

Deltonin Ameliorates Cerebral Ischemia/Reperfusion Injury in Correlation with Modulation of Autophagy and Inflammation

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Introduction: Deltonin, an active component extracted from *Dioscorea zingiberensis* C.H. WRIGHT, was widely utilized in traditional Chinese medicines. It has been shown to have anti-cancer functions such as colon cancer, breast cancer, and head and neck squamous carcinoma. Herein, we will investigate the role of deltonin in cerebral ischemia/reperfusion injuries.

Methods: Ly294002 and anisomycin were used as inhibitors to monitor the effects of deltonin. Middle cerebral artery occlusion I/R model was constructed. Infarct volumes, neurological deficits and brain water contents were evaluated under different conditions. Rotarod test, ELISA, and Western blotting were carried to investigate the effects in vitro.

Results: We found that deltonin in ischemia/reperfusion (I/R) rats greatly enhanced brain damages as well as neurological functions through up-regulating p-Akt and p-mTOR as well as inhibiting the expressions of LC3-II/LC3-I, Beclin-1, IL-1, TLR4, and p-p38. Deltonin exerted neuroprotection effect through relieving autophagy activity by regulating PI3K/Akt/mTOR signaling. Deltonin suppressed inflammation reactions through modulation TLR4/p38/MAPK signaling as well.

Conclusion: Overall, our data suggested that deltonin could suppress ischemic brain injury by regulating autophagy and inflammation during I/R. Deltonin can be a potential therapeutic method for patient with I/R.

Keywords: deltonin, cerebral I/R, MCAO/R, autophagy, inflammation

Introductions

Ischemic stroke is resulted from the occlusion of major cerebral arteries. It is a devastating disease, with a major cause of death and disability worldwide. Deltonin is a kind of steroidal saponin, with a chemical structure of C₄₅H₇₂O₁₇. Deltonin, an active component extracted from *Dioscorea zingiberensis* C.H. WRIGHT, was widely utilized in traditional Chinese medicines. It has been shown to have anti-cancer functions such as colon cancer,¹ breast cancer,² and head and neck squamous carcinoma.³ Xie et al demonstrated that deltonin was associated with the cell apoptosis and autophagy in head and neck squamous carcinoma FaDu cell.³ Deltonin may have a strong connection with cell apoptosis and autophagy. Herein, we will investigate the role of deltonin in cerebral ischemia/reperfusion injuries.

Autophagy is a cellular function that is related to cell homeostasis, defenses and adaptations to different environments. Regarding ischemia, many previous reports have revealed that autophagy participates actively in the developments of cerebral ischemia,⁴ including global ischemia,⁵ focal ischemia,⁶ and also

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hypoxia-ischemia.^{5,7} Neuroinflammation caused by ischemia/reperfusion injuries brings damages to the neuron cells. Therefore, the inhibitions of autophagy and inflammation are critical to prevent the neuro-degenerative damages after focal cerebral I/R.⁸

PI3K/Akt/mTOR pathway has been proved to play central roles in ischemia-reperfusion (I/R) injuries. For instance, Zhang reported that sevoflurane post-conditioning protected rat hearts against ischemia-reperfusion injury through the activation of PI3K/AKT/mTOR signaling.⁹ Chen also reported the neuroprotective effects of brain-derived neurotrophic factor mediated by autophagy through the PI3K/Akt/mTOR pathway.¹⁰ In addition, TLR4/p38/MAPK pathway was also reported to be involved in the neuro inflammations in microglia. In 2016, Swaroop reported that HSP60 could regulate IL-1 β -related microglial inflammatory reactions through TLR4-p38/MAPK. In this paper, we aim to reveal the functional mechanisms of deltonin, PI3K/Akt/mTOR pathway TLR4-p38 MAPK pathway in the autophagy and inflammation caused by cerebral ischemia/reperfusion injury, as well as their inner associations.

Methods and Materials

Reagents

Deltonin with a purity of over 98% was generated as previously described and its purity determined by high-performance liquid chromatography (>98%) according to Tong et al.¹ Figure 1A shows the chemical structure of deltonin.

LY294002, anisomycin and TTC reagents were obtained from Sigma, USA. Assays to detect IL-6, IL-10, TNF- α , and iNOS were provided by Jiancheng Bio. Ins., China. Rabbit anti-Akt, anti-Phospho-Akt (p-Akt), anti-mTOR, anti-Phospho-mTOR (anti-p-mTOR), anti-P38, anti-Phospho-p38 (anti-p-P38), anti-LC3, anti-Becclin-1, anti-microtubule-associated protein (anti-MAP-2), anti-TLR4 and anti-IL-1 were obtained from Abcam, USA). Rabbit anti-GAPDH was purchased from Beijing Zhongshan Jinqiao Bio., China. Horseradish peroxidase linked goat anti-rabbit IgG was provided by Santa Cruz Biotechnology, US.

Animals

Male Sprague-Dawley rats (200–220g) were bought from the Experimental Animal Center of Fu-Ning People's Hospital, Yancheng, China. Rats were maintained at 22–24°C with a 12-hr light and 12-h dark cycle. They were free to eat and drink. Animal Care and Use Committee of Fu-Ning People's Hospital approved our researches, which were conducted by strictly following the NIH Guidelines for the Care and Use of Laboratory Animals.

Focal Cerebral I/R Model

MCAO/R (middle cerebral artery occlusion I/R), 2/24h¹¹ was conducted firstly by anesthetizing the rats with 10% (w/v) chloral hydrate (350 mg/kg) intraperitoneally at 37°C. After incising the skins and muscles, left common

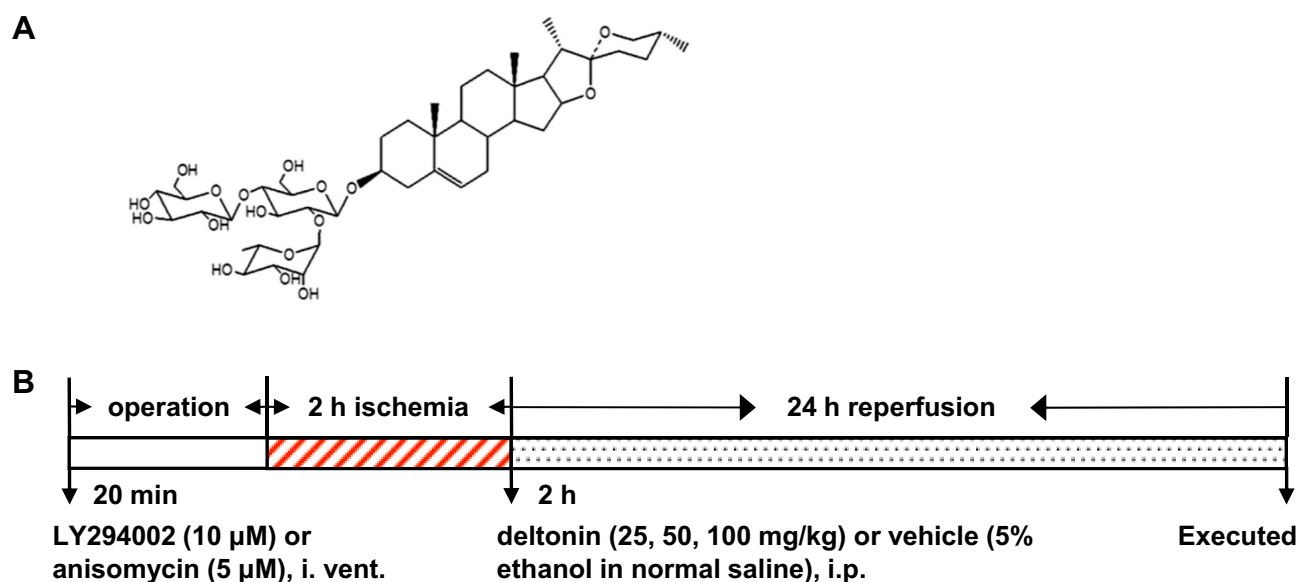


Figure 1 Deltonin's chemical structure (A) and experiment protocol (B).

carotid artery (CCA) was clipped, and external carotid artery (ECA) was ligatured. A monofilament was inserted from CCA to internal carotid artery (ICA). After 2 hrs' MCAO, reperfusion was conducted via removing the monofilament. The sham group had I/R surgery, without the insertion of nanofilament.

Groups and Drug Administration

300 rats were divided to 7 groups: (1) sham ($n = 60$), rats had I/R procedures, without the insertion of nanofilament to CCA; (2) I/R ($n = 60$), the rats had cerebral ischemia by ligation for 2 hrs, and reperfusion for 1 day; (3) 25 mg/kg deltonin ($n = 30$), (4) 50 mg/kg deltonin ($n = 30$), and (5) 100 mg/kg deltonin ($n = 60$). Rats were injected intraperitoneally with 25, 50, 100 mg/kg deltonin at 2 hrs after ischemia; (6) 100 mg/kg deltonin + 10 μ M LY294002 ($n = 30$), the rats were slowly injected intraventricularly with 10 μ L 10 μ M LY294002 at 20 mins prior to ischemia; (7) 100 mg/kg deltonin + 5 μ M anisomycin ($n = 30$), the rats were slowly injected intraventricularly with 10 μ L 5 μ M anisomycin at 20 mins prior to ischemia. Deltonin was dissolved in ethanol and saline to reach an ethanol concentration of 5%.¹² LY294002 and anisomycin were dissolved in 5% ethanol. Rat in sham and I/R groups had same volume of 5% ethanol in normal saline. After 24 hrs reperfusion, rats were anesthetized and decapitated. [Figure 1B](#) shows the experiment protocols.

Assessments of Infarct Volumes, Neurological Deficits and Brain Water Contents

After reperfusion, the rats ($n = 6$) were anesthetized and decapitated. The brains were dissected and sliced (5 slices of 1.5-mm tissues). They were stained with 1% TTC for 30 min and fixed by 4% paraformaldehyde. We recorded the stained parts and calculated the volumes by ImageJ. Neurological deficits were evaluated: 0, rat had normal behavior; 1, rat cannot fully stretch left front leg; 2, rat turns around to a circle; 3, rat falls to the left; 4, rat cannot move, and lost consciousness. Brain water contents ($n = 6$) were detected at 24 hrs after reperfusion. We measured infarct brain hemispheres as wet weight, dried them overnight at 105 to get dry weight. Brain water was calculated as $[(\text{wet weight} - \text{dry weight})/\text{wet weight}] \times 100\%$.

Rotarod Test

Rotarod test system (TSE, Germany) evaluated neurological functions.¹³ Rat was trained for 3 days prior to surgeries. The speed was accelerated from 4 to 40 rpm in 5 mins. Prior to or after I/R/R, each rat was detected for triplicates. We recorded the time needed for them to run on the rods.

ELISA

ELISA was utilized to measure the protein expressions of IL-6, IL-10, TNF- α , and iNOS. Firstly, we extracted the brain tissues from ischemic area, homogenized them (10%, w/v), and had centrifugation at 2,500 r/min for 10 mins. In the supernatants, the protein concentration was detected by ELISA. We visualized the results through the Multiskan MK3 (Thermo Scientific, USA).

Western Blotting

We isolated the hippocampus and measured the protein concentration via BCA detection kit (Beyotime, China). The proteins were separated via 10 SDS-PAGE and electro-transferred to a nitrocellulose membrane. The membrane was blocked by 5% skim milk. After washing, it was treated with anti-Akt (1:500), anti-p-Akt (1:500), anti-mTOR (1:500), anti-mTOR (1:500), anti-P38 (1:600), anti-p-P38 (1:600), anti-LC3 (1:1000), anti-Becclin-1 (1:1000), anti-MAP-2 (1:1000), anti-TLR4 (1:1000) and anti-IL-1 (1:1000). All were purchased from Abcam, USA. Then, it was incubated with secondary antibody for 2 hrs. We visualized the signals via Odyssey system (LI-COR, USA) and analyzed by ImageJ.

Statistical Analysis

We showed the data as the means $\pm$ standard deviation (S.D.). The comparisons were carried out by one-way ANOVA with Holm-Sidak post hoc test. Differences were regarded to be significant as $P < 0.05$.

Results

Deltonin Improved Neurological Function and Reduced Cerebral Infarction

Compared with the sham, a great deficit was detected in I/R ($P < 0.01$), which were markedly relieved by deltonin ($P < 0.05$, $P < 0.01$) ([Figure 2A](#) and [B](#)). From [Figure 2C](#) and [D](#), we observed that cerebral I/R greatly

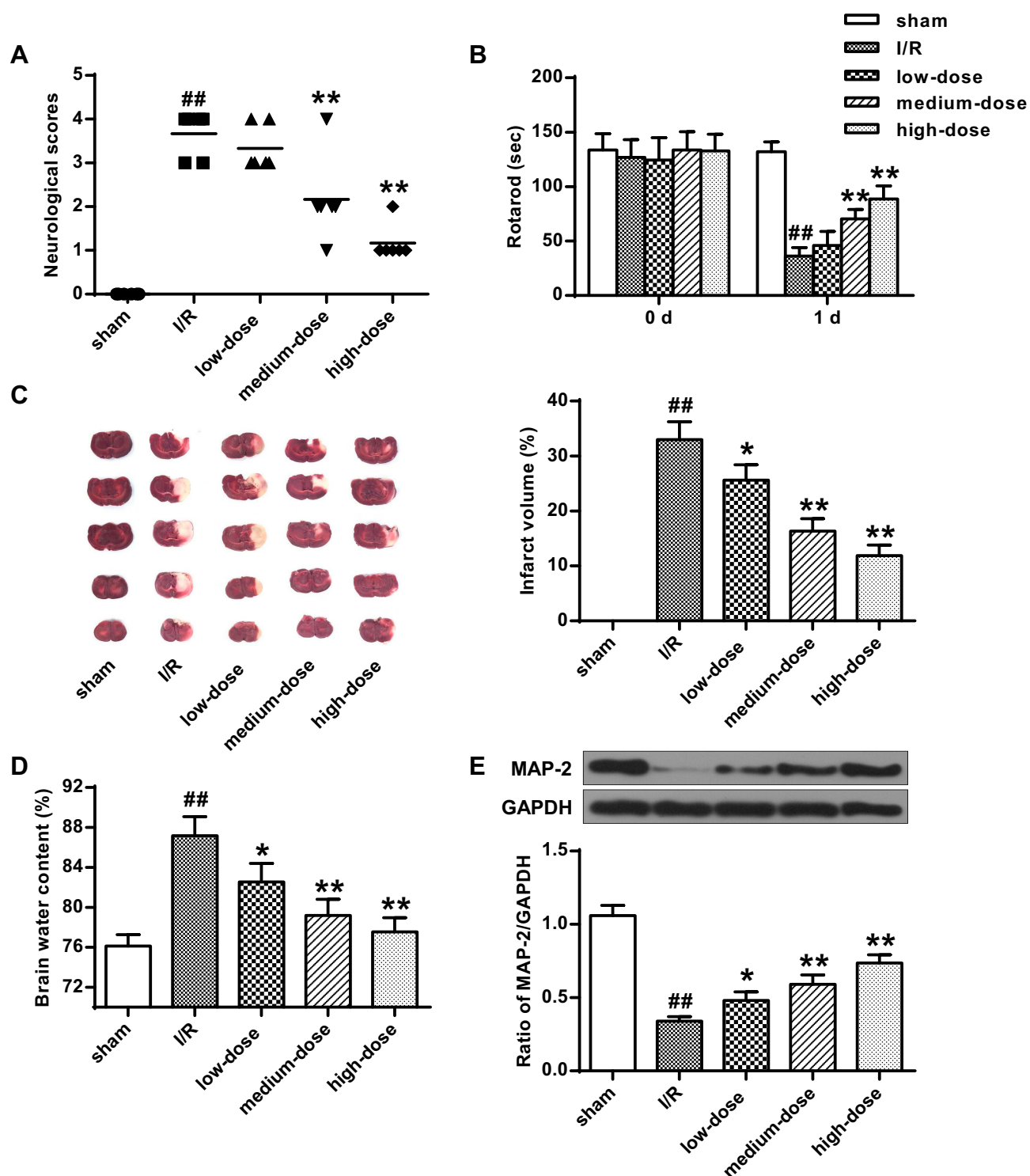


Figure 2 Deltonin improved neurological function and reduced cerebral infarction. For groups of sham, I/R, low-dose deltonin (25 mg/kg), medium-dose deltonin (50 mg/kg), and high-dose deltonin (100 mg/kg): (A) Neurological scores. (B) Rotarod periods prior to or after treatments. (C) Pictures on the infarct volumes and infarct volume percentage. (D) Brain water contents. (E) Western blot of ratios of MAP-2/GAPDH. ^{##} $P < 0.01$ vs sham; ^{*} $P < 0.05$, ^{**} $P < 0.01$ vs I/R.

elevated infarct volumes and brain water contents ($P < 0.01$); however, this effect was rescued by deltonin ($P < 0.05$, $P < 0.01$). Figure 2E shows the Western blot results. We found that MAP-2 in I/R was much lower

compared with sham ($P < 0.01$). Deltonin elevated concentrations of MAP-2 ($P < 0.05$, $P < 0.01$). It was obvious that deltonin improved neurological function and reduced cerebral infarction in the rats after I/R.

Deltonin Reduced Autophagy Activities Through PI3K/Akt/mTOR Signaling

In **Figure 3A**, it was demonstrated that LY294002 (inhibitor of PI3K/Akt) was markedly decreased in the neurological score, when comparing with deltonin group ($P < 0.05$). **Figure 3B** shows the infarct volumes from the groups of sham, I/R, high-dose deltonin, and high-dose deltonin + LY294002. It was obvious that deltonin could reduce the infarct volumes. **Figure 3C** and **D** showed the Western blot. No obvious difference was observed in expressions of Akt and mTOR ($P > 0.05$). Compared with sham, I/R decreased the expressions of p-Akt and p-mTOR, and increased the levels of LC3II and Beclin-1. Due to this reason, ratios of LC3-II/LC3-I and Beclin-1/GAPDH were increased ($P < 0.01$) (**Figure 3D**). However, deltonin had a great elevation in p-Akt and p-mTOR in contrast with I/R ($P < 0.01$) (**Figure 3D**). In addition, the LC3-II/LC3-I and Beclin-1/GAPDH ratios reduced greatly by deltonin ($P < 0.01$) (**Figure 3D**). However, LY294002 greatly decreased p-Akt

and pmTOR expressions and elevated the ratios of LC3-II/LC3-I and Beclin-1/GAPDH than the I/R + deltonin ($P < 0.05$) (**Figure 3D**). The data indicated that deltonin attenuated autophagies and had neuroprotection to rats with I/R.

Deltonin Suppressed Inflammations Through TLR4/P38/MAPK in I/R Rats

From **Figure 4**, the ultrastructural changes among sham, I/R, high-dose deltonin, and high-dose deltonin + LY294002 showed that deltonin reduced autophagy activities during cerebral I/R injury. **Figure 5A** demonstrated that anisomycin (activator of p38/MAPK) decreased the neurological scores than deltonin group ($P < 0.05$). **Figure 5B** showed that deltonin reduced the infarct volume, but anisomycin attenuated the effect. **Figure 5C** showed the protein expressions of TLR4, p-38, p-p38, and IL-1. No significant difference was found in the expressions of p38 in all groups ($P > 0.05$), but TLR4, p-p38, and IL-1 were elevated greatly in I/R ($P < 0.01$). Reversely, TLR4, p-p38, and IL-1 reduced by deltonin

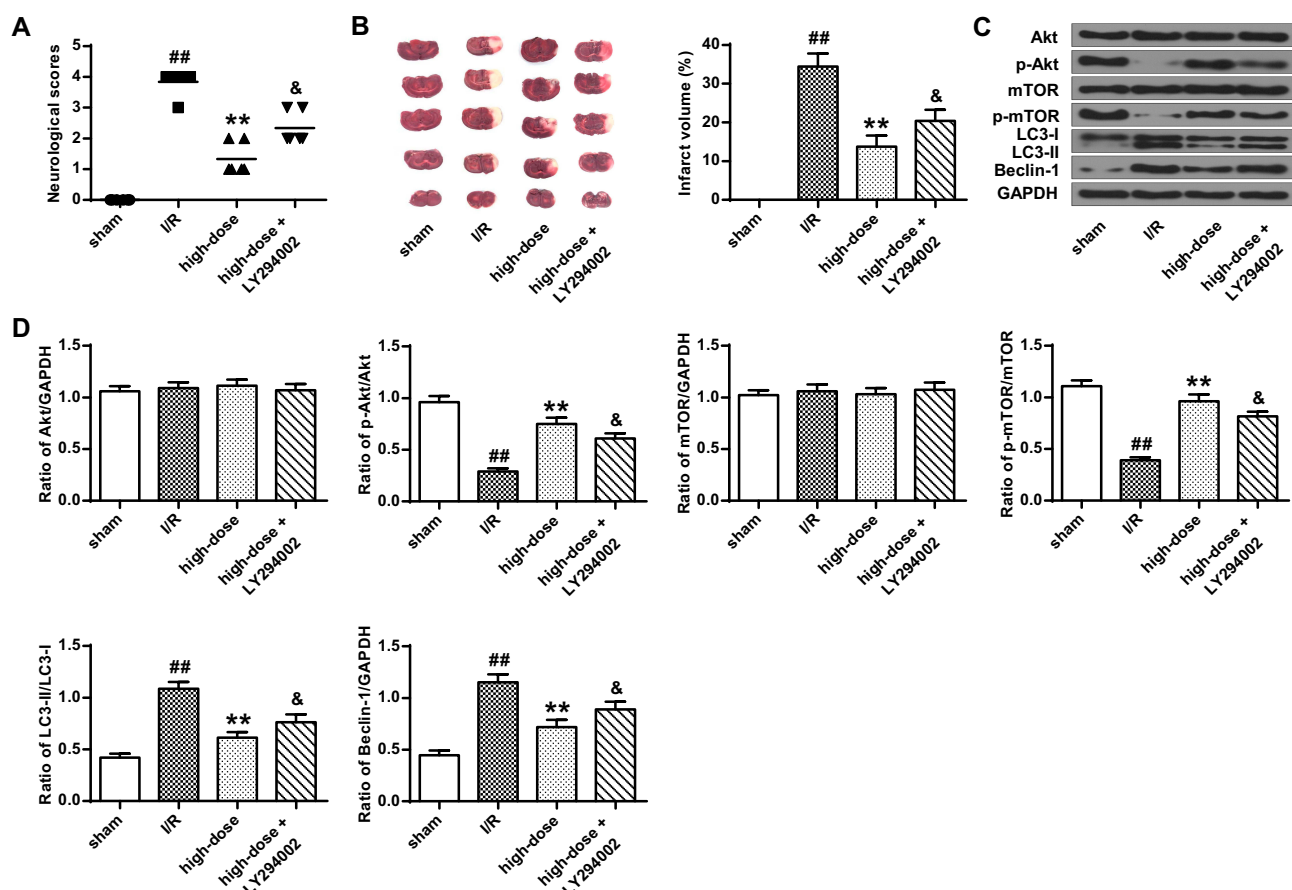


Figure 3 Deltonin reduced autophagy activities through PI3K/Akt/mTOR signaling. For groups of sham, I/R, high-dose deltonin (100 mg/kg) and high-dose + LY294002: (A) Neurological scores. (B) Infarct volumes. (C) and (D) Western blot images for protein expressions and ratios of Akt/GAPDH, p-Akt/GAPDH, mTOR/GAPDH, p-mTOR/GAPDH, LC3-II/GAPDH, LC3-I/GAPDH and Beclin-1/GAPDH. ^{##} $P < 0.01$ vs sham; ^{**} $P < 0.01$ vs I/R; [&] $P < 0.05$ vs deltonin.

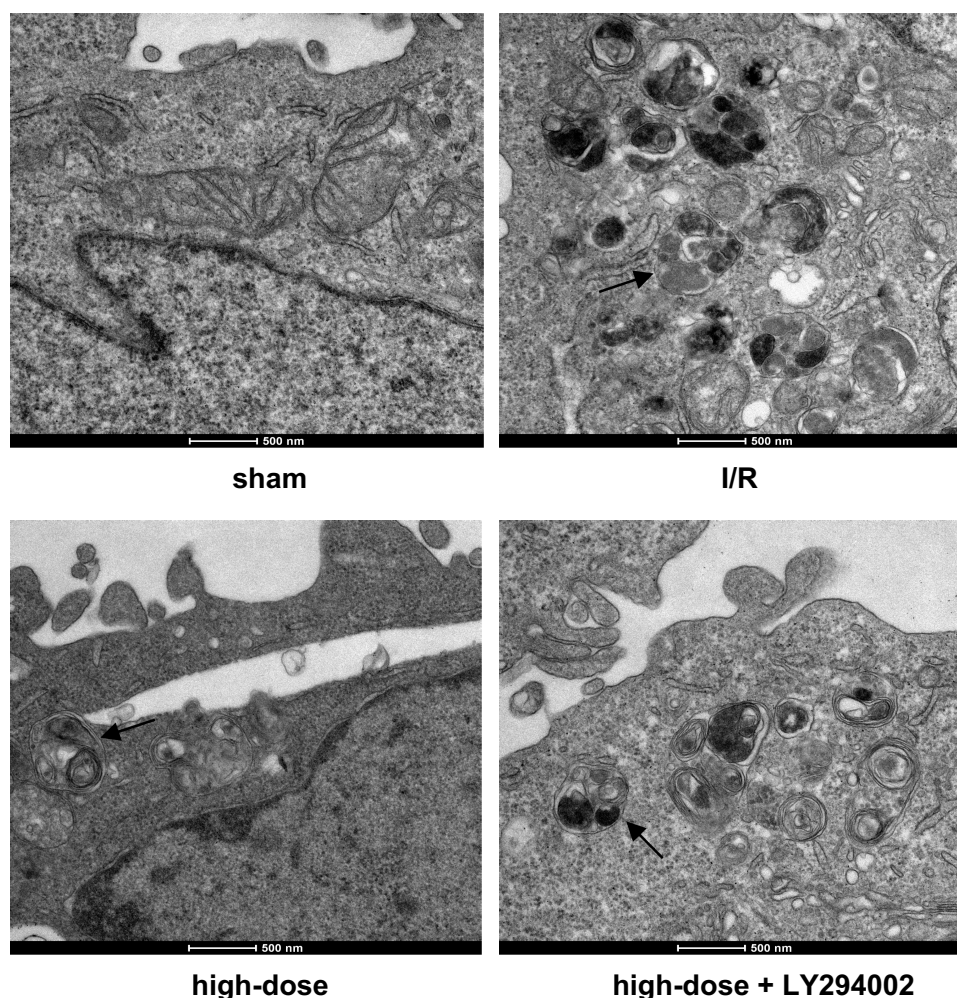


Figure 4 TEM images showing the ultrastructural changes. The ultrastructural changes among sham, I/R, high-dose deltonin, and high-dose deltonin+LY294002 showed that deltonin reduced autophagy activities during cerebral I/R injury.

($P < 0.01$) (Figure 5C and D). However, anisomycin up-regulated the expressions of TLR4, p-p38, and IL-1 ($P < 0.05$).

Figure 6 showed the ELISA results about inflammatory factors of IL-6 (Figure 6A), IL-10 (Figure 6B), TNF- α (Figure 6C), and iNOS (downstream to TLR4) (Figure 6D). It was noted that IL-6, TNF- α , and iNOS were elevated greatly after I/R ($P < 0.01$). But IL-10 was markedly inhibited than sham ($P < 0.01$). With 100 mg/kg deltonin, IL-6, TNF- α , and iNOS were greatly decreased, but IL-10 was markedly promoted than I/R rats ($P < 0.01$). But LY294002 and anisomycin up-regulated the IL-6, TNF- α , and iNOS and down-regulated IL-10 ($P < 0.01$). It was identified that deltonin attenuated the inflammation impacts of I/R in rats.

Discussions

It was well established that neurological functions, infarct volumes and brain water contents are the major indicators

for cerebral ischemia/reperfusion injuries. An effective treatment could usually pose an impact in the three factors. For example, Y. Lin reported the neuroprotection effects from resveratrol on I/R injuries in rat by TRPC6/CREB.¹⁴ Resveratrol lowered the neurological disorders and decreased brain infarctions at 1 day after reperfusion. Microtubule-Associated Protein-2 (MAP-2) is a neuron-specific phosphoprotein which is measured as a marker of ischemic injury following cerebral ischemia.¹⁵ In this study, we found that MAP-2 in I/R was much lower compared with sham, and deltonin elevated concentrations of MAP-2. Our study results are in agreement with the results of the studies by previous researches which represented that MAP-2 decreased during cerebral I/R.^{14,16} In our experiments, we noticed a great deficit was detected in I/R, which were markedly relieved by deltonin, compared with the sham. We observed that cerebral I/R greatly

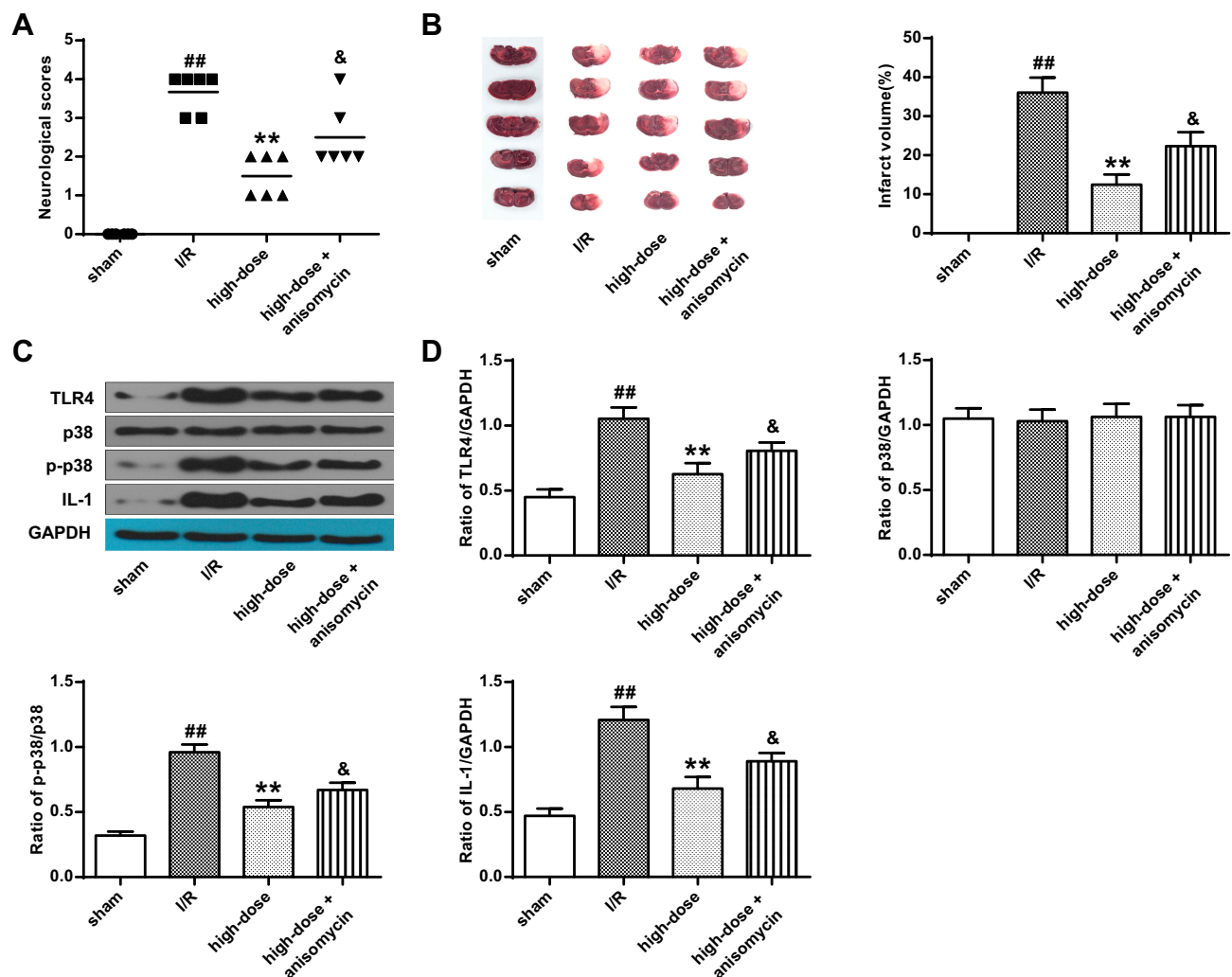


Figure 5 Deltonin suppressed inflammations through TLR4/p38/MAPK in I/R rats. For groups of sham, I/R, high-dose deltonin (100 mg/kg) and high-dose+ anisomycin: **(A)** Neurological scores. **(B)** Infarct volumes. **(C)** Western blot images for protein expressions of TLR4, p38, p-P38, IL-1, and GAPDH. **(D)** Ratios of TLR4/GAPDH, p38/GAPDH, p-P38/GAPDH, and IL-1/GAPDH. ###P<0.01 vs sham; **P<0.01 vs I/R; &P<0.05 vs deltonin.

elevated infarct volumes and brain water contents; however, this effect was rescued by deltonin. It was obvious that deltonin improved neurological function and reduced cerebral infarction in the rats after I/R.

According to Sai Ma in 2015, autophagy activation during cardiac I/R could either antagonize cardiac pathogenesis or contribute to further myocardium damage.¹⁷ In our experiments, I/R increased the levels of LC3II and Beclin-1, which were 2 autophagy indicators. I/R also decreased the expressions of p-Akt and p-mTOR. However, deltonin had a great elevation in p-Akt and p-mTOR in contrast with I/R, and in addition, reduced the expressions of LC3-II and Beclin-1 greatly. Moreover, LY294002, an indicator of PI3K/Akt, greatly elevated the ratios of LC3-II Beclin-1. The data indicated that deltonin reduced autophagy and had

neuroprotection to rats with I/R through PI3K/Akt/mTOR signaling.

TLR4/p38/MAPK signaling pathway was widely known to participate in the activation of inflammations. For instance, J. Li demonstrated that oridonin protected against the inflammatory response in diabetic nephropathy by inhibiting the TLR4/p38-MAPK and TLR4/NF- κ B signaling pathways.¹⁸ In 2018, JP. Dai also reported that oxymatrine inhibited influenza A virus replication and inflammation via TLR4, p38 MAPK and NF- κ B Pathways.¹⁹ There were no significant differences found in the expressions of p38 in all groups, but TLR4, p-p38, and IL-1 were elevated greatly in I/R group. Reversely, TLR4, p-p38, and IL-1 reduced by deltonin. But anisomycin, that could activate p38/MAPK, up-regulated the expressions of TLR4, p-p38, and IL-1.

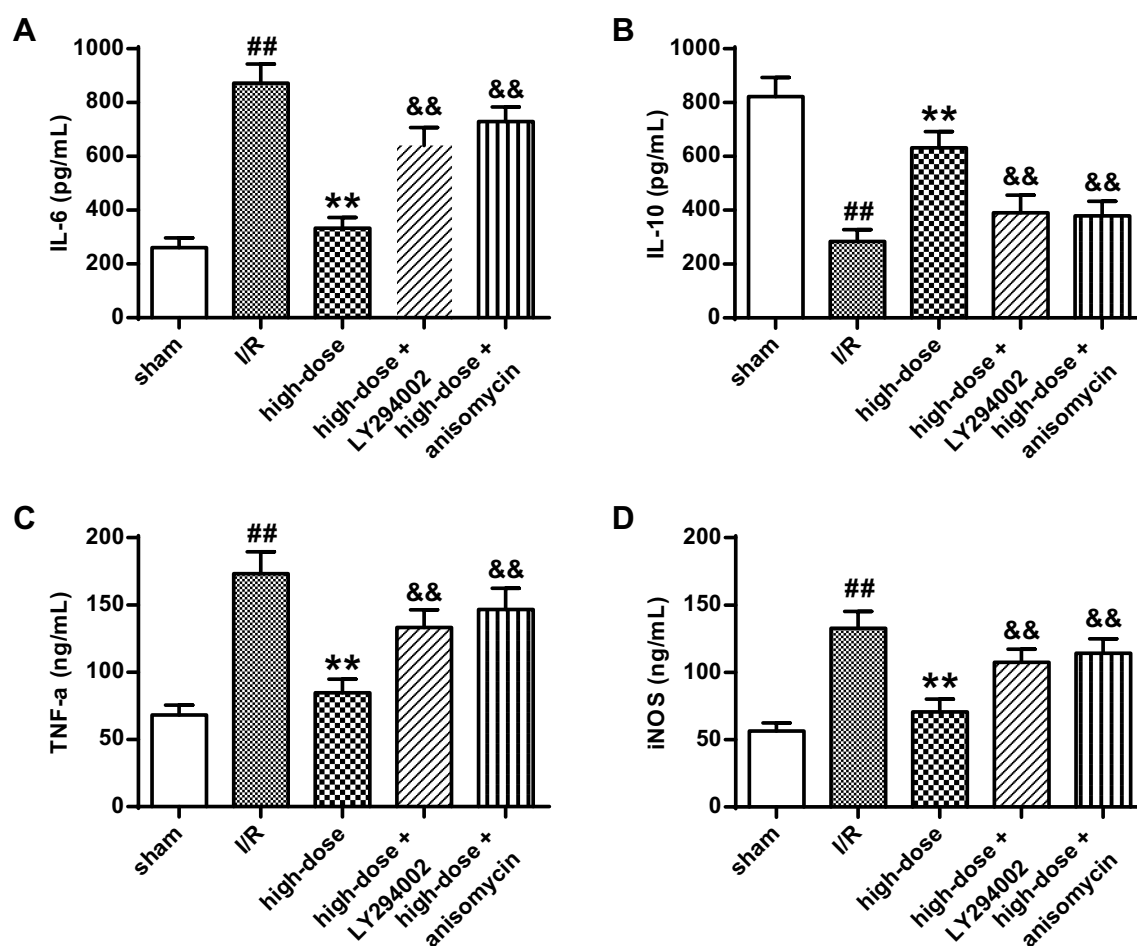


Figure 6 Protein expressions of IL-6 (A), IL-10 (B), TNF- α (C), and iNOS (D) for groups of sham, I/R, high-dose deltonin (100 mg/kg), high-dose + LY294002, and high-dose + anisomycin. ###P<0.01 versus sham group; **P<0.01 versus I/R group; &&P<0.01 versus deltonin group.

In addition, we detected the protein expressions of inflammatory factors were elevated greatly by I/R, and IL-10 was markedly reduced. By 100 mg/kg deltonin, IL-6, TNF- α , and iNOS were greatly decreased, but IL-10 was markedly promoted than I/R rats. But LY294002 and anisomycin up-regulated the IL-6, TNF- α , and iNOS and down-regulated IL-10 in the I/R+LY294002+deltonin and I/R+anisomycin+deltonin groups than the I/R+deltonin group. Obviously, deltonin attenuated the inflammation impacts of I/R through TLR4/p38/MAPK signaling pathway.

Conclusions

Deltonin could suppress ischemic brain injury by regulating autophagy and inflammation during I/R. Deltonin can be an efficient therapeutic method for patient with ischemia.

Data Sharing Statement

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Ethics Approval

The present study was approved by the Ethics Committee of Fu-Ning People's Hospital.

Author Contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors declare that they have no conflicts of interest in this work.

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