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ORIGINAL RESEARCH

Lupeol Alleviates Cerebral Ischemia–Reperfusion Injury in Correlation with Modulation of PI3K/Akt Pathway

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Background/Aim: This study aimed to investigate the effect and mechanism of lupeol on cerebral ischemia–reperfusion injury in rats.

Methods: The effects of lupeol on cerebral infarction, cerebral water content, neurological symptoms and cerebral blood flow in rats were evaluated. Nissl staining was carried out to assess the neuronal damage of ischemic brain after I/R in rats. Apoptosis of ischemic brain neurons after I/R was detected by TUNEL staining. Western blotting was carried out to detect the effects of lupeol on the expression of p-PDK1, p-Akt, pc-Raf, p-BAD, cleaved caspase-3 and p-PTEN.

Results: Lupeol significantly increased cerebral blood flow after I/R in rats, reduced brain water content and infarct volume, and decreased neurological function scores. It significantly reduced neuronal damage after I/R in rats, and significantly reduced neuronal cell loss. PI3K inhibitor (LY294002) can eliminate the effect of lupeol on I/R in rats. In addition, lupeol significantly increased the protein expression of p-PDK1, p-Akt, pc-Raf, p-BAD, and down-regulated the expression of cleaved caspase-3. LY294002 reversed the effects of lupeol on the expression of PI3K/Akt signaling pathway-related proteins and cleaved caspase-3 after I/R in rats.

Conclusion: Lupeol had significant neuroprotective effects on brain I/R injury and neuronal apoptosis, and its mechanism may be related to the activation of PI3K/Akt signaling pathway.

Keywords: lupeol, PI3K/Akt, apoptosis, cerebral ischemia/reperfusion (I/R) injury

Introduction

Ischemic cerebrovascular disease has a high incidence, mortality and disability rate, and is one of the diseases that seriously endanger human health.[1,2] Studies have been investigating the mechanisms of ischemic cerebrovascular disease, with great progress been made in the treatment of this disease. Combination therapy of vascular and cellular mechanisms for ischemic brain injury is likely to have significant impact on the prognosis of stroke.[3] Early thrombolysis is a treatment for blood vessels. With the development of thrombolytic therapy for ischemic cerebral infarction, reperfusion injury after blood circulation reconstruction has received extensive attention. Once reperfusion injury occurs, neuroprotective therapy is the preferred treatment strategy.[4,5]

In recent years, studies using animal models of cerebral ischemia have provided evidence of apoptosis in cerebral ischemia–reperfusion injury from the perspectives of morphology, biochemistry, pharmacology and gene regulation.[6,7] Apoptosis is involved in all stages of cerebral ischemia–reperfusion injury, such as delayed neuronal death, penumbra and cerebral infarction volume expansion.[8,9] The PI3K/

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Akt signaling pathway is a critical signaling pathway that promotes the repair and survival of ischemic nerve cells in cerebral ischemia.[10] Therefore, the development of effective PI3K/Akt activators could be future research focus on ischemic cerebrovascular disease. In the nervous system, PI3K is involved in the survival and differentiation of neurons and glial cells. Activated PI3K can regulate the expression of downstream target genes through AKT activation. In recent years, increasing numbers of studies aim to investigate the roles of PI3K/AKT in neural stem cells. Some studies suggested that the survival of neural stem cells depends on the activation of PI3K/AKT pathway. LY294002 is a widely used PI-3K inhibitor, which can specifically inhibit the catalytic activity of PI3Kp110 subunit, but it has a certain toxic effect on cells. Studies have shown that LY294002 can make ovarian cancer cells more sensitive to Taxol.[11]

Lupeol is a triterpenoid widely found in plants such as peppers, tomatoes, olive oil, figs, mangoes, strawberries, red grapes, and a variety of Chinese herbal medicines.[12,13] Its molecular formula is $C_{30}H_{50}O$ and the molecular weight is 426.7174 g/mol.[14] Biological effects of lupeol include antihypertensive, anti-fatigue and anti-tumor.[15,16] Lupeol antagonizes excitatory neurotransmitters during neurological damage, and its regulation at various aspects such as antioxidative damage and secretion of neuroprotective factors also received increasing attention.[17] The influence of lupeol on the expression of apoptosis-regulating genes is unknown. Therefore, this study was carried out to investigate whether lupeol can exert its anti-apoptotic effect by activating the expression of PI3K/Akt signaling pathway using the animal model of cerebral ischemia–reperfusion, and the pharmacological mechanism of treating cerebrovascular diseases was further explored. Our findings will provide a new research direction for finding new drugs to treat cerebral ischemic injury.

Materials and Methods

Reagents

Lupeol (purity ≥99%) (chemical structure is shown in Figure 1A).

Animal

A total of 86 healthy male Sprague Dawley rats that are 7–8 weeks old and weighing 200–220 g were obtained from the Experimental Animal Center of the First Hospital of Hebei Medical University. All experiments were approved by the First Hospital of Hebei Medical University Animal Care and Use Committee and conducted in accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines.

Focal Cerebral Ischemia/Reperfusion (I/R) Model

After 1 week of adaptive feeding, the rats were in good condition and were divided into sham operation group (sham group), model group (I/R group) and lupeol group (12.5, 25, 50, 100 mg/kg), lupeol + LY294002 group. The rats of lupeol group, lupeol + LY294002 group and lupeol + vehicle group were given lupeol 30 min before surgery. Dose of LY294002 and vehicle referred to relevant literature.[18] The rats of sham group and the I/R group were given an equal volume of intraperitoneal injection of normal saline, and it was only isolated from the artery in the sham group. The middle cerebral artery occlusion and reperfusion (MCAO) models were established in the model group, the lupeol group, and the lupeol + LY294002 group. An olive oil group was also established and no adverse or beneficial effects on this dose (data not shown) were found. The operation was smooth and there were no dead animals. After the operation, each group was fed in a single cage and had free access to drinking water. The specific experimental design was shown in (Figure 1B).

Evaluation of Infarct Volume, Neurological Deficits, Brain Water Content and Cerebral Blood Flow

The brain tissue was stained with 2,3,5-triphenyltetrazolium chloride (TTC) dye for 30 minutes. The staining results showed that white was infarct focus and red was normal brain tissue. After taking pictures, the area of cerebral infarction was measured as described in literature.[19] After 24 h of cerebral ischemia–reperfusion in rats, neurological deficits were scored according to the 5-point standard established in literature.[20]

Brain water contents (n = 6) were determined 24 h after reperfusion. Infarct brain hemispheres were quantified with an electronic scale (wet weight), dried overnight at 105 °C in a desiccating oven, and weighed (dry weight). The total brain water was calculated as [(wet weight - dry weight)/wet weight] × 100%. Cerebral blood flow (CBF) (n = 6) was measured using laser Doppler flowmetry. A computer-controlled optical scanner directed a low-power laser beam over the exposed cortex. The scanner head was positioned in parallel to the cerebral cortex at

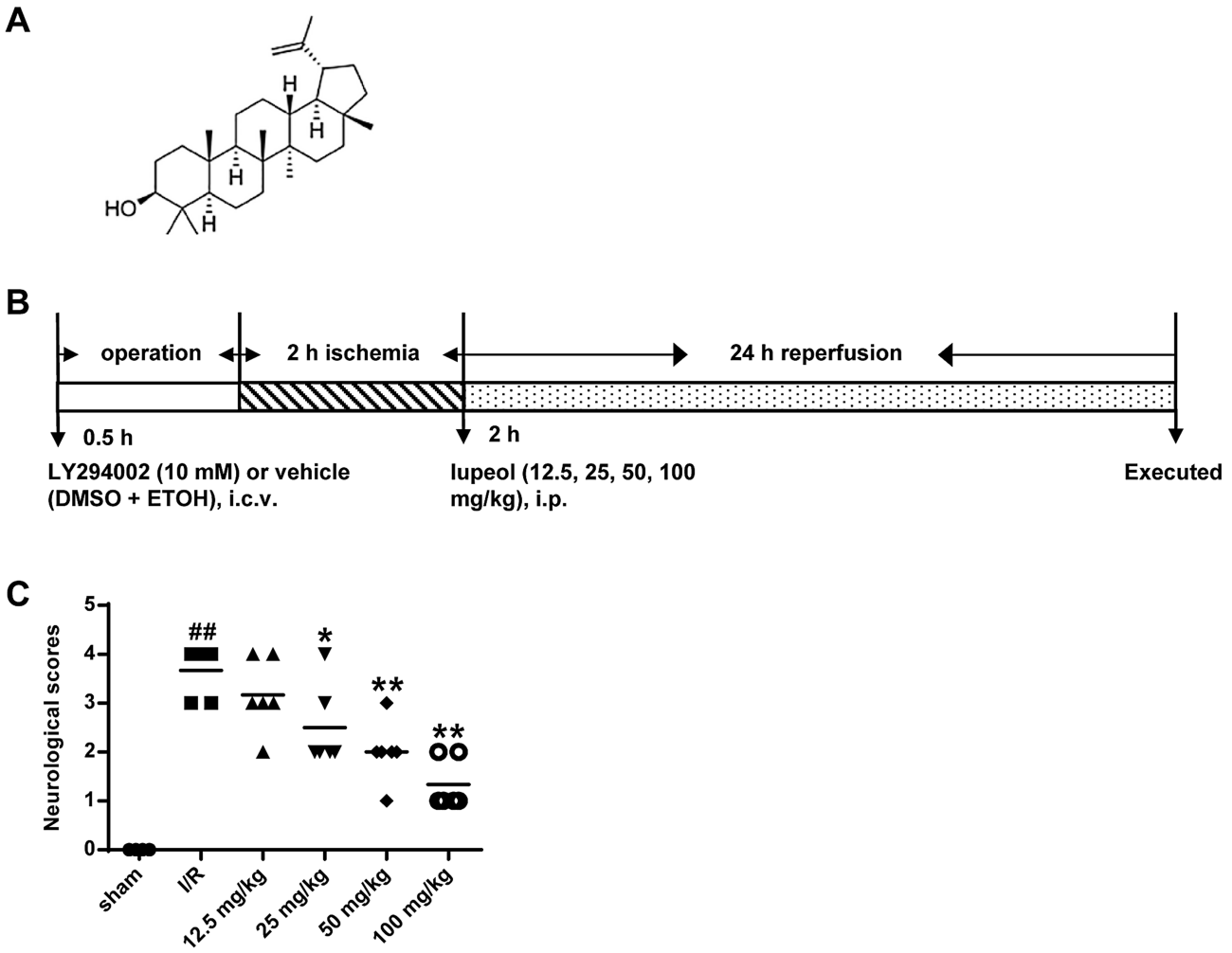

Figure 1 Effects of different doses of lupeol on neurological deficits score. (**A**) Chemical structure of lupeol ($C_{30}H_{50}O$, molecular weight: 462.72). (**B**) Schematic diagram of the experimental protocols. As shown in this diagram, the rats in I/R and lupeol-treated groups were subjected to cerebral I/R and lupeol were administered i.p. to rats 2 h after the onset of ischemia in lupeol-treated groups, while rats in the sham group only underwent the same surgical operation without an inserted suture and were given the distilled water. In some groups, lupeol-treated rats were handled by intracerebroventricular injection of LY294002 or vehicle 0.5 h before operation. After 24 h of reperfusion, the rats were anesthetized and then decapitated. (**C**) Effects of different doses of lupeol on neurological deficits score. Data were presented as the mean ± SD, n = 6. $^{\#\#}P < 0.01$ vs sham group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs I/R group.

a distance of about 20 cm from the onset of ischemia to reperfusion for 24 h.

Nissl Staining and TUNEL Staining

The rats were decapitated and perfused with 4% paraformaldehyde. The brain tissue was fixed in a fixing agent for 1 d. The brain sections were then subjected to Nissl staining, and the neuronal death of the rats was assessed under a light microscope. The experiment was conducted as described in literature.[21]

Western Blot Analysis

Western blot analysis was used to detect the expression levels of proteins involved in PI3K/Akt pathway at 24 h after reperfusion. Total proteins in ischemic penumbra were extracted from the fresh cerebral cortex (n = 6). The protein assay kit from Beyotime (Haimen, Jiangsu, China) was used to measure protein concentrations. Equal amounts of protein samples were separated by electrophoresis on polyacrylamide gels and electro-transferred onto the poly-vinylidene fluoride membranes (PVDF, Millipore). The membranes were blocked in 5% non-fat dry milk in Tris-buffered saline with 0.05% Tween-20 (TBST), followed by incubation in primary antibody PTEN (#9188, Cell Signaling Technology), p-PTEN (#9551, Cell Signaling Technology), PDK1 (#5662, Cell Signaling Technology), p-PDK1 (#3438, Cell Signaling Technology), Akt (#4685, Cell Signaling Technology), p-Akt (#4060,Cell Signaling Technology), BAD (#9292, Cell Signaling Technology), p-BAD (#5284, Cell Signaling Technology), cleaved caspase-3 (#9661, Cell

Signaling Technology), c-Raf (#53745, Cell Signaling Technology), p-c-Raf (#9421, Cell Signaling Technology), GAPDH (#5174, Cell Signaling Technology) and HRP (#7076; Cell Signaling Technology (Beverly, MA, USA) at 4 °C for overnight, rinsed with TBST for 10 min for three times, and finally incubated with horse-radish peroxidase-conjugated secondary anti-rabbit antibody for 2h. Chemiluminescence substrate (ECL Plus) was used to incubate the blots and band intensities were detected by Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA). Phosphorylation levels of the targeted proteins were evaluated by comparing with corresponding total proteins. GAPDH protein was used as an internal control.[22]

Statistical Method

The monitoring data were analyzed by SPSS19.0 statistical software. The data were shown as mean ± standard deviation (SD). Differences among multigroup data were explored by one-way ANOVA. LSD test is used for subsequent analysis. $P < 0.05$ was considered as significant difference.

Results

Effects of Lupeol on Neurological Deficits Following Cerebral I/R Injury

As shown in (Figure 1C), the rats in the control group had no neurological damage and cerebral infarction. The neurological injury scores in the model group were significantly improved ($P < 0.01$). Lupeol could alleviate the neurological damage in rats. With the increase of concentration, the score of neurological impairment in rats was decreased gradually, and lupeol had the best effect at 100 mg/kg (Figure 1C). Therefore, the molecular mechanism of the neuroprotective effect of 100 mg/kg lupeol on focal brain I/R injury in rats was further analyzed.

Effects of Lupeol on Infarct Volume, Neurological Deficits, Brain Water Content, and Cerebral Blood Flow Following Cerebral I/R Injury

Compared with the sham group, the area of cerebral infarction in the I/R group was significantly raised ($P < 0.05$). The infarct size of the rat in the lupeol group was significantly reduced ($P < 0.01$) compared with that in the I/R group (Figure 2A and B). However, compared with the lupeol +vehicle group, the area of cerebral infarction was significantly increased in the lupeol + LY294002 group ($P < 0.05$). As shown in (Figure 2C and D), compared with the sham group, the neurological deficit score and brain water content in the I/R group were significantly increased ($P < 0.05$). The neurological deficit score and brain water content in the lupeol group were significantly decreased ($P < 0.01$) compared with that in the I/R group. However, compared with the lupeol + vehicle group, brain water and the neurological deficit score content of lupeol + LY294002 group were significantly raised ($P < 0.05$).

Compared with the sham group, the neurological deficit score and brain water content in the I/R group were significantly raised ($P < 0.05$). Compared with I/R group, the neurological deficit score and brain water content in lupeol group were significantly decreased ($P < 0.01$) (Figure 2C and D). In addition, the cerebral blood flow in the I/R group was significantly lower compared with that in the sham group ($P < 0.05$) (Figure 2E). The cerebral blood flow in the lupeol group was significantly higher than that in the I/R group ($P < 0.01$). Compared with the lupeol + vehicle group, cerebral blood flow was significantly raised in the lupeol + LY294002 group ($P < 0.05$) (Figure 2E).

Effects of Lupeol on Neuronal Injury Following Cerebral I/R Injury

Compared with the sham group, in the I/R group, the interstitial space of the ischemic penumbra increased, and most of the Nissl neurons disappeared, the nucleus was condensed, and I/R-induced damage was typical ($P < 0.01$). In the lupeol group, the neuronal cell contours were clear and the interstitial space of the ischemic penumbra became smaller ($P < 0.01$). LY294002 blocked the protective effect of the lupeol group on neuronal injury, increased the cell gap in the ischemic penumbra, and decreased the number of Nissl neurons in the neurons ($P < 0.01$) (Figure 3).

Effects of Lupeol on Neuron Apoptosis Following Cerebral I/R Injury

Compared with the sham group, a large number of TUNEL-positive cells were expressed in the brain I/R injury model group ($P < 0.01$). Compared with the brain I/R model group, the number of TUNEL-positive cells of the ischemic penumbra of the lupeol group was significantly lower ($P < 0.01$). However, compared with the lupeol + vehicle group, the number of TUNEL-positive

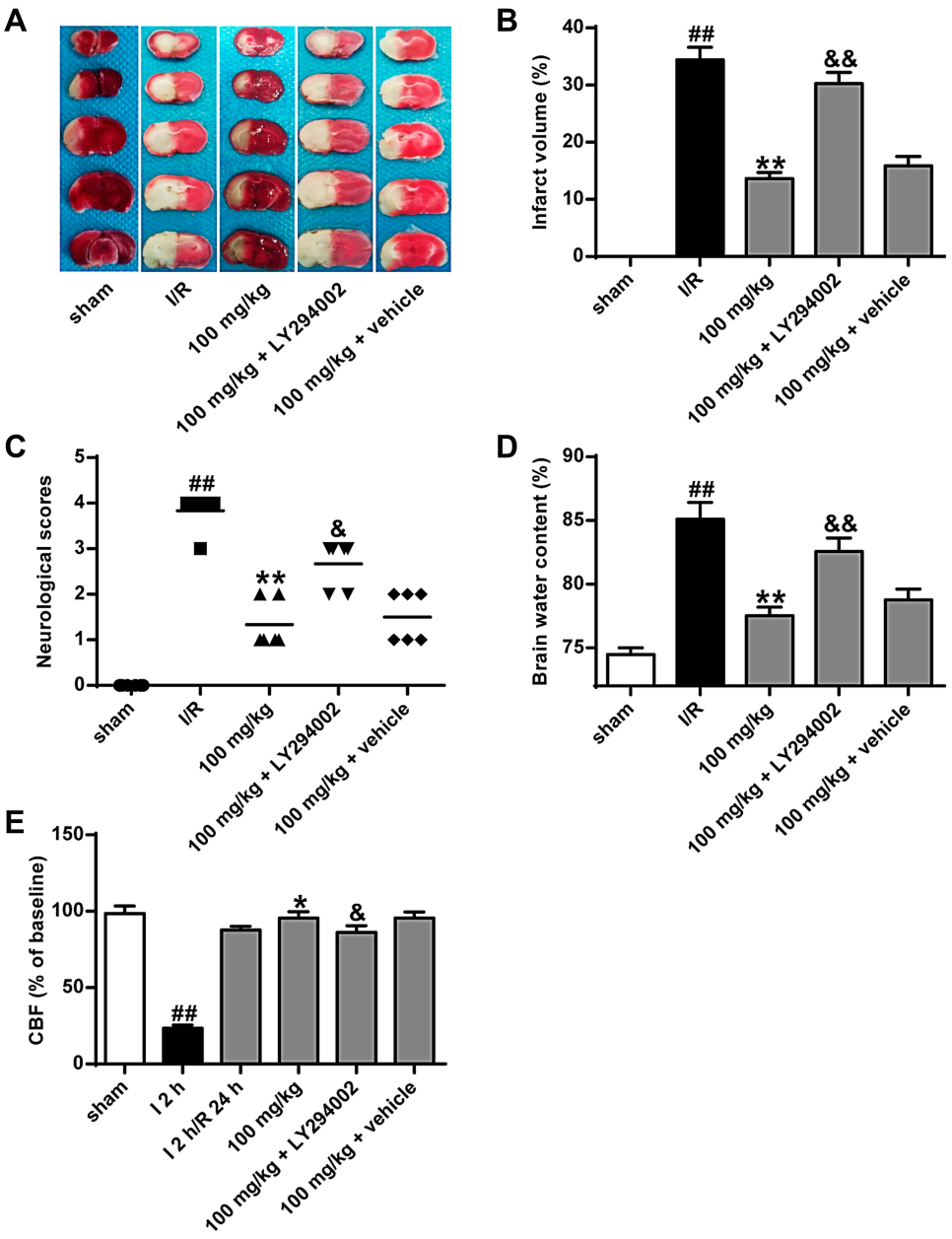

Figure 2 Effect of lupeol on infarct volume (**A** and **B**), neurological deficit (**C**), rain water content (**D**), and cerebral blood flow (**E**) after brain I/R injury. $^{\#\#}P$ <0.01 vs the sham operation group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs I/R group; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ vs lupeol + vehicle group.

cells in the lupeol + LY294002 group was significantly increased ($P < 0.01$) (Figure 4).

Lupeol Mediated the Expression of Proteins in PI3K/Akt Pathway

As shown in (Figure 5A and B), compared to the sham group, the expression levels of p-PTEN (Ser380) and p-PDK1 (Ser241) were significantly lower ($P < 0.01$). Compared to the I/R group, the expression levels of p-PTEN (Ser380) and p-PDK1 (Ser241) were significantly increased in the lupeol group ($P < 0.01$). While the expression levels of p-PDK1 (Ser241) in the lupeol + LY294002 group were significantly reduced ($P < 0.01$) compared with that in the lupeol + vehicle group. The expression of p-PTEN (Ser380) did not change significantly.

As shown in (Figure 6A and B), compared to the sham group, the expression levels of p-Akt (Ser473), p-BAD (Ser136), and pc-Raf (Ser259) were significantly decreased in the I/R group, the expression levels of cleaved caspase-3 were significantly increased ($P <$

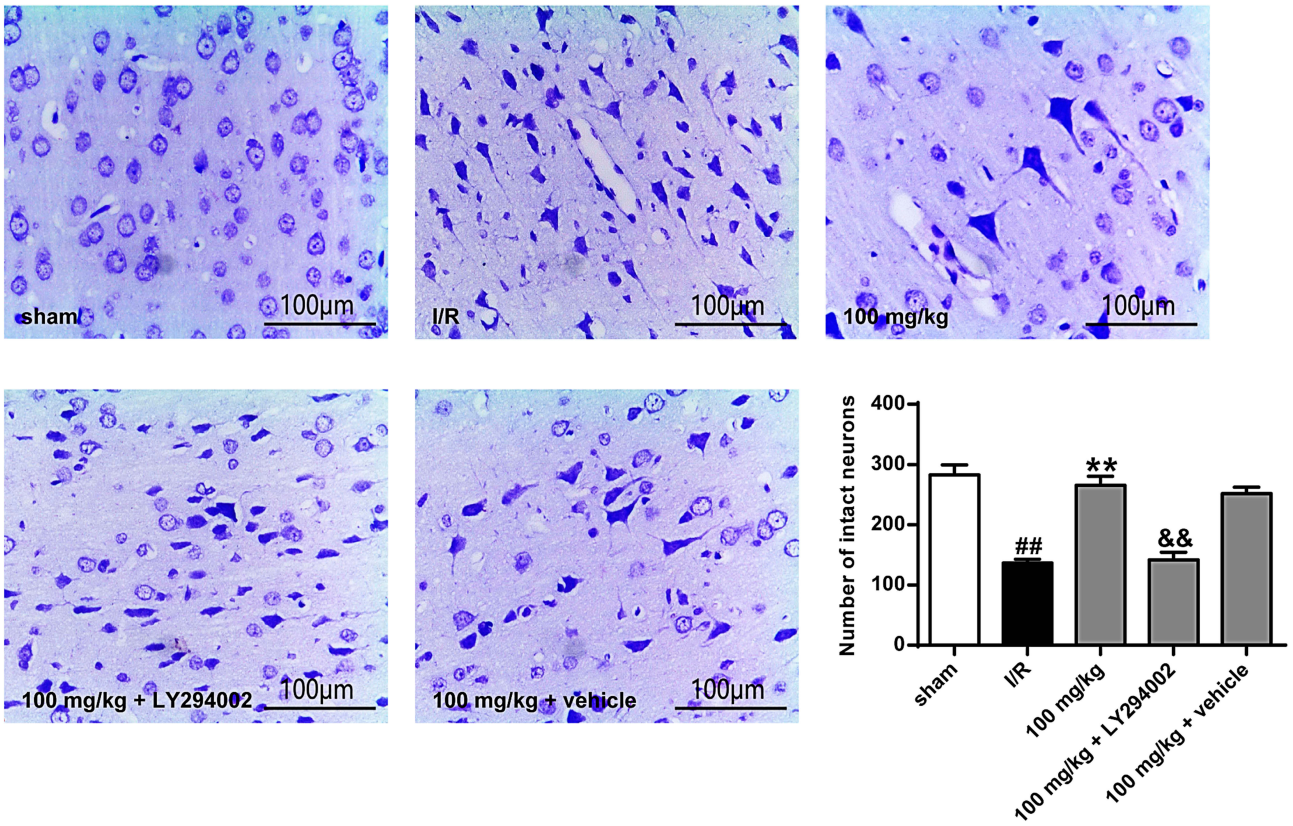

Figure 3 Effects of lupeol on neuronal injury following cerebral I/R injury. Representative pictures of Nissl staining and quantitative analysis of intact cells at 24 h after reperfusion (400× magnification). Data were presented as the mean ± SD, n = 6. $^{\#\#}P < 0.01$ vs sham group; $^{**}P < 0.01$ vs I/R group; $^{\&\&}P < 0.01$ vs lupeol + vehicle group.

0.01). However, the expression levels of p-Akt (Ser473), p-BAD (Ser136), and pc-Raf (Ser259) were significantly higher in the lupeol group than that in the I/R group (Figure 6C and D). The expression levels of cleaved caspase-3 were significantly decreased ($P < 0.01$). Compared to the lupeol + vehicle group, the expression levels of p-Akt (Ser473), p-BAD (Ser136), pc-Raf (Ser259) in lupeol + LY294002 group were significantly decreased ($P < 0.01$), while the expression levels of cleaved caspase-3 were significantly increased ($P < 0.01$).

Discussion

Stroke is a common disease that seriously endangers human health.[23] With the increase of aging rate, the prevalence of cerebrovascular diseases in the elderly is increasing. With the extensive development of cardiac surgery in vitro, cardiac transplantation and other cardiovascular surgery, the incidence of cerebral infarction is increasing, which has become an important cause of cerebral infarction.[24] There is currently no effective intervention, so it is important to find new drugs to reduce cerebral ischemia–reperfusion injury. The present study indicated that lupeol could significantly protect brains from I/R injury through suppression of apoptosis and inflammation. Accordingly, FRE, as a preventive agent, is significant to decrease the incidence of ischemic stroke.

The anti-oxidative stress of lupeol has been reported.[25] Studies have found that lupeol enhances the antioxidant activity of rat liver by scavenging free radicals.[26] In addition, studies have found that supplementation of lupeol can effectively reduce acetaminophen-induced oxidative stress, restore antioxidant enzyme activity, and reduce lipid peroxidation.[27] However, the molecular mechanisms of lupeol neuroprotection have not been fully elucidated.

The PI3K/Akt signaling pathway plays important roles in promoting cell survival. In cerebral ischemia, it can promote the repair and survival of ischemic nerve cells.[28] Short-term ischemic preconditioning can alleviate ischemic brain damage in rats and up-regulate the expression of p-Akt in brain tissues. Intraventricular injection of PI3K/Akt inhibitor LY294002 inhibits the expression of p-Akt and the neuroprotective effect of ischemic preconditioning. It suggests that the PI3K/Akt signaling pathway

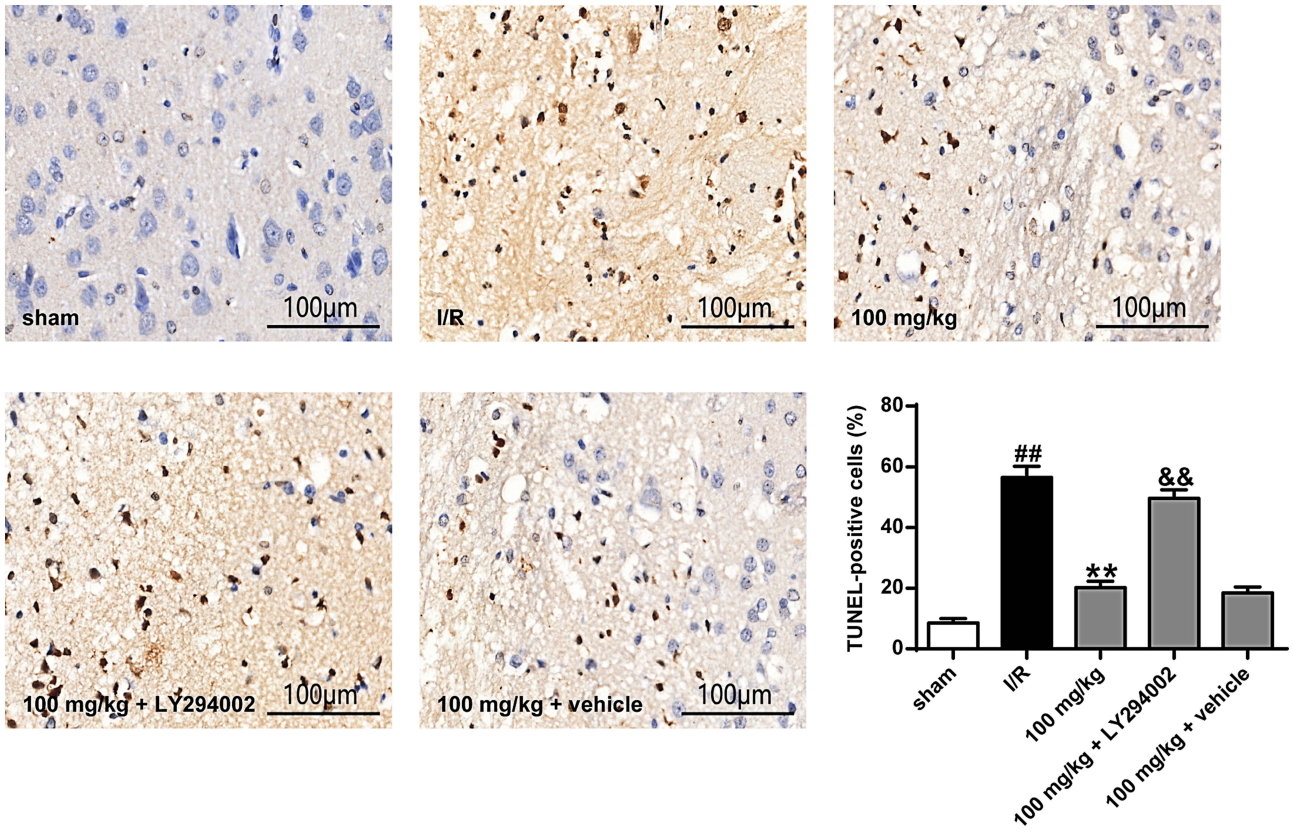

Figure 4 Effects of lupeol on neuron apoptosis following cerebral I/R injury. Representative pictures of TUNEL staining and quantitative analysis of neuronal apoptosis at 24 h after reperfusion. Data were presented as the mean ± SD, n = 6. $^{\#\#}P < 0.01$ vs sham group; $^{**}P < 0.01$ vs I/R group; $^{\&\&}P < 0.01$ vs lupeol + vehicle group.

is involved in regulating the neuroprotective effect of ischemic preconditioning on cerebral ischemia.[29] Studies have found that leptin can reduce the volume of neurological dysfunction and ischemic brain edema, thereby alleviating ischemic brain damage in rats. Injection of PI3K/Akt inhibitor LY294002 into caudal vein inhibits the neuroprotective effect of lupeol. Cerebral ischemia induces high expression of p-Akt, lupeol further promotes

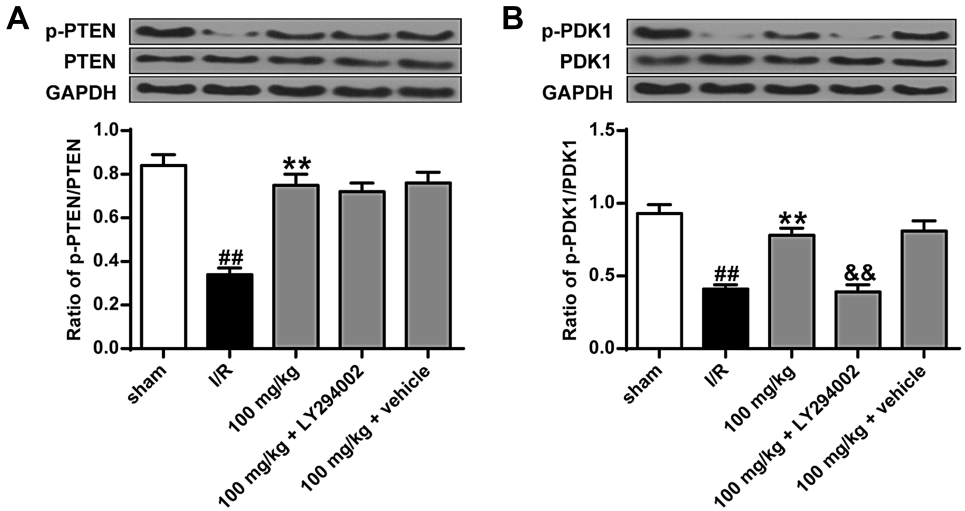

Figure 5 Effect of lupeol on phosphorylation levels of PTEN and PDK1. (**A**) Protein expression level p-PTEN (Ser380) and PTEN were detected by Western blot. (**B**) Protein expression level p-PDK1 (Ser241) and p-PDK1 were detected by Western blot. $^{\#\#}P < 0.01$vs the sham operation group; $^{**}P < 0.01$ compared with the I/R group; $^{\&\&}P < 0.01$ vs the lupeol + carrier group.

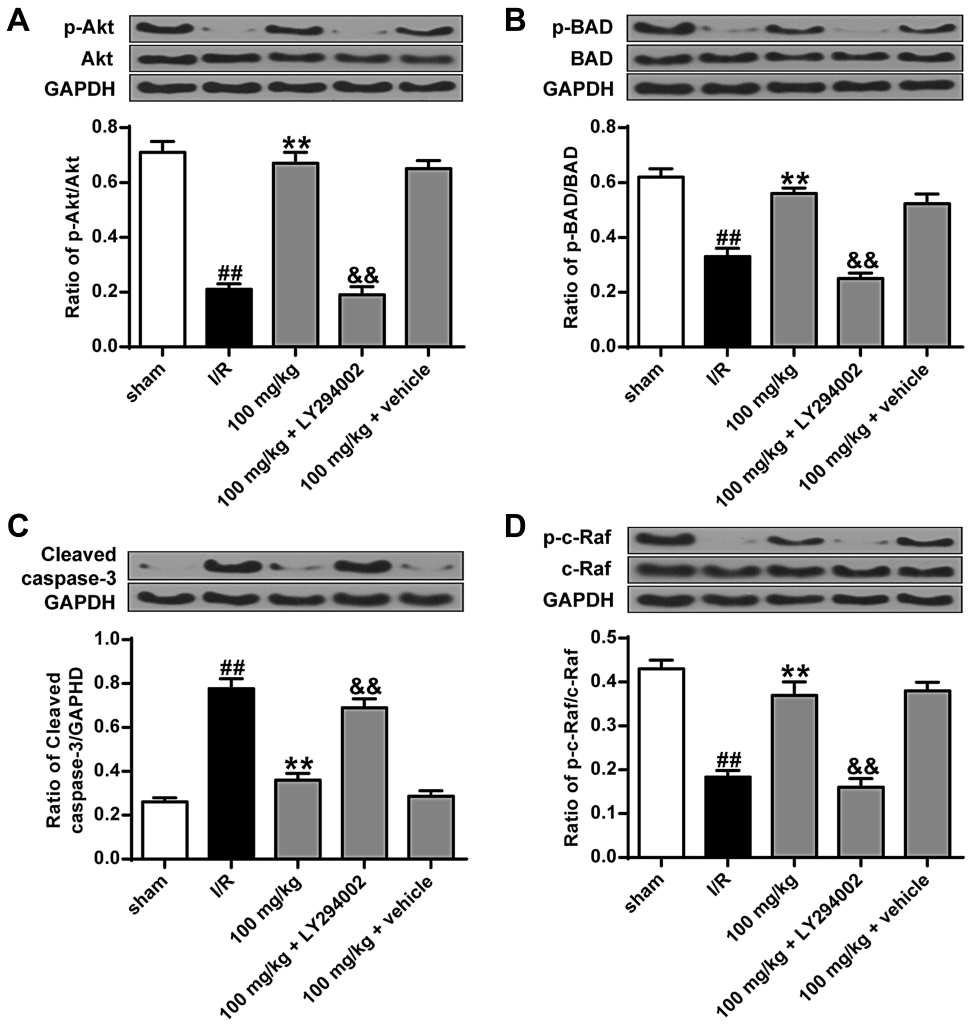

Figure 6 Effects of lupeol on phosphorylation levels of Akt, c-Raf, BAD, and cleaved caspase-3 at 24 h after reperfusion. (**A**) Western blot and quantitative analysis of the level of p-Akt (Ser473) by normalization to level of the Akt. (**B**) Western blot and quantitative analysis of the level of p-BAD (Ser136) by normalization to level of the BAD. (**C**) Western blot and quantitative analysis of the level of cleaved caspase-3 by normalization to level of the GAPDH. (**D**) Western blot and quantitative analysis of the level of p-c-Raf (Ser259) by normalization to level of the c-Raf. GAPDH is the internal control. $^{\#\#}P < 0.01$ vs sham group; $^{**}P < 0.01$ vs I/R group; $^{\&\&}P < 0.01$ vs lupeol + vehicle group.

the expression of p-Akt in cerebral ischemia, while LY294002 inhibits the expression of p-Akt. It was reported that lupeol protects the mouse from cerebral ischemic injury via the PI3K/Akt signaling pathway.[30]

Caspase 3 played a pivotal role in apoptosis initiated by various factors.[31,32] Our results confirmed that lupeol decreased the expression levels of caspase 3, increased the expression levels of p-PTEN (Ser380), p-PDK1 (Ser241) p-Akt (Ser473), p-BAD (Ser136) and pc-Raf (Ser259). LY294002, a known PI3K/Akt signaling inhibitor, has been widely used in different studies including ischemic stroke. Therefore, LY294002 was chosen here for comparison with lupeol to determine the possible effects of lupeol on the PI3K/Akt signaling pathway.[33] Lupeol inhibited caspase 3 via the PI3K/Akt signaling pathway. Intraventricular injection of LY294002 inhibited the effect of lupeol on the expression of caspase 3 and PI3K/Akt signaling pathways in rat cerebral ischemia. These results suggested that lupeol can alleviate the caspase 3 in cerebral ischemia by activating the PI3K/Akt signaling pathway.

The results showed that lupeol can improve IR injury by activating PI3K/Akt, which provides a theoretical basis for nutritional intervention of neurological injury induced by ischemic stroke. In future studies, the expression levels of other proteins in the pathway could further explore the molecular mechanisms of lupeol to improve IR injury. In future research, we would try to assess the possible effects of lupeol on oxidative stress during cerebral I/R.

Conclusion

Lupeol attenuated cerebral ischemia–reperfusion injury and the expression of caspase 3 via the PI3K/Akt signaling pathway. As a pro-survival signaling pathway, PI3K/Akt may become a therapeutic target for ischemic cerebrovascular disease in the future.

Data Sharing Statement

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

Disclosure

The authors report no conflicts of interest in this work.

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