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Butylphthalide regulation of nerve cells in rats with cerebral infarction through jnk/p38mapk signaling pathway

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Abstract: To investigate the regulatory effect of n-butylphthalide (NBP) on the nerve cells of rats with cerebral infarction (CI) through the JNK/p38MAPK signaling pathway, 135 SPF SD male rats were and randomly assigned into the control group (n=45, sham surgery + peanut oil gavage), model group (n=45, CI model + peanut oil gavage), and NBP group (n= 45, CI model + NBP gavage). The comparison of the neurological function score between the model group and the NBP group, as well as the integrated locomotor ability score, Slit2 expression level, blood-brain barrier permeability, microvessel density (MVD), CI volume, neuronal apoptosis rate of the brain tissue and expression levels of brain tissue p-JNK and p-p38MAPK protein among three groups was conducted. NBP inhibits the expression of JNK/p38MAPK signaling pathway, promotes the expression of Slit2 in CI rats, improves the neurological function and locomotor ability of CI rats, while promoting micro vascularization of the brain tissue, protecting the blood-brain barrier, reducing the volume of CI and the apoptosis of nerve cells.

Keywords: N-butylphthalide, JNK/p38MAPK, cerebral infarction, rat, nerve cell.

INTRODUCTION

Cerebral infarction (CI) is a cerebrovascular disease caused by cerebral ischemia and hypoxia and persistent apoptosis of brain neurons attributable to cerebral blood flow disorders due to various factors, with high incidence, disability rate and mortality, posing a great threat to people's life and health (Deguchi *et al*., 2020; Aberg *et al*., 2020). The main mechanism of brain injury induced by CI is thought to be the apoptosis of cerebral neurons. The main physiological damage is thought to be neuronal necrosis in the early stage of cerebral ischemia and irreversible brain neuronal apoptosis in the late stage of cerebral ischemia. Since brain neuron apoptosis is closely related to the expression of signaling pathway, the inhibition of the expression of relevant signaling pathways is crucial for attenuating neuronal apoptosis and brain injury (Hou *et al*., 2020; Jang *et al*., 2020). Butylphthalide, a natural compound derived from celery seeds, has shown promising neuroprotective effects in preclinical and clinical studies. Previous research has suggested that butylphthalide can regulate multiple cellular processes involved in neuronal survival and recovery, including reducing oxidative stress, inhibiting apoptosis, and enhancing neurotrophic support. N-butylphthalide (NBP), namely butylphthalide (chemical name: 3-butyl-1 (3H) -isobenzofuranone), with its main component of racemic 1-3-n-butylphthalide, is a successfully developed anti-cerebral ischemia drug in China, which can penetrate the blood-brain barrier, improve free radicals and reduce ischemia reperfusion, and then relieve cerebral blood flow disorders (Tian *et al*., 2020). Mitogen-activated protein kinase (MAPK) bears the transmission of signals from the cell surface to the inside of the nucleus, primarily including three signaling pathways such as JNK, p38 and ERK (Yan *et al*., 2017). To investigate whether JNK and p38 signaling pathways were involved in the therapeutic effect of NBP on CI, the regulatory effect of NBP on nerve cells of CI rats through JNK/p38 MAPK signaling pathway was analyzed in the study and the results are reported below.

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MATERIALS AND METHODS

Animals and group assignment

A total of 135 SPF SD male rats were selected as the objects of study. These rats were supplied by the Experimental Animal Center of Sichuan Academy of Chinese Medical Sciences (Animal certificate No.: SCXK (Sichuan 2008-19)); rats were aged 4-6 weeks and weighed 205-265g. The interior environment was regulated to a temperature of 20-22^{o}C, humidity of about 50% and light of 12 L: 12 D. These rats were housed for 7 days in an SPF barrier system to acclimatize themselves to the environment and supplied by a complete pellet feed manufactured by the Laboratory Animal Center of Sichuan Academy of Chinese Medical Sciences. All rats were randomly divided into the control group (n=45), model group (n=45) and NBP group (n=45). This study conformed to animal ethics regulations.

Reagents and instruments

Reagents: NBP Sodium Chloride Injection (Shijiazhuang Pharmaceutical Group, approval number: GYZZ H20100041, strength: 100 ml: NBP 25 mg and sodium chloride 0.9 g); p-p38MAPK, p- JNK antibody, goat anti-rabbit IgG secondary antibody (Abcam, USA); HE

staining kit, TUNEL kit (Shenglog, Shanghai); goat anti-rabbit SABC kit, rabbit anti-rat endothelial cell adhesion molecule, DAB chromogenic kit (Wuhan Boster Biological Technology Co. Ltd.).

Instruments: optical microscope (Olympus); gel electrophoresis (Beijing Zeping Bioscience & Technologies Co., Ltd.); flow cytometer (BD); ELISA analyzer (Perlong Medical).

Control group

Sham surgery and intragastric administration of peanut oil were performed. (1) Sham surgery is defined as sham surgical procedures with separation only without ligation at the time of the CI model establishment in the following model groups. (2) Peanut oil gavage: Starting from the 1st day after sham surgery, peanut oil was intragastrically administered for 2 weeks at a dose of 80 mg kg- 1 d- 1.

Model group

The CI model was established, and peanut oil was intragastrically administered (Gao *et al*., 2019). (1) The surgical method was set up for the CI model: (1)The focal cerebral ischemia model in rats was constructed by the modified Zea Longa method. (2) The rats were maintained in the fasting state for 12 hours before the operation, and then anaesthetized by isoflurane inhalation (1~1.5%); (3) The rats were positioned supinely and immobilized. It is required to select central neck incision, avoid skin tissue, expose and separate the external and internal carotid arteries and the left common carotid artery. (4) The distal end of the external carotid artery was ligated, and the common carotid artery and internal carotid artery were clamped with a clip. At this time, the external carotid artery was directly cut, and a fishing line was inserted into the direction of the internal carotid artery. When the depth of suture insertion was approximately 17 mm, this operation could be stopped if resistance occurred. (5) Finally, the external carotid artery stump was ligated and the fishing line was fixed. (6) After the operation, the incision was sutured and incandescent lamp irradiation was used to maintain the anal temperature at 37°C. (6) The rat tail suspension test was positive, the ipsilateral eye fissure was reduced, the contralateral limb was paralyzed and it turned to the ground, which indicated that the modeling was successful. (2) Peanut oil gavage was the same as the control group.

NBP group

The CI model was established, and NBP was intragastrically administered. (1) The CI model was established, and the surgical method was the same as the model group. (2) NBP gavage: NBP was diluted with peanut oil to a concentration of 7 g/L and NBP solution was administered by gavage for 2 weeks at a dose of 80mg·kg-1·d-1 starting on the 1st day after the establishment of the CI model.

Neurological function score, integrated locomotor ability score, and detection of Slit2 expression level

(1) Five rats were taken from the three groups at 3 h after surgery and at 1 d, 3 d and 7 d after gavage, and their neurological status was assessed using the Zea Longa scoring method, with scores ranging from 0 to 4, including 4 points: freely walking but unclear consciousness, 3 points: falling to the left while walking, 2 points: rotating to the left while walking, 1 point: abnormal extension of the left forelimb, and 0 point: insignificant neurological-related symptoms. (2) After neurological function scores were performed on rats in the model and NBP groups at 1d, 3d, and 7d after gavage as described above, their integrated locomotor ability was then tested by a rotational barometer and the time of the rats to drop at 100 r/min (maximum) rpm was recorded and measured at intervals of 20 min for a total of three times. (3) The expression level of Slit2 was detected after the above scores were performed for rats in the three groups at 1d, 3d and 7d after gavage: the brain tissue on the infarcted side of the right cerebral hemisphere was collected after anesthesia with inhalation of 1~1.5% isoflurane, placed in an embedding box containing OCT glue, loaded into a freezer tube, quickly frozen with liquid nitrogen for 2h and then cut into slices using a cryostat microtome at -20°C, blow dried, fixed in 4% paraformaldehyde for 20 min and washed 5 min/time for 15 min in total, blow dried again, sealed and stored at -80°C; the enzyme-linked immunosorbent assay (ELISA) was used for the measurement and the kit was supplied by Shanghai HengYuan Biological Technology Co., Ltd. (1)The microwell plate was coated with purified rat Slit2 antibody, added with Slit2 one by one and then reacted with HRP (horseradish peroxidase) -labeled Slit2 antibody to form antibody-antigen-enzyme-labeled antibody complex; (2) The complex was washed, TMB (3,3′,5,5′-tetramethylbenzidine) was added for color development; (3)The darker the color, the higher the Slit2 level. The absorbance value at 450 nm was measured by the microplate reader and the expression level of Slit2 in the sample was calculated by the standard curve.

Determination of blood-brain barrier permeability

(1) At 71 h after surgery, 10 rats from each group were given a tail vein injection with 20 g/L of Evans blue 3 mL/kg, followed by inhalation of 1~1.5% isoflurane after 1 hour to anesthetize the rats; (2)The aorta was pierced from the left ventricle and normal saline at 37^{o}C was injected until there was clear fluid outflow from the right atrial appendage of the rat; (3)The brain was decapitated, and about 200 mg of cortex around the infarcted tissue was isolated and placed in 3mL DMF (dimethyl formamide) for 2 days in a 37°C warm bath, and then centrifugated at 1500 r/min for 10 min; (4) The supernatant was retained, the absorbance at 630 nm was detected using an enzyme-labeled analyzer and the content of Evans blue was obtained from the standard curve.

Determination of MVD

At two weeks after intragastric administration, ten rats were taken from each group and the brain tissues were collected and fixed with 4% paraformaldehyde in PBS buffer to make sections; the operation method was based on the ABC method and the immunohistochemistry method was used for the assay to master the protein expression status of CD31 antigen (specific marker) in brain endothelial cells of rats. Weidner method was used for the assessment and counting of micro vessels. Finally, immunopositive micro vessel density (MVD) of CD31 in brain tissues was obtained.

CI volume and neuronal apoptosis in brain tissue and expression levels of p-JNK and p-p38 MAPK protein in brain tissue

(1) At two weeks after intragastric administration, 10 rats were taken from each group to observe their CI volume by TTC (2,3,5-triphenyltetrazolium chloride) staining method. These rates were sacrificed. Later, the brain tissue was aseptically removed and stored at -80°C for 5 min, after which a 2 mm frontal pole posterior to the coronal plane was removed, stored in 1% TTC solution, and incubated at 37°C, protected from the light; the brain tissue was turned every 5 min to stain, the white was the infarcted tissue, and the red was the normal brain tissue; (2) Neuronal apoptosis in brain tissue: TUNEL (TdT-mediated dUTP Nick-End Labeling) method was used to detect neuronal apoptosis in rats. Paraffin sections of CI tissues were prepared and stained with TUNEL to detect neuronal apoptosis. The detection method was adopted in strict accordance with the instructions of the TUNEL kit; the color of apoptosis was tan as seen by optical microscopy. (2) The expression levels of p-JNK and p-p38 MAPK proteins in brain tissue were detected by Western blotting (immunoblotting). The lysate was taken to soak the brain tissue and then centrifugated at 4°C for separation for 0.5 h; the overall protein concentration was detected by SCA (human anti-steroid cell antibody) protein detection kit; The samples were treated with SDS-PAGE electrophoresis and switched to PVDF membranes, and incubated with anti-GAPDH, p-p38MAP and Kp-JNK antibodies overnight at 4°C. After washing, the secondary antibody (horseradish peroxidase conjugated) was selected for incubation for 1 h. After cooling, the expression levels of p-JNK and p-p38MAPK proteins were calculated, and the data were analyzed using Nuance software. Finally, ECL (electrochemiluminescence) was used for developing, shooting and storage.

Observation indicators

(1) Comparison of neurological function scores in the model group and NBP group: The higher score represented a poorer locomotor ability. (2) Integrated locomotor ability score: The comprehensive ability such as balance ability, coordination ability, physical strength and endurance of rats was assessed and the higher score represented the milder neurological impairment. (3) Expression level of Slit2. (3) Blood-brain barrier permeability and brain microvasculature. (5) CI volume and neuronal apoptosis rate of the brain tissue: (1) The image of white infarct tissue of rats was processed and analyzed using professional image analysis software and the percentage of CI volume of rats was calculated; (2) Myocardial apoptosis rate = the number of apoptotic cells/the total number of cells × 100%. (6) Expression levels of p-JNK and p-p38MAPK proteins in brain tissue.

Ethical approval

The study was approved by the ethics committee of Cangzhou Hospital of Integrated Traditional Chinese and Western Medicine, No.2018032577, and was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from patients involved in the study.

STATISTICAL ANALYSIS

SPSS 20.0 software was used to analyze the data. The quantitative data were expressed as $\bar{x} \pm s$ and analyzed by t-test or F-test. The repeated measures analysis of variance was performed for the comparison of the data at different time points. $P<0.05$ suggested a statistically significant difference.

RESULTS

Comparison of neurological function scores in the model group and NBP group

Apart from the fact that the neurological function score in the NBP group was higher than that in the model group at 3 h after surgery, the neurological function score in the NBP group was lower than that in the model group at 1d, 3d, and 7d after intragastric administration (inter-group effect: $F=16.200$, $P<0.001$). The neurological function scores in the two groups have a tendency to decrease over time (time effect: $F=25.350$, $P<0.001$) and the grouping and time have an interactive effect (interactive effect: $F=5.209$, $P=0.0048$) (table 1).

Comparison of integrated locomotor ability scores among the three groups

At 1d, 3d and 7d after intragastric administration, the integrated locomotor ability score in the NBP group was higher than that in the model group but was lower than that in the control group, and the difference was statistically significant ($P<0.05$) (table 2).

Comparison of the expression level of Slit2 among the three groups

At 1d, 3d and 7d after intragastric administration, the expression level of Slit2 in the model group and NBP group was higher than that in the control group ($P<0.05$), while it was higher in the NBP group than in the model group ($P<0.05$), as shown in fig 1 below.

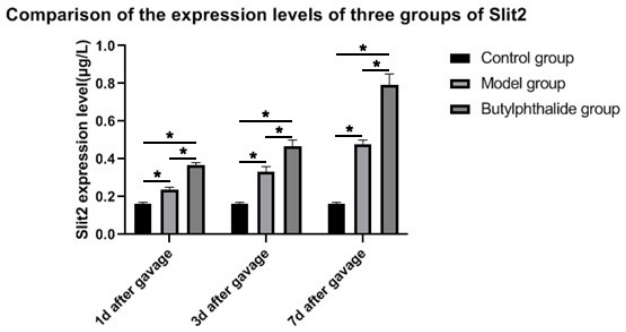

Fig 1 Comparison of the expression level of Slit2 among the three groups, *P<0.05.

Comparison of blood-brain barrier permeability and brain microvasculature among the three groups

The blood-brain barrier permeability in the model group and the NBP group was higher than that in the control group, and the statistically significant difference was observed (tmodel group vs. control group=64.080, tNBP group vs. control group=54.870; P<0.001), whereas it was lower in the NBP group than in the model group, and a significant difference was observed (tNBP group vs. model group=6.384, P<0.001); MVD level in the model group and NBP group was higher than that in the control group, and a significant difference was found (tmodel group vs. control group=5.791, tNBP group vs. control group=21.430; P<0.001), while it was higher in the NBP group than in the model group and the statistically significant difference was noted (tNBP group vs. model group=18.630, P<0.001) (table 3).

Comparison of CI volume and neuronal apoptosis rate of brain tissue

The CI volume in the model group and the NBP group was higher than that in the control group, and there was a statistically significant difference (tmodel group vs. control group=19.06, tNBP group vs. control group=16.94; P<0.001), while it was lower in the NBP group than in the model group, and the difference was statistically significant (tNBP group vs. model group=6.627, $P<0.001$); the neuronal apoptosis rate of brain tissue in the model group and NBP group was higher than that in the control group and the statistically significant difference was observed (tmodel group vs. control group=26.210, tNBP group vs. control group=16.560; P<0.001), while it was lower in the NBP group than in the model group (tNBP group vs. model group=11.980, P<0.001) (table 4).

Comparison of expression of p-JNK and p-p38MAPK proteins in brain tissue among the three groups

The expression of p-JNK protein in the brain tissue in the model group and NBP group was higher than that in the control group (tmodel group vs. control group =6.172, tNBP group vs. control group = 7.317; P<0.001), while it was lower in the NBP group than in the model group, and a significant difference was noted (tNBP group vs. model group =2.688, P=0.015). The expression of p-p38MAPK protein in the brain tissue in the model group and NBP group was higher than that in the control group (tmodel group vs. control group =26.53, tNBP group vs. control group =9.000; P<0.001), whereas it was lower in the NBP group than in the model group (tNBP group vs. model group = 16.400, $P<0.001$). See table 5.

DISCUSSION

Cerebral infarction (CI) has the potential to result in prolonged hypoxemia in the brain tissue of rats, which in turn causes severe impairment of cerebral function. According to relevant research data, JNK/p38 MAPK signaling pathway is closely associated with CI, even involved in the pathological process of CI. It acts as an active regulator of neuronal differentiation and apoptosis, and affects the occurrence and development of other cerebral diseases (Tamai *et al*., 2019). At present, the focus of clinical practice in the treatment of CI is to prevent ischemic reperfusion. The cerebral nerve cell damage was controlled usually by drug medication, which is of great significance to protect cerebral nerves, tissues and cells.

Our results suggested that (1) the expression levels of p-JNK and p-p38 MAPK proteins were increased in the NBP group and model group compared with the control group, while they were lower in the NBP group than in the model group; the neurological function score in the NBP group was lower than that in the model group at 1 d, 3 d, and 7 d after gavage (inter-group effect: F = 16.200, P < 0.001), suggesting NBP reduces the expression levels of p-JNK and p-p38 MAPK in the brain tissue of CI rats and improves the neurological function. Meanwhile, the neurological deficit was most severe in the model group compared with the control group, and the neurological impairment was alleviated in the NBP group; it was shown that NBP improves the effect of CI neurological impairment. The analysis results are as follows: JNK and p38 MAPK belong to the category of downstream factors of MAPK signaling, which can promote the occurrence and development of CI; thanks to the blockage of blood flow in the cerebral vessels of CI body, it has been reported to promote the activation of JNK/p38 MAPK signaling, so that it sends out regulatory signals (such as nitric oxide, inflammatory factors, etc.), which not only triggers neurological dysfunction, but also promotes further ischemic necrosis of the tissues in the cerebral perfusion area, and the severe development of the disease (Yan *et al*., 2017). Therefore, p-p38 MAPK is involved in the process of CI injury. Once activated, it contributes to the release of some inflammatory factors, thereby aggravating the CI. NBP can remarkably enhance the activity of antioxidant enzymes in the body, reduce the concentration of intracellular calcium ions, and then

Table 1: Comparison of neurological function scores among three groups (points, x ± s)

Group	Model group (n=5)	NBP group (n=5)	t	*P*
3h post-operation	1.48±0.29	1.71±0.23	1.389	0.202
1d after gavage	3.21±0.72	2.29±0.37	2.541	0.035
3d after gavage	2.86±0.67	1.96±0.25	2.814	0.023
7d after gavage	2.31±0.32	1.14±0.31	5.872	<0.001

Table 2: Comparison of integrated locomotor ability scores among the three groups (points, x ± s)

Group	N	1d after gavage	3d after gavage	7d after gavage
Control group	5	265.23±45.12	263.46±47.54	268.34±44.36
Model group	5	52.35±10.03	49.28±10.13	88.67±14.31
NBP group	5	77.26±11.39	80.62±16.27	115.29±17.96
F		89.660	76.390	56.520
P		<0.001	<0.001	<0.001

Table 3: Comparison of blood-brain barrier permeability and brain microvasculature among the three groups (x ± s)

Group	N	MVD	Blood-brain barrier permeability (μg/g)
Control group	10	27.04±3.01	22.79±5.04
Model group	10	45.22±9.46	320.86±13.82
NBP group	10	226.87±29.34	281.13±14.01
F		381.800	1904.000
P		<0.001	<0.001

Table 4: Comparison of CI volume and neuronal apoptosis rate of brain tissue among the three groups (%, x±s)

Group	N	CI volume	Neuronal apoptosis rate of brain tissue
Control group	10	0.00±0.00	3.47±0.59
Model group	10	31.46±5.22	70.54±8.07
NBP group	10	18.37±3.43	33.21±5.65
F		192.100	347.900
P		<0.001	<0.001

Table 5: Comparison of expression levels of p-JNK and p-p38MAPK proteins in brain tissue among the three groups (x ± s)

Group	N	p-JNK	p-p38MAPK
Control group	10	0.28±0.09	0.91±0.07
Model group	10	1.31±0.52	2.53±0.18
NBP group	10	0.83±0.22	1.54±0.21
F		24.380	245.800
P		<0.001	<0.001

hinder the function of inflammatory factors, whereas a large number of inflammatory factors produced in the body are differentiated by the JNK/p38 MAPK signaling pathway, indicating that NBP may serve as an inhibitor of the JNK/p38 MAPK signaling pathway, and repair neurological function (Li *et al*., 2019). (2) At 24h, 48h and 72h after gavage, the integrated locomotor ability score in the NBP group was significantly higher than that in the model group, indicating that the integrated locomotor ability of CI rats may be upgraded by NBP. This may be related to the mechanism by which NBP can reconstruct the circulation in the ischemic area, improve the energy metabolism of the tissue in the ischemic area, inhibit the release of oxygen free radicals and arachidonic acid, and protect the normal function of mitochondria (Sahib *et al*., 2019; Sun *et al*., 2017). (3) The increase in the expression level of Slit2 in the NBP group was significantly greater than that in the model group after gavage (P<0.05), indicating that NBP greatly promotes the expression of Slit2.

Slit2 is the most important matrix protein present extra cellularly in mammals and regulates the direction of neuronal axon growth. Studies have shown that Slit2 is

able to accelerate angiogenesis (Chen *et al*., 2020). Newly produced capillaries establish collateral circulation to help the body restore normal blood perfusion in the infarcted tissue area as soon as possible, which is conducive to the survival and prognosis of neurons after CI; NBP can promote the construction of collateral circulation and improve microcirculation and energy metabolism and neurological deficits after CI, but there is no consensus on the mechanism of action of NBP, which may be based on promoting the expression of Slit2, promoting vascular regeneration and collateral establishment, attenuating brain injury, relieving cognitive dysfunction and protecting cranial nerve tissue (Zhang *et al*., 2020). (4) The increase in blood-brain barrier permeability in the NBP group was significantly lower than that in the model group, while the increase in MVD level in the NBP group was significantly higher than that in the model group ($P<0.05$); these results suggested that NBP markedly reduces the blood-brain barrier permeability in CI rats while raising the MVD level. (1) Blood-brain barrier is a membrane structure that separates brain tissue, blood and cerebrospinal fluid. Under normal physiological state of the body, the blood-brain barrier permeability is relatively low. Moreover, while ensuring a stable environment in the central nervous system, it can also effectively prevent the intrusion of harmful substances from outside the body (Wu *et al*., 2020) The blood-brain barrier can be affected by inflammatory reactions, ischemia, hypoxia and other risk factors in the body and cause structural damage, resulting in increased permeability, so that macromolecules such as plasma proteins penetrate the blood-brain barrier into the brain tissue, promote the increase of colloid osmotic pressure in the brain tissue, form brain edema, and endanger the brain nerve function of rats (Li *et al*., 2019). NBP can reduce the degree of blood-brain barrier permeability and the neurological impairment caused by ischemia in CI rats (2) NBP is able to increase the MVD level. Considering that it can increase the content of prostacyclin and nitric oxide on the cerebrovascular endothelium of the body, and reduce the phenomenon of calcium overload in cells, thereby resulting in decreased glutamate secretion, indirectly inhibiting arachidonic acid release, resisting free radicals, and enhancing the activity of antioxidant enzyme; at the same time, it can prevent mitochondrial function damage and finally, the number of capillaries in the ischemic area of the brain tissue increases, and the microcirculation is improved (Zhang *et al*., 2017). (5) The CI volume and neuronal apoptosis were reduced in the NBP group compared with the model group, indicating that NBP medication effectively reduces the CI volume and neuronal apoptosis in rats as well as the degree of neurological impairment. (1) According to the data survey, NBP can serve as the guarantee for the normal function of mitochondria in the brain cells of the body, increase the brain microcirculation of rats in the model group, enhance energy metabolism and then reduce the CI volume of rats (Qin *et al*., 2019). (2) After intragastric administration of NBP in CI rats, the number of nerve cells increased, which may be closely related to the inhibition of J and related receptors of nerve apoptosis by NBP, or NBP may reduce nerve cell apoptosis by reducing the number of platelet activation and inhibiting the release of inflammatory factors from p-p38 MAPK (Li *et al*., 2019). It was consistent with the findings of another study (Liao *et al*., 2019). This study may have a small sample size resulting in certain bias in the results and data. Therefore, it is required to be further confirmed by studies with a larger sample size.

CONCLUSION

In summary, NBP holds potential in promoting Slit2 expression, improving neurological function and motor ability in CI rats via inhibiting JNK/p38 MAPK signaling pathway expression, while promoting brain micro angiogenesis, protecting the blood-brain barrier and reducing CI volume and neuronal apoptosis.

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