Osthole prevents cerebral ischemia/reperfusion injury via Notch signaling pathway

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2 **signaling pathway**

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11 **Abstract**

12 Stroke is a common cerebrovascular disease in aged population with the second leading
 13 mortality and the leading cause of permanent disability, and the ischemic stroke is the primary
 14 form. Osthole is a coumarin derivative extracted from the fruits of *Cnidium monnieri* (Linn.) Cuss.
 15 In this study, we established the rat middle cerebral artery occlusion/reperfusion (MCAO/R)
 16 model *in vivo*, and found that MCAO/R caused cerebral infarction, hippocampus neuronal injury
 17 and apoptosis, and also activated the Notch 1 signaling pathway. However, the treatment of
 18 Osthole further enhanced the activity of Notch 1 signaling, and reduced the cerebral infarction,
 19 hippocampus neuronal injury and apoptosis induced by MCAO/R in a dose-dependent manner.
 20 The same results were observed in primary neuronal oxygen glucose deficiency/reperfusion
 21 (OGD/R) model *in vitro*, and also the effect of Osthole could be blocked by a inhibitor of Notch 1
 22 signaling, DAPT. Therefore, we demonstrated that Osthole injection prevented rat
 23 ischemia/reperfusion injury via activating Notch 1 signaling pathway *in vivo* and *in vitro* in a
 24 dose-dependent manner, which may be of great significant for clinical treatment of ischemic
 25 stroke.

26

27 **Keywords:** Osthole; stroke; ischemia/reperfusion; hippocampus neuron; Notch

28

29 Introduction

30 The World Health Organization (WHO) reported in 2014 that stroke was the second leading
31 cause of mortality with 6.7 million deaths each year around the world (WHO 2014). Stroke is also
32 a leading cause of permanent disability (Kim et al. 2016). Stroke is categorized into two major
33 subtypes, ischemic stroke and hemorrhagic stroke, wherein the former accounts for 80-85% (Kim
34 et al. 2016). Ischemic stroke is caused by the formation of a blood clot in a cerebral artery, which
35 is associated with hypertension, hyperlipidemia, diabetes mellitus and arteriosclerosis
36 (Rosamond et al. 2007). Continuous ischemia leads to lesions and necrosis of brain tissue, and
37 recovery of blood supply can rescue the reversible damage in brain tissue caused by ischemia
38 (Schaller and Graf 2004). However, ischemia/reperfusion may result in additional injuries.
39 Pathogenic edema and intracranial hemorrhage occurred in some ischemic stroke patients after
40 thrombolysis. In several animal ischemic stroke models, reperfusion after ischemia for a long time
41 would result in more serious cerebral infarction than permanent vascular ligation (Aronowski et
42 al. 1997; Yang and Betz 1994). The mechanism of ischemia/reperfusion has not been completely
43 illuminated, but it has been associated with reactive oxygen species (ROS), inflammation,
44 mitochondria dysfunction, Ca^{2+} overload, apoptosis and autophagy.

45 Osthole (7-methoxy-8-isopentenoxycoumarin) is a coumarin derivative extracted from a
46 Chinese herbal medicine, the fruits of *Cnidium monnieri* (Linn.) Cuss. Since its anti-oxidant,
47 anti-inflammation and anti-tumor potential, Osthole has been used for gynecopathy and
48 andrology diseases for many years. Recently, it has been reported that Osthole can ameliorate
49 brain damage of mice and rats after ischemia via different signaling pathways (Chao et al. 2010;
50 Chen et al. 2015; Li et al. 2012; Li et al. 2016; Mao et al. 2011).

In this study, we established rat middle cerebral artery occlusion/reperfusion (MCAO/R) model, and probed whether Osthole intervene the ischemia/reperfusion damage and the underlying mechanism.

Methods

Ethical statement

Animals in our study were taken care of according to Guide for the Care and Use of Laboratory Animals (1996, published by National Academy Press, 2101 Constitution Ave. NW, Washington, DC 20055, USA) and Laboratory Animal Care and Use Standard of China Medical University. Animal treatment and experimental procedures were approved by the Ethics Committee of China Medical University.

Animal model

Healthy adult male SD rats (250-280g) (Vital River, Beijing, China) were randomly divided into 5 groups: Sham, MCAO/R, MCAO/R+Ost(25), MCAO/R+Ost(50) and MCAO/R+Ost(100) (n=6 per group). The rats in MCAO/R+Ost(25), MCAO/R+Ost(50) and MCAO/R+Ost(100) groups were intraperitoneally injected with Osthole (Aladdin, Shanghai, China) of 25 mg/kg, 50 mg/kg and 100 mg/kg, and the rats in Sham and MCAO/R groups were injected with the same volume of 10% Tween 80 as the control. 30 min later, all rats were anesthetized with 10% chloral hydrate (3.5 mg/kg), and ischemia for 120 min by Suture Embolization Method according to previous description (Jiang et al. 2016). The rats in Sham group were treated with the same operation but without ischemia. Then the reperfusion was induced, and rats in MCAO/R+Ost(25), MCAO/R+Ost(50) and MCAO/R+Ost(100) groups were intraperitoneally injected with Osthole of 25 mg/kg, 50 mg/kg and 100 mg/kg again, with the rats in Sham and MCAO/R groups injected

73 with the same volume of 10% Tween 80. After reperfusion for 24 h, all rats were sacrificed with
74 10% chloral hydrate (5 mg/kg) through intraperitoneal injection, and the brain tissues were
75 collected for the subsequent experiments.

76 ***TTC staining***

77 The fresh brain tissues were frozen at -20 °C for 30 min and cut into sections of 2 mm. The
78 sections were stained in 1% 2,3,5-triphenyl tetrazolium chloride (TTC) at 37 °C for 15 min. During
79 the staining process, the sections were turned over continually to be stained evenly. Thereafter,
80 the percentage of infarction area was measured with ip6.0. Five samples were estimated in each
81 group, and the average value was calculated.

82 ***Nissl staining***

83 The hippocampus tissue was fixed with 10% formaldehyde (Sinopharm) overnight, washed
84 with water for 4 h, and dehydrated with ethanol of 70% for 2 h, 80% for 2 h, 90% for 2 h and 100%
85 for 1 h twice. After permeated with xylene (Sinopharm) for 30 min, the tissue was embedded
86 with paraffin at 60 °C and cut into sections of 5 µm. The sections were dried at 60 °C, dewaxed
87 with xylene for 15 min twice, and rehydrated with ethanol of different concentrations (100% for 5
88 min twice, 95% for 2 min, 85% for 2 min and 75% for 2 min) and water for 3 min. Subsequently,
89 the sections were stained with 0.5% cresol purple (Sinopharm) at temperature room for 10 min,
90 soaked with 0.25% acetic acid/ethanol (Kermel, Tianjin, China) for several seconds, and
91 dehydrated with anhydrous ethanol (Sinopharm) for 5 min twice and xylene for 10 min twice.
92 After wiping off residual liquid, the sections were mounted with half one drop of gum, and
93 photographed with a microscope (Olympus, Tokyo, Japan) at 200× magnification.

94 ***TUNEL assay***

95 The hippocampus tissue was fixed with 10% formaldehyde (Sinopharm) at temperature
96 overnight and washed with flow water for 4 h. Subsequently, the tissue was dehydrated with
97 ethanol of different concentrations (70% for 2 h, 80% overnight, 90% for 2 h and 100% for 1 h
98 twice), permeated with xylene (Sinopharm) for 30 min, embedded with paraffin at 60 °C and
99 cut into sections of 5 µm. The sections were dried on glass slides at 60 °C, dewaxed with xylene
100 for 15 min twice, hydrated with ethanol of 100% for 5 min twice, 95% for 2 min, 85% for 2 min,
101 75% for 2 min and water for 2 min. Then the sections were permeated with 0.1% Triton X-100
102 (Beyotime, Haimen, Jiangsu, China) for 8 min, blocked with 3% H₂O₂ (Sinopharm) for 10 min and
103 incubated with TUNEL reagent (Roche, Basel, Switzerland) at 37 °C in dark for 60 min. After
104 rinsing with PBS, the sections were incubated with Converter-POD (Roche) at 37 °C for 30 min,
105 reacted with DAB reagent (Solarbio, Beijing, China), stained with hematoxylin (Solarbio) for 3 min,
106 soaked in 1% hydrochloric acid/ethanol for 3 s, and washed with flow water for 20 min.
107 Thereafter, the sections were dehydrated with ethanol of different concentrations (75% for 2 min,
108 85% for 2 min, 95% for 2 min and 100% for 10 min twice) and xylene for 10 min twice, and
109 mounted with half one drop of gum. Finally, the sections were photographed with a microscope
110 (Olympus) at 400× magnification.

111 ***Immunofluorescence (IF) assay***

112 The hippocampus tissues were made into paraffin according to the previous description. The
113 sections were dried at 60 °C, dewaxed with xylene for 15 min twice, and hydrated with ethanol
114 of 95% for 1 min, 85% for 1 min and 75% for 1 min. After washing with PBS for 5 min 3 times, the
115 sections were repaired with antigen retrieval buffer for 10 min, and washed with PBS for 5 min
116 twice. After blocking with goat serum at room temperature for 30 min, the sections were

117 incubated with antibodies of mouse anti-NeuN (1:200) (Abcam, Cambridge, UK) and rabbit
118 anti-cleaved caspase-3 (1:200) (CST, Boston, MA, USA) together at 4 °C overnight. After washing
119 with PBS for 5 min 3 times, the sections were incubated with goat anti-mouse IgG labeled with
120 Cy3 (1:200) (Beyotime) and goat anti-rabbit IgG labeled with FITC (1:200) (Beyotime) at room
121 temperature for 90 min in dark. After washing with PBS for 5 min 3 times, the sections were
122 counter-stained with DAPI, mounted in the presence of Antifade Mounting Medium (Solarbio),
123 and photographed with a fluorescence microscope (Olympus) at 400× magnification.

124 ***Western blot***

125 The total protein was extracted from hippocampus tissues or primary neurons by RIPA lysis
126 buffer (Beyotime). After degenerating via boiling, the protein sample was separated by SDS-PAGE
127 and transferred onto PVDF membrane (Millipore, Boston, MA, USA). After blocking with skim milk
128 (YILI, Hohhot, Inner Mongolia, China) at room temperature for 1 h, the PVDF membrane was
129 incubated with the following antibodies at 4 °C overnight: rabbit anti-cleaved caspase-3 (Abcam,
130 Cambridge, UK) (1:1000), rabbit anti-Bax (Boster) (1:400), rabbit anti-Bcl-2 (Boster, Wuhan, Hubei,
131 China) (1:400), rabbit anti-Notch 1 (Abcam) (1:500), rabbit anti-NICD1 (Sangon, Shanghai, China)
132 (1:500), and rabbit anti-Hes 1 (Boster) (1:400). After washing with PBS, the PVDF membrane was
133 incubated with goat anti-rabbit IgG-HRP (Beyotime) (1:5000) at 37 °C for 45 min, and explored
134 with ECL reagent (7 sea, Shanghai, China). After removing the antibodies with stripping buffer
135 (Beyotime), the PVDF membrane was incubated with mouse anti- β -actin (Santa Cruz, CA, USA)
136 (1:1000) and goat anti-mouse IgG-HRP (Beyotime) (1:5000) to detect the internal control, β -actin.

137 ***Primary neurons isolation and culture***

138 The hippocampus tissue isolated from neonatal SD rats was washed with PBS twice and cut

139 into pieces of 1-3 mm³. Subsequently, the pieces were digested with trypsin at 37 °C for 30 min
140 and centrifuged at 2500 rpm for 10 min. After removing the supernatant, the precipitation was
141 resuspended with PBS, and centrifuged at 2500 rpm for 10 min. After removing the supernatant,
142 the precipitation was resuspended with Neurobasal medium (Gibco BRL, Gaithersburg, MD, USA),
143 and filtered with 200-mesh sieve to remove the tissue masses. The filtrate was centrifuged at
144 1000 rpm for 10 min, the cells in the precipitation was collected, washed with PBS twice, seeded
145 into plates and cultured with Neurobasal medium added with 2% B27 (Gibco) at 37 °C in 5% CO₂.

146 Establishment of the oxygen glucose deficiency/reperfusion (OGD/R) model: the neurons were
147 cultured with Neurobasal medium (no glucose) in incubator with 5% CO₂ and 95% N₂ for 2 h, and
148 then re-cultured in normal condition.

149 **MTT assay**

150 The neurons were seeded into 96-well plates at a density of 4×10³ per pore and cultured at
151 37 °C with 5% CO₂. 22 h later, the neurons were treated with Osthole of different
152 concentrations for 2 h. Subsequently, the neurons were treated with OGD/R according to the
153 previous description. After treating with OGD/R, the cells were added with MTT (0.2 mg/ml)
154 (Sigma, St. Louis, MO, USA) to incubate for 4 h and centrifuged at 1000 rpm for 10 min. The
155 supernatant was discarded, and 200 µl of dimethyl sulfoxide (DMSO) (Sigma) was added to
156 dissolve the formazan crystal. Subsequently, the optical density of the solution was detected with
157 a microplate reader (BioTek, VT, USA) at 490 nm.

158 **Lactic dehydrogenase (LDH) assay**

159 The LDH content in the cell culture supernatant was detected with LDH assay kit (Jiancheng,
160 Nanjing, Jiangsu, China) according to the manufacturer's protocol.

Flow cytometry

The neurons were seeded into plates and cultured with Neurobasal medium supplemented with 2% B27. After culturing for 22 h, the neurons were treated with Osthole of different concentrations for 2 h, and treated with OGD/R. Thereafter, the neurons were collected, centrifuged at 800 rpm for 5 min. After removing the supernatant, the cells were washed with PBS and centrifuged at 800 rpm for 5 min twice. Then the cells were treated with Annexin V-FITC/PI Apoptosis Detection Kit (KeyGEN, Nanjing, Jiangsu, China) according to the manufacturer's instruction, and detected with flow cytometry (BD, Franklin Lakes, NJ, USA).

Statistical analysis

The data in this study were presented as mean $\pm$ standard deviation (SD), and analyzed by one-way ANOVA test. The data of animal tissues were collected from six individual experiments, and cell data were obtained from three individual experiments. It was considered statistically significant when $P < 0.05$. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns: no significance)

Results**Osthole reduced ischemia-post cerebral infarction and hippocampus neuronal apoptosis**

The rat MCAO/R model was established by Suture Embolization Method and some rats were treated with Osthole of 25 mg/kg, 50 mg/kg and 100 mg/kg. Subsequently, TTC staining was used to detect the cerebral infarction of rats with different treatments. The results showed that MCAO/R led to an infarction of 39.4% in cerebra, and Osthole decreased the infarction by 3.3%, 22.8% and 32.8% (Fig. 1A and B). Then Nissl staining results revealed that MCAO/R caused hippocampus neurons injury and the injury was mitigated by the treatment of Osthole (Fig. 1C and E). The similar results were observed in TUNEL assay, namely MCAO/R induced hippocampus

neuronal apoptosis and Osthole alleviated the apoptosis (Fig. 1D and F). Thereafter, IF was performed to measure apoptosis by testing the apoptosis executor, cleaved caspase-3, and a neuronal marker, NeuN. As shown in Fig. 2, MCAO/R increased the level of cleaved caspase-3 and decreased the level of NeuN significantly, which were rescued by Osthole (Fig. 2A-C). Finally, we measured the expression levels of cleaved caspase-3, Bax and Bcl-2, by western blot. As shown in Fig. 2, after MCAO/R, cleaved caspase-3 was increased by 3.2-folds, Bax was elevated by 3.41-folds, and Bcl-2 was decreased by 70% (Fig. 2D and E). However, the treatment of Osthole of 25 mg/kg, 50 mg/kg and 100 mg/kg descended cleaved caspase-3 by 41.6%, 50.8% and 54.8%, reduced Bax by 27.6%, 37.7% and 52.2%, and ascended Bcl-2 by 1.4 folds, 2.32 folds and 2.6 folds (Fig. 2D and E), respectively.

Osthole enhanced the activity of Notch 1 signaling pathway *in vivo*

To investigate the relationship among MCAO/R, Osthole and Notch 1, we detect the expression levels of Notch 1, Notch intracellular domain (NICD) 1 and a canonical target of Notch 1, Hes 1 after treating with MCAO/R or MCAO+Osthole. As shown in western blot results, MCAO/R increased the expression of Notch 1 by 1.86-folds, NICD by 1.34-fold and Hes 1 by 1.6-fold (Fig. 2F and G). Moreover, after treatment with Osthole, the level of Notch 1 further increased by 1.06-fold, 1.17-fold and 1.45-fold, the level of NICD 1 further increased by 1.1-fold, 1.26-fold and 1.65-fold, and the level of Hes 1 further increased by 1.03-fold, 1.13-fold and 1.5-fold (Fig. 2F and G), respectively, comparing with MCAO/R groups. These results discovered that MCAO/R activated the Notch 1 signaling pathway and the Osthole strengthened the activity in a dose-dependent manner.

Osthole increased the viability of primary neuron

205 The above results proved that MCAO/R induced cerebral infarction and neuronal injury, and
206 activated Notch 1 signaling pathway *in vivo*, next we explored whether Osthole reduced neuronal
207 injury via Notch 1 signaling pathway *in vitro*. The primary neurons were isolated from
208 hippocampus tissues of fetal rats, and cultured in glucose-free medium without O₂ to simulate
209 the ischemia/reperfusion *in vitro*. After pre-treating with Osthole of 10 μ M, 50 μ M and 100 μ M,
210 the neurons were detected by MTT assay to measure the viability. The results showed that
211 OGD/R decreased the neuronal viability by 43%, and the pre-treatment of Osthole recovered the
212 viability by 19%, 87% and 84% (Fig. 3A). Then a common cerebral damage marker, LDH, was
213 measured. As exhibited in the results, OGD/R enhanced the LDH activity by 3.8-fold, but the
214 Osthole of 10 μ M, 50 μ M and 100 μ M weakened the LDH activity by 96%, 65% and 59% (Fig. 3B).
215 To probe whether Osthole played a role via Notch 1 pathway, a γ -secretase inhibitor,
216 N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) was used with 1 μ M.
217 Considering the effect of Osthole of different concentrations, 50 μ M was selected as the
218 experimental concentration in the subsequently experiments. Then we detected the neuronal
219 viability and found that DAPT inhibited the function of Osthole in some degree (Fig. 3C), and
220 similar results were observed in LDH enzymatic activity assay results (Fig. 3D).

221 **Osthole inhibited the neuronal apoptosis induced by OGD/R and the inhibition was reversed by**
222 **DAPT**

223 Next, we tested the apoptosis of primary neurons *in vitro* by flow cytometry. The results
224 revealed that OGD/R enhanced the apoptosis rate of primary neurons by 9.2%, but the
225 enhancement was inhibited 52% by Osthole, however, the inhibition was neutralized by DAPT
226 partly (Fig. 4A and B). Then the expression levels of cleaved caspase-3, Bax and Bcl-2 in the

primary neurons were detected by western blot. As similar to the results *in vivo*, after treating with OGD/R, the levels of cleaved caspase-3 and Bax were increased, and the level of Bcl-2 was decreased, which were recovered by Osthole. However, the recovery was relieved by DAPT partly (Fig. 4C and D), suggesting that Osthole inhibited the ischemia/reperfusion-induced apoptosis of neurons by activating Notch pathway.

Osthole promoted the activity of Notch 1 signaling pathway *in vitro*

To verify the hypothesis that Osthole alleviated the neuronal apoptosis induced by ischemia/reperfusion via Notch 1 signaling pathway, we detect the expression levels of Notch 1, NICD 1 and Hes 1 in the primary neurons with different treatments. As expected, OGD/R increased the levels of Notch 1 by 1.6-fold, NICD 1 by 1.9-fold and Hes 1 by 1.5-fold, and Osthole further promoted the expression of Notch 1 by 1.4-fold, NICD 1 by 1.3-fold and Hes 1 by 1.5-fold. However, the increase was suppressed by DAPT, an inhibitor of Notch pathway (Fig. 4E and F).

Discussion

Notch is a class of highly conserved cell surface receptors. There are four isomers of Notch in mammals, Notch 1-4. Notch is a transmembrane protein, including extracellular domain and intracellular domain. In the canonical Notch signaling pathway, when the extracellular domain binds to the ligand, the intracellular domain is hydrolyzed twice by tumor necrosis factor (TNF)- α converting enzyme (TACE) and γ -secretase into the activated form, Notch intracellular domain (NICD). Subsequently, NICD translocates into the nuclear, and binds to the transcriptional factor CBF-1/suppressor of hairless/LAG-1 (CSL), to activate the expression of downstream genes (Crabtree et al. 2016). CSL is a transcriptional inhibitor when it exists alone, but induces the transcription of corresponding genes after combining with NICD (Kovall and Blacklow 2010).

249 Notch 1 is highly expressed in neural stem cells, and regulates neural development and
250 dendrite morphology (Ding et al. 2016). What's more, Notch 1 signaling pathway was activated
251 after ischemia *in vivo and in vitro*, with a neural repair function. The activation of Notch 1 could
252 promote the proliferation and differentiation of neural progenitor cells after ischemic stroke
253 (Wang et al. 2009a). Increased activation of Notch 1 enhances neurogenesis in the subventricular
254 zone (SVZ) of ischemic rat brain, and inhibiting Notch 1 signaling results in a reduction of
255 proliferating cells SVZ cells in the SVZ (Wang et al. 2009b). It has also been reported that Notch
256 signaling promotes macrophage activation and dendritic cells differentiation after stroke (Brifault
257 et al. 2015; Shang et al. 2016). Notch signaling contributes to the maintenance of NF- κ B
258 activation, and enhances the inflammatory response (Cai et al. 2016; Zhang et al. 2015). In
259 addition, Notch 1 activates microglia after focal cerebral ischemia, which is able to mediate the
260 inflammation, clear the damaged neurons and repair the injury after ischemia (Wei et al. 2011).
261 In addition, ischemic injury could be alleviated by several drugs via Notch 1 signaling (Chen et al.
262 2008; Liu et al. 2016; Yuan et al. 2015). In this study, we established rat MCAO/R model and
263 found that MCAO/R caused cerebral infarction, neuronal injury and apoptosis, and activated
264 Notch 1 signaling *in vivo*. However, Osthole further enhanced the activity of Notch 1 signaling,
265 and reduced the cerebral infarction, neuronal injury and apoptosis caused by MCAO/R in a
266 dose-dependent manner. The same results were observed in primary neurons treating with
267 OGD/R *in vitro*.

268 Ischemia/reperfusion injury is a complex process, including oxidative stress, inflammation and
269 calcium overload, involving in multiple signaling pathways. As a well-studied pathway, Notch
270 plays a crucial role in organ development, immune regulation and tumorigenesis (Hitoshi et al.

271 2002; Rooman et al. 2006; Shang et al. 2016). On the other hand, since extracting from a
 272 traditional Chinese medicine, Osthole has been used for many years with its anti-inflammation,
 273 bacteriostat and anti-tumor functions. In this study, we demonstrated that Osthole ameliorated
 274 decreased cerebral infarction, restrained neural apoptosis induced by ischemia/reperfusion *in*
 275 *vivo* and *in vitro* via Notch signaling pathway. These findings may provide some research basic for
 276 clinical treatment of ischemia stroke.

277 Conclusion

278 In this study, we established rat MCAO/R model and demonstrated that Osthole prevented
 279 cerebral infarction, hippocampus neuronal injury and apoptosis induced by MCAO/R by activating
 280 Notch 1 signaling pathway in a dose-dependent manner *in vivo*. Similar results were observed in
 281 OGD/R-treated primary neurons *in vitro*. These findings may provide some research basic for
 282 clinical therapy of ischemia/reperfusion.

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286 **Conflict of interest**

287 There is no conflict of interest between authors of this article, neither between authors and
288 any other institutions or individuals.

Draft

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369

370 **Figure legends**

371 **Fig. 1 Osthole reduced the cerebral infarction and hippocampus neuronal injury induced by**
372 **middle cerebral artery occlusion/reperfusion (MCAO/R)**

373 (A) TTC staining was performed to detect cerebral infarction of rats suffering from MCAO/R or
374 Osthole. The scale bar represented 4 mm. (B) The cerebral infarction volume percentage of rats in
375 each group. (C) Nissl staining was used to detect the hippocampus neuronal injury of rats in each
376 group. The scale bar represented 100 μ m. (D) TUNEL assay was carried out to test hippocampus
377 neuronal apoptosis of rats in each group. The scale bar represented 50 μ m. The representative
378 image was taken at 600 $\times$ magnification, and the TUNEL-positive cells were indicated with arrows.
379 (E) Quantification of Nissl staining of hippocampus neurons. (F) Quantification of TUNEL-positive
380 cells. (* $P<0.05$, *** $P<0.001$, ns: no significance)

381 **Fig. 2 Osthole alleviated MCAO/R-induced hippocampus neuronal apoptosis and enhanced the**
382 **activity of Notch 1 signaling pathway *in vivo***

383 (A) Immunofluorescence (IF) was used to measure the neuronal apoptosis by testing cleaved
384 caspase-3 and NeuN in hippocampus tissue of rats with different treatments. The scale bar
385 represented 50 μ m. (B) Quantification of NeuN fluorescence signal. (C) Quantification of cleaved
386 caspase-3 fluorescence signal. (D) The expression levels of cleaved caspase-3, Bax and Bcl-2 in the
387 hippocampus tissues were detected by western blot. (E) The western blot bands of cleaved
388 caspase-3, Bax and Bcl-2 in the hippocampus tissues. (F) The expression level of Notch 1, Notch
389 intracellular domain (NICD) 1 and Hes 1 in the hippocampus tissues was detected by western blot.
390 (G) The western blot bands of Notch 1, Notch intracellular domain (NICD) 1 and Hes 1 in the
391 hippocampus tissues. (* $P<0.05$, ** $P<0.01$, *** $P<0.001$, ns: no significance)

392 **Fig. 3 Osthole increased the viability of primary neuron**

393 (A) The MTT assay was performed to measure the viability of primary neurons treated with
394 Osthole of 10 μ M, 50 μ M and 100 μ M. (C) The viability of primary neuron suffering from different
395 treatments was tested at 490 nm. (B) The enzymatic activity of lactic dehydrogenase (LDH) in
396 primary neurons culture supernatant treated with Osthole of 10 μ M, 50 μ M and 100 μ M. (D) The
397 LDH activity in culture supernatant of primary neurons under different treatments was detected.
398 (** $P < 0.01$, *** $P < 0.001$, ns: no significance)

399 **Fig. 4 Osthole inhibited OGD/R-induced neuronal apoptosis and promoted the activity of Notch**
400 **1 signaling pathway *in vitro***

401 (A) Flow cytometry was performed to measure primary neuronal apoptosis with different
402 treatments. (B) Apoptosis rate of neurons in each group. (C) The expression levels of cleaved
403 caspase-3, Bax and Bcl-2 in primary neurons were tested by western blot. (D) The western blot
404 bands of cleaved caspase-3, Bax and Bcl-2 in primary neurons. (E) Western blot was used to
405 measure the expression levels of Notch 1, NICD 1 and Hes 1 in primary neuron with different
406 treatments. (F) The western blot bands of Notch 1, NICD 1 and Hes 1 in primary neuron with
407 different treatments. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

408

figure 1

199x285mm (300 x 300 DPI)

figure 2

171x295mm (300 x 300 DPI)

figure 3

186x174mm (300 x 300 DPI)

figure 4

185x247mm (300 x 300 DPI)