

## Original Research Article

# Protective effect of furofuranone against cerebral ischemic stroke via activation of PI3k/Akt/GSK 3 $\beta$ signaling pathway

Pan Gu, Jing Feng, Xiaokun Geng, Hongpeng Zhang, Huishan Du\*

Department of Neurology, Luhe hospital, Capital Medical University, Beijing 101100, China

\*For correspondence: **Email:** DellaMasonebo@yahoo.com; **Tel:** 0086-010-69531069

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### Abstract

**Purpose:** To study the protective effects of furofuranone on oxygen and glucose-deprived damage to brain microvascular endothelial cells (BMECs) *in vitro*, and *in vivo* in cerebral ischemic stroke rat model.

**Methods:** BMECs were isolated from the Sprague Dawley rats and deprived of oxygen and glucose. The effect of 10, 20, 30, 40, 50 and 100  $\mu$ M furofuranone on the oxygen/glucose-deprived BMECs was studied using Transwell chamber method. A rat cerebral ischemic stroke model was established using middle cerebral arterial occlusion method. Caspase-3 and other proteins, inflammatory cytokines, and other parameters of the brain tissue were evaluated by enzyme-linked assay (ELISA), polymerase chain reaction (PCR) and Western blot as appropriate. Further studies on the brain tissues was carried out by immunochemical analysis and hematoxylin and eosin staining.

**Results:** Furofuranone decreased caspase 3 levels in a dose-based manner in rat BMECs, and significantly reduced the release of lactate dehydrogenase (LDH) in ischemic stroke rat model ( $p < 0.05$ ). It also led to marked increases in the levels of p PI3k, p Akt and p GSK3 $\beta$  in cerebral ischemic stroke rats. Growth-associated protein-43 (GAP-43) and microtubule-associated protein 2 (MAP-2) levels increased in the cerebral ischemic stroke rat brain tissues, in addition to marked increase in ionized calcium-binding adaptor protein (IBA-1) and glial fibrillary acidic protein (GFAP) ( $p < 0.05$ ). Furofuranone treatment reduced the population of microtubule-associated protein light chain 3 (MAP1LC3A) and Beclin 1-positive cells, and significantly downregulated L selectin, leptin, monocyte chemotactic protein-1 (MCP-1) and tumor necrosis factor (TNF)- $\alpha$  ( $p < 0.05$ ). The release of tissue inhibitor of metalloproteinases 1 (TIMP-1) was enhanced in the cerebral ischemic stroke rats by furofuranone treatment.

**Conclusion:** Furofuranone treatment prevents cerebral ischemic stroke-induced damage in rats via phosphorylation of PI3k, Akt and GSK3 $\beta$  proteins, and reduction of inflammatory cytokine levels. Therefore, furofuranone may be useful as chemotherapeutic agent for cerebral ischemic stroke.

**Keywords:** Ischemia, Cytokines, Phosphorylation, Diterpenoid, Neurite outgrowth

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## INTRODUCTION

The incidence of stroke ranges from 260 to 310 cases in every 100,000 people throughout the world, with a mortality of 30 % [1]. In developed

countries of the world, stroke is the 3<sup>rd</sup> leading cause of mortality [1]. The incidence of stroke is expected to increase further due to ageing of the world population [2]. Moreover, sedentary lifestyle and unhealthy food habits are believed to contribute significantly to the increase in the

incidence of stroke and the associated mortality in patients [2]. Ischemic cerebral infarction alone accounts more than 80% of the cases of stroke globally [3]. The major cause of ischemic stroke is blockage of blood flow to the brain due to clot formation and thickening of veins [4]. In most cases, stroke patients suffer from irreversible and life-long disabilities because of the very poor regenerative potential of nervous tissues [4].

Neovibsanins are diterpenoid compounds containing multiple functional groups. These compounds were isolated initially from *Viburnum awabuki* in 1996 by Fukuyama *et al* [5]. Investigations have revealed their potential as neurite outgrowth-promoting agents in rat PC12 cells. This suggests that neovibsanin-type compounds may be useful in the development of new strategies for treating neurological diseases [6]. It has been reported that natural and synthetic analogues of neovibsanins possess promising neurite outgrowth-enhancing properties. Stereochemistry has limited effect on biological activity of these compounds [7]. The present study investigated the effect of furofuranone on oxygen/glucose-deprived brain microvascular endothelial cells *in vitro*, and *in vivo* on cerebral ischemic stroke rat model.

## EXPERIMENTAL

### Sprague-Dawley rats

Sprague-Dawley rats aged 5-6 weeks (mean weight = 260 ± 3 g) were obtained from the Animal Laboratory of Beijing Vital River Laboratory Animal Technology Co. Ltd (Beijing, China). The rats were placed under controlled laboratory conditions at 23±2°C and 60% humidity in an environment with 12-h light/12-h dark cycle. Standard laboratory chow and drinking water were freely made available to the rats. The experimental procedures were approved by the Animal Ethics Committee of Luhe Hospital, Capital Medical University (approval no. SXXSS00716). The rats were handled in accordance with the guidelines for Care and Use of Laboratory Animals, National Institute of Health (NIH), China [8].

### Isolation of brain microvascular endothelial cells (BMECs)

BMECs were isolated from the Sprague-Dawley rats as reported in a previous study [9]. The rats were sacrificed by injection of air through the ear vein. The brains were excised from the rats to isolate the cerebral cortices which were subsequently digested with collagenase II (0.1

%) and DNase I (30 U/mL) for 3 h at 37 °C. The tissue pellets formed were re-suspended in bovine serum albumin (20 %) and then subjected to centrifugation at 4 °C for 30 min at 1,200 x g. The microvessel pellets present in the lower layer were subjected to digestion with collagenase/dispase (0.2 %) and DNase I (30 U/mL) for 2 h at 37 °C. The cells were incubated for 24 h at 37 °C under 5 % CO<sub>2</sub> and 95 % humidity, and the BMECs were identified with fluorescence microscopy and immunofluorescence labelling using anti-factor VIII antibody.

### Oxygen/glucose deprivation BMECs model

The effect of 10, 20, 30, 40, 50 and 100 µM furofuranone on the oxygen/glucose-deprived BMECs was studied using Transwell chamber method. The oxygen/glucose-deprived microvascular endothelial cell model was established by growing the cells in DMEM mixed with a balanced salt solution devoid of free glucose for 8 h at 37°C in a hypoxic chamber. The atmosphere in the chamber contained 95% nitrogen and 5% carbon dioxide. BMECs (density = 3 x 10<sup>6</sup> cells/well) were treated with 10, 20, 30, 40, 50 and 100 µM furofuranone in the upper compartments of the Transwell inserts. The BMECs in the untreated group were subjected to oxygen/glucose deprivation but were not treated with furofuranone.

### Establishment of cerebral ischemic stroke rat model

The middle cerebral arterial occlusion method was used for the establishment of a rat model of cerebral ischemic stroke. The rats were given 10% chloral hydrate (360 mg/kg) anaesthesia through intraperitoneal route. An operating microscope was used for inducing cerebral ischemia in the rats via middle cerebral arterial occlusion. After insertion into the right carotid artery, the nylon filament was advanced through until it occluded the proximal stem of middle cerebral artery. The rats were randomly divided into five groups, each containing 10 rats: normal control, untreated, and three treatment groups (20, 50 and 100 mg/kg furofuranone). Rats in the treatment groups were given 20, 50 or 100 mg/kg furofuranone intraperitoneally after 1 h of cerebral ischemic stroke, while those in untreated and normal control groups were given normal saline only. On the 4<sup>th</sup> day post-cerebral ischemic stroke, the rats were sacrificed by injection of air through the ear vein, and the brain samples were collected for determination of pathological changes.

### Determination of caspase-3 activity

Caspase-3 activity was determined colorimetrically using commercially available caspase 3 activity assay kit. BMECs exposed to furofuranone were lysed with lysis buffer, and the lysates were subjected to centrifugation at 12,400 g for 25 min at 4°C. The supernatant fractions were collected. Acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) was added to each well and the plates were shaken for 10 min. The plates were then incubated for 5 h at 37°C, followed by measurement of absorbance at 403 nm in a microplate reader.

### Determination of cytotoxicity

The effect of furofuranone on oxygen/glucose-deprived BMECs was determined by lactate dehydrogenase (LDH) release assay using CytoTox-one Homogeneous Membrane Integrity Assay Kit (Promega Corporation, Madison, WI, USA) in accordance with the manufacturer's instructions.

### Reverse transcription quantitative polymerase chain reaction (RT-qPCR) in brain tissue

The brain samples of the cerebral ischemic stroke rats treated with furofuranone were excised after sacrifice of the rats. A standard methodology was used for determination of PI3k, Akt and GSK-3 $\beta$  mRNA in the tissue samples [10]. Total RNA was isolated from the brain samples using TRIzol reagent (Thermo Fisher Scientific, Inc.) in accordance with the kit manufacturer's protocol. The RNA was subjected to reverse transcription to cDNA using Reverse transcription kit (Tiangen Biotech, Co. Ltd, Beijing, China) according to the manufacturer's procedure.

**Table 1:** Sequence of the primers used

Gene	Forward	Reverse
PI3K	5'-CTC TCC TGT	5'-GCT CTC GGT
	GCT GGC TAC	TGA TTC CAA
	TGT-3'	ACT-3'
Akt	5'-TCA GGA TGT	5'-CTG CAG GCA
	GGA TCA GCG	GCG GAT GAT
	AGA-3'	AA-3'
GSK-3 $\beta$	5'-TTC TCG GTA	5'-GAC TCA TCG
	CTA CAG GGC	TAC TCC TGC
	ACC A-3'	TTG CTG-3'
$\beta$ -Actin	5'-GGA GAT TAC	5'-GAC TCA TCG
	TGC CCT GGC	TAC TCC TGC
	TCC TA-3'	TTG CTG-3'

SYBR Green PCR Master mix using ABI 7300 PCR Instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used for qPCR. The

conditions used for cycling consisted of polymerase activation for 8 min at 93 °C, at 93 °C for 25 s, and at 58 °C for 25 s (Table 1). The level of  $\beta$ -actin expression was taken as internal control.

### Western blot analysis

Ischemic penumbra tissues were treated with lysis buffer (50 mM Tris, pH 7.6 containing 150 mM NaCl, 10% Triton-X 100, 100x Roche protease inhibitor cocktail protease, and phosphatase inhibitors) to obtain the lysates. The tissue lysates were subjected to centrifugation at 13,400 x g for 5 min at 4 °C, and the protein contents of the lysates supernatant were determined using BCA assay kit (Promega Corporation). Protein samples (30- $\mu$ g) were subjected to 12 % SDS-polyacrylamide gel electrophoresis for 3 h, and then transferred to PVDF membranes which were blocked with 5 % BSA in TBST overnight at 4°C. The membrane was incubated with antibodies for p-PI3k, p-Akt, p-GSK3 $\beta$  and GAPDH (from Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4°C, and thereafter were washed with PBS, followed by incubation for 2 h with horseradish-peroxidase-conjugated secondary antibodies at room temperature. The visualization of protein bands was performed using an ECL kit according to the instructions from manufacturer.

### Determination of inflammatory cytokines

The levels of inflammatory cytokines in the brain tissues were measured using commercially available Multiplexed Sandwich ELISA Quantitative array- kit (RayBiotech, Inc., Norcross, GA, USA) in accordance with the manual protocol. The levels of the cytokines leptin, L-selectin, MCP-1, TIMP-1 and TNF- $\alpha$  were determined using commercially available kits.

### Hematoxylin and eosin (H&E) staining

The excised brain tissues were fixed for 24 h with 4 % paraformaldehyde at room temperature. Then, the tissues were cut into 2- $\mu$ m sections and put onto the slides coated with gelatin. The sections were rehydrated in distilled water, followed by staining for 15 min with hematoxylin and eosin (H & E) at room temperature. The sections were rehydrated in gradient of ethyl alcohol before suspending in xylene. Finally, the stained tissues were examined for pathological changes under a light microscope.

## Immunohistochemical analysis

On the day 4 post-cerebral ischemic stroke, brain tissues were subjected to immunohistochemical analysis using antigen retrieval method [11]. The brain tissues were incubated overnight with primary antibodies against microtubule-associated protein-2 (MAP-2), growth associated protein-43 (GAP-43), synaptophysin, glial fibrillary acidic protein (GFAP), ionized calcium binding adaptor protein (IBA-1), microtubule-associated protein light chain-3 (MAP1LC3A) and Beclin-1 at 4°C. Following primary antibody incubation, the tissues were subjected to incubation with goat anti-rabbit immunoglobulin G, and then with streptavidin-peroxidase at room temperature for 50 min. Image J software version 1.48 was used for the analysis of data. Visualization of the tissue samples was carried out in 5-randomly selected fields at x250 magnification.

## Statistical analysis

The data are expressed as mean  $\pm$  standard deviation of three experiments. One-way analysis of variance (ANOVA) and least significant difference post hoc test were used for comparison among groups. All statistical analyses were carried out using SPSS software (17.0; SPSS, Inc., Chicago, IL, USA). Differences were taken as significant statistically at  $p < 0.05$ .

## RESULTS

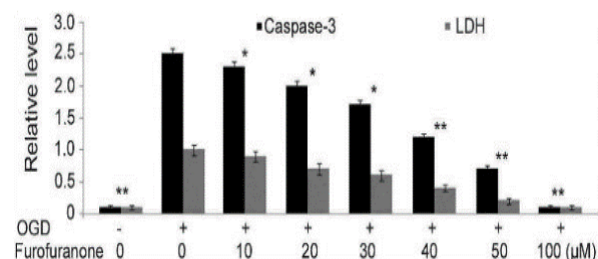
### Furofuranone suppressed caspase-3 and reduced LDH release

The level of caspase-3 was decreased dose-dependently in the BMECs of rats treated with furofuranone (Figure 1). Caspase level in BMECs of untreated rats was higher than that in BMECs of rats treated with 100  $\mu$ M of furofuranone. The cytotoxicity inhibitory effect of furofuranone in the rat BMECs was determined by assaying LDH release from damaged cells (Figure 1). Treatment of the rats with furofuranone significantly ( $p < 0.05$ ) reduced the release of LDH. On treatment with 100  $\mu$ M furofuranone, the release of LDH in rat BMECs was almost completely inhibited.

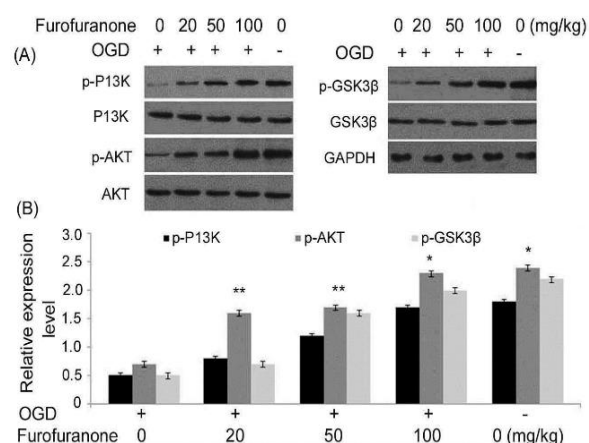
### Effect of furofuranone on phosphorylation of PI3k/Akt/GSK-3 $\beta$

Western blotting showed that furofuranone marked upregulated the protein expressions of p-PI3k, p-Akt and p-GSK3 $\beta$  in the cerebral ischemic stroke rats, relative to the untreated

group (Figure 2 A). The expressions of mRNAs corresponding to p-PI3k, p-Akt and p-GSK3 $\beta$  were also higher in the furofuranone-treated rats (Figure 2 B).



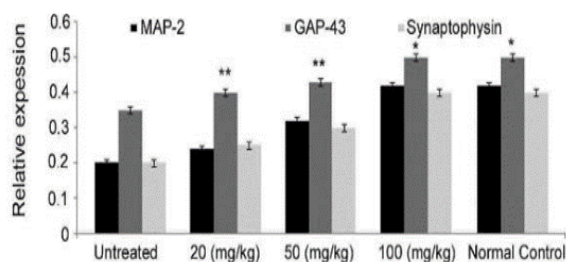
**Figure 1:** Effect of furofuranone on caspase-3 level and LDH release in rat BMECs. The rat BMECs were exposed for 72 h to 10, 20, 30, 40, 50 and 100  $\mu$ M furofuranone, followed by determination of caspase-3 levels and LDH release (\* $p < 0.05$ ; \*\* $p < 0.02$  vs. untreated BMECs)



**Figure 2:** Effect of furofuranone on activation of PI3k, Akt and GSK3 $\beta$  in cerebral ischemic stroke rats. (A) The activation levels of PI3k, Akt and GSK3 $\beta$  proteins were determined using western blotting, with GAPDH as internal control. (B) Results of RT-PCR analysis of PI3k, Akt and GSK3 $\beta$  mRNA levels. The values are expressed as mean  $\pm$  standard deviation. \* $p < 0.05$ ; \*\* $p < 0.01$  vs. untreated group

### Effect of furofuranone on GAP-43, MAP-2 and synaptophysin in rat brain tissues

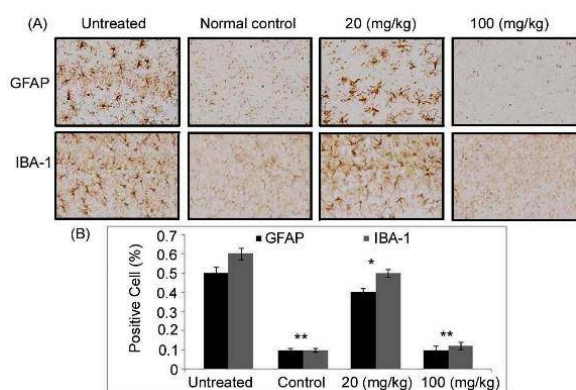
Immunofluorescence staining showed markedly lower levels of GAP-43, MAP-2 and synaptophysin in the cerebral ischemic stroke rat brain tissues (Figure 3). However, dose-dependent increases in GAP-43, MAP-2 and synaptophysin levels were evident in the brain tissues of cerebral ischemic stroke rats treated with furofuranone. The levels of GAP-43, MAP-2 and synaptophysin were highest in the rats treated with furofuranone at the dose of 100 mg/kg.



**Figure 3:** Effect of furofuranone on the expressions of GAP-43, MAP-2 and synaptophysin in rat brain tissues, as assayed via immunofluorescent staining. The relative expressions of GAP-43, MAP-2 and synaptophysin in brain tissues of cerebral ischemic stroke rats are shown. \**p* < 0.05; \*\**p* < 0.02 vs. untreated rats.

**Effect of furofuranone on pathological changes in rats**

The level of expression of IBA-1 in the microglia and that of GFAP in the astrocytes of untreated rats were markedly lower than the corresponding expressions in the normal group (Figure 4). However, treatment of the cerebral ischemic stroke rats with furofuranone led to marked increases in immunofluorescent staining for IBA-1 and GFAP.

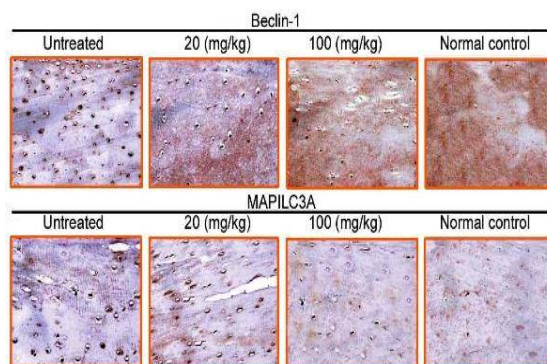


**Figure 4:** Effect of furofuranone treatment on immunoreactive cell count in brain tissue of cerebral ischemic stroke rats. (A) Immunofluorescent staining for GFAP and IBA-1. (B) Relative proportions of GFAP and IBA-1 in the positively-stained cells (x250; \**p* < 0.05; \*\**p* < 0.01 vs. untreated rats)

**Furofuranone decreased MAP1LC3 and Beclin-1-positive neurons in cerebral ischemic stroke rats**

In the untreated cerebral ischemic stroke rats, the percentage of neurons that were positive for MAP1LC3 and Beclin-1 staining was markedly higher than that in the normal group (Figure 5). However, treatment of the cerebral ischemic stroke rats with furofuranone led to dose-dependent reductions in MAP1LC3 and

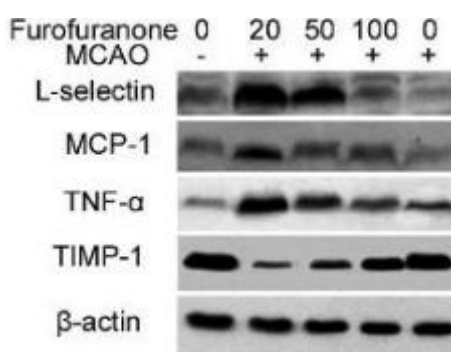
Beclin-1. The MAP1LC3 and Beclin-1 positively-stained cells were almost completely absent in the group treated with furofuranone at the dose of 100 mg/kg.



**Figure 5:** Effect of furofuranone on autophagy marker levels in cerebral ischemic stroke rats. Beclin-1 and MAP1LC3A-positive cell populations were determined using immunofluorescence staining

**Furofuranone suppressed release of inflammatory cytokines**

The levels of L-selectin, leptin, MCP-1 and TNF $\alpha$  were markedly higher in the rats with cerebral ischemic stroke than in rats in the normal control group (Figure 6). Interestingly, treatment of the cerebral ischemic stroke rats with furofuranone led to marked reductions in L-selectin, MCP-1 and TNF $\alpha$ . Moreover, there was enhanced release of TIMP-1 in the cerebral ischemic stroke rats on treatment with furofuranone. The increase in TIMP-1 release in the furofuranone-treated rats was maximum in the 100 mg/kg treatment group.



**Figure 6:** Effect of furofuranone on cytokine levels in rats with cerebral ischemic stroke. The rats were treated with furofuranone at doses of 20, 50 and 100 mg/kg. The levels of TIMP-1, L-selectin, MCP-1 and TNF $\alpha$  were assayed using western blot assay

**DISCUSSION**

The present study investigated the effect of furofuranone on cerebral ischemic stroke rat

model *in vivo*. The study determined the changes in levels of phosphoinositide 3-kinase, protein kinase b and glycogen synthase kinase-3 $\beta$  in cerebral ischemic stroke rats on treatment with furofuranone. The results indicated that furofuranone exhibited protective effect against cerebral ischemic stroke-induced brain damage in rats through phosphorylation of PI3k/Akt/GSK-3 $\beta$ , and inhibition of pro-apoptotic proteins, autophagy proteins and inflammatory cytokines.

Previous studies on the effect of furofuranone on cerebral ischemic stroke were done mainly in the hippocampal region of human adult brains [12]. The hippocampus is associated with the regulation of various signalling pathways which control proliferation, migration, differentiation and integration of the signals [13]. Therefore, the hippocampus is considered a vital target in the development of new treatments for various neurological disorders. Apoptosis is a cellular process which leads to death of the cells in a programmed manner. Stroke-induced apoptosis of cells is accompanied by the release of cytochrome c and activation of the caspase cascade [14]. In the present study, treatment of the rat BMECs with furofuranone caused marked reduction in caspase-3 level *in vitro*, when compared to the control cells. Cell membrane damage leads to release of LDH, which is taken as an indicator of cytotoxicity. In the present study, treatment of the rat BMECs with furofuranone significantly reduced the release of LDH. Autophagy is activated by ischemia through the formation of autophagosome in the animal models. The two important members of autophagy signaling pathway which stimulate the formation of autophagosome and initiate autophagy are MAP1LC3 and Beclin-1 [15]. In the present study, the populations of Beclin-1 and MAP1LC3-positive puncta were markedly higher in the cerebral ischemic stroke rats than in the normal group. The populations of MAP1LC3 and Beclin-1 positive puncta were dose-dependently and markedly reduced in the rats on treatment with furofuranone.

The cytoskeletal phosphoprotein, MAP-2 is involved in the formation of microtubules and postsynaptic densities [16]. Another phosphoprotein, GAP-43 is associated with the growth of axons, synaptogenesis, and release of neurotransmitters [17]. The presynaptic vesicles bearing neurotransmitters in the membranes contain a calcium-binding glycoprotein known as synaptophysin which is used at the presynaptic terminal as the specific protein marker [18]. The density and number of synapses in the rat brains

are measured via the expression of synaptophysin [19]. Immunofluorescence staining showed markedly low levels of GAP-43 and MAP-2 in the cerebral ischemic stroke rat brain tissues. However, furofuranone treatment led to dose-dependent increases in GAP-43 and MAP-2 levels in the cerebral ischemic stroke rat brain tissues.

Studies have demonstrated that the immunomodulatory effects of chemotherapeutic agents are associated with suppression of GFAP and IBA1-positive cells [20-22]. In the present study, furofuranone treatment of the rats caused marked reductions in the GFAP and IBA1-positive cells, when compared to the untreated group. Treatment of various neurological disorders like brain injury and Parkinson's disease involves inhibition of inflammatory processes [11]. The current study showed that expressions of L-selectin, leptin, MCP-1 and TNF $\alpha$  in the cerebral ischemic stroke rats were inhibited by furofuranone.

## CONCLUSION

This study has demonstrated that furofuranone treatment prevents cerebral ischemic stroke-induced damage in rats via phosphorylation of PI3k, Akt and GSK3 $\beta$  proteins, and also by upregulation of GAP-43 and MAP-2. These results indicate that furofuranone treatment prevents cerebral ischemic stroke in the rat model. Therefore, furofuranone has potential for use as a chemotherapeutic agent for cerebral ischemic stroke.

## DECLARATIONS

### *Conflict of interest*

No conflict of interest is associated with this work.

### *Contribution of authors*

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Huishan Du designed the study and wrote the paper. Pan Gu, Jing Feng, Xiaokun Geng and Hongpeng Zhang performed the experimental work. Pan Gu and Jing Feng carried out the literature study and compiled the data. Xiaokun Geng and Hongpeng Zhang performed literature survey, analyzed the data and compiled the data. The manuscript was thoroughly read by all the authors for publication.

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