased in the infarction site compared to the contralateral side at 24 h after injury. These results suggest that early activation of MMP-9 is correlated with alteration of BBB permeability in the ischemic brain [17].

Rutin is a kind of bioflavonoid glycoside, and it is found in buckwheat, fruits, vegetables, tea, and wine [15,16]. Rutin is known as vitamin P and has anti-oxidative, anti-inflammatory, anti-viral, anti-carcinogenic, and anti-hypertensive properties [14,16]. The mechanisms of protective effects of rutin against ischemic tissue injury have been focused on the enzymes that reduce oxygen free radicals, such as superoxide dismutase (SOD). Bhandary et al. [28] demonstrated that rutin has consistent protective effects to myocardial tissue in ischemic/reperfusion-associated hemodynamic alteration by enhancing SOD and 1,1-diphenyl-2-picrylhydrazyl (DPPH) activities. Wei et al. [29] suggested that rutin protects the tests from ischemia–reperfusion injury by scavenging ROS through increasing SOD and catalase activities. However, no previous studies have been conducted regarding the control effects of rutin on MMP-9 expression and activities in ischemic organ injury. In this study, rutin treatment attenuated not only MMP-9 expression and activity but also the amount of Evans blue extravasation after photothrombosis ischemic insult in rats. Therefore, these findings suggest that oxidative stress by free radicals might be densely associated with activation of the MMP-9 pathway, the subsequent protection by rutin against BBB disruption after cerebral ischemia through MMP-9 activity via reduction of oxidative stress by scavenging oxygen free radicals. In this study, rutin was shown to be effective against ischemic brain injury when rutin administration was initiated 1 h after the ischemic event. The results of this study demonstrate that delayed rutin treatment by 1 h after injury markedly decreased MMP-9 levels, activities, and BBB disruption. Moreover, administration of rutin significantly improved functional deficits from photothrombosis focal cerebral ischemia. These facts support the possible use of rutin as a therapeutic agent to ameliorate the cerebral ischemic process. However, it remains to be precisely defined how long after an ischemic episode the start of ‘window of opportunity’ could be delayed, and for how long a treatment should be given to achieve beneficial outcome.

Photothrombosis is a well-established stroke model [25,30–33]. Cortical lesions can be produced in a precise location and size, with low mortality. The features associated with this model include severe endothelial cell damage, BBB disruption and edema formation [33]. A specific feature of the photothrombosis model is the relatively restricted involvement of the penumbral area due to the associated end arterial occlusion. However, there is pharmacological evidence demonstrating up to 85% neuroprotection using this model [33]. Zou et al. monitored regional cerebral blood flow (CBF) in the ischemic penumbra at different time points using a laser Doppler flow meter in a photothrombotic stroke model in mice, and penumbral CBF was shown to decrease progressively in all animals [34]. These data suggest the presence of a salvageable penumbral area with photothrombosis. Therefore, the photothrombotic stroke model might be suitable to determine the functional aspects of lesion-associated cerebral ischemia and for the evaluation of potential neuroprotective and therapeutic agents in human stroke.

In conclusion, administration of rutin starting 1 h after injury significantly reduced BBB disruption via downregulation of MMP-9 expression and activity in the photothrombosis cerebral ischemic model of rats. Moreover, rutin administration was shown to improve functional outcomes. This study supports the usefulness of rutin as a therapeutic agent to ameliorate the ischemic process. Additional studies are required to find out the effectiveness of rutin in relation to various treatment start times after the onset of stroke and further to elucidate the protective effect of rutin on the BBB.

Conflict of interest

None.

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