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ORIGINAL ARTICLE

Chrysoeriol Promotes Functional Neurological Recovery in a Rat Model of Cerebral Ischemia

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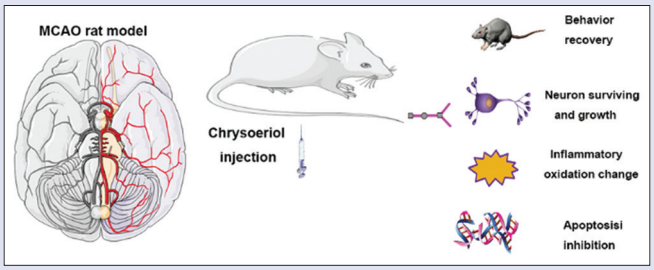
ABSTRACT

Background: Stroke is among the leading causes of death worldwide, but current treatment options for cerebral ischemia are limited. Chrysoeriol is a natural isolated agent with bioactive pharmacological properties. **Materials and Methods:** Sprague–Dawley rats were subjected to transient middle cerebral artery occlusion (MCAO) surgery and chrysoeriol was administrated for the subsequent 3 days. Neurological deficit scores, apoptosis, oxidant stress, and inflammatory cytokines were then measured and the effects on the Wnt/β-catenin pathway were evaluated. **Results:** Behavioral tests revealed that chrysoeriol promoted recovery from neurological deficits in rats. Hematoxylin and eosin staining demonstrated that chrysoeriol alleviated neurological damage. Triphenyl tetrazolium chloride staining demonstrated that chrysoeriol reduced the area of ischemia. An enzyme-linked immune sorbent assay (ELISA) demonstrated that chrysoeriol inhibited excessive pro-inflammatory cytokine production (tumor necrosis factor-α, interleukin [IL]-1β, and IL-6) and regulated oxidative stress (malondialdehyde, superoxide dismutase, and glutathione). Caspase-3 immunofluorescence and a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay showed that chrysoeriol reduced neuronal apoptosis. The ELISA assay and Nissl staining showed that chrysoeriol promoted neuronal survival and growth. Western blotting and an immunofluorescence assay demonstrated that the Wnt/β-catenin signaling pathway plays a crucial role in the neuronal effects of chrysoeriol. The Wnt inhibitor, Dickkopf-related protein-1, validated the role of the Wnt/β-catenin signaling pathway in the neuroprotective effects of chrysoeriol. **Conclusion:** Chrysoeriol exerts neuroprotective effects in a rat model of MCAO. These effects are mediated by the Wnt/β-catenin signaling pathway.

Key words: Apoptosis, inflammatory, middle cerebral artery occlusion, oxidant stress, stroke

SUMMARY

- Stroke is among the leading causes of death worldwide, yet current treatment options for cerebral ischemia are limited. Chrysoeriol is a natural isolated agent with bioactive pharmacological effects. In this study, we examined the neuroprotective effects of chrysoeriol on a stroke model of Sprague–Dawley rats. Chrysoeriol was found to promote recovery from neurological deficits and alleviate neurological damage in rats. Chrysoeriol also reduced the area affected by ischemia. It inhibited excessive pro-inflammatory cytokine production, regulated oxidative stress, reduced neuronal apoptosis, and promoted neuronal survival and growth. The Wnt/β-catenin signaling pathway was found to play a crucial role in the neuronal effects of chrysoeriol. Our results showed chrysoeriol to have promising neuroprotective effects in a rat middle cerebral artery occlusion model. Further exploration of the pharmacological actions of chrysoeriol is warranted.



Abbreviation used: MCAO: Middle cerebral artery occlusion; TNF-α: Tumor necrosis factor-α; IL-1β: interleukin-1β; IL-6: Interleukin-1β; ELISA: Enzyme-linked immune sorbent assay; IF: Immune fluorescent; IL-10: Interleukin-10; MDA: Malondialdehyde; SOD: Superoxide dismutase; GSH: Glutathione; NGF: Nerve growth factor; BDNF: Brain-derived neurotrophic factor; bFGF: Basic fibroblast growth factor; CCA: Common carotid artery; ICA: Internal carotid artery; ECA: External carotid artery; TTC: Triphenyl tetrazolium chloride; TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling

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INTRODUCTION

Stroke is a cerebrovascular disease and the second leading cause of mortality worldwide.[1] With an increasing number of aging populations globally, the high incidence of stroke represents a major public health issue.[2] Most strokes are triggered by cerebral ischemia, an acutely restricted blood supply to the brain.[3] The mechanisms of cerebral ischemic stroke commonly include excessive oxidative stress, inflammation of the brain, elevated serum levels, and metabolic imbalance.[4] Reperfusion following ischemic stroke causes further neuronal injury, such as molecular cascade activation, and neuronal death. Several factors are involved in the pathophysiology of neuronal injury. Ischemic stroke activates immune-competent cells, such as granulocytes, microglia, and astrocytes, in the brain. Ischemic stroke

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causes an inflammatory response through the excessive secretion of pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α, and downregulation of the anti-inflammatory cytokine, IL-10.[5] The IL-1β is upregulated immediately after ischemic stroke and aggravate stroke pathology by regulation of caspase-1.[6] The IL-6 plays a role of neurotrophic element to modulate neuronal homeostasis.[7] The TNF-α is also a macrophage immune-regulatory cytokine which affects neuronal plasticity in acute and chronic ischemia in brain.[8] By contrast, IL-10 binds to IL-10 receptors to inhibit the inflammatory response and apoptosis.[9] Meanwhile, inflammation aggravated an imbalance of endogenous antioxidant and oxidant molecules, resulting in oxidative stress in the brain. This interferes with levels of malonaldehyde (MDA), superoxide dismutase (SOD), and glutathione (GSH).[10] Ischemia–reperfusion causes the activation of xanthine oxidase, which then produces superoxide anion free radicals. The resultant oxidative damage can consume intracellular antioxidants (GSH and SOD), converting alkane radicals to excess MDA. The inflammation, oxidative stress, and harmful secretions cause the activation of caspase-3 and gradual apoptosis of neurons. Neuronal growth factors, such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and basic fibroblast growth factor (bFGF), promote the generation of new neurons and neurogenesis, which facilitate the recovery of motor functions. BDNF enhances neuronal survival, protects synaptic function, and improves neuronal connectivity.[11] NGF promotes neurite outgrowth.[12] BFGF contributes to basal transferrin secretion. When dietary restrictions are implemented, it can also intensify neurogenesis.[13] Several signaling pathways are involved in the pathogenesis of ischemic stroke and recovery from it. These include the protein kinase B-AKT (PI3K/AKT) pathway,[14] nuclear factor-erythroid factor 2-related factor 2,[15] the toll-like receptor 4/nuclear transcription factor κB pathway,[16] AMP-activated protein kinase,[17] extracellular signal-regulated kinase,[18] and the wingless-related integration site (Wnt) signaling pathway.[19] These pathways contribute to numerous processes, including inflammation regulation, neuronal growth promotion, and oxidative stress modulation. Despite great advances in the last few decades, clinically effective pharmacological options are still limited and new strategies for the treatment and prevention of cerebral ischemic stroke are urgently needed.

Chrysoeriol is a bioactive flavone isolated from the roots and leaves of plants of the *Phyllanthus* genus. Various studies have shown chrysoeriol to possess multiple medicinal properties. It has shown anti-inflammatory effects in acute inflammatory skin conditions[20] and the treatment of arthritis,[21] and has shown promise as an antioxidant[22,23] and an anticancer drug.[24] Moreover, chrysoeriol exerts growth-promoting or protective effects in many diseases. It was also shown to prevent neurotoxicity in a cell model of Parkinson's disease.[25] Chrysoeriol promotes the proliferation and differentiation of osteoblastic cells.[26] It is an effective inhibitor of the cytochrome P450 enzyme (P450)[27] and therefore has hepatoprotective abilities.[28] Most importantly, chrysoeriol 7-O-[b-D-glucuronopyranosyl-(1-2)-O-b-D-glucuronopyranoside] has been found to alleviate myocardial ischemia–reperfusion injury in a rat model.[29] This suggests that it has potential as a treatment for stroke. Middle cerebral artery occlusion (MCAO) is widely employed *in vivo* ischemic stroke model. Based on chrysoeriol's previously reported pharmacological abilities, it is predicted that this substance can promote recovery from stroke. Therefore, this study was designed to explore the effects of chrysoeriol on cerebral ischemic stroke in a rat model of MCAO/reperfusion. The intrinsic mechanism of Wnt/β-catenin signaling pathway regulation was also explored.

MATERIALS AND METHODS

Animals and model establishment

The behavioral test, drug administration, and final sacrifice in this study were approved by the Animal Ethics Committee of Fudan University. Male Sprague–Dawley (SD) rats (220–240 g) were provided by the Shanghai SIPPR-Bk Lab Animal Co. Ltd. The rats were housed in an animal center at a constant temperature, with free access to specific-pathogen-free-grade water and food.

Middle cerebral artery occlusion model and chrysoeriol administration

The animal study was conducted following the procedures used previously.[30] The rats were divided into six groups: a sham operation group (sham), an MCAO model group (MCAO), an MCAO + chrysoeriol single dose (3 mg/kg) group, an MCAO + chrysoeriol double dose (6 mg/kg) group, an MCAO + chrysoeriol triple dose (9 mg/kg) group, and an MCAO + chrysoeriol triple dose + Dicckkopf-1 (9 + DKK-1) group.

All the rats except for those in the sham group were subjected to transient MCAO surgery following the method used in previous studies.[31] Each rat was anesthetized intraperitoneally with an initial dose of pentobarbital sodium (40 mg/kg) and a maintenance mixture of O_2 and isoflurane (2.5%) using a small animal anesthesia machine (R-500, RWD, Shenzhen, China). The rat was then intubated and given ventilation. Most importantly, each rat was placed on a heating pad (HP-30, Cinontech, Beijing, China) to maintain the body temperature at 37°C during the surgical procedure, while the blood pressure was monitored by another team member. The left common carotid artery (CCA), internal carotid artery, and external carotid artery (ECA) were carefully exposed. The left CCA and ECA were ligated. Then, a 4-0 monofilament nylon thread (Cinontech, Beijing, China) with an enlarged rounded tip was inserted. The distance from the suture end to CCA bifurcation was 18.5–19.5 mm. The duration of left MCAO was 2 h. The filament was taken out carefully and withdrawn gently to allow reperfusion of the ischemic artery. The efficacy of the MCAO was verified by a third-party animal sonography operator using Doppler sonography. The technician was blind to this animal study. The rats in the sham group underwent an identical procedure but without the insertion of the nylon filament.

For the three MCAO + chrysoeriol groups, chrysoeriol (Nature-Standard Corporation, Shanghai, China) was dissolved in 1% dimethyl sulfoxide/saline. This was administered via intraperitoneal injection twice a day (8 am and 6 pm) for 3 days after waking from the surgery at the dose for the group they were in. The 9+DKK-1 group underwent the same MCAO surgery and postsurgical chrysoeriol treatment (9 mg/kg) and received DKK-1 (5 μg/kg) (Med Chem Express, NJ, USA) via intraperitoneal injection 30 min before the surgery.[32]

Neurological deficit score assessment

Rats were subjected to neurological deficit score (NDS) assessments on three occasions: 1 day before the MCAO, 2 h after the MCAO (when they were awake), and 6 h after the last chrysoeriol administration. Investigator who is blinded to this experiment evaluate the animal behavior according to the Zea-longa scoring method described in literature:[33] where 0 = no apparent deficits, 1 = unable to extend right forelimb, 2 = walk in circles, 3 = falling to right, 4 = can not walk or comma.

Triphenyl tetrazolium chloride staining

After the behavior test, the rats were quickly anesthetized and sacrificed. 2,3,5- triphenyl tetrazolium chloride (TTC) staining

was performed to examine the ischemic infarct volume.[34-36] The rat's brains were removed carefully, immersed in ice-cold water for 3 min, and cut into serial coronal slices of 2 mm thickness using a brain slice matrix (BS-300C, Cinontech). The sections were stained in TTC solution (Sigma Aldrich) for 15 min at room temperature. The presence of infarction was determined by the area that was not stained by TTC (ImageJ, National Institutes of Health).

Morphological assays

Hematoxylin and eosin (H and E) staining and Nissl staining were used for the morphological investigations. Each harvested brain was fixed in 4% phosphate-buffered paraformaldehyde, dehydrated in ascending ethanol, and cut into coronal slices of 5 μm thickness. For H and E staining, the slices were stained with hematoxylin solution (C0105M, Beyotime, Haimeng, China) for 10 min and then immersed in eosin solution for 1 min. For Nissl staining, the slices were stained with Nissl solution (C0117, Beyotime) for 10 min. Morphological differences between the neurons and other tissue in the ischemic penumbra of the different groups were compared under an optical microscope (MF31, Mshot, Guangzhou, China).

Terminal deoxynucleotidyl transferase dUTP nick-end labeling assay

A terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) kit (C1091, Beyotime) was used to investigate apoptosis in areas of cortical ischemia.[37] The brain tissue was incubated with proteinase K, H_2O_2, terminal deoxyribonucleotidyl transferase enzyme, and then with peroxidase-conjugated antibody and 3,3′-diaminobenzidine. Images were captured randomly using a light microscope (MF31, Mshot). TUNEL-positive cells in the ischemia penumbra were counted and the positive cell ratio was quantified using Image-Pro Plus (Media Cybernetics, MD, USA).

Immunofluorescence staining

Primary antibodies were used for deoxyribonucleotidic immunofluorescence staining at a 1:100 dilution. Rat brains were prepared by freezing them in liquid nitrogen and then cut into slices of 5 μm thickness. The slices were cultured with primary antibodies (Wnt3a: Abcam, no.: Ab234099; β-catenin: Abcam, no.: Ab32572) overnight at −4°C. On the 2nd day, the slices were re-incubated with secondary antibodies (Alexa Fluor 568-conjugated, 1:100–1:300 dilutions, Invitrogen) for 2 h at room temperature. The sections were stained with 4',6-diamidino-2-phenylindole for 5 min. Immunofluorescent images of the cortical ischemia were captured randomly using a fluorescence microscope (3DHistech Ltd., Budapest, Hungary). The immunofluorescent intensity was quantified using Image-Pro Plus (Media Cybernetics).

Enzyme-linked immune sorbent assay

Enzyme-linked immune sorbent assay and bicinchoninic acid (BCA) kits were obtained from Beyotime. The ipsilateral ischemic brain of each rat was collected, tissues were homogenized in phosphate-buffered saline, the homogenates were centrifuged at 4°C, 12,000 rpm/min for 10 min, and supernatants were collected and determined. The proteins in the brains were quantified by BCA kits on a microplate reader (Cayman Chemical, Ann Arbor, MI, USA). Levels of the inflammatory cytokine TNF-α, IL-1β, IL-6, and IL-10 were determined according to methods reported previously.[38] The MDA, GSH, and SOD concentrations in the brain were measured according to the manufacturers' instructions. NGF, BDNF, and bFGF concentrations in the brain were measured using the same equipment with separate absorbance following methods reported previously.[39]

Western blotting

A western blot analysis was performed to investigate protein expression in the brain. Proteins were extracted on ice using an extraction kit and adjusted using BCA kits. The protein was transferred to polyvinylidene difluoride membranes, blocked using a blocking kit, and incubated with primary antibodies (Wnt3a: Abcam, no.: Ab234099; β-catenin: Abcam, no.: Ab32572) overnight at −4°C. On the 2nd day, the slices were re-incubated with secondary antibodies for 2 h at room temperature. Then, proteins were detected using an electrochemiluminescence kit and photographed. Protein expression was quantified using ImageJ software (National Institutes of Health).

Statistical analyses

Data were presented as the mean ± standard deviation using GraphPad Prism 6.0 (GraphPad Software corporation, San Diego, CA, USA). The statistical analyses were conducted using SPSS 22.0 SPSS 22.0 (IBM Corp, Armonk, NY, USA) to perform a one-way analysis of variance. $P < 0.05$ between groups was considered statistically significant.

RESULTS

Chrysoeriol promotes the behavioral recovery of rats

Before the formal experiment, researchers practiced the MCAO surgery many times to control the mortality of rats and to ensure the consistency of the cerebral vascular embolism. Special attention was paid to temperature control of the body, and the location and time of embolization. All the rats survived during the formal experiment.

A dose–response preliminary experiment was performed using the administration method described in the methods section. As shown in Figure 1a, the 0 mg/kg control group was not treated with chrysoeriol, and groups administered 1 and 2 mg/kg chrysoeriol in the preliminary experiment demonstrated no response in their NDS values ($P > 0.05$). However, chrysoeriol doses of 5, 10, 20, 40, and 60 mg/kg lowered the NDS values ($P < 0.05$). Statistically significant differences ($P < 0.05$) were seen between the 5 mg/kg and the 10, 20, 40, and 60 mg/kg groups ($P < 0.05$). Although doses of 10, 20, 40, and 60 mg/kg reduced the NDS significantly compared with the 0 mg/kg group, there was no statistical difference between the 10 mg/kg and the 20, 40, and 60 mg/kg groups ($P > 0.05$). Therefore, 3, 6, and 9 mg/kg were selected as suitable chrysoeriol dosages for this experiment.

NDSs of the rats in all six groups at three time points are shown in Figure 1b. After the MCAO surgery and reperfusion, the NDS of the MCAO, chrysoeriol 3 mg/kg, chrysoeriol 6 mg/kg, and chrysoeriol 9 mg/kg groups increased significantly compared with the sham group. This demonstrated the successful establishment of the MCAO model. There were no differences between the MCAO group and the 3, 6, or 9 mg/kg groups on day 0. After chrysoeriol administration, the NDSs of the 3, 6, and 9 mg/kg chrysoeriol groups all decreased in a dose-dependent manner compared with the MCAO group. This demonstrated that chrysoeriol had promoted the behavioral recovery of the rats following cerebral ischemia and reperfusion injury.

Chrysoeriol alleviates neurological damage in a middle cerebral artery occlusion rat model

TTC staining results indicated that the brain infarction areas increased significantly in the MCAO group. This is shown in Figure 1c. However,

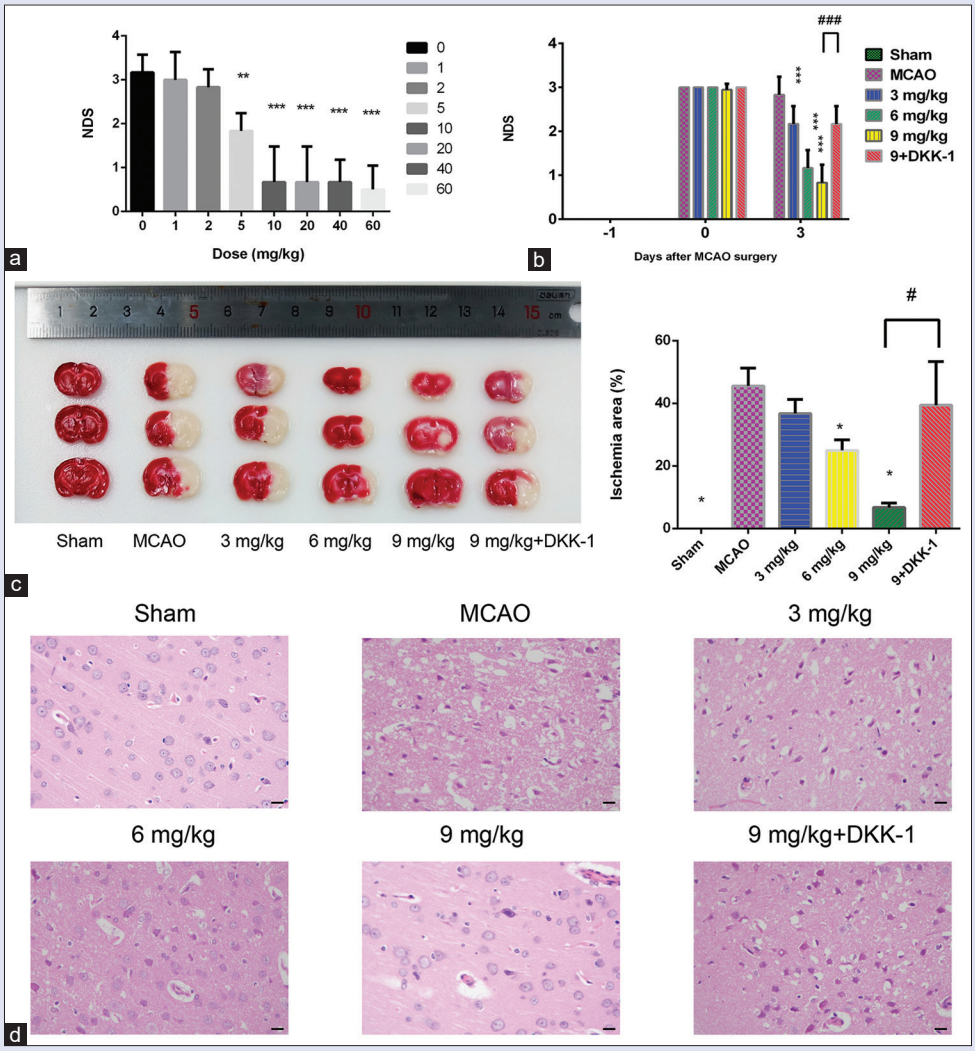

Figure 1: Chrysoeriol promoted behavioral recovery and alleviated neurological damage in a rat middle cerebral artery occlusion model. (a) Neurological deficits scores in dose–response experiments. (b) The Neurological deficits scores of the six groups at 3 time points. (c) Representative triphenyl tetrazolium chloride staining of brain tissues and quantification of normalized brain infarct area. (d) Representative H and E staining of brain tissue. Scale bar = 25 μm. The statistical difference between the middle cerebral artery occlusion group (0 mg/kg in A and middle cerebral artery occlusion in other pictures) and the other groups was considered significant at the levels of $^{*}P < 0.05$, $^{**}P < 0.01$, or $^{***}P < 0.001$. The statistical difference between the 9 mg/kg chrysoeriol group and the 9 mg/kg chrysoeriol group with DKK-1 was significant at the level of $^{\#}P < 0.05$ or $^{\#\#\#}P < 0.001$

the brain infarction areas in two of the chrysoeriol groups (6 and 9 mg/kg) were reduced.

H and E staining indicated the morphological changes of neurons in the ischemic penumbra. As shown in Figure 1d, normal structures, such as clear outlines of dense stoma, were seen in the sham group. In the MCAO group, cell membranes were damaged, and edema, pyknosis, and deformation were apparent in neurons. The number of neurons was reduced and the remaining neurons showed obvious degeneration. Chrysoeriol alleviated these morphological changes. In the chrysoeriol groups, the vacuoles were decreased, especially in the chrysoeriol 9 mg/kg group. Greater numbers of normal and newly generated neurons were seen in the chrysoeriol groups.

Chrysoeriol regulates inflammatory and oxidative cytokines

There was a marked rise in TNF-α, IL-1β, and IL-6 levels in the brain of the MCAO group, suggesting that the cerebral ischemia and reperfusion injury had elicited the production of pro-inflammatory cytokines, as shown in Figure 2a-c, respectively. After chrysoeriol administration, there was a significant reduction in these cytokines in chrysoeriol groups compared with their levels the MCAO group. An opposite trend occurred with IL-10, an anti-inflammatory cytokine, with greater expression in the chrysoeriol groups. This was dose dependent [Figure 2d]. These results demonstrated that chrysoeriol administration after MCAO inhibits the production of pro-inflammatory cytokines and promotes the production of anti-inflammatory cytokines.

Ischemia/reperfusion enhanced the MDA levels in the MCAO rats, as shown in Figure 2e. GSH and SOD levels were reduced significantly in the brains of the MCAO rats, as shown in Figure 2f and g. Chrysoeriol administration ameliorated the parameters of oxidative stress in the brain. MDA, GSH, and SOD levels were attenuated after chrysoeriol administration.

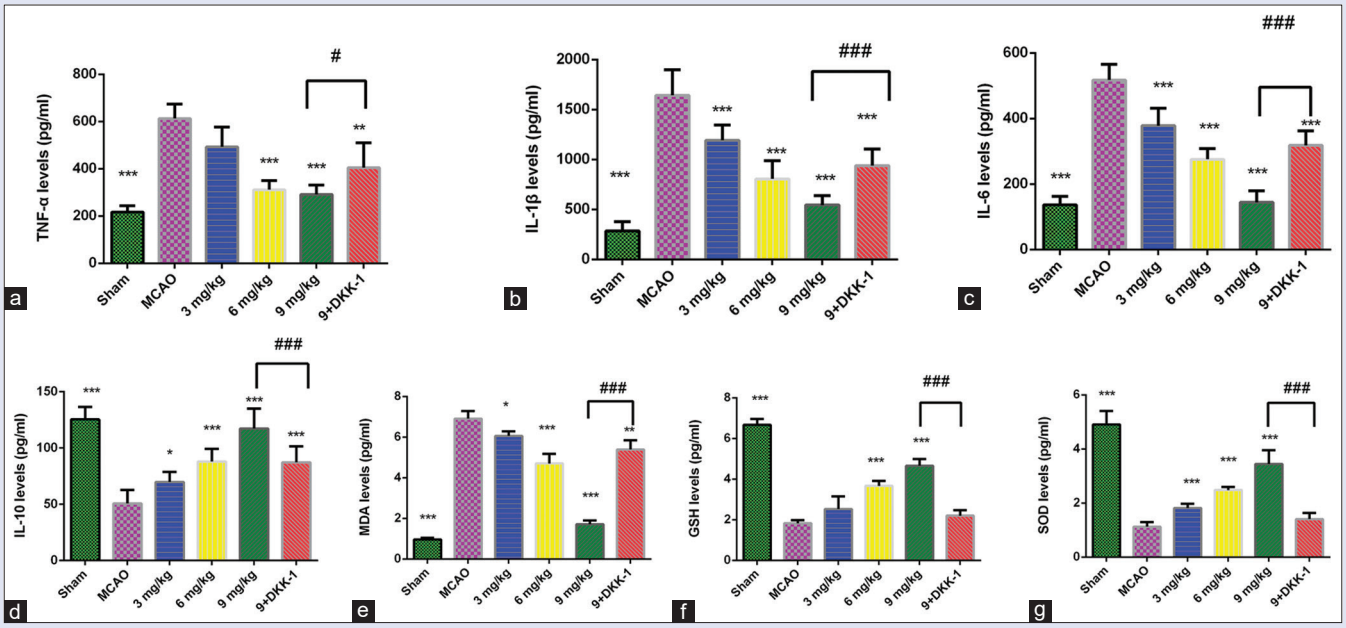

Figure 2: Chrysoeriol regulated inflammatory and oxidative cytokines. (a) Tumor necrosis factor-alpha levels in the brain. (b) Interleukin-1β levels in the brain. (c) Interleukin-6 levels in the brain. (d) Interleukin-10 levels in the brain. (e) Malonaldehyde levels in the brain. (f) Glutathione levels in the brain. (g) Superoxide dismutase levels in the brain. The statistical difference between the middle cerebral artery occlusion group and the other groups was considered significant at the levels of $^{*}P < 0.05$, $^{**}P < 0.01$, or $^{***}P < 0.001$. The statistical difference between the 9 mg/kg chrysoeriol group and the 9 mg/kg chrysoeriol group with DKK-1 was considered significant at the levels of $^{\#}P < 0.05$, or $^{\#\#\#}P < 0.001$

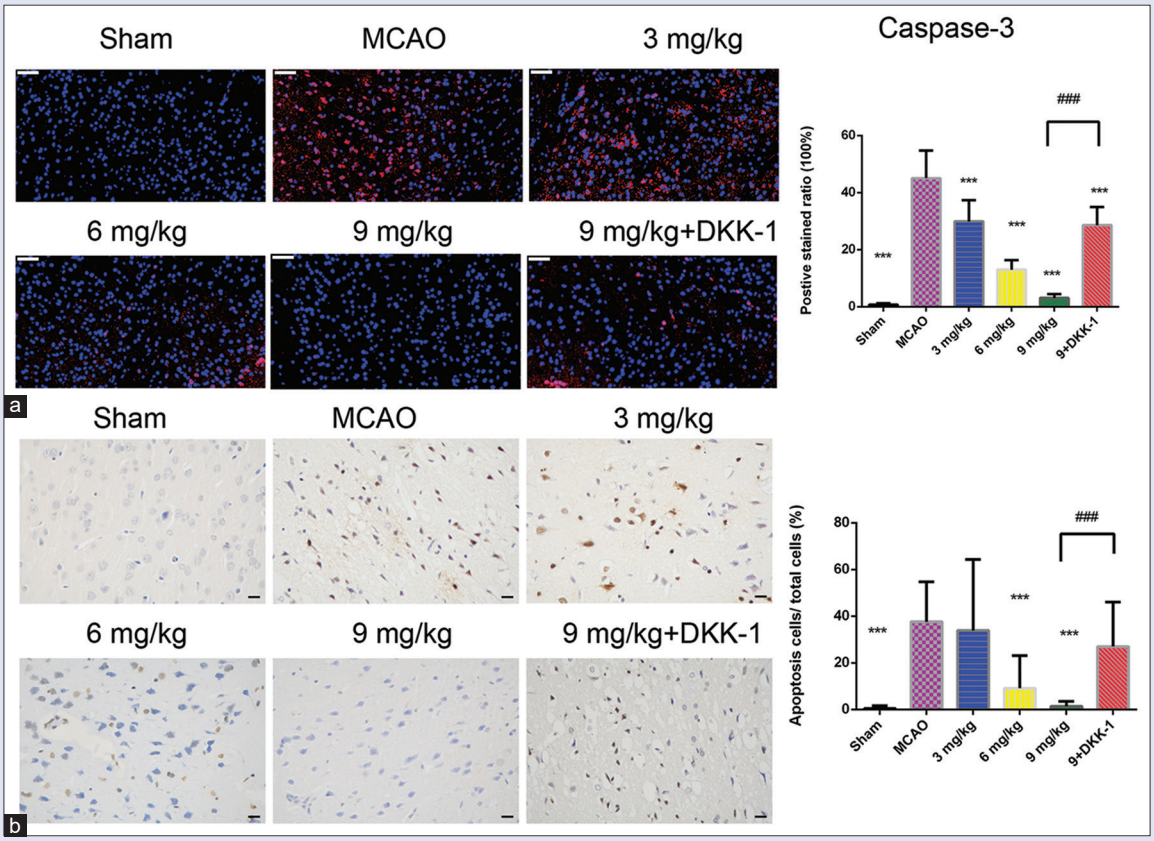

Figure 3: Chrysoeriol alleviated neuronal apoptosis in the brain (ischemic penumbra). (a) Representative immunofluorescence assay of caspase-3 and quantification. Scale bar = 50 μm. (b) Representative TUNEL staining and quantification. Scale bar = 25 μm. The statistical difference between the middle cerebral artery occlusion group and the other groups was considered significant at $^{***}P < 0.001$. The statistical difference between the 9 mg/kg chrysoeriol group and the 9 mg/kg chrysoeriol group with DKK-1 was considered significant at $^{\#\#\#}P < 0.001$

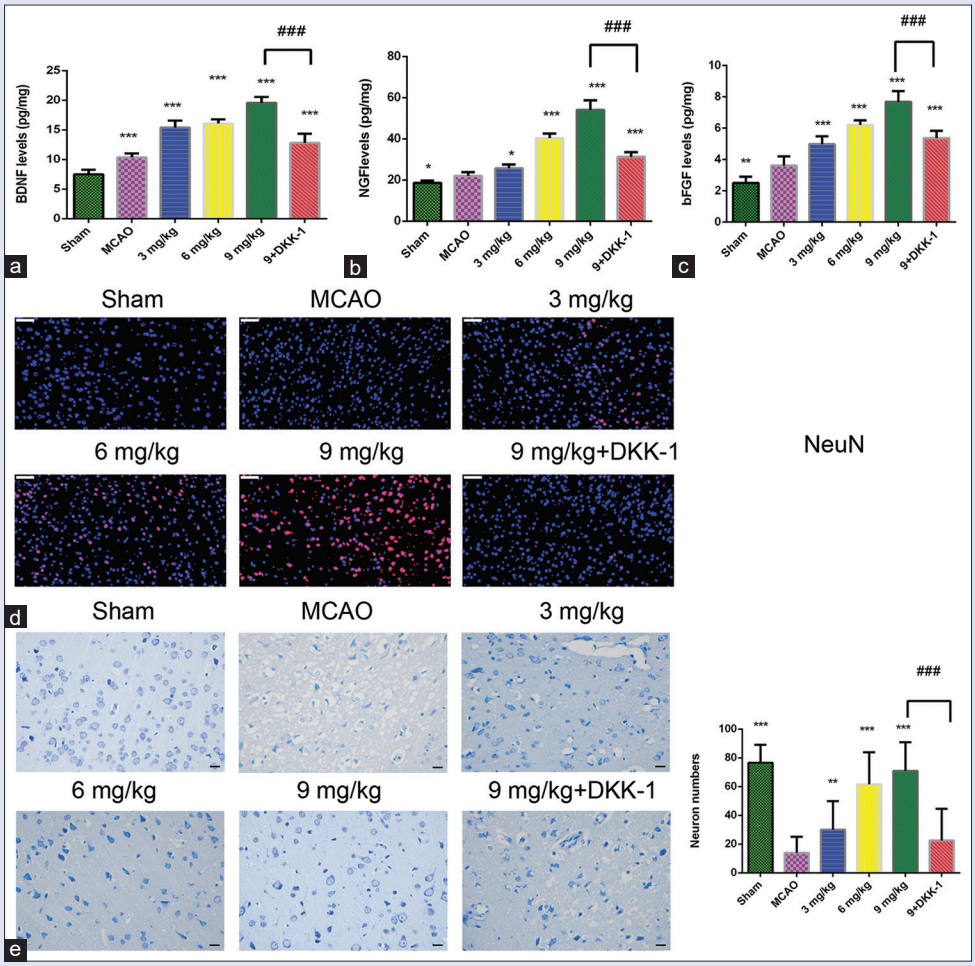

Figure 4: Chrysoeriol accelerated nerve growth in the brain (ischemic penumbra). (a) Brain-derived neurotrophic factor levels of the brain. (b) Nerve growth factor levels of the brain. (c) Basic fibroblast growth factor levels of the brain. (d) Representative immunofluorescence of NeuN. There was none quantification of NeuN. Scale bar = 50 μm. (e) Representative Nissl staining on surviving neurons and quantification. Scale bar = 25 μm. The statistical difference between the middle cerebral artery occlusion group and the other groups was considered significant at $^{***}P < 0.001$. The statistical difference between the 9 mg/kg chrysoeriol group and the 9 mg/kg chrysoeriol group with DKK-1 was considered significant at $^{###}P < 0.001$

Chrysoeriol alleviates neuronal apoptosis

As shown in Figure 3a, caspase-3 fluorescence levels in the ischemic cortex were enhanced significantly in the MCAO group, demonstrating that MCAO surgery elicits neuronal degeneration and apoptosis. Chrysoeriol administration decreased the caspase-3 fluorescence levels. Furthermore, the TUNEL-positive cell ratio in the ischemic cortex was enhanced in the MCAO group [Figure 3b], suggesting that ischemia/reperfusion was the cause of neuron death. Chrysoeriol administration decreased the TUNEL-positive cell ratio in the ischemic cortex in a dose-dependent manner.

Chrysoeriol promotes neuron growth

BDNF, NGF, and bFGF are key NGFs. As shown in Figure 4a-c, BDNF, NGF, and bFGF levels in the brain were increased in the MCAO group, suggesting that ischemia/reperfusion triggered new neuron growth. After chrysoeriol administration, BDNF, NGF, and bFGF levels increased further the NeuN increase as shown in Figure 4d. These results reveal that chrysoeriol promotes nerve growth. The Nissl body is a unique neuronal structure. Nissl staining results revealed that the number of surviving neurons in the MCAO group was reduced markedly [Figure 4e]. After chrysoeriol administration, the number of surviving neurons increased in a dose-dependent manner.

Chrysoeriol influences the expression of the Wnt/β-catenin signaling pathway

The Wnt/β-catenin signaling pathway commonly participates in the promotion of neuron survival in ischemia/reperfusion. In this study, western blot analyses demonstrated greater inhibition of Wnt and β-catenin proteins in the MCAO group than in the sham group, as shown in Figure 5a and b. However, chrysoeriol administration increased the expression of Wnt and β-catenin protein levels compared with the MCAO group. The immunofluorescence analyses supported this finding. The Wnt and β-catenin proteins were activated significantly less in the MCAO group than in the sham group. However, their activation was increased in the chrysoeriol groups, as shown in Figure 5c and d.

To further determine the role of the Wnt/β-catenin signaling pathway in the effects of chrysoeriol, DKK-1, a specific inhibitor of the Wnt signaling pathway was injected in one group of rats. First, DKK-1 inhibited the expression of the Wnt/β-catenin protein [Figure 5a-d]. Second, pro-inflammatory cytokine levels in the 9+DKK-1 group were enhanced compared with those in the 9 mg/kg group [Figure 2].

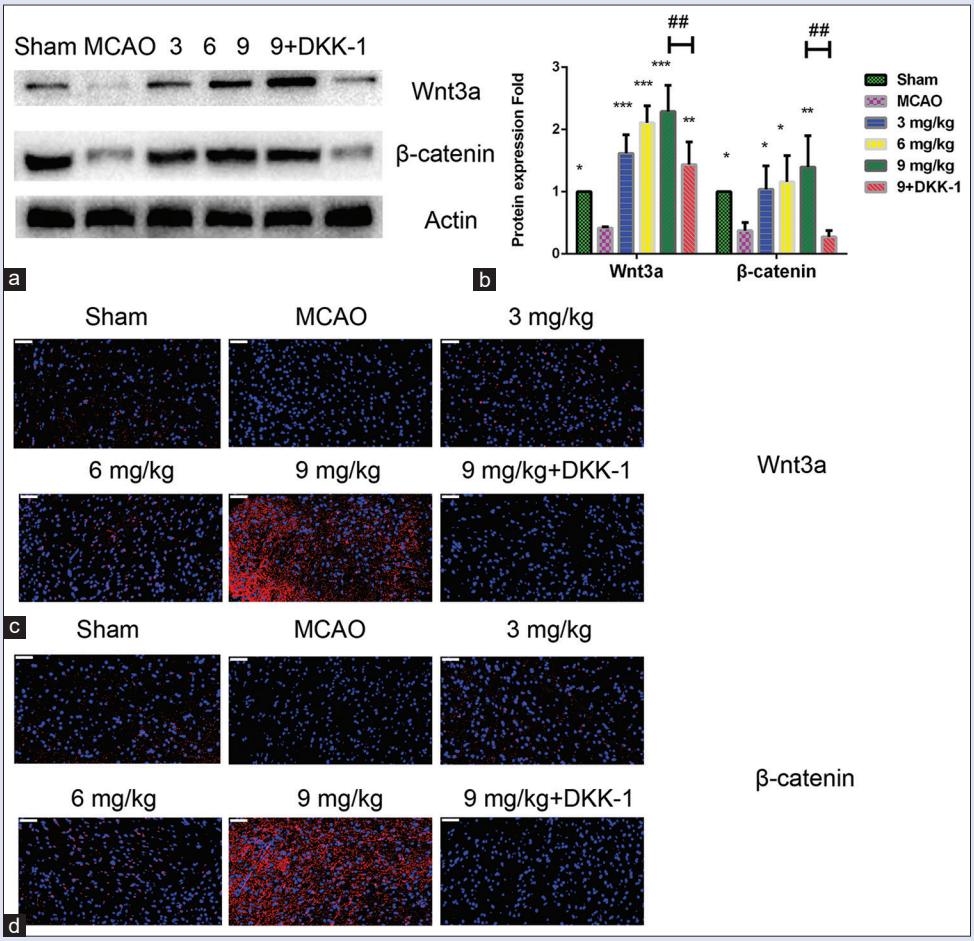

Figure 5: The role of the noncanonical Wnt signaling pathway in the neuroprotective effects of chrysoeriol. (a) Representative western blots of Wnt3a and β-catenin. (b) Quantification of Wnt3a and β-catenin. (c) Representative immunofluorescence of Wnt3a. (d) Representative immunofluorescence of β-catenin. Scale bar = 50 μm. The statistical difference between the middle cerebral artery occlusion group and the other groups was considered significant at ***$P < 0.001$. The statistical difference between the 9 mg/kg chrysoeriol group and the 9 mg/kg chrysoeriol group with DKK-1 was considered significant at $^{\#\#}P < 0.01$

Third, apoptosis rates in the 9+DKK-1 group were enhanced [Figure 3] and the neuron count was reduced compared to that in the 9 mg/kg group [Figure 4]. Most importantly, DKK-1 decreased the NDS and increased the ischemic area compared with the 9 mg/kg group, as shown in Figure 1. These results demonstrated that the Wnt/β-catenin pathway is widely involved in the neuroprotective effects of chrysoeriol.

DISCUSSION

Cerebral ischemia and the consequent blood reperfusion elicit the excess production of toxic metabolites, such as pro-inflammatory cytokines, glutamate, excitatory amino acids, and oxygen-free radicals.[40] These toxic metabolites further aggravate brain injury after reperfusion. Among these, pro-inflammatory cytokines, such as IL-6, IL-1β, and TNF-α, are closely linked to the inflammatory cascade and neuroinflammatory damage of cerebral ischemia–reperfusion.[41] Meanwhile, cerebral ischemia–reperfusion commonly results in the excess production of reactive oxygen species, causing excess H_2O_2 in the brain. This can attack cellular proteins and result in neuron death.[42] Previous studies revealed that chrysoeriol has anti-inflammatory properties,[43] and can act as an anti-oxygen free radical agent.[22] In this study, the IL-6, IL-1β, and TNF-α cytokine levels were significantly higher in the MCAO group. Chrysoeriol administration reduced the IL-6, IL-1β, and TNF-α cytokine levels. Reducing the pro-inflammatory cytokine levels can stabilize the physiological environment in the brain. This is one of the protective mechanisms of chrysoeriol against damage from cerebral ischemia. Our findings are consistent with those of previous research showing chrysoeriol to have anti-inflammatory properties.[20] Inflammation is commonly accompanied by oxidative stress, which also mediated the neurological dysfunction in our MCAO rats. In this study, chrysoeriol administration modulated GSH, SOD, and MDA levels and inhibited the excessive production of oxygen free radicals. This is consistent with previous reports that chrysoeriol has antioxidant properties.[23,44]

Previous research on this treatment has tended to focus on the anti-inflammatory abilities of chrysoeriol. However, the new findings in this study show that it also has neuroprotective abilities. Chrysoeriol administration reduced brain infarction, relieved neurobehavioral deficits, and attenuated neuronal apoptosis. The most common form of stroke is that arising from arterial occlusion, which leads to acute ischemia in the brain. This results in apoptosis and other types of neuron death in the ischemic area. Both our clinical and preclinical experiments demonstrated that chrysoeriol alleviates such apoptosis. This is a key factor for effective stroke treatment.[45,46] Caspase-3 promotes cell death. Chrysoeriol administration inhibited

caspase-3 expression and further inhibited neuron death. A TUNEL assay showed caspase-3 activation and neuron death to be attenuated by chrysoeriol.

Numerous signaling pathways are involved in the pathogenesis of, and recovery from, ischemic stroke. A previous study has demonstrated the growth-promoting effects of chrysoeriol on osteoblastic cells.[26] Researches have also demonstrated its capacity to protect and regulate mitochondria.[23,25] There is tight cross-talk between mitochondria and the Wnt signaling pathway that acts as a feed-forward loop; Wnt activation adjusts mitochondrial function and activates the Wnt signaling pathway.[47] The Wnt signaling pathway also manages mitochondrial distribution and dynamics.[48] Therefore, this signaling pathway was explored in the current study. Our results indicated that the Wnt/β-catenin signaling pathway plays a crucial role in the pharmacological effects of chrysoeriol.

Wnt/β-catenin signaling makes several contributions to recovery from stroke. First, the Wnt/β-catenin signaling pathway is closely linked to neurogenesis, neural differentiation, and central nervous system plasticity.[49] Wnt3a promotes the activation of neuronal nuclear antigens (NeuN) in neurons.[50] β-catenin translocates from the cytoplasm to the nucleus under the stimulation of Wnt proteins. It then combines with T-cell factors/lymphoid enhancer factors, such as neurogenin2, Pax6, and Tbr2, to facilitate angiogenesis and neurogenesis.[51] Growth factors are important indicators of neurogenesis in the restoration of ischemic areas. NGF and BDNF levels were decreased significantly in the CA1 neurons of the animal model of ischemic stroke. NGF and BDNF are key factors in the growth of neurons and neurogenesis. Increased bFGF levels also promote neurogenesis, which occurred in the chrysoeriol groups of this study. Therefore, we can conclude that chrysoeriol activates the Wnt signaling pathway, leading to neurogenesis and new neuron growth reflected by the NeuN, Nissl staining, and growth factor levels in the rat brain.

The Bcl-2/Bax complex is crucial for apoptosis regulation. Research has shown that β-catenin regulates Bcl-2 expression, indirectly inhibiting Bax and thereby regulating the Bcl-2 to Bax ratio[52] and activating caspase-3 to induce apoptosis. Activation of the Wnt/β-catenin signaling pathway reduces neuronal apoptosis.[53] In this study; chrysoeriol treatment activated the Wnt/β-catenin signaling pathway, attenuating caspase-3 activation and neuron death, reflected by the TUNEL assay. The Wnt/β-catenin signaling pathway is also involved in the neuroinflammation seen in ischemic stroke. Wnt-3a alleviates neuroinflammation after ischemic stroke by modulating the responses of microglia, macrophages, and astrocytes.[54] This inflammation is linked to oxidative stress in cellular homeostasis. The inflammatory process can elicit oxidative stress, for example, when polymorphonuclear neutrophils produce reactive oxygen species through the NADPH oxidase enzyme pathway.[55] Therefore, neuroinflammation inhibition helps oxidative stress regulation. As Wnt-3a activation by chrysoeriol inhibited the production of pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β) in this study, we can infer the additional inhibition of oxidative stress. To sum up, Wnt activation by chrysoeriol inhibits the inflammatory response, modulates oxidative stress, promotes neurogenesis, and reduces neuronal apoptosis via the Wnt signaling pathway. Moreover, the pharmacological effects of chrysoeriol were blocked by the specific Wnt inhibitor, DKK-1, further validating the intrinsic involvement of the Wnt signaling pathway.

CONCLUSION

This study demonstrated that chrysoeriol administration alleviates the damage caused by cerebral ischemia and reperfusion in MCAO model rats. Chrysoeriol administration reduced the area of brain infarction, relieved neurobehavioral deficits, inhibited the production of pro-inflammatory cytokines, reduced neuronal apoptosis, and promoted nerve growth. This neuroprotective mechanism was strongly linked to the activation of the Wnt/β-catenin signaling pathway. In conclusion, this study revealed that chrysoeriol is a promising agent in stroke treatment.

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Conflicts of interest

There are no conflicts of interest.

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