International Journal of Pharmacology

ISSN 1811-7775

science alert

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Asian Network for Scientific Information

OPEN ACCESS International Journal of Pharmacology

ISSN 1811-7775
DOI: 10.3923/ijp.2024.573.581

Research Article
Butylphthalide Improves Neurological Function in Acute Cerebral Infarction Patients via Netrin/DCC/VEGF Signaling Pathway

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Abstract

Background and Objective: Ischemia in acute cerebral infarction leads to brain tissue damage. Butylphthalide improves blood flow, reduces edema and protects mitochondria, benefiting patient prognosis, but its precise mechanism requires further study. This study aimed to evaluate the role of Netrin/DCC/VEGF signaling pathway in the prognosis of patients with acute cerebral infarction and treated with butylphthalide. **Materials and Methods:** The 131 patients with acute cerebral infarction from July, 2019 to February, 2021 were selected and grouped according to the randomized control principle. The control group (CG) (65 cases) received administration of atorvastatin calcium while the experimental group (EG) (66 cases) was treated with atorvastatin calcium+butylphthalide. The cerebral perfusion indexes, serum indexes, neurological function, daily life activities and self-care capacity, Netrin/DCC/VEGF signaling pathway-related protein and prognosis of 2 groups were compared. **Results:** After treatment in the EG, CVR, bilateral peak systolic velocity and mean blood flow velocity, serum BDNF, 3-MST, fibulin-5 levels and Netrin-1, DCC and VEGF protein expression levels were significantly higher than those in the CG, while PI, ICAM-1, GFAP, β-actin, S100β, HMGB1 and PTX3 levels were significantly lower than those in the CG ($p<0.05$). **Conclusion:** Butylphthalide can regulate cerebral blood perfusion, reduce neuronal damage, reduce inflammatory cascade, repair cerebrovascular damage, enhance the ability of daily living and improve prognosis in patients with acute cerebral infarction.

Key words: Acute cerebral infarction, butylphthalide, prognosis, Netrin/DCC/VEGF signaling pathway, neurological function

Citation: Cao, W., 2024. Butylphthalide improves neurological function in acute cerebral infarction patients via Netrin/DCC/VEGF signaling pathway. Int. J. Pharmacol., 20: 573-581.

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Competing Interest: The author has declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Acute cerebral infarction (ACI) is a prevalent condition characterized by restricted blood flow in the brain, leading to ischemic necrosis or softening of specific brain tissue due to impaired blood supply causing ischemia and hypoxia[1,2]. The ACI affects primarily middle-aged and elderly people and patients often present with sudden limb movement disorder and impaired consciousness, resulting in high disability and mortality rate and a poor prognosis during acute phase[3]. Currently, ACI treatment options aimed at reducing the infarct area, inhibiting thrombosis formation, protecting brain cells, restoring cerebral perfusion in ischemic areas, improving cerebral microcirculation and preventing brain cell apoptosis[4]. Statins have antioxidant, antithrombotic, anti-inflammatory effects and could promote plaque calcification. The early use of a statin can reduce the occurrence of endpoint adverse events and improve neurological function. However, some patients still have atherosclerosis that develops into plaque and ruptures and the condition relapses again while adverse reactions such as muscle aches and gastrointestinal reactions can occur during treatment[5]. Butylphthalide demonstrates a range of target neuroprotective roles, including the augmentation of capillary numbers in the ischemic region, enhancement of microcirculation and blood flow, reduction of cerebral edema and support for mitochondrial preservation while inhibiting neuronal apoptosis[6]. Nonetheless, further clarification of its specific mechanism is necessary.

The Netrin-1 protein is a neuronal axon guidance factor that regulates cell migration and axon growth as well as promotes the expression of downstream related signaling pathways through attracting spinal commissural axons and repelling trochlear axons, playing an key role in endothelial cell proliferation, neurological injury and apoptosis following cerebral ischemic and hypoxic injury[7]. It has been demonstrated that Netrin-1 binding to its receptor DCC enhances activation of c-Jun amino-terminal kinase, increases neuronal activity, promotes neurological recovery and inhibits neuronal apoptosis[8]. Animal studies found that upregulation of Netrin-1 expression in neurons of brain-injured rats and its binding to DCC activated ERK signaling pathway and regulated apoptosis and cycle arrest[9]. It was also found that the use of exogenous VEGF promotes neovascularization, improves neuronal cell survival, stimulates axonal growth, reduces brain infarct size and protects neuronal cells[10]. It can be speculated that the Netrin-1/DCC/VEGF signaling pathway may have a relationship with neurological recovery in ACI patients, but there is a lack of enough evidence. This study aimed to evaluate the role of Netrin/DCC/VEGF signaling pathway in improving the prognosis of patients with acute cerebral infarction treated by butylphthalide, with the aim of providing new targets for clinical treatment.

MATERIALS AND METHODS

Clinical data: This study was a prospective randomized controlled trial, which was approved by the Medical Ethics Committee of the First Affiliated Hospital of Anhui University of Chinese Medicine. One hundred and thirty-one patients with acute cerebral infarction in the First Affiliated Hospital of Anhui University of Chinese Medicine from July, 2019 to February, 2021 were enrolled, including 73 males and 58 females, aged 38-76 years with a mean age of (59.92±3.73) years. (1) Inclusion criteria: Patients who met the relevant diagnostic criteria in the 2018 edition of the Chinese Guidelines for Diagnosis and Treatment of Acute Ischemic Stroke[11] and confirmed by cranial CT and magnetic resonance imaging, Time from onset to admission <72 hrs, first onset, not receiving anti-fibrinolytic, thrombolytic, or anticoagulant therapy before admission, clear consciousness at admission, patients and family members voluntarily signed the informed consent and (2) Exclusion criteria: Acute cerebral infarction due to trauma, with cerebral hemorrhage, intracranial occupying lesions, previous history of cerebral infarction and craniocerebral trauma, comorbid with hematologic diseases, autoimmune diseases, malignant tumors, coronary heart disease, abnormal liver and kidney function, with intracranial aneurysm or other cerebrovascular diseases, presence of swallowing dysfunction, impaired consciousness, history of allergy to drugs used in this study, during pregnancy or lactation.

Methods: Routine treatment, all patients were given conventional treatment according to the Chinese Guidelines for the Diagnosis and Treatment of Acute Ischemic Stroke, i.e., nutritional support, stabilization of vital signs, active correction of water-electrolyte disorders, regulation of blood glucose and blood pressure levels, oxygenation, respiratory support, etc. Symptomatic medications that could improve cerebral circulation, brain function and anti-platelet aggregation were administered according to the patients' conditions.

Control group (CG, n = 65), patients were orally administrated with atorvastatin calcium (20 mg, Guangdong Dongyang Pharmaceutical Co. Ltd., H20213513), 80 mg/day, 1 time/day, for 2 weeks.

Experimental group (EG, n = 66), on the basis of the CG, butylphthalide soft capsule (0.1g, Shiyapharm Group Enbip Pharmaceutical Co., Ltd., H20050299) were administrated t.i.d 0.2 g/time for 2 weeks.

Outcome measurement

Cerebral blood perfusion: Cerebrovascular reserve capacity (CVR), PI, bilateral PSV and bilateral MBV were measured by color ultrasound transcranial Doppler flow analyzer (KJ-2V2M, Nanjing WSP Industrial Co. Ltd.,) before treatment and after 2 weeks of treatment.

Laboratory indices: The 3 mL of fasting peripheral blood was collected from patients before and after 2 weeks of treatment, centrifuged at 3000 r/min for 10 min. The supernatant was stored in -20°C refrigerator for measurement. Intercellular Adhesion Factor-1 (ICAM-1), Glial Fibrillary Acidic Protein (GFAP), β-actin, Brain-Derived Neurotrophic Factor (BDNF), calcium-binding Protein (S100β), High Mobility Group Protein 1 (HMGB1), Fibulin-5, 3-Mercaptopyruvate Sulfotransferase (3MST) and Pentraxin 3 (PTX3) levels were measured by Enzyme-Linked Immunosorbent Assay (ELISA) (kit purchased from Shanghai Jianglai Biotechnology Co. Ltd.).

Scales: The NIH Stroke Scale/Score (NIHSS) is composed of 11 items including level of consciousness, visual field, facial palsy, motor arm and motor leg, ranging 0-42 points. Low scores indicated low level of neurological deficits, the modified Rankin scale ranges 0-6 points, with low scores indicated better neurological recovery, Barthel index contains 10 items such as eating, bathing, toileting, dressing, ranging 0-100 points, with low scores indicating poor self-care ability in daily life. The scales were evaluated before treatment and after 2 weeks of treatment.

Western blot: Western blot method to determine the protein expression related to Netrin/DCC/VEGF signaling pathway: As 3 mL of fasting peripheral blood was collected from patients before and after 2 weeks of treatment, anticoagulated with heparin. The peripheral blood lymphocytes were isolated and single nucleated cells were extracted, washed twice with 1640 culture medium, resuspended and the peripheral blood single nucleated cells were collected and lysed in cell lysis solution (Beijing Solarbio Technology Co. Ltd.). The supernatant was harvested by centrifugation to obtain total protein and the concentration of protein was determined by Bradford method[12]. The membrane was transferred at 200 mA using 10% SDS-PAGE gel electrophoresis (Solexpro, China) and incubated overnight at 4°C with primary antibody (Netrin-1, DCC, VEGF were purchased from CST, USA) after being closed with 5% skim milk powder for 2 hrs. The membranes were washed 3 times with Tris-Buffered Saline Tween 20 (TBST) after 1 hr of incubation. Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was used as an internal reference and the protein bands were measured using chemiluminescence imager (Type: UVP BioSpectrum, UVP LLC, Upland, California, USA) and quantitative analysis was performed using ImageJ software V1.8.0.112.

Prognosis: The prognosis of patients after 3 months of treatment was assessed using the modified Rankin scale. Complete recovery: 0 points; good recovery: 1 point; mild disability: 2 points; moderate disability: 3 points; severe disability: 4 points; severe disability: 5 points; death: 6 points; good prognosis: ≦ 2 points.

Statistical analysis: The SPSS 24.0 statistical analysis software was used and the measurement data conforming to normal distribution were $\bar{\chi}\pm$sec examined by t-test, the count data were expressed by rate and examined by χ^2 test, $p<0.05$ was considered statistically significant difference.

RESULTS

Baseline data: There was no significant difference in baseline data between EG and the CG ($p>0.05$), indicating that the 2 groups were well balanced and comparable (Table 1).

Cerebral blood perfusion: The differences in CVR, PI, bilateral PSV and MBV before treatment did not differ significantly between the two groups ($p>0.05$); After treatment, the EG exhibited higher CVR (Fig. 1a), lower PI (Fig. 1b), higher bilateral PSV (Fig. 1c) and higher bilateral MBV (Fig. 1d) than the CG ($p<0.05$), indicating that butylphthalide could improve cerebral perfusion in patients with acute cerebral infarction.

Levels of neuronal injury markers: Before treatment, serum ICAM-1, GFAP, β-Actin, BDNF and S100βlevels in the EG were

Table 1: Comparison of the clinical data between the two groups (n, $\bar{\chi}\pm$S)

Group	Male/ female	Age (years)	Body mass index (kg/m²)	Time from onset to hospital admission (hrs)	Infarct area (cm²)	Infarct type A/B/C/D	Comorbidity E/F/G	Site of lesion Cerebellum/basal/brainstem
Control group (n = 65)	35/30	59.62±3.15	23.02±2.45	21.03±2.05	25.34±3.02	20/23/17/5	12/13/9	13/30/22
Experimental group (n = 66)	38/28	60.92±2.77	22.96±1.86	20.95±2.41	26.73±2.94	18/22/18/8	10/16/12	10/29/27

A: Total anterior circulation, B: Partial anterior circulation, C: Posterior circulation, D: Luminal, E: Hypertension, F: Hyperlipidemia and G: Diabetes mellitus

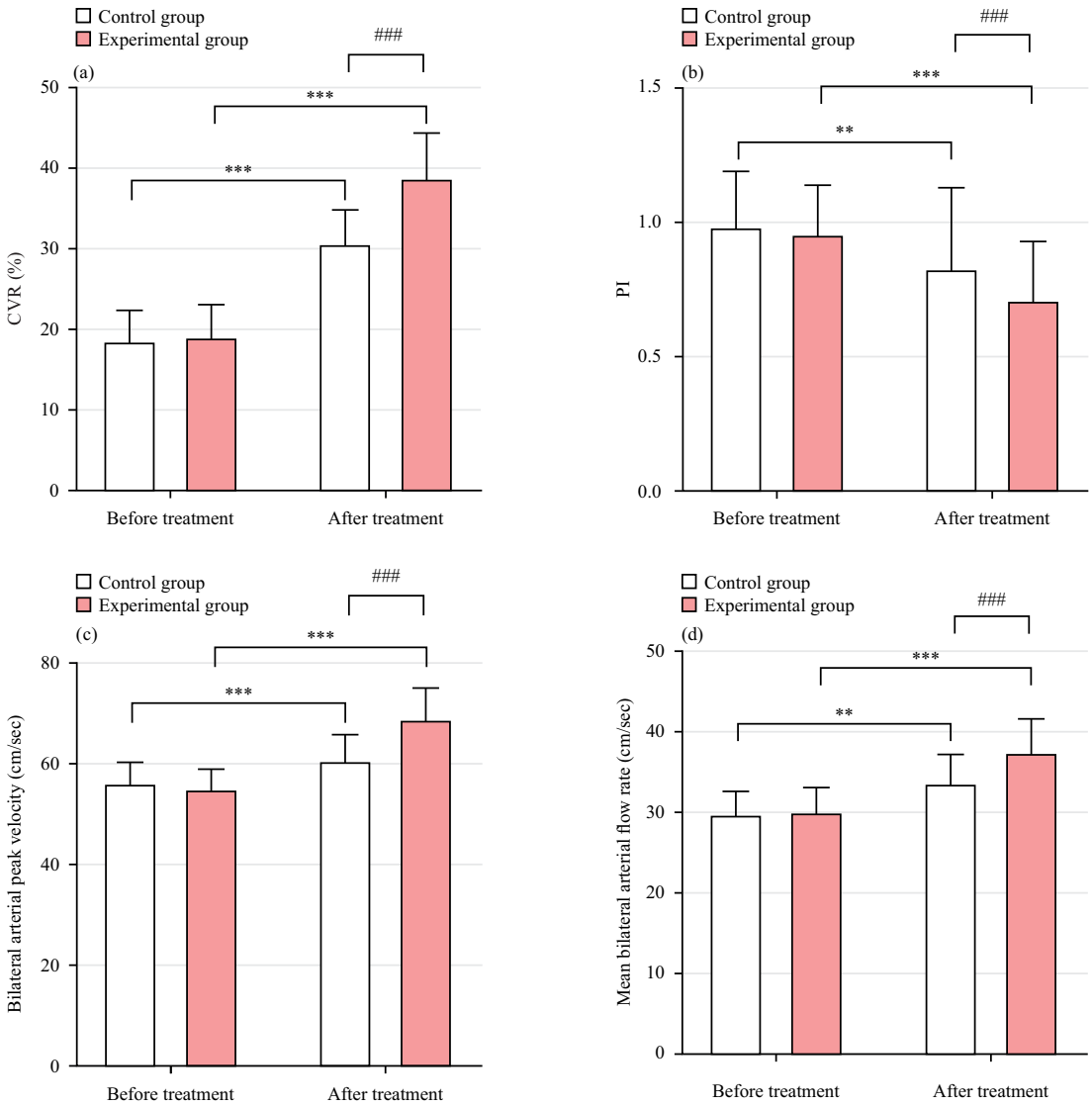

Fig. 1(a-d): Comparison of cerebral blood perfusion between the two groups, after treatment, the experimental group exhibited higher, (a) CVR, (b) PI, (c) Bilateral PSV and (d) Bilateral MBV than that of the control group

Compared with control group ###p<0.001, Compared within the same group, **p<0.01 and ***p<0.001

not statistically significant compared with the CG (p>0.05); After treatment, the EG exhibited lower ICAM-1 (Fig. 2a), lower GFAP (Fig. 2b), lower β-Actin (Fig. 2c), higher BDNF (Fig. 2d) and lower S100β (Fig. 2e) levels than the CG (p<0.05), indicating that butylphthalide could regulate the level of neuronal injury markers and reduce the degree of neuronal damage in patients with acute cerebral infarction.

Serum PTX3, HMGB1, 3-MST, Fibulin-5 levels: Before treatment, serum HMGB1, 3-MST, fibulin-5 and PTX3 levels in the EG were not statistically significant compared with the CG (p>0.05); After treatment, serum PTX3 (Fig. 3a) and HMGB1 (Fig. 3b) levels were lower and 3-MST (Fig. 3c) and fibulin-5 (Fig. 3d) levels were higher in the EG than in the CG (p<0.05), indicating that butylphthalide can reduce inflammatory cascade and repair cerebrovascular damage in patients with acute cerebral infarction.

Scale scores: The differences between the NIHSS scale, modified Rankin scale and Barthel index scores before treatment did not differ significantly between the two groups (p>0.05), the Barthel index scores after treatment were higher while the NIHSS scale and modified Rankin scale was lower in the EG than in the CG (p<0.05), indicating that butylphthalide

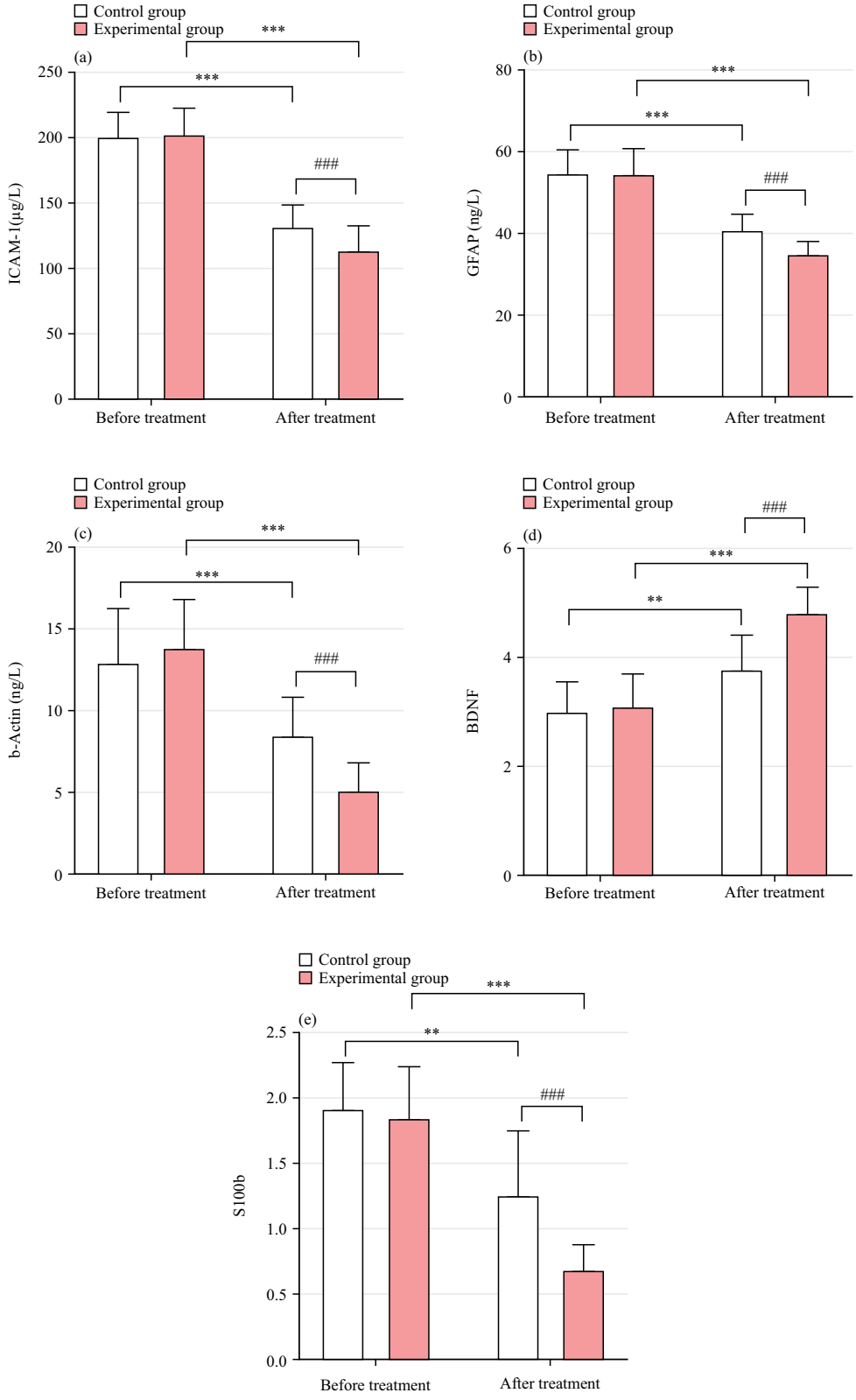

Fig. 2(a-e): Comparison of neuronal injury marker levels, experimental group showed lower, (a) ICAM-1, lower, (b) GFAP, lower, (c) β-Actin, higher, (d) BDNF and lower and (e) S100β levels than the control group after treatment

Compared with control group ###p<0.001, Compared within the same group, **p<0.01 and ***p<0.001

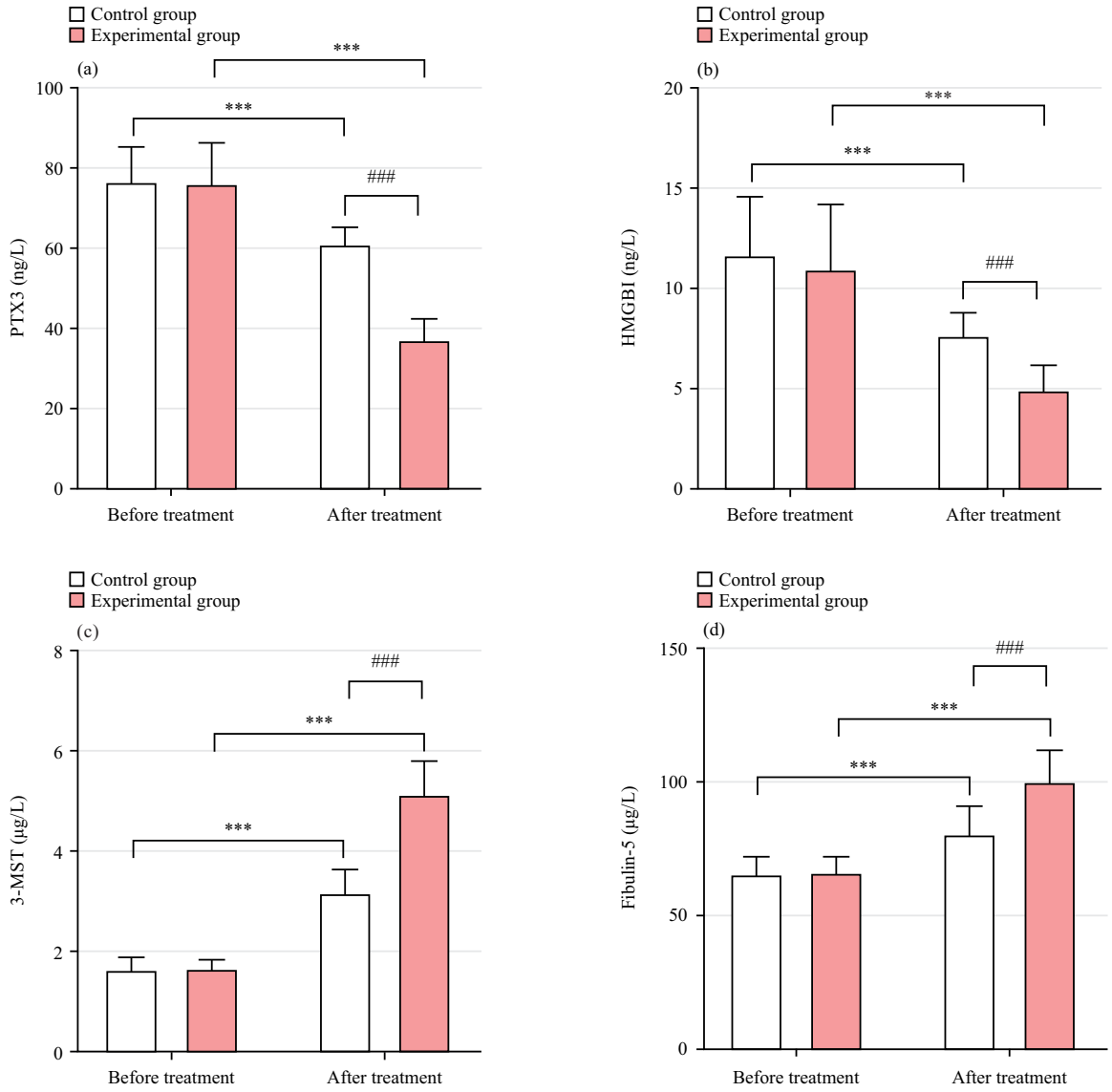

Fig. 3(a-d): Comparison of serum, (a) PTX3, (b) HMGB1, (c) 3-MST and (d) Fibulin-5 levels, serum levels were higher in the experimental group than in the control group after treatment

Compared with control group ###p<0.001, Compared within the same group, **p<0.01 and ***p<0.001

Table 2: Comparison of NIHSS scale, modified Rankin scale and Barthel index scores ($\bar{\chi}\pm S$, points)

	NIHSS scale		Modified rankin scale		Barthel index	
Group	Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment
Control group (n = 65)	8.69±2.54	6.12±1.27***	3.56±0.85	2.02±0.42***	66.02±10.46	78.85±9.95***
Experimental group (n = 66)	9.12±2.34	4.08±1.03###***	3.68±0.76	1.37±0.27###***	65.39±9.64	84.16±6.08###***

Compared with control group ###p<0.001, Compared within the same group, **p<0.01 and ***p<0.001

Table 3: Comparison of protein expression related to Netrin/DCC/VEGF signaling pathway between the two groups ($\bar{\chi}\pm S$)

	Netrin-1		DCC		VEGF	
Group	Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment
Control group (n = 65)	0.28±0.06	0.47±0.16***	0.16±0.08	0.36±0.08***	0.23±0.08	0.37±0.09***
Experimental group (n = 66)	0.25±0.08	0.62±0.13###***	0.17±0.11	0.51±0.03###***	0.21±0.05	0.59±0.15###***

Compared with control group ###p<0.001, Compared within the same group, **p<0.01 and ***p<0.001

Table 4: Comparison of patient prognosis between the two groups n (%)

Group	Complete recovery	Good recovery	Mild disability	Moderate disability	Severe disability	Extreme disability	Death	Good prognosis
Control group (n = 65)	0	16 (24.62)	31 (47.69)	14 (21.54)	4 (6.15)	0	0	47 (72.31)
Experimental group (n = 66)	4 (6.06)	22 (33.33)	33 (50.00)	6 (9.09)	1 (1.52)	0	0	59 89.39##

Compared to the control group and ##p<0.01

can reduce the degree of neurological impairment, promote the recovery of neurological function and enhance the self-care ability of daily life in patients with ACI (Table 2).

Netrin/DCC/VEGF signaling pathway-related protein expression: The Netrin-1, DCC and VEGF protein expression before treatment did not differ significantly between the 2 groups (p>0.05), these indexes in the EG after treatment were higher than those in the CG (p<0.05), indicating that butylphthalide could activate the Netrin/DCC/VEGF signaling pathway in peripheral blood lymphocytes of patients with acute cerebral infarction (Table 3).

Prognosis: The good prognosis rate of the EG (89.39%) was higher than that of the CG (72.31%) (p<0.05), indicating that butylphthalide can improve the prognosis of patients with acute cerebral infarction and promote recovery (Table 4).

DISCUSSION

The ACI is traditionally treated with antiplatelet and statin drugs which could repair neurological function, restore cerebral tissue perfusion and inhibit platelet aggregation. However, patients are susceptible to inflammatory reactions, free radical damage, intracellular calcium overload and apoptosis during the restoration of brain tissue perfusion, which increase the risk of ischemia-reperfusion and aggravation of neuronal cell injury[12,13]. Therefore, it is particularly important to find ways to inhibit brain cell apoptosis, reduce neuronal damage and decrease inflammatory cascade.

The results of this study found that butylphthalide combined with atorvastatin calcium could improve cerebral perfusion, reduce inflammatory cascade, repair neuronal and cerebrovascular damage and enhance the ability to care for oneself in daily life. The reasons may be related to the following mechanisms of action of butylphthalide: (1) Butylphthalide can reduce intracellular calcium concentration, increase cerebrovascular endothelial PGI2 and NO levels and then inhibit glutamate release, superoxide anion formation and glutamate release, reduce arachidonic acid levels, enhance oxidase activity, reduce microvascular spasm, inhibit platelet aggregation and antithrombotic formation[14,15]. (2) Butylphthalide enhances biological behaviors such as vascular endothelial cell proliferation, activation and migration, accelerates the establishment of collateral circulation, reconstructs and improves microcirculation in ischemic areas, increases blood flow to cerebral tissues at ischemic sites, promotes angiogenesis and alleviates regional dysfunction induced by perfusion[16,17]. (3) Butylphthalide can enhance the activity of mitochondrial ATPase and mitochondrial complex enzyme IV, maintain the stability of mitochondrial membrane, inhibit cytochrome C release, enhance tissue energy metabolism and attenuate caspase-3-mediated apoptosis. Li *et al.*[18] reported that butylphthalide promoted the functional recovery of damaged neuronal cells in acute cerebral infarction rats by inhibiting caspase-3 protein expression in brain tissue, It also inhibited neuronal apoptosis by blocking the JNK/p38MAPK signaling pathway. (4) Butylphthalide can activate the cMet/PI3K/AKT signaling pathway and enhance mTOR activity through upregulation of hepatocyte growth factor levels, which protects against brain injury induced by ischemia-reperfusion and attenuates neuronal apoptosis[19]. (5) Butylphthalide reduces inflammatory oxidative stress, inhibits oxygen free radicals and increases the activity of antioxidant enzymes, attenuates the ischemic cascade, improves the damaged state of vascular endothelial cells and thus reduces the degree of damage to the ischemic penumbra. Xiao-Xuan *et al.*[20] found that butylphthalide reduced serum TNF-α and IL-6 levels and inhibited NF-κB p65 protein expression in brain tissue of ischemic stroke model rats.

Neuronal apoptosis can exacerbate the inflammatory response and neurological deficits, so inhibition of neuronal apoptosis is particularly critical in the protection of damaged brain tissue. Netrin1 protein mainly binds to the DCC receptor family to play a biological role in transmitting chemoattraction, repulsion and thus mediating neuronal cell migration and establishment of neurotransmission pathway[21]. Tang *et al.*[22] found that after the establishment of an ischemic stroke model, the autophagy of rat brain tissue and neurons was enhanced and Netrin1 protein could reduce ischemic brain tissue damage, inhibit brain tissue and neuronal autophagy and enhance hypoxic neuronal viability through the intracellular phosphatidyl alcohol kinase pathway. Wang *et al.*[23] established a rat middle cerebral

artery ischemia-reperfusion model and 1 day after cerebral ischemia-reperfusion, the expression of Netrin1 and DCC increased in rats, which was positively correlated with the time of axon regeneration and it was speculated that Netrin1 might promote axon growth after ischemia-reperfusion through DCC receptors. Kang *et al.*[24] found that Netrin-1/DCC could induce rearrangement of the actin cytoskeleton through Src kinase activation of PLCγ1, mediating attractive axonal growth and affecting structural brain development. Hu *et al.*[25] established a model of transient middle cerebral artery occlusion in rats and after successful model establishment, Netrin and its receptors DCC and VEGF were downregulated in rats and fluoxetine could promote neovascularization, restore the long-term function of ischemic stroke and improve the prognosis by upregulating the expression of HIF-1α-Netrin/VEGF cascade protein. However, no studies explored the role of the Netrin/DCC/VEGF signaling pathway in patients with acute cerebral infarction Therefore, in this study, western blot was used to determine the protein expression related to patients before and after treatment and found that the protein expression of Netrin-1, DCC and VEGF increased in both groups after treatment and the protein expression of the EG was higher than that of the CG and the good prognosis rate of the EG was higher than that of the CG, indicating that butylphthalide can activate the Netrin/DCC/VEGF signaling pathway in peripheral blood lymphocytes of patients with acute cerebral infarction, reducing neuronal damage and inhibiting neuronal cell apoptosis, thus improving the prognosis of patients.

CONCLUSION

Butylphthalide can regulate cerebral perfusion, reduce neuronal damage, reduce inflammatory cascade, repair cerebrovascular damage, improve the ability of daily living as well as prognosis in patients with acute cerebral infarction and the mechanism of action may be related to the activation of Netrin/DCC/VEGF signaling pathway by butylphthalide. However, this study still has limitations such as low sample size, single sample source, short follow-up time and the lack of *in vitro* model experiments, so further research is needed on how butylphthalide activates the Netrin/DCC/VEGF signaling pathway and the protective mechanism of neurological function.

SIGNIFICANCE STATEMENT

This study emphasizes the critical need to address complications arising during cerebral infarction treatment. By combining butylphthalide with atorvastatin calcium, our research highlights a novel approach that enhances cerebral perfusion, mitigates inflammatory responses and fosters neuronal repair. The observed activation of the Netrin/DCC/VEGF signaling pathway elucidates a promising mechanism for reducing neuronal damage and inhibiting apoptosis, thereby improving patient prognosis. These findings emphasize the potential of butylphthalide in modulating neurological function following acute cerebral infarction, although further investigations are warranted to delve deeper into its mechanistic nuances.

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