Int J Clin Exp Med 2017;10(10):14519-14525
www.ijcem.com /ISSN:1940-5901/IJCEM0045790

Original Article

Role of GSK-3β inhibitor TWS119 in protecting neurons against oxygen-glucose deprivation injury

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Received October 20, 2016; Accepted March 31, 2017; Epub October 15, 2017; Published October 30, 2017

Abstract: Aim: Glycogen synthase kinase-3 (GSK-3) is one of the few signaling molecules that regulates a truly astonishing number of critical intracellular signaling pathways.TWS119 is a GSK-3β inhibitor with IC50 of 30 nM. This study investigated the effects of the above GSK-3β inhibitor with respect to protecting neurons against oxygen-glucose deprivation (OGD)-induced injury. Methods: Cell viability assay was performed using MTT. Neurite length quantification was processed by image J in neurons exhibiting microtubule-associated protein 2 (MCP-2)-positive staining. Bax, Bcl-2 and Mcl-1 protein expression levels were analyzed by Western blotting. Inflammatory cytokine expression was investigated by a multiplexed sandwich ELISA-based quantitative array. Results: OGD-induced neurotoxicity was significantly inhibited in neurons pre-treated with different concentrations of the GSK-3β inhibitor. The GSK-3β inhibitor also attenuated OGD-induced Bcl-2/Bax ratio reductions in neurons. We noted a significant reduction in inflammatory cytokine and pro-apoptotic protein levels in 2 h OGD-treated neurons. Conclusions: Our findings suggest that the GSK-3β inhibitor plays a critical neuro-protective role in OGD-treated cells through its anti-inflammatory and anti-apoptotic effect, and indicate that it may be a promising novel therapy in ischemic stroke.

Keywords: Oxygen-glucose deprivation, neuron, GSK-3β, treatment

Introduction

Ischemic stroke, which is characterized by disruption of the blood supply to the brain, is a severe worldwide health threat [1]. The incidence and prognosis of ischemic stroke vary greatly for people of different races, ethnicities and geographic areas [2]. As the aging profile of our population becomes more advanced in the coming decades, the total number of deaths caused by ischemic stroke is predicted to increase to six million per year [3]. However, there are no effective pharmaco therapies for treating ischemic injury [3]. Since ischemic stroke (IS) is caused by a reduction in blood flow to the brain, which leads to decreases in glucose and oxygen availability in brain cells. In previous studies, *in vitro* oxygen-glucose deprivation models have been used to mimic the ischemic injury [4, 5].

Glycogen synthase kinase-3 (GSK-3) is a serine threonine kinase that phosphorylates glycogen synthase (GS). Extensive investigations on the neuroprotective effects of GSK-3 have been conducted [6-8]. It is constitutively active and ubiquitously expressed in body tissues, especially in neurons [9]. GSK-3β is expressed in all brain regions and exhibits variable mRNA expression levels. GSK-3β activity is controlled by complex mechanisms, dependents on signaling pathways, such as the PI3K/AKT/GSK-3, p53, JNK, c-jun, mTOR, hedgehog, notch, and ERK-1,2 pathways, as well as many other pathways [10]. GSK-3β inhibition has shown to have beneficial effects in ischemia; however, the molecular mechanisms underlying these effects still need further investigation. In the present study, we conducted a neurotoxicity assay and apoptosis-related protein and inflammatory cytokine quantification to determine whether GSK-3β inhibition could influence the pathology of ischemic stroke.

Materials and methods

Primary culture of neuron

The hippocampus of 17-18 day Sprague-Dawley rat fetuses (Second Military Medical Uni-

versity Experimental Animal Center) was isolated under an anatomical microscope (by peeling off the meninges and large blood vessels). Hippocampus tissue samples were cut into sections approximately 1 mm × 1 mm × 1 mm in size using tissue scissors and then dissociated enzymatically (0.125% trypsin, 37°C for 20 min) and mechanically (via repeated pipetting for 20 times). Then, the samples were centrifuged at 800 rpm for 4 minutes before being filtered through a 100 μm nylon mesh. The neurons were subsequently plated on a poly-L-lysine-coated dish and cultured in neurobasal medium with B27 and 1% penicillin/streptomycin.

Oxygen-glucose deprivation model

The cells were incubated for 2 h with glucose-free balanced salt solutionina cell incubator that was perfused with 95% N_2 and 5% CO_2 at 37°C. To examine drug efficacy, we treated the cells with TWS119 for 12 h before oxygen-glucose deprivation. Cells that were not exposed to oxygen-glucose deprivation served as external controls, and those that were exposed to oxygen-glucose deprivation conditions but were not treated with any drugs served as internal controls (OGD controls). Each experiment was repeated at least three times, so every data point was the mean value of at least three measurements.

GSK-3β inhibitor administration

A stock solution of 4,6-disubstituted pyrolopyrimidine TWS119, a potent GSK-3β inhibitor with an IC50 of 30 nM (Selleckchem, USA), was prepared in dimethyl sulfoxide (DMSO) and diluted in neurobasal medium to obtain a final DMSO concentration less than 0.1%. The cells were treated with TWS119 for 12 hours before OGD. Different concentrations were tested during the MTT assay.

MTT assay

The cells were plated at a density of 2,000/well in 96-well plates and incubated for 24 hours. The GSK-3β inhibitor was added to the cells in the treatment group, and normal medium was added to the control group. MTT assay was performed according to the reagent kit instructions (Abcam, USA). Briefly, 20 μl of MTT reagent was added to 200 μl of medium, and the mixture was incubated at 37°C for 4 hours. Then, the supernatant was aspirated and discarded, and 150 μL of formazan reagent was added to the mixture. The optical density value was assessed at 570 nm until all the formazan dissolved using an ELISA plate reader (Tecan, Switzerland).

Immunofluorescence and neurite length quantitation

The cells were fixed with 4% paraformaldehyde solution. Non-specific staining was blocked by the addition 500 μL of blocking buffer, after which the cells were incubated for 15 minutes at room temperature. The appropriate unconjugated primary antibody (Sigma, USA) was diluted in dilution buffer (1:1000), and the cells were incubated with this antibody overnight at 4°C. Negative control cells were incubated in incubation buffer with no primary antibody to identify non-specific secondary reagent staining. Then, the appropriate secondary antibody was diluted (1:4000) in dilution buffer, and the cells were incubated at room temperature for 1 hour in the dark. Then, 300 μl of diluted DAPI solution (1:2000) was added to each well, and the cells were incubated 2-5 minutes at room temperature.

Western blot analysis

The cells were washed with PBS, and then ice-cold lysis buffer was added to the plates. The cells were subsequently centrifuged in a microcentrifuge at 12000 rpm for 1 minute 4°C. The protein concentration was determined using a BCA assay kit. The gel was loaded and run for 2 hours at 120 V. Transferring the protein from the gel to the membrane. Incubate the membrane with 1:1000 dilution of primary antibody in blocking buffer overnight at 4°C. The membranes were then incubated with the appropriate conjugated secondary antibody, diluted 1:4000, in blocking buffer for 1 h at room temperature. Primary antibodies against Mcl-1, Bcl-2 and Bax (Sigma-Aldrich, Taufkirchen, Germany), as well as the appropriate secondary antibodies, were used.

Inflammatory cytokine quantitation

Cytokine secretion was measured in conditioned media using a Multiplexed Sandwich ELISA-based Quantitative array-quanti body

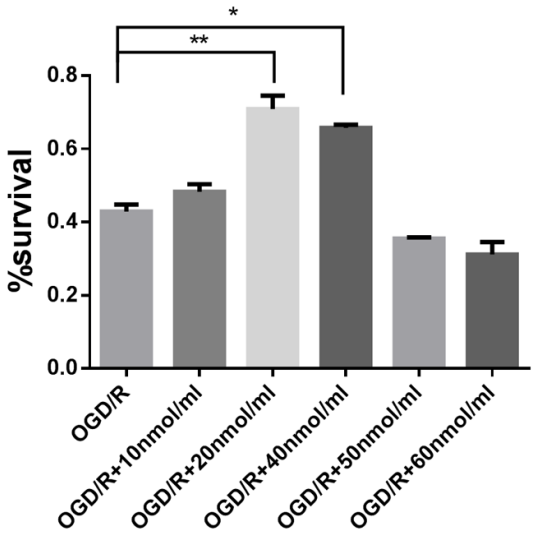

Figure 1. The effect of TWS119 on the viability of the OGD-treated neurons. The neurons were treated with TWS119 at concentrations of 10, 20, 40, 50, and 60 nmol/ml before they were subjected to OGD-injury. Cell viability was analyzed using MTT assay. Data are presented as mean ± S.D. (n = 3). Cell viability was significantly improved after pre-treatment with TWS119 at concentrations of 20 nmol/ml and 40 μM. Quantification of the neuron viability in TWS119 treated groups were normalized to control groups, *P < 0.05, **P < 0.01 (ANOVA with Tukey's post-hoc analysis).

Rat Cytokine Array 2 (Raybiotech, Norcross, USA). Protein molecule was performed according to the manufacturer's instructions.

Statistical analyses

All data were obtained using cells from at least three different experiments. Statistical significance across the groups was analyzed using one-way analysis of variance and Tukey's post hoc test. Data are shown as the mean ± S.E. Differences were considered as significant when $P < 0.05$. Statistical analysis was performed with SPSS, version 19.0 (IBM Inc).

Results

GSK-3β inhibition improved cell viability

Tetrazolium dye reduction is dependent on NAD(P)H-dependent oxidoreductase enzymes that are largely localized in the cytosolic compartment of the cell. Therefore, reducing MTT and other tetrazolium dyes depends on cellular metabolic activity caused by NAD(P)H flux. **Figure 1** shows that OGD significantly reduced cell viability (by approximately 54%), as evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, in primary cultured neurons subjected to 2 hours of OGD. However, neuronal viability was improved after pre-treatment with different concentrations of TWS119. These findings indicate that inhibiting GSK-3 activity exerted neuro-protective effects after OGD.

GSK-3β inhibition increased neurite length

MAP-2 is a protein that belongs to the microtubule-associated protein family whose expression is enriched in dendrites, indicating that it plays a role in determining and stabilizing dendrite shapes during neuron development. MAP2 illustrated in **Figure 2**, reflects neuron health and was increased in the GSK-3β inhibitor pre-treatment group compared to the OGD control group. We calculated total dendrite length and observed that the dendrites of treated neurons were approximately 35% longer than those of internal control neurons, All the neurons (n = 15-16) were obtained from three individual preparations. TWS-119 has a positive impact on dendritic development in OGD-treated cells.

GSK-3β inhibition exerted effects apoptosis-related protein expression levels

TWS119 remarkably diminished the down-regulation of the anti-apoptotic proteins Mcl-1 and Bcl-2 but decreased the expression level of the pro-apoptotic protein Bax, thus increasing the Bcl-2/Bax ratio (**Figure 3**). Thus, TWS119 treatment ameliorated the serious effects induced by OGD.

GSK-3β inhibition reduced inflammatory cytokine levels

As ischemic stroke is accompanied by increased inflammatory cytokine section, we evaluated basal cytokine section in the culture medium. We used a protein array in which different cytokines were quantified simultaneously. Secretion of the anti-inflammatory cytokine interleukin-10 (IL-10) was increased in TWS119 treated neurons, whereas release of the inflammatory cytokines IL-1β, IL-6, and ICAM-1 was decreased. We noted no apparent change in IFN-γ secretion (**Figure 4**). These findings show

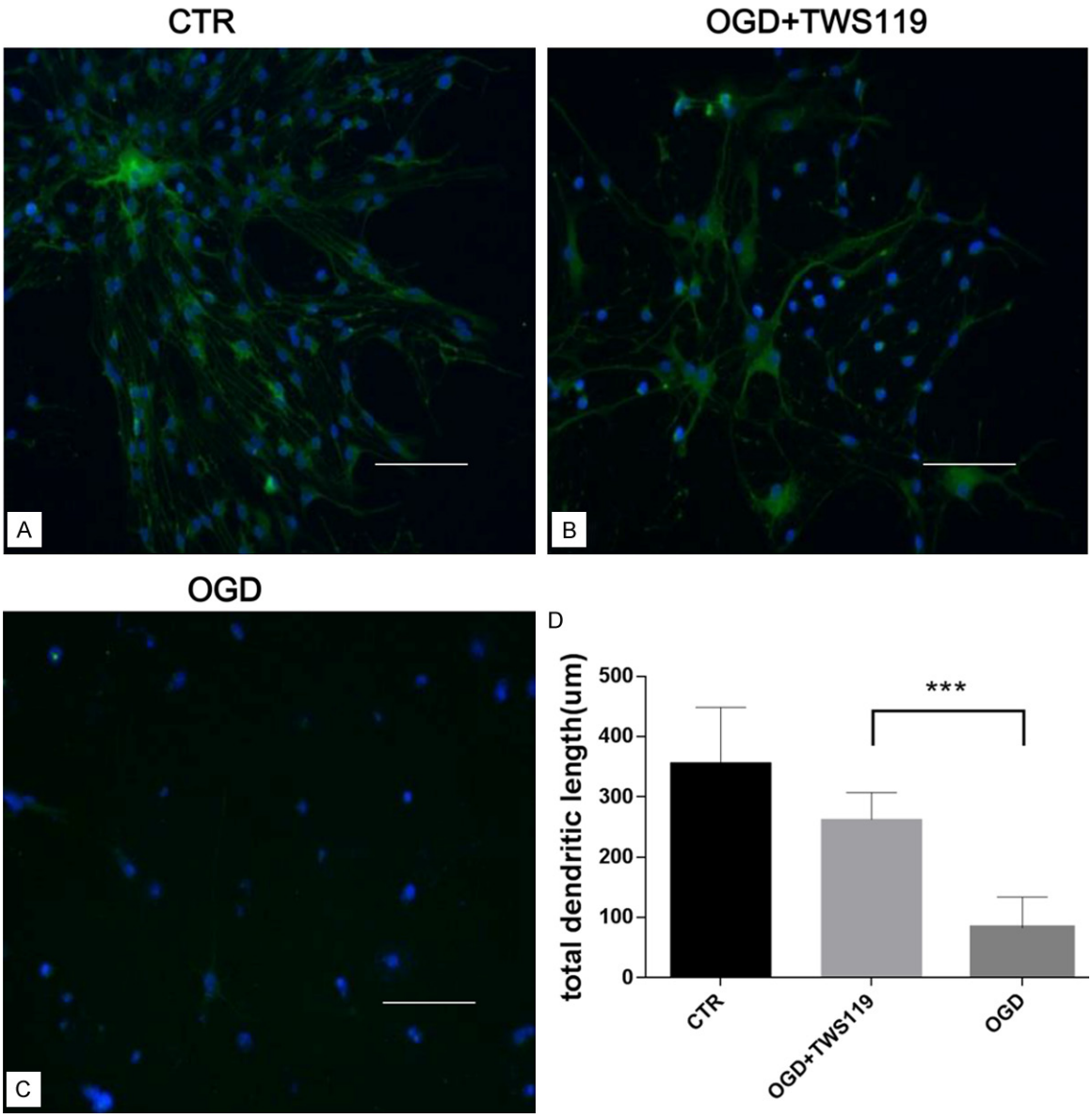

Figure 2. Measurement of dendritic length after treatment of OGD. A. Immunofluorescence staining of MCP-2 in control group. B. Immunofluorescence staining of MCP-2 in experimental group. C. Immunofluorescence staining of MCP-2 in OGD group. D. Total dendritic length per cell measured in different groups, neurons (n = 15-16) comes from three individual preparations. MAP2 immunoreactivity was increased in GSK-3β inhibitor pre-treatment group compared to the OGD control group. ($F(2,24) = 49.06.3$; $P < 0.0001$; ANOVA). ***$P < 0.001$ (ANOVA with Tukey's post-hoc analysis). All images are at 200x magnification (scale bar, 50 μm).

the powerful ability of GSK3 inhibitors to shift the balance of the inflammatory response from pro-inflammatory to anti-inflammatory.

Discussion

Ischemic stroke results from a transient or permanent reduction in cerebral blood flow that is mainly caused by cerebral artery occlusion [11]. With an incidence of approximately 250-400 cases in 100000 people and a mortality rate of approximately 30%, stroke remains the third leading cause of death in industrialized countries [12]. Stroke therapies have been largely ineffective with respect to reducing morbidity and mortality [1].

Numerous drug trials have reported disappointing results regarding rescuing neurons within ischemic brains, suggesting that, neuroprotec-

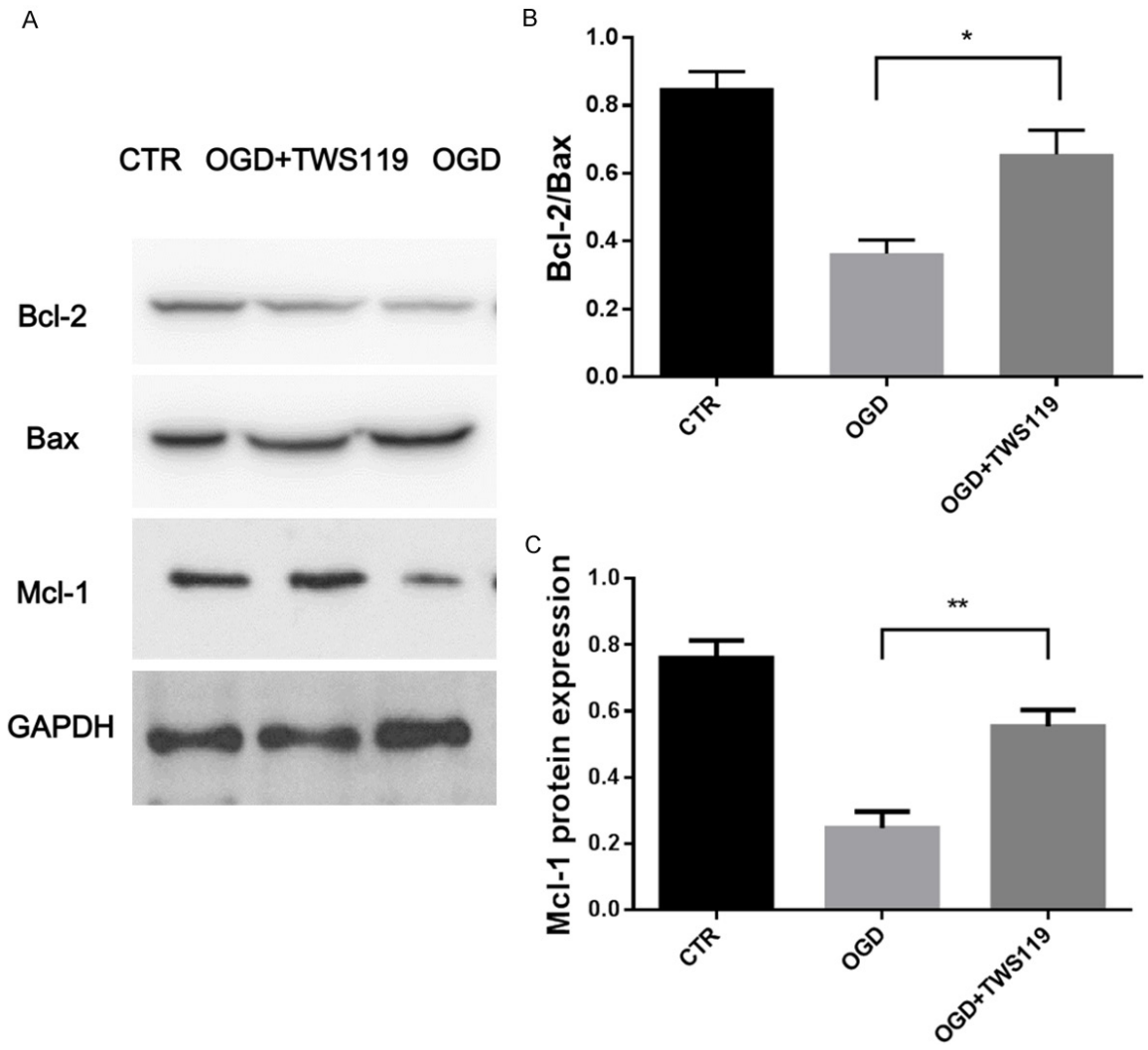

Figure 3. Western blot analysis of the protein expression levels of Mcl-1, Bcl-2 and Bax. A. The expressions of Mcl-1, Bcl-2 and Bax were shown in western blot, GAPDH acts as loading control. B. Analysis of the ratio of Bcl-2/Bax. Data are presented as mean ± S.D. (n = 3). The Bcl-2/Bax ratio was remarkably increased by TWS119. *P < 0.05 (ANOVA with Tukey's post-hoc analysis). C. Analysis of the relative expression levels of Mcl-1. Data are presented as mean ± S.D. (n = 3). The down-regulation of Mcl-1 protein was significantly diminished after treatment with TWS119. **P < 0.01 (ANOVA with Tukey's post-hoc analysis).

tion may not be as straightforward in stroke patients as it is in experimental animals [1, 12, 13]. However, experimental work will still be greatly important for finding new methods of treating stroke in the future. The injury-inducing mechanisms established using *in vitro* and *in vivo* models are related to corresponding mechanisms observed in the human body. Since ischemic stroke is caused by a reduction in blood flow to the brain, which leads to decreased glucose and oxygen availability in brain cells, *in vitro* oxygen-glucose deprivation models have been used to mimic ischemic injury [14-16].

GSK-3 has been identified as a kinase for over forty different proteins in a variety of different pathways [17]. In mammals, GSK-3 is encoded by two known genes, GSK-3 alpha (GSK3α) and GSK-3 beta (GSK3β) [18]. GSK-3 has recently been the subject of much research because it has been implicated in a number of diseases and is involved in a great number of signaling pathways, including pathways related to type II diabetes (diabetes mellitus type 2), Alzheimer's disease, inflammation, cancer, and bipolar disorder [10, 19]. Our work showed that OGD-induced neurotoxicity was significantly inhibited in neurons pre-treated with the GSK-3β

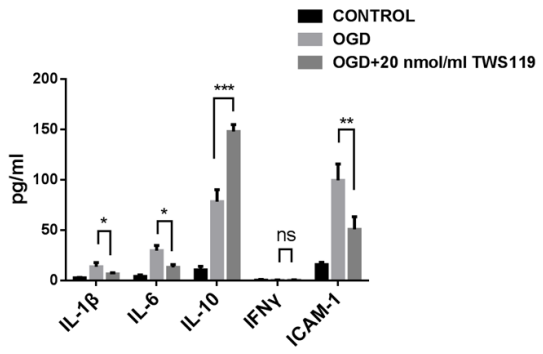

Figure 4. GSK-3β inhibition reduced inflammatory cytokine levels. Among the analyzed cytokines, secretion of the anti-inflammatory cytokine interleukin-10 (IL-10) was increased in TWS119-treated neurons, whereas release of the inflammatory cytokines IL-1β, IL-6 and ICAM-1 was decreased. We noted no apparent changes in IFN-γ secretion. *P < 0.05, **P < 0.01, **P < 0.001 (ANOVA with Tukey's post-hoc analysis).

inhibitor TWS119. GSK-3β inhibitor also attenuated OGD-induced Bcl-2/Bax ratio reductions in neurons. However there was a significant reduction in inflammatory cytokines and pro-apoptotic protein levels in 2 h OGD-treated neurons. In the present study, the ability of the GSK-3β inhibitor TWS119 to protect neurons against oxygen-glucose deprivation was discussed. this protein plays a role in the major pathogenic mechanisms underlying stroke-induced brain injury, including inflammation and programmed cell death [20].

From a pharmacologic standpoint, GSK3 may represent a new target in stroke therapy. It is believed that more preclinical studies involving small-molecule inhibitors are warranted to determine if GSK-3 inhibitors can be used in the clinic in the management of ischemic stroke, however, since TWS119 is still a type of chemical used only for research purposes, developing bio-safe drugs, finding appropriate delivery routes and defining treatment timing will be the keys to determining whether therapeutic outcomes can be achieved with respect to stroke treatment and whether the possible adverse effects of stroke treatment can be avoided or limited [16, 21].

Acknowledgements

This work was supported in part by grant 201440022 from Shanghai municipal health and family planning commission. The content is solely the responsibility of the authors.

Disclosure of conflict of interest

None.

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