# Neuroprotective effect of, a flavonoid, sudachitin in mice stroke model

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**Abbreviations used**: 4-HNE, 4-hydroxynonenal; CARR U, Carratelli units; CC3, cleaved caspase-3; DAB, diaminobenzidine; HPDLC, human periodontal ligament cells; LPS, lipopolysaccharides; MAP-2, microtubule-associated protein-2; MMP, Matrix metalloproteinase; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PGC, peroxisome proliferator-activated receptor-gamma coactivator; ROS, reactive oxygen species; sec, seconds; Sirt1, silent mating type information regulation 2 homolog 1; TNF, tumoral necrosis factor; tMCAO, transient middle cerebral artery occlusion.

**ABSTRACT**

A flavonoid, sudachitin, has been reported to show some beneficial health effects, including as an anti-inflammatory in LPS-stimulated macrophages, as well as improving glucose and lipid metabolism in mice fed a high-fat diet. In this study, we investigated the neuroprotective effect of sudachitin in the transient middle cerebral artery occlusion (tMCAO) mouse model. After daily pre-treatment of vehicle or sudachitin (5 or 50 mg/kg) for 14 days, mice ($n = 76$) were subjected to a sham operation or tMCAO for 45 min, and on the following days, they were treated daily with vehicle or sudachitin. The administration of sudachitin significantly reduced ($p < 0.05$) cerebral infarct volume and attenuated apoptosis, 5 days after tMCAO. Neurological impairment improved, the expression of an oxidative stress marker, 4-HNE, decreased, and the Sirt1/PGC-1α pathway was activated 5 days after tMCAO in the sudachitin-treated group. This is the first report to demonstrate the neuroprotective effect of sudachitin in cerebral ischemia/reperfusion injury mice model, probably by activating the Sirt1/PGC-1α axis. Sudachitin may be a promising supplement or therapeutic agent for reducing injury caused by ischemic strokes.

Key words: Sudachitin, transient middle cerebral artery occlusion, ischemia, apoptosis, oxidative stress.

# 1. INTRODUCTION

In 2019, it was estimated that there were 12.2 million incident cases and 101 million prevalent cases of stroke. Stroke was the second cause of death worldwide after ischemic heart disease and the third cause of death and disability combined, after neonatal disorders and ischemic heart disease. Ischemic stroke accounted for 62.4% of all new strokes in 2019 (7.63 million), intracerebral hemorrhage accounted for 27.9% (3.41 million), and subarachnoid hemorrhage accounted for 9.7% (1.18 million) (Fergin et al., 2021). Brain tissue can be rescued if blood flow is recovered rapidly after the onset of an acute ischemic stroke, and the intravenous administration of recombinant tissue plasminogen activator and endovascular thrombectomy using stent retrievers can significantly improve the disability-free recovery rate (Tawil et al., 2017). However, a delay in the initial treatment or other contraindications may limit the effectiveness of these treatments, indicating the need for new treatments.

*Citrus sudachi* (Rutaceae) is an evergreen tree that is found mainly in Tokushima prefecture, Japan (Nakasugi et al., 2000; Nakagawa et al., 2001). One of the flavonoids extracted from *C. sudachi* is sudachitin (5,7,4'-trihydroxy-6,8,3'-trimethoxyflavone) (Nakasugi et al., 2000; Nakagawa et al., 2006). Sudachitin improves glucose and lipid metabolism (Tsutsumi et al., 2014), blocks lipopolysaccharides (LPS)-induced inflammatory bone destruction (Ohyama et al., 2018), possesses an anti-inflammatory property in LPS-stimulated macrophages (Yuasa et al., 2012), and inhibits matrix metalloproteinases (MMP)-1 and MMP-3 production in tumoral necrosis factor (TNF)-α-stimulated human periodontal ligament cells (HPDLCs) (Hosokawa et al., 2019). Sudachitin also inhibited inflammatory cytokines and MMP production in interleukin (IL)-1β-stimulated HPDLCs (Hosokawa et al., 2021). In addition, sudachitin treatment increased the expression of peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1α and silent mating type information regulation 2 homolog 1 (Sirt1), which participate in neuroprotection in in vivo (Fu et al., 2014; Gao et al., 2020; Li et al., 2021) and in vitro models (Lv et al., 2015). However, there are no reports on the effects of sudachitin in cerebral ischemia/reperfusion injury.

Therefore, the objective of this study was to investigate the therapeutic effects of sudachitin in a transient middle cerebral artery occlusion (tMCAO) mouse model and its possible signaling pathway related to the Sirt1/PGC-1α axis.

## 2. Results

*2.1 A low dose of sudachitin improved body weight and the Bederson score*

Prior to evaluation, there were 1 and 9 dead mice in the sacrificed 1 day and 5 days after tMCAO subgroups (Fig. 1), respectively. In the sacrificed 1 day after tMCAO subgroup, there was a significant difference in body weight between sham (sham + vehicle) and vehicle groups (tMCAO + vehicle) ($p < 0.0001$) (Fig. 2 A). In the sacrificed 5 days after tMCAO subgroup, there was a concomitant reduction in body weight of each tMCAO group (vehicle, 88.3% ± 12.9; LD group (tMCAO + low dose of sudachitin (5 mg/kg of body weight per day)), 94.3% ± 4.1; HD group (tMCAO + high dose of sudachitin (50 mg/kg of body weight per day)), 89.3% ± 10.3). Weight recovery in the LD group was not significantly reduced (Fig. 2 B).

In the subgroup sacrificed 1 day after tMCAO, the Bederson score of each tMCAO group increased (vehicle, 1.7 ± 0.9; LD, 1.2 ± 0.8 and HD, 1.3 ± 1.2). There was significant difference between sham and vehicle groups ($p < 0.001$), while the LD group was not significantly different ($p = 0.051$) to the vehicle group. In the sacrificed 5 days after tMCAO subgroup, the Bederson score of each tMCAO group increased (vehicle, 1.9 ± 0.7; LD, 1.3 ± 0.8 and HD, 1.7 ± 1.2). There was a significant difference between sham and vehicle groups ($p < 0.0001$) while the LD group was not significantly different ($p = 0.29$) to the vehicle group (Fig. 2 C, D). In the hangwire and rotarod tests, there were no significant differences between groups or subgroups (Fig. 2 E - H).

*2.2 Serum oxidant and antioxidant markers*

There were no significant differences in serum oxidants, as evaluated by the d-ROM test (Fig. 3 A, B), or in serum antioxidant capacity as evaluated by the OXY-adsorbent test (Fig. 3 C, D), between any of the subgroups at 1 or 5 days after tMCAO.

*2.3 A low dose of sudachitin decreased cerebral infarct volume*

Nissl staining and MAP2 immunohistochemistry were conducted to evaluate cerebral infarct volume. In the subgroup sacrificed 1 day after tMCAO, Nissl staining indicated no significant differences between any of the groups (Fig. 4 A, B). In the mice sacrificed 5 days after tMCAO, there was a significant reduction in cerebral infarct

volume in the LD group compared with the vehicle group ($p < 0.05$; vehicle, 11.4 ± 8.1 $mm^3$; LD, 3.5 ± 1.3 $mm^3$; HD, 12.1 ± 17.9 $mm^3$) (Fig. 4 C, D).

In the mice sacrificed 1 day after tMCAO, MAP2 staining indicated no significant differences between any sudachitin-treated group compared with the vehicle group (Fig. 4 E, F). In the mice sacrificed 5 days after tMCAO, there was a significant reduction in cerebral infarct volume in the LD group compared with the vehicle group ($p < 0.05$; vehicle, 8.7 ± 6.3 $mm^3$; LD, 2.4 ± 1.7 $mm^3$; HD, 8.4 ± 7.2 $mm^3$) (Fig. 4 G, H).

*2.4 A low dose of sudachitin attenuated apoptosis and reduced oxidative stress*

CC3 immunohistochemistry was used to evaluate apoptosis in mice brains. Every positive cell in the ischemic/reperfusion ipsilateral hemisphere (Fig. 5 A) was counted from five sections (Bregma+1 mm, Bregma+0.5 mm, Bregma, Bregma-0.5 mm and Bregma-1 mm) of each mouse brain sample, and the average was calculated. The staining pattern was dominant in nuclei. In the subgroup sacrificed 1 day after tMCAO, the LD group had insignificantly fewer CC3-positive cells than the vehicle group ($p = 0.14$; sham, 7.7 ± 5.5 cells; vehicle, 70.4 ± 39.6 cells; LD, 47.9 ± 56.7 cells; HD, 49.9 ± 38.5 cells) (Fig. 5 C, D). In the subgroup sacrificed 5 days after tMCAO, the LD group had significantly fewer CC3-positive cells than the vehicle group ($p < 0.005$; sham, 9.0 ± 6.8 cells; vehicle, 82.9 ± 51.1 cells; LD, 15.4 ± 6.3 cells; HD, 66.8 ± 64.5 cells) (Fig. 5 E, F). To confirm apoptosis, a TUNEL assay was performed. Positive cells were counted in four random penumbra areas of the cortex and striatum (Fig. 5 B) of one section per brain sample. In the sacrificed 1 day after tMCAO subgroup there was a decreasing tendency in LD compared to the vehicle group was although the difference was not significant ($p = 0.09$; sham, 2 ± 0.5 cells; vehicle, 17.3 ± 10.2 cells; LD, 7.3 ± 6.2 cells; HD, 13.7 ± 13.1 cells) (Fig. 5 G, H). In the sacrificed 5 days after tMCAO subgroup there was no significant difference between the sudachitin and vehicle groups (sham, 6.6 ± 1.8 cells; vehicle, 19.7 ± 11.7 cells; LD, 12.9 ± 10.9 cells; HD, 19.5 ± 18.7 cells) (Fig. 5 I, J). In addition to not finding any significant differences in these results, the pattern between CC3 and TUNEL-positive cells tended to be similar. To evaluate the expression of oxidative stress in brain tissue, 4-hydroxynonenal (4-HNE), a residue of lipid peroxidation that shows a cytoplasmic staining pattern, was used in immunohistochemistry. The average number of 4-HNE-positive cells in three random

penumbra areas (Fig. 5 B) of three sections (Bregma+1 mm, Bregma, and Bregma-0.5 mm) per brain was calculated. In the subgroup sacrificed 1 day after tMCAO, LD and HD groups had insignificantly fewer 4-HNE-positive cells than the vehicle group ($p = 0.07$ and 0.12 respectively; sham, 4.8 ± 3.5 cells; vehicle, 8.7 ± 3.8 cells; LD, 5.7 ± 2.8 cells; HD, 5.8 ± 4 cells) (Fig. 5 K, L). Similarly, in the subgroup sacrificed 5 days after tMCAO, the HD group had insignificantly fewer 4-HNE-positive cells than the vehicle group ($p = 0.10$; sham, 3.3 ± 4.5 cells; vehicle, 12.1 ± 3.8 cells; LD, 8.6 ± 4.8 cells; HD, 6.8 ± 3.4 cells) (Fig. 5 M, N).

*2.5 A low dose of sudachitin increased Sirt1 and PGC-1*α

To evaluate the possible sudachitin signaling pathway, Sirt1/PGC-1α, Sirt1 and PGC-1α immunohistochemistry were performed. The average number of Sirt1/PGC-1α-positive cells in three random penumbra areas (Fig. 5 B) of three sections (Bregma+1 mm, Bregma, and Bregma-0.5 mm) per brain was calculated. In the mice sacrificed 1 day after tMCAO, there were no significant differences in Sirt1-positive cells between any group (Fig. 6 A, B). However, in the mice sacrificed 5 days after tMCAO, the LD group had significantly more Sirt1-positive cells than the vehicle group ($p < 0.05$; sham, 159.5 ± 4.1 cells; vehicle, 116.8 ± 63 cells; LD, 184.3 ± 59.8 cells; HD, 146.2 ± 23.3 cells) (Fig. 6 C, D).

In the mice sacrificed 1 day after tMCAO, LD and HD groups showed an insignificant increase in PGC-1α-positive cells compared with the vehicle group ($p = 0.21$ and 0.12, respectively; sham 15.6 ± 3.9 cells, vehicle, 11.4 ± 7.6 cells; LD, 14.8 ± 3.6 cells; HD, 16 ± 6.1 cells) (Fig. 6 E, F). In the mice sacrificed 5 days after tMCAO, the LD group had significantly more PGC-1α-positive cells than the vehicle group ($p < 0.05$; sham, 12.8 ± 4.9 cells; vehicle, 16.4 ± 8 cells; LD, 35.8 ± 9.9 cells; HD, 33.2 ± 16.9 cells) while the difference between the HD and vehicle groups was not significant ($p = 0.08$) (Fig. 6 G, H).

## 3. Discussion

The present study is the first report to show the beneficial effects of sudachitin against cerebral ischemia/reperfusion injury in the tMCAO mouse model. The principal findings were that the administration of a low dose of sudachitin significantly decreased cerebral infarct volume and suppressed apoptosis 5 days after tMCAO (Fig. 4 D, 4 H and 5 D). This study also showed that the Sirt1/PGC-1α pathway was activated ($p < 0.05$) by a low dose of sudachitin 5 days after tMCAO (Fig. 6 D and 6 H). In this study we did not perform TTC staining for two reasons: 1) infarct area was evaluated by immunostaining; 2) all brain sections were required for different immunohistochemical assays.

Mice in the low-dose sudachitin group received 97.1% sudachitin at 5 mg/kg per dose, which was a similar dose and concentration employed in previous publications (Tsutsumi et al., 2014; Kobayashi et al., 2017; Nii et al., 2019). In the present study, a low dose of sudachitin had better effects than a high dose (Fig. 4 D, H; 5 D; and 6 D and H), although we are unable to offer a precise explanation for this observation. However, one possibility is that an overdose of sudachitin might activate some detrimental signaling pathways for cell survival or polarize some pathways to become proapoptotic. Some treatments showed toxic effects when they exceed the recommended dose, such as N nitro-L-arginine, a nitric oxide inhibitor, which was used to reduce infarct volume in a mouse model (Spinnewyn et al., 1999). The bell-shaped dose effect explains that some treatments or compounds can show favorable efficacy when they are administrated at an appropriate dose rather than at a higher dose. The results of the present study suggest that sudachitin shows a bell-shaped dose effect.

In a previous paper, RT-PCR clearly showed that the expression levels of *Ucp1*, *Ucp3*, *Fas*, *Acc1*, *Acc2*, *PGC-1α* and *Sirt1*, which are related to antiinflammation, antioxidation, and mitochondrial functioning and biogenesis, increased in murine adipose/liver tissue of mice fed sudachitin (Tsutsumi et al., 2014). In addition, there is evidence supporting that the Sirt1/PGC-1α signaling pathway improves cell survival after ischemia/reperfusion injury (Liu et al., 2015; Fangma et al., 2021; Li et al., 2021). That evidence suggests that the Sirt1/PGC-1α signaling pathway may be related to the therapeutic effect of sudachitin, by protecting neuronal cells after ischemia/reperfusion injury.

In this study, we evaluated the effect of sudachitin as a pretreatment and as an orally administered treatment. In a previous paper, a mice group fed with a normal diet and another mice group fed with a high-fat diet were treated with sudachitin, with a significant difference in weight detected only in the latter group (Tsutusmi et al., 2014). In *Sirt1* knockout mice, sepsis-induced lung inflammatory injury was aggravated, displaying an increase in caspase-3 and caspase-7 expression (Gao et al., 2015). In LPS-pretreated murine macrophage cells and human primary peripheral blood mononuclear cells, proinflammatory cytokines increased and suppressed the expression of SIRT1, PGC-1α, and PPAR-γ (Wang et al., 2021). Sudachitin might attenuate an inflammatory environment by enhancing/promoting the Sirt1/PGC-1α signaling pathway. Nowadays, there are already some polymethoxyflavone-based products that are being used as supplements against obesity. Following additional evidence-based assessments, we propose that sudachitin be used as a supplement in patients with risk factors for stroke as a way to potentially prevent a future stroke.

The present study has several limitations: 1) sudachitin was not tracked to confirm the target cell(s) in the brain; 2) oxygraphy could not be performed and OXPHOS markers could not be evaluated; 3) additional studies are needed to evaluate the accurate downstream mechanism.

Taken together, we conclude that sudachitin plays a critical role in decreasing final brain infarct volume by reducing apoptotic cell number, possibly through the Sirt1/ PGC-1α signaling pathway. However, additional studies are needed to clarify the impact of sudachitin on lower downstream signaling pathways as well as on other pathways.

## 4. Experimental Procedure

*4.1 Animals*

Adult male C57BL/6J Jms Slc mice (weight: 23–27 g, 8 weeks old) were purchased from Shimizu Laboratory Supplies (Kyoto, Japan). The mice were maintained in a temperature-regulated room (23–25 °C) under a 12/12 hour light-dark cycle with constant humidity and allowed free access to food and water. All experimental procedures were approved by the Animal Committee of the Okayama University Graduate School of Medicine (OKU-2022705) and conducted in accordance with ARRIVE guidelines as well as the Okayama University guidelines on the Care and Use of Laboratory Animals.

*4.2 Sudachitin treatment and experimental group*

Sudachitin, in a powdered form at a concentration of 97.1%, was purchased from DKS Co. Ltd. (Lot No. 220620; Kyoto, Japan). Sudachitin, which was suspended in 0.5% (w/v) methyl cellulose (133-17815; FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan), was administered to each mouse using oral gavage (feeding gavage PTFE; Fuchigami, Japan).

Sample size was calculated based on a report by Momoko et al. (2017). To detect the attenuation of brain infarcts at a two-sided 5% significance level and 80% power, a sample size of 8 mice per group was necessary, as assessed by using G*Power 3.1.9.4 software (Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany). The obtained group number was multiplied by the successful surgical technique rate and the survival rate. Four groups of mice were studied. The sham group (n = 8) received methyl cellulose orally once a day for 14 days and continued to receive it after a sham operation. The vehicle group (n = 22) received methyl cellulose orally for the same period and continued to receive it after tMCAO. Sudachitin doses were set at 5 mg/kg because previous papers showed positive health effects at this dose (Tsutsumi et al., 2014; Kobayashi et al., 2017; Nii et al., 2019), while 50 mg/kg was also evaluated to appreciate if an increased dose could improve the effects. The LD group (n = 24) and the HD group (n = 22) received sudachitin orally for the same period. Each group was divided in two subgroups: one was sacrificed 1 day after tMCAO or the sham operation while the other was sacrificed 5 days after tMCAO or the sham operation (Fig. 1). Total sample number was 76 mice. Mice that had skin injuries observed during the pretreatment period, mice that died before the

sacrificed time point (n = 10), and mice with a Bederson score of 0 after tMCAO were excluded.

*4.3 Focal cerebral ischemia*

On the 14th day (10 weeks old), mice were anesthetized with a mixture of nitrous oxide: oxygen: isoflurane (69: 30: 1%, v/v/v) via an inhalation mask. The right carotid bifurcation was exposed, and a 7-0 nylon (ELP, Akiyama Medical Co., Ltd., Tokyo, Japan) filament thread with silicon coating was introduced through the right common carotid artery to occlude the right middle cerebral artery. After 45 min, the nylon thread was slowly withdrawn to recover cerebral blood flow in the MCA (Abe et al., 1992; Yamashita et al., 2006). During surgery, rectal temperature was maintained at 37.0 ± 0.3 °C by placing the mouse on a heating bed (Model BWT-100; Bio Research Centre, Nagoya, Japan). Sham operation mice were prepared with a cervical incision but without inserting a thread.

*4.4 Neurobehavioral analysis*

The following neurobehavioral tests were evaluated daily: Bederson score 0–4 (Yamashita et al., 2009), Bederson score 0–5 (De Meyer et al., 2011), the hang wire test (Carlson et al., 2010; Mustafa et al., 2013), and the rotarod test (Kawai et al., 2010). Bederson score 0–4 with minor modifications (Yamashita et al., 2009) was assessed as follows: 0, no observable neurological deficits; 1, failure to extend the left forepaw; 2, circling to the contralateral side; 3, falling to the left; 4, unable to walk spontaneously. Bederson score 0–5 (De Meyer et al., 2011) was assessed as follows: 0, no deficits; 1, forelimb flexion; 2, decreased resistance to lateral push; 3, unidirectional circling; 4, longitudinal spinning; 5, no movement.

To perform the hangwire test (Carlson et al., 2010; Mustafa et al., 2013) and the rotarod test (Kawai et al., 2010), mice were trained for 3 days before tMCAO or the sham operation. In the hangwire test, mice were placed on a metal wire mesh, which was then inverted. The hanging time was defined as the time that the mouse held on to the mesh before they fell; maximum hanging time was set as 180 seconds (sec). In each session, the test was repeated three times or until the mouse managed to hold on for 180 sec (Carlson et al., 2010). The longest hanging time served as the data. The rotarod test was performed on each mouse by placing it on top of a rotating rod (type 7650, No 40993; Ugo Basile; Gemonio Varese, Lombardia, Italy). The time that it could last on the rod was

measured in each mouse until it fell or reached the maximum period (300 sec). In each session, the procedure was repeated three times or until the mouse managed to balance for 300 sec. The longest balancing time served as the data.

*4.5 Sample collection and preparation of sections*

Just before sacrifice, a blood sample was collected from the left ventricle of each mouse's heart. Serum samples were separated by centrifugation (3,000 rpm, 4 °C, 10 min) with a S500FR centrifuge (Kubota, Tokyo, Japan) and stored at -80ºC. For histological analysis, mice were deeply anesthetized using isoflurane and then transcardially perfused with chilled phosphate-buffered saline (PBS, pH 7.4), followed by 4% paraformaldehyde (PFA) in PBS. The whole brain was removed and postfixed in 4% PFA overnight at 4 ºC. Fixed brains were incubated in 10%, 20% and 30% (w/v) sucrose in PBS for 24 h in a 4 °C room. Coronal brain sections (20 μm thickness) were cut on a cryostat (HM525 NX; Thermo Fisher Scientific; MA, USA) set at -20 °C between 1.5 mm anterior and 1.5 mm posterior to the bregma. Sections were mounted on silane-coated glass slides (BM5116, Muto Pure Chemicals Co., Ltd., Tokyo, Japan) and covered with cover slip.

*4.6 Measurement of reactive oxygen species and antioxidant capacity in serum*

The presence and level of reactive oxygen species (ROS) in serum were examined by the d-ROMs test (Redoxlibra, Wismerll, Tokyo, Japan). ROS levels were expressed as arbitrary "Carratelli units" (CARR U), with 1 CARR U corresponding to 0.08 mg per 100 mL of $H_2O_2$. Total serum antioxidant capacity was assayed by the OXY-Adsorbent test (Redoxlibra). The d-ROMs and OXY-Adsorbent tests were performed using a spectrophotometer (DI-701M, Redoxlibra) (Tamaki et al., 2011).

*4.7 Histology and immunohistochemistry*

To quantify infarct volume, brain sections (20 μm) were stained with cresyl violet as Nissl staining and images were captured by a microscope (SZX-12; Olympus Optical, Tokyo, Japan). Infarct areas were measured in five sections using Image J software (National Institutes of Health, Bethesda, MD, USA). Final infarct volume was calculated by multiplying each infarct area by 0.5 mm and summing the resulting volumes.

For immunohistochemistry, brain sections were first immersed in citric acid buffer then incubated in 0.3% $H_2O_2$/methanol for 30 min to block endogenous peroxidase activity. Next, sections were incubated in 5% bovine serum albumin containing 0.1%

Triton X for 1 h, then incubated in primary antibody at 4 °C overnight. Primary antibodies used were: Microtubule-associated protein-2 (MAP-2) antibody (1:1000, ab32454; Abcam, Cambridge, UK), Cleaved caspase 3 (CC3) antibody (1:400, 9661; Cell Signaling Technology, Danvers, MA, USA), 4-HNE antibody (1:100, MHN-020P; JaICA, Shizuoka, Japan), silent mating type information regulation 2 homolog 1 (Sirt1) antibody (1:200, 8469; Cell Signaling Technology), peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1α antibody (1:400, NBP1-04676; Novus Biologicals, Centennial, CO, USA). Negative control sections were stained in the same way, but without primary antibody. Sections were then washed in PBS and incubated with anti-mouse or anti-rabbit IgG secondary antibodies at 1:500 for 2 h at room temperature. Sections were then incubated with the avidin-biotin-peroxidase complex (PK-6101, PK-6102; Vectastatin ABC kit, Funakoshi, Japan) for 30 min and finally visualized by the 3, 3'-diaminobenzidine (DAB) reaction. For TdT-mediated dUTP nick end labeling (TUNEL), an in situ Cell Death Detection Kit (Takara, Kusatsu, Japan) was used and TUNEL-positive cells were labeled with DAB.

For CC3 immunostaining, every positive cell in the ischemic/reperfusion ipsilateral hemisphere was counted from three sections of each mouse brain sample, and these were summed. For 4-HNE, TUNEL assay, Sirt1 and PGC-1α, the average number of positive cells in random penumbra areas of three sections per brain was calculated.

*4.8 Statistical analysis*

Data are expressed as the mean ± standard deviation. Statistical analysis was performed using GraphPad Prism version 9.5.0 (GraphPad Software LCC, Boston, MA, USA). A nonparametric statistical analysis was performed followed by the Kruskall-Wallis test. In all statistical analyses, significance was set at $p < 0.05$.

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**Conflict of interest**

T.Y. and F.Y. have patents for the supplemental/therapeutic use of sudachitin for ischemic stroke. The other authors declare no conflicts of interest.

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Neuroscience Letters* 2021; 749: 135736

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## Figure legends

Fig. 1 Experimental design. Mice were divided into four groups: Sham + vehicle (sham) (n = 8), tMCAO + vehicle (vehicle) (n = 22), tMCAO + low dose sudachitin (LD) (n = 24) and tMCAO + high dose sudachitin (HD) (n = 22). Sham and vehicle groups received methyl cellulose (vehicle) as pretreatment and after tMCAO. LD and HD groups received sudachitin as pretreatment and after tMCAO. Black arrow heads indicate the administration of oral sudachitin/vehicle. Each group was subdivided into two subgroups: sacrificed 1 day after tMCAO and sacrificed 5 days after tMCAO.

Fig. 2 Body weight and neurobehavioral test. Body weight of mice was measured before tMCAO and daily thereafter until the day of sacrifice (A, B). Three neurobehavioral tests were administered: Bederson score (C, D), the hangwire test (E , F), and the rotarod test (G, H). Values are means ± S.D. (***p < 0.0005 and ****p < 0.0001 versus the sham group).

Fig. 3 Quantitative analysis of oxidants and antioxidants. Serum level of ROS metabolites (oxidants) expressed by CARR U in 1 day after tMCAO (A) and in 5 days after tMCAO (B) subgroups. Serum levels of antioxidant capacity in 1 day after tMCAO (C) and in 5 days after tMCAO (D) subgroups. There were no significant differences between subgroups in both assays.

Fig. 4 Ischemic infarct volume. Nissl staining in 1 day after tMCAO (A) and in 5 days after tMCAO (C) subgroups with their respective quantitative analyses (B and D). MAP2 staining in 1 day after tMCAO (E) and in 5 days after tMCAO (G) subgroups with their respective quantitative analyses (F and H). Note that infarct volumes decreased significantly in the low-dose sudachitin group compared with the vehicle group 5 days after tMCAO in both staining conditions. Values are means ± S.D. Scale bar: 50 μm. *p < 0.05, **p < 0.005 and ***p < 0.001 versus the vehicle group.

Fig. 5 Apoptosis and oxidative markers in tMCAO mice brains. (A) All the CC3-positive cells from the ipsilateral hemisphere were counted. (B) To count TUNEL-, 4-HNE-, Sirt1- and PGC-1α-positive cells, photos were taken from the penumbra in the cortex and striatum areas. Immunohistochemical staining of apoptotic marker CC3 in 1 day after

tMCAO (C) and in 5 days after tMCAO (D) subgroups with their respective quantitative analyses (D and F). Note that apoptotic cells decreased significantly in LD compared with the vehicle group 5 days after tMCAO. TUNEL assay in 1 day after tMCAO (G) and in 5 days after tMCAO (I) subgroups with their respective quantitative analyses (H and J). Note that TUNEL-positive cells show a decreased tendency in LD compared with the vehicle group 1 day after tMCAO ($p = 0.09$). Immunohistochemical staining of oxidative stress marker 4-HNE in 1 day after tMCAO (K) and in 5 days after tMCAO (M) subgroups with their respective quantitative analyses (L and N). There were no significant differences in LD and HD when compared with the vehicle group 1 day ($p = 0.07$ and 0.12, respectively) and 5 days ($p = 0.44$ and 0.10, respectively) after tMCAO. Arrows indicate positive cells in each staining condition. Values are means ± S.D. Scale bar: 50 μm. *$p < 0.05$ and **$p < 0.005$ versus the vehicle group.

Fig. 6 Sudachitin signaling pathway markers in tMCAO mice brains.
Immunohistochemical staining of Sirt1 in 1 day (A) and in 5 days after tMCAO (C) subgroups with their respective quantitative analyses (B and D). Immunohistochemical staining of PGC-1α in 1 day (E) and in 5 days after tMCAO (G) subgroups with their respective quantitative analyses (F and H). Note that both Sirt1 and PGC-1α expression increased significantly compared with the vehicle group ($p < 0.05$) 5 days after tMCAO. Values are means ± S.D. Scale bar: 50 μm. *$p < 0.05$ and **$p < 0.01$ versus the vehicle group.