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Original Research

A Preliminary Finding: N-butyl-phthalide Plays a Neuroprotective Role by Blocking the TLR4/HMGB1 Pathway and Improves Mild Cognitive Impairment Induced by Acute Cerebral Infarction

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Abstract

Background: Most acute cerebral infarctions (ACI) may develop vascular dementia (VD), which involves almost all types of cognitive impairment. Unfortunately, there is currently no effective treatment for VD. Most patients exhibit mild cognitive impairment (MCI) before the development of VD. N-butyl-phthalide (NBP) is used to treat ACI and improve cognitive function. The oxygen and glucose deprivation (OGD) model of neurons is an *in vitro* model of ischemia, hypoxia, and cognitive dysfunction. **Methods**: We conducted clinical studies and *in vitro* experiments to investigate the clinical efficacy and mechanism of action of NBP for treating ACI-induced MCI. Patients with ACI-induced MCI were randomly divided into control (Ctrl) and NBP groups. We assessed various indicators, such as clinical efficacy, montreal cognitive assessment scale (MOCA), activities of daily living (ADL), and cerebral infarct size in both groups before and after treatment. We observed the morphology of neurons and detected the survival rate, action potentials (APs), expression of high mobility group box 1 (HMGB1), toll-like receptor 4 (TLR4), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α), and the interaction between TLR4 and HMGB1. **Results**: The MOCA and ADL scores increased significantly after treatment in the NBP group. A OGD model of neurons was established, and the neurons were divided into Ctrl and NBP groups. We observed that the survival rate and APs amplitude of the neurons were significantly increased in the NBP group, whereas TNF-α expression was decreased. Furthermore, the interaction between TLR4 and HMGB1 decreased in the NBP group. **Conclusion**: NBP plays a neuroprotective role by inhibiting the TLR4/HMGB1 pathway and ameliorating ACI-induced MCI.

Keywords: acute cerebral infarction; mild cognitive impairment; glucose and oxygen deprivation; HMGB1; toll-like receptor 4; action potentials

1. Introduction

The morbidity rate of cognitive impairment in patients with acute cerebral infarction (ACI) is 41% [1]. Most patients may develop vascular dementia (VD), which involves almost all types of cognitive impairment [2]. Unfortunately, no effective treatment for VD is found. Before the development of VD, most patients exhibit mild cognitive impairment (MCI) [3]. The clinical symptoms of MCI are mainly a progressive decline in memory or other cognitive functions, such as computation, orientation, attention, and executive function; however, they do not affect daily life functioning. Cognitive impairment associated with MCI is inconsistent with age and educational level, and the disease does not meet the diagnostic criteria for dementia [4,5]. Lamb *et al.* [6] confirms that early treatment of patients with MCI can preserve cognitive ability and reduce the rate of progression to dementia.

The pathogenesis of ACI-induced MCI remains unclear. ACI can cause cognitive impairment because it leads to cerebral ischemia and hypoxia. Neurons are in a state of hypoxia and hypoglycemic metabolism, which triggers a cellular inflammatory response [7]. The toll-like receptor 4/high mobility group box 1 (TLR4/HMGB1) pathway, containing TLR4 and HMGB1 proteins, is a crucial mechanism and pathway of cellular inflammation and is the primary molecular mechanism of cognitive impairment [7]. During ischemia and hypoxia state, the TLR4/HMGB1 pathway can be activated [4], which promotes the release of inflammatory transmitters, including interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) [8]. Moreover, inflammatory transmitters promote amyloid deposition which is considered as a key factor in cognitive dysfunction [9].

N-butyl-phthalide (NBP) is a safe, fat-soluble drug used to treat ACI and improve cognitive function [10]. Nevertheless, it is not clear whether NBP inhibits inflammation by acting on the TLR4/HMGB1 pathway. To identify an effective therapy for MCI and to study the pathogenesis of MCI, we treated ACI patients with MCI and explored the relationship between TLR4/HMGB1 and MCI. NBP has been used to treat patients with ACI and MCI, demonstrating that it can improve cognitive function in these patients. Furthermore, we developed an oxygen and sugar depriva-

tion (OGD) model using primary cultured neurons to investigate the effects of NBP on TLR4/HMGB1, which has been proven to be a model of ischemia hypoxia [11] and cognitive dysfunction [12]. Additionally, we explored the mechanism of HMGB1-TLT4 and the protective effect of NBP on neurons in the OGD model.

2. Methods and Materials

2.1 Population

This was a case-control study. From March 2019 to March 2020, 86 patients who were hospitalized in the Neurology Department of Jiangbin Hospital and initially diagnosed with ACI-induced MCI were enrolled in our study. The diagnosis of ACI conforms to the diagnostic criteria of the Guidelines for The management of Ischemic Stroke and Transient Ischemic Attack 2008 [13]. All patients had evidence of ACI on nuclear magnetic resonance (MRI) or computed tomography (CT). Based on the results of MRI/CT, the territories of cerebral infarct were divided into three, the territory of the anterior cerebral artery (ACA), the territory of the middle cerebral artery (MCA) and the territory of posterior circulation (PC). In this study, all patients had a single territory of cerebral infarct. The diagnosis of MCI follows Petersen *et al.*'s criteria [14]. The exclusion criteria were as follows: patients with multiple territories of cerebral infarct; patients with diabetes mellitus, malignant tumor, metabolic disorders, recurrent stroke, or massive ischemic stroke with severe impairment of consciousness. This study adhered to the principles of the Declaration of Helsinki. Ethical approval was granted by the institutional review board of Jiangbin Hospital (No. LW-2022-09). All patients were randomly divided into the control (Ctrl) and NBP groups. Informed consent was obtained from patients or their guardians.

2.2 Treatment

Patients in both groups received conventional symptomatic treatment, including blood pressure and blood glucose control, hypolipidemic, anticoagulants, and microcirculation improvement drugs, supplemented with cognitive function rehabilitation training. Patients in the Ctrl group received a placebo. Patients in the NBP group received NBP soft capsule (H20050299, CSPC-NBP Pharmaceutical Co., Ltd., Shijiazhuang, Hebei, China) (0.2 g orally thrice daily). Efficacy was evaluated after eight weeks of therapy.

2.3 Evaluation of the Therapeutic Effect

The brains of the two groups were scanned by MRI (GE Signa HDXT 1.5T MR, General Electric Company, Boston, MA, USA)/CT (SIEMENS SOMATOM Definition FLash CT, SIEMENS, Amberg, Germany) before and after treatment, and the changes in lesions were compared. The montreal cognitive assessment scale (MOCA) was used to assess cognitive function in both the groups before and after treatment. Cognitive function was evaluated based on memory, attention, orientation, language impairment, naming ability, visual-spatial executive ability, abstract thinking, and delayed memory. A total score of 30, 26, or above was considered normal; 18–26, MCI; 10–17, moderate impairment; and $<$10, severe impairment. Activities of daily living (ADL) were assessed in both groups before and after treatment. The total scores were 100, 100 represent normal, $>$60 was mild disorder, 41–60 was moderate disorder, 20–40 was severe disorder, and $<$20 was disability.

Table 1. Baseline characteristics.

	Ctrl	NBP
Male, n (%)	20 (51.28)	26 (48.72)
Female, n (%)	19 (60.47)	17 (39.53)
Age, years	66.00 $\pm$ 9.10	62.44 $\pm$ 8.88
Disease course, days	5.97 $\pm$ 2.33	60.9 $\pm$ 2.16
Hypertension, n (%)	31 (79.49)	33 (76.74)
Smoking, n (%)	13 (33.33)	16 (37.21)
ACA, n (%)	12 (30.77)	10 (23.26)
MCA, n (%)	19 (48.72)	28 (65.12)
PC, n (%)	8 (20.51)	5 (11.63)

Abbreviation: Ctrl, control; NBP, N-butyl-phthalide; MCA, middle cerebral artery; ACA, anterior cerebral artery; PC, posterior circulation.

2.4 Primary Neuron Culture and OGD Model

Neurons (CP-R107, Procell Life Science & Technology Co., Ltd., Wuhan, Hubei, China) were cultured according to the protocol described by Wang *et al.* [15]. Leica microscopes (DMi1, Leica, Shanghai, China) were used to observe cell morphology. OGD model was performed following the method of Tasca *et al.* [16]. After 24 h of exposure to OGD, neurons were replaced with normal media. The cells were divided into OGD and OGD + NBP groups. The drugs and concentrations added to each group were as follows: no drugs were added to the OGD group, and NBP was added to the OGD + NBP group at concentrations of 0.1, 1, 10, 50, and 100 µM. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) kit (AR1156, Boster, Wuhan, Hubei, China) was used to calculate the cell survival rate after 24 h. The optimal concentration was selected for subsequent experiments.

2.5 Neuronal Electrophysiology

The patch-clamp system was used to obtain the action potentials (APs). The primary method described by Li *et al.* [17]. The amplitude and frequency of neuronal APs were collected and analyzed.

2.6 Immunoblotting (IB)

Protein extraction, protein concentration detection, sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), membrane transfer, antibody incubation, and visualization of immunoreactive bands were performed as

Table 2. Population of cognitive impairment.

	Normal	Mild cognitive impairment (18–26)	Moderate impairment (10–17)	Severe impairment (<10)	Total-population
Ctrl-before treatment	0	0	13	30	43
Ctrl-after treatment	0	0	15	28	43
NBP-before treatment	0	1	14	24	39
NBP-after treatment	0	2	20	17	39

described by Li *et al*. [18]. The primary antibodies used were anti-TLR4 antibody 1:1000 (ab8376, Abcam, Shanghai, China), anti-HMGB1 antibody 1:1000 (ab190377, Abcam, Shanghai, China), anti-interleukin (IL-6) antibody 1:1000 (ab259341, Abcam, Shanghai, China), anti-tumor necrosis factor-alpha (TNF-α) antibody 1:1000 (ab205587, Abcam, Shanghai, China), and anti-GAPDH antibody 1:10000 (D110016, Sangon Biotech, Shanghai, China). Subsequently, the nitrocellulose (NC) membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody 1:8000 (Proteintech, SA00001-2, Wuhan, Hubei, China) for 1 h at room temperature. Protein levels were determined by normalizing to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) loading controls. Each experiment was conducted in triplicates. The original figures of Western Blot can be found in the **Supplementary Materials-IB**.

2.7 Co-Immunoprecipitation (Co-IP)

Protein extraction, protein concentration detection, construction of antigen-antibody complexes, and immunoblotting (IB) were performed as described by Li *et al*. [18]. The antigen-antibody complex was constructed using an anti-TLR4 receptor antibody 1:30 (Abcam, ab252430). The primary antibodies for IB were anti-TLR4 antibody 1:1000 (Abcam, ab8376) and anti-HMGB1 antibody 1:1000 (Abcam, ab18256). The Immunoprecipitation (IP) protein band for TLR4 was used to normalize the pull-down protein levels obtained from Co-IP. The co-precipitated protein levels between each group were then compared. The original figures of Western Blot can be found in the **Supplementary Materials-IP**.

2.8 Immunofluorescence (IF)

The fixation, penetration, and blocking of the neurons were based on the methods described by Li *et al*. [17,18]. A multiplex fluorescent immunohistochemistry kit (abs50012, Absin, Shanghai, China) was used to stain neurons [18]. The primary antibodies for Immunofluorescence (IF) were anti-TLR4 antibody 1:100 (Abcam, ab8376) and anti-HMGB1 antibody 1:100 (Abcam, ab18256). A Leica 8.0 laser confocal microscope (TCS SP8, Leica, Wetzlar, Germany) was used to acquire fluorescence signals of TLR4 (green) and HMGB1 (red). The ImageJ plug-in (version 1.54b; University of Wisconsin, Madison, WI, USA) was used to calculate the Pearson's correlation coefficients (PCCs) between TLR4 and HMGB1.

2.9 Statistical Analysis

Statistical analysis was conducted using Statistical Package for Social Sciences (SPSS, version 25.0, IBM, Chicago, IL, USA) software. The measurement data are expressed as mean $\pm$ standard deviation. The independent sample *t*-test, paired samples *t*-test, and One-Way analysis of variance (ANOVA) were used to assess the significance of the differences between the groups. The chi-square test assessed the significance of the difference in percentage. Statistical significance was set at $p < 0.05$.

3. Results

3.1 Baseline Characteristics

The baseline characteristics were exhibited in Table 1. The Ctrl group included 20 male and 19 female participants aged between 43 and 78 (66.00 $\pm$ 9.10) years. The disease course ranged from 1 to 10 days (5.97 $\pm$ 2.33 days). The number of the patients with history of hypertension was 31 (79.49%). The number of the patients with history of smoking was 13 (33.33%). The number of the patients with the territory of ACA was 12 (30.77%). The number of the patients with the territory of MCA was 19 (48.72%). The number of the patients with the territory of posterior circulation (PC) was 8 (20.51%).

The NBP group comprised 26 male and 17 female participants aged between 44 and 76 (62.44 $\pm$ 8.88) years. The disease course ranged from 1 to 10 days (6.09 $\pm$ 2.16 days). The number of the patients with history of hypertension was 33 (76.74%). The number of the patients with history of smoking was 16 (37.21%). The number of the patients with the territory of the ACA was 10 (23.26%). The number of the patients with the territory of the MCA was 28 (65.12%). The number of the patients with the territory of posterior circulation 5 (11.63%). There were no differences in the baseline characteristics of the Ctrl and NBP groups, such as gender, age, disease course, hypertension, smoking and the territories of cerebral infarct, between the two groups ($p > 0.05$).

3.2 Effect of NBP on Improving Cognitive Function in ACI Patients with MCI

Before treatment, no significant differences in MOCA were observed between the control and NBP groups. Table 1 presents the pretreatment composition of the Ctrl

Table 3. The score of MOCA.

	Ctrl			NBP		
	Before treatment	After treatment	*p* value (before versus after)	Before treatment	After treatment	*p* value (before versus after)
Memory	0.81 ± 0.66	0.86 ± 0.64	0.16	0.64 ± 0.71	1.23 ± 0.74	0.00
Attention	0.81 ± 0.66	0.84 ± 0.65	0.32	0.69 ± 0.69	1.85 ± 0.71	0.00
Orientation	0.98 ± 0.89	0.98 ± 0.89	-	0.90 ± 0.91	1.18 ± 0.88	0.00
Language	0.53 ± 0.85	0.58 ± 0.91	0.16	0.59 ± 0.85	0.72 ± 0.86	0.06
Naming ability	0.53 ± 0.85	0.56 ± 0.85	0.32	0.59 ± 0.85	0.64 ± 0.84	0.16
Visual-spatial	2.05 ± 0.79	2.09 ± 0.78	0.16	2.28 ± 1.52	2.46 ± 1.52	0.01
Abstract thinking	0.74 ± 0.66	0.79 ± 0.64	0.16	0.46 ± 0.64	0.54 ± 0.64	0.08
Delayed memory	2.05 ± 0.79	2.12 ± 0.79	0.08	2.28 ± 1.52	2.44 ± 1.64	0.03
Total points	8.51 ± 2.30	8.81 ± 2.44	0.00	8.44 ± 3.83	10.79 ± 3.79	0.00

Abbreviation: MOCA, montreal cognitive assessment scale.

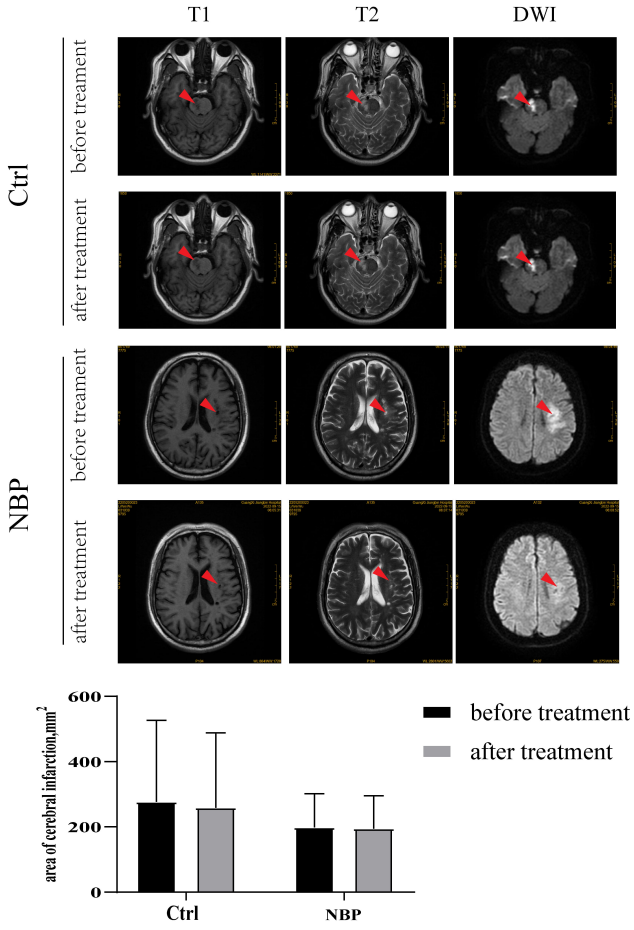

Fig. 1. Changes in cerebral infarct size: the infarct size of the Ctrl group was 276.81 ± 249.90 mm^2, while the infarct size of the NBP group was 198.25 ± 103.32 mm^2. After treatment, infarct size did not change significantly in the Ctrl and NBP groups. Red arrows: infarct size. DWI, diffusion weighted imaging.

group, including 0 normal patients, 0 patients with MCI, 13 with moderate cognitive impairment, and 30 with severe cognitive impairment. After treatment, there were 0 normal patients, 1 patient with MCI, 15 with moderate cognitive impairment, and 28 with severe cognitive impairment in the Ctrl group (Table 2). The pretreatment NBP group comprised 0 normal patients, 1 patient with MCI, 12 moderate cognitive impairment patients, and 24 severe cognitive impairment patients. After treatment, there were 0 normal patients, 2 patients with MCI, 20 with moderate cognitive impairment, and 17 with severe cognitive impairment in the NBP group. The Ctrl group achieved a MOCA score of 8.81 ± 2.44 after treatment compared to 8.51 ± 2.30 before treatment (p = 0.000, Table 3). Similarly, the NBP group achieved a MOCA score of 10.79 ± 3.79 after treatment compared to 8.44 ± 3.83 before treatment (p = 0.000, Table 3). The Ctrl group revealed no significant improvement in memory, attention, orientation, language impairment, naming ability, visuospatial executive ability, abstract thinking, or delayed memory (Table 3). Conversely, the patients in the NBP group demonstrated significant improvements in memory, attention, orientation, and delayed memory (Table 3). Before treatment, there were no significant differences in ADL between the Ctrl and NBP groups (Ctrl versus NBP, $p > 0.05$). Before treatment, there were 0 normal patients, 12 patients with mild disorder, 17 with moderate disorder, 4 with severe disorder, and 10 with disability in the Ctrl group. After treatment, there were 0 normal patients, 12 patients with mild disorder, 17 with moderate disorder, 6 with severe disorder, and 8 with disability (Table 4). Before treatment, there were 3 normal patients, 4 with mild disorder, 19 with moderate disorder, 2 with severe disorder, and 11 with disability in the NBP group. After treatment, there were 3 normal patients, 4 with mild disorder, 20 with moderate disorder, 2 with severe disorder, and 10 with disability in the NBP group (Table 4). The Ctrl group achieved an ADL score of 49.19 ± 27.45 after treatment compared to 47.44 ± 28.12 before treatment (p = 0.004, Table 5). The NBP group improved the ADL score from 42.18 ± 31.03 pretreatment to 45.13 ± 28.20 post-treatment (p = 0.000, Table 5). Patients in the Ctrl group revealed no significant improvement in stool, urination, grooming, toilet use, eating, transfer, movement,

Table 4. Population of disorder.

	Normal (100)	Mild disorder (60–100)	Moderate disorder (42–60)	Severe disorder (20–40)	Disability (<20)	Total-population
Ctrl-before treatment	0	12	17	4	10	43
Ctrl-after treatment	0	12	17	6	8	43
NBP-before treatment	3	4	19	2	11	39
NBP-after treatment	3	4	20	2	10	39

Table 5. The score of ADL.

	Ctrl			NBP		
	Before treatment	After treatment	*p* value (before versus after)	Before treatment	After treatment	*p* value (before versus after)
Excrement	5.93 ± 3.32	6.05 ± 3.19	0.32	5.26 ± 4.13	5.51 ± 3.94	0.16
Urinate	5.35 ± 3.68	5.35 ± 3.68	-	4.74 ± 3.80	4.74 ± 3.80	-
Grooming/personal hygiene	4.88 ± 3.86	5.12 ± 3.70	0.16	3.85 ± 3.53	4.49 ± 3.40	0.02
Toileting	5.35 ± 3.68	5.47 ± 3.59	0.32	4.74 ± 3.80	5.00 ± 3.63	0.16
Eating	4.88 ± 3.86	5.12 ± 3.86	0.16	3.46 ± 3.28	3.97 ± 3.07	0.04
Transfer	4.65 ± 3.68	4.88 ± 3.53	0.16	4.49 ± 3.59	4.74 ± 3.43	0.16
Activity	4.30 ± 3.71	4.53 ± 3.59	0.16	3.97 ± 3.48	4.36 ± 3.28	0.08
Dressing	4.19 ± 3.61	4.53 ± 3.42	0.08	3.59 ± 3.23	3.97 ± 3.07	0.08
Up and down stairs	4.65 ± 3.68	4.77 ± 3.61	0.32	4.49 ± 3.59	4.74 ± 3.43	0.16
Bath	3.26 ± 3.25	3.37 ± 3.22	0.57	3.59 ± 3.23	3.59 ± 3.23	-
ADL-total points	47.44 ± 28.12	49.19 ± 27.45	0.00	42.18 ± 31.03	45.13 ± 28.20	0.00

Abbreviation: ADL, activities of daily living.

dressing, ascending and descending stairs, and bathing after treatment (Table 5). However, patients in the NBP group demonstrated improvements in grooming and eating abilities after treatment (Table 5).

3.3 Changes in Cerebral Infarct Size

Before treatment, CT and MRI scans revealed distinct cerebral ischemic lesions in both groups without statistically significant differences in the ischemic area. The Ctrl group exhibited an infarct size of 276.81 ± 249.90 mm^2, whereas the NBP group exhibited an infarct size of 198.25 ± 103.32 mm^2 (versus Ctrl, $p = 0.064$; Fig. 1). After treatment, no significant alterations in infarct size were observed in the Ctrl group (versus before treatment, $p = 0.084$; Fig. 1) or the NBP group (versus before treatment, $p = 0.073$; Fig. 1).

3.4 The Protective Effect of NBP on Neurons

In the OGD group, cellular edema was observed in the neurons, and their number was also reduced. The OGD + 1 μM NBP, OGD + 10 μM NBP, and OGD + 50 μM NBP groups exhibited decreased edema and increased number of neurons (Fig. 2A). To further clarify the protective effects of NBP on neurons, we performed an MTT assay. The changes in neuronal survival were most significant in the OGD + 1 μM NBP, OGD + 10 μM NBP, and OGD + 50 μM NBP groups (versus OGD, $p > 0.05$; Fig. 2B). The survival rate of neurons was highest in the OGD + 10 μM NBP group. Therefore, we performed electrophysiological tests on this group of neurons using whole-cell patch-clamp recordings. The data revealed that the amplitudes of the APs were higher in the neurons of the OGD + 10 μM NBP group versus those of the OGD group ($p = 0.001$; Fig. 2C). However, there were no differences in the frequency of neuronal APs between OGD group and OGD + 10 μM NBP group ($p > 0.05$; Fig. 2C).

3.5 NBP Alleviates Neuronal Inflammation by Inhibiting the TLR4/HMGB1 Pathway

We extracted total protein from neurons in the OGD and OGD + 10 μM NBP groups for the IB assay. The results demonstrated that TLR4, HMGB1, and IL-6 expression in the OGD + 10 μM NBP group did not change significantly. However, TNF-α expression was significantly decreased (versus OGD, $p = 0.00$; Fig. 3A). We identified the relationship between TLR4 and HMGB1 using the Co-IP assay. These findings revealed a significant decrease in the interaction between TLR4 and HMGB1 (versus OGD, $p = 0.000$; Fig. 3A). We observed co-expression of TLR4 and HMGB1 in neurons using an IF assay. To further verify the relationship between TLR4 and HMGB1, PCC was used to evaluate the interactions between these proteins. In the OGD + 10 μM NBP group, the PCC between TLR4 and HMGB1 was decreased (versus OGD, $p = 0.000$; Fig. 3B).

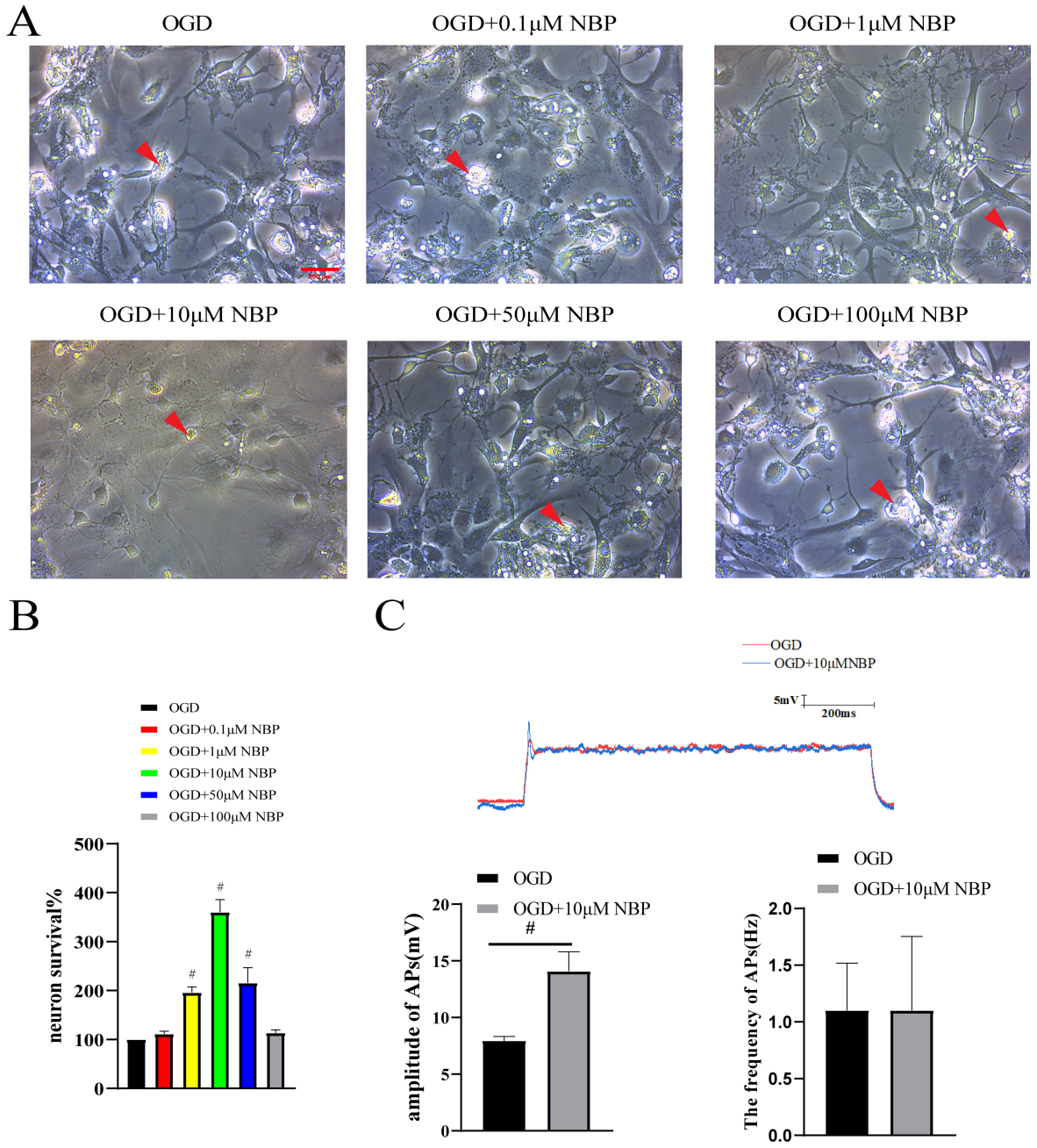

Fig. 2. Morphology, survival and action potentials of neurons. (A) Neuronal morphology: in the OGD group, cellular edema was observed in the neurons, and the number was also reduced. The OGD + 1 μM NBP, OGD + 10 μM NBP, and OGD + 50 μM NBP groups exhibited decreased edema and increased number of neurons. Red arrows: swollen and ruptured neurons. Scale Bar: 500 μm. (B) MTT assay: the changes in neuronal survival were most significant in the OGD + 1 μM NBP, OGD + 10 μM NBP, and OGD + 50 μM NBP groups (versus OGD, #, $p < 0.01$). The survival rate of neurons was highest in the OGD + 10 μM NBP group. (C) The amplitudes of the APs were higher in the neurons of the OGD + 10 μM NBP group (versus OGD, #, $p < 0.01$). There were no differences in the frequency of neuronal APs between OGD group and OGD + 10 μM NBP group. OGD, oxygen and glucose deprivation; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; APs, action potentials.

4. Discussion

MCI is a pathological condition that lies between normal brain aging and dementia. MCI occurs in 60% of patients with stroke, and 30% of patients ultimately develop VD. The incidence of MCI is higher in patients with ACI than in the general elderly [19]. Nevertheless, treatments for ACI-induced MCI are limited. To investigate the therapeutic effect of NBP on ACI-induced MCI, the clinical data of patients in the control group and the NBP group was analyzed. No significant difference in age, gender, disease

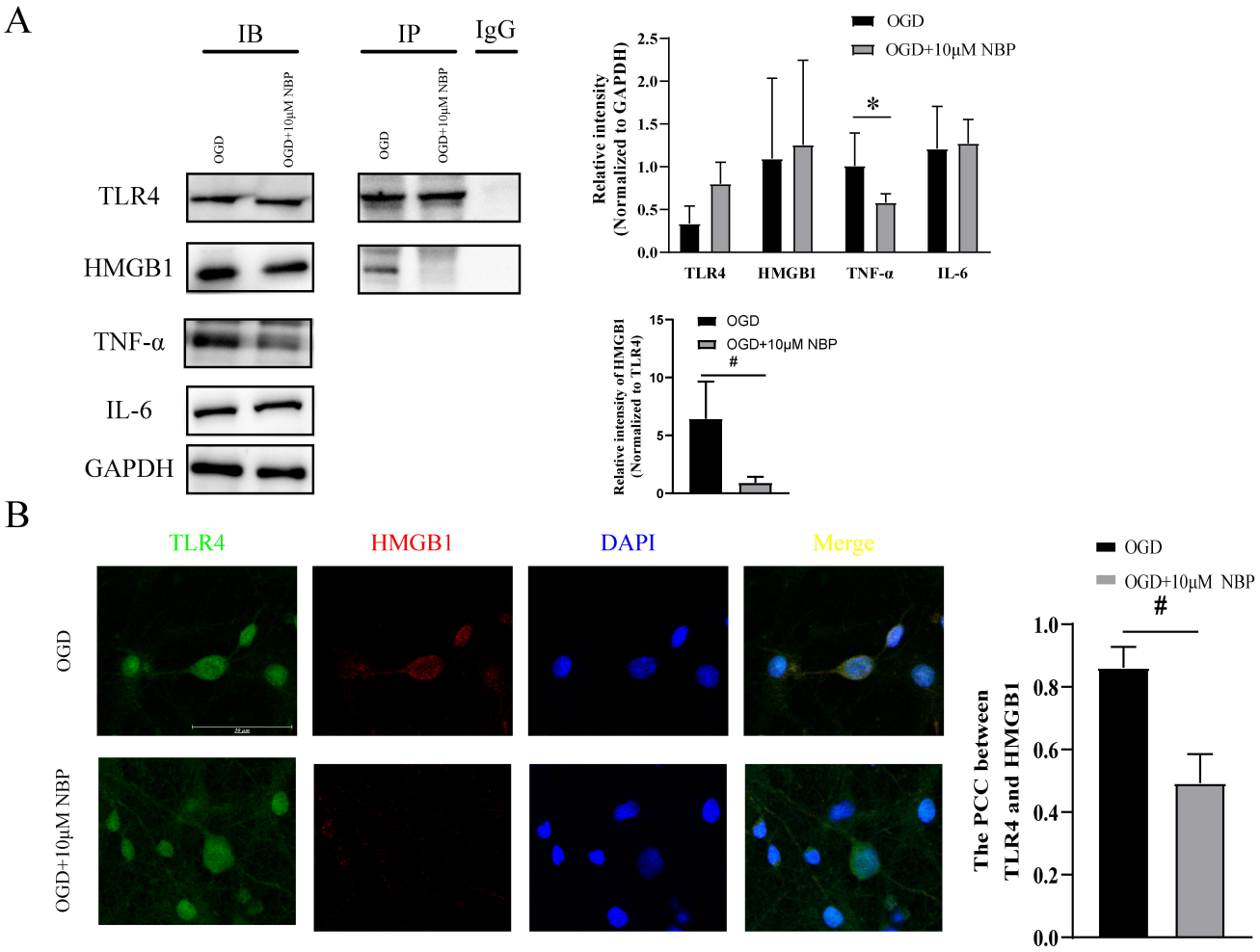

Fig. 3. The TLR4/HMGB1 pathway was inhibited by NBP. Neuronal inflammation and the TLR4/HMGB1 pathway between OGD group and OGD+10 μM NBP group (A) The IB results indicated that TLR4, HMGB1, and IL-6 expressions did not change significantly in the OGD + 10 μM NBP group, but TNF-α expression was significantly decreased (versus OGD, *, $p < 0.05$). The results of the Co-IP assay revealed that the interaction between TLR4 and HMGB1 decreased (versus OGD, #, $p < 0.01$). (B) The PCC between TLR4 and HMGB1 decreased (versus OGD, #, $p < 0.01$). Scale Bar: 50 μm. TLR4, toll-like receptor 4; HMGB1, expression of high mobility group box 1; IL-6, interleukin-6; TNF-α, tumor necrosis factor-alpha; PCC, Pearson's correlation coefficient; Co-IP, Co-Immunoprecipitation; IB, Immunoblotting; IgG, immunoglobulin G; GADPH, Glyceric acid phosphate dehydrogenase; DAPI, 4′,6-diamidino-2-phenylindole.

course, the history of hypertension and smoking was found between the two groups, which could rule out the effect of these factors on the results. Arboix *et al.* [20] demonstrate that cerebral infarcts in the territory of the ACA have a better prognosis than infarcts in the territory of the MCA. However, no difference in the territory of ACA, MCA and PC was observed between Ctrl group and NBP group, indicating that the location of vascular cerebral topography could not influence the results.

Preventive interventions may have modest effects at the individual level. Interventions included lifestyle modifications, control of vascular risk factors (hyperlipidemia, hypertension, and diabetes), treatment of concomitant vascular disease, and proper aerobic exercise [21]. This study improved MOCA and ADL scores in the Ctrl group by controlling blood pressure, glucose, and lipids, as well as by using appropriate rehabilitation exercises. However, conventional treatment did not improve the specific functions of MOCA and ADL. After treatment, the patients in the NBP group demonstrated significant improvements in memory, attention, orientation, and delayed memory. Moreover, patients in the NBP group indicated improved grooming and eating abilities after treatment with NBP. These results suggest that NBP can enhance cognitive function and daily living activities in patients with MCI. Some researchers have suggested that the key to VD treatment is to improve brain blood supply and brain metabolism [6] and to inhibit inflammation during MCI [22]. NBP is considered an effective drug for treating ACI [23]. In addition, we also examined cerebral infarct size in patients using MRI and CT scans. However, there was no significant change in the infarct size of patients in either the Ctrl or NBP group after

treatment. Imaging evidence suggests that NBP does not reduce infarct size.

To confirm the pharmacological mechanism of NBP, an *in vitro* model of OGD, proven to be a model of ischemia, hypoxia [11], and cognitive dysfunction [12]. Abnormal morphology of neurons was observed in the OGD group, which was mainly characterized by cell edema and fragmentation. After NBP treatment, the morphology of OGD neurons significantly improved, and edema and fragmentation were reduced. An MTT assay was performed to confirm that NBP could effectively protect neurons after OGD. The MTT assay revealed a significant increase in neuronal survival. Furthermore, neuronal survival was most significant at 10 μM NBP. 10 μM of NBP may be considered the optimal concentration for neuronal therapy. The concentration of NBP is insufficient to play a neuroprotective role in the $<$10 μM NBP group. Changes in osmotic pressure may be responsible for neuronal death in the $>$10 μM NBP group. Consequently, 10 μM NBP was selected as the next step. Electrophysiology is the most commonly used measure of neuronal activity [24]. The AP is a significant indicator of neuronal activity [25]. Therefore, we measured the APs of the neurons and revealed that the amplitude of neurons was increased in the OGD + 10 μM NBP group, which suggested that neuronal activity was improved after treatment with NBP.

The pathogenesis of ACI-induced MCI remains unclear. When the brain is in ischemia and hypoxia state, the TLR4/HMGB1 pathway can be activated after neuronal injury [4], which promotes the release of IL-6, TNF-α, and other inflammatory transmitters [8]. Moreover, inflammatory transmitters promote amyloid deposition [9]. This process may increase cholinesterase activity, inhibiting choline acetylase activity and resulting in brain cell damage, nerve damage, and cognitive impairment [26]. Previous research indicated NBP improve the neuronal activity by inhibiting inflammatory responses [27]. Considering that NBP can inhibit inflammatory response, we investigated the inflammatory factors. IB results indicated that NBP could inhibit IL-6 in the OGD + 10 μM NBP group, implying that it can inhibit the inflammatory response; however, the underlying mechanism remains unclear. TLR4 and HMGB1 are important proteins [28] that instigate inflammatory responses by binding to each other and triggering an inflammatory cascade [29]. To further investigate whether NBP acts on the TLR4/HMGB1 pathway to inhibit the inflammatory response, we examined the interaction between these two proteins using Co-IP and colocalization assays. Co-IP data demonstrated a significant reduction in the pull-down of the HMGB1 in the OGD + 10 μM NBP group. The PCC between TLR4 and HMGB1 decreased in the OGD + 10 μM NBP group. These results suggest that NBP could inhibit the TLR4/HMGB1 pathway to exert anti-inflammatory effects, even without reducing the expression of TLR4 and HMGB1.

Nevertheless, several limitations should be acknowledged. Firstly, the small number of the sample is a potential limitation of the study. We will increase the sample number in future studies in order to consolidate the results. Secondly, the *in vivo* model of ACI-induced MCI should be constructed to explore more treatments for ACI-induced MCI. Finally, the underlying mechanisms of the connection between glial cells and neurons in ACI-induced MCI should be further investigated.

5. Conclusion

In conclusion, this study demonstrated that NBP can effectively improve the cognitive function of patients with ACI-induced MCI and effectively rescue neurons by inhibiting the inflammatory response induced by the TLR4/HMGB1 pathway.

Abbreviations

ACA, anterior cerebral artery; ACI, Acute Cerebral Infarction; ADL, Activities of Daily Living; AP, action potential; Co-IP, Co-immunoprecipitation; Ctrl, control; OGD, oxygen and glucose deprivation; HMGB1, high mobility group box 1; IB, Immunoblotting; IF, Immunofluorescence; IL-6, interleukin-6; MCA, middle cerebral artery; MCI, mild cognitive impairment; MOCA, Montreal cognitive assessment scale; NBP, N-butyl-phthalide; PC, posterior circulation; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor-α; VD, vascular dementia.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

HZ and SL conceptualized and designed the study. HZ investigated the clinical efficacy of the NBP and wrote the manuscript. SL performed the patch-clamp and wrote the manuscript. CH and YC finished the primary neuron culture and OGD model. LW and JL performed the IB and Co-IP. YL performed IF and contributed to the analysis the results of the work. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study adhered to the principles of the Declaration of Helsinki. Ethical approval was granted by the institutional review board of Jiangbin Hospital (No. LW-2022-09). Informed consent was obtained from patients or their guardians.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.jin2308158.

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