Neuroprotective effects of TongLuoJiuNao in neurons exposed to oxygen and glucose deprivation

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ABSTRACT

Ethnopharmacological relevance: TongLuoJiuNao (TLJN) is an herb extract that mainly contains ginsenoside Rg1 and geniposide, which are clinically used for treating ischemic damages in the brain.

Aim of the study: In the stroke, cerebral ischemia followed by oxygen reperfusion induced apoptosis in hippocampal neurons, while extension of axons and dendrites in neurons may compensate for and repair damages of neuronal network in the hypoxia brain. In this study, we investigated whether TLJN can protect neurons against damages by ischemia in brain vasculature.

Materials and methods: We measured cell viability and lactate dehydrogenase (LDH) release from primary culture of rat hippocampal neurons before and after the neurons were deprived of oxygen and glucose (OGD). In addition, the effects were evaluated with cell viability and neurite outgrowth before or after OGD.

Results: We found that TLJN could play a neuroprotective role to cultured primary rat hippocampal neurons under both normal and oxygen/glucose-deprivation (OGD) conditions. TLJN could protect both cultured primary rat hippocampal neurons and brain microvascular endothelial cells (BMECs) from cell death under both normal and oxygen/glucose-deprivation (OGD) conditions. Moreover, under the same conditions, BMECs-conditioned media pretreated by TNJN could also promote neuron viability and neurite outgrowth, indicating that TLJN stimulated BMECs to secret some neuroprotective/neurotrophic factors.

Conclusion: These findings suggest that TLJN has a marked neuroprotective and neurotrophic roles by either direct or indirect operation, and provide insight into the mechanism of clinical efficacy of this drug against stroke.

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1. Introduction

Stroke always induces acute neuropathological events by neuron death in the affected area of the brain, which leads to loss of motor, sensory and cognitive function. Antithrombotic agents in the form of thrombolytic therapy are the primary treatment of stroke patients (Akasofu et al., 2003). These drugs, however, only slow down the progression of hypoxia rather than restore brain function. Regardless of the types of stroke, its consequences mainly result from neuronal degeneration and atrophy. To against the damages on brain by these diseases, the attempts for neuroprotection have lately been considered attractive (Dietrich et al., 1991; Broderick and William, 2004; Yoshida et al., 2006). Although it is difficult to repair neurons or to achieve neuronal regeneration after neurodegeneration in the central nervous system, new synapses could possibly be formed through the activation of remaining immature and mature neurons. Since synaptic formation is based on neurite outgrowth and maturation, drugs activating these steps may help recovery of brain function. On the other hand, during focal ischemia, the cerebral microvasculature alters rapidly and dynamically (Kim, 2005). As neurons and their vascular supply are arranged in and behave in a unitary fashion, the neurovascular unit. One strategy for achieving amelioration after stroke could be again the damages to the brain through activating of microvasculature. We observed the condition medium of brain microvascular endothelial cells (BMECs) can affect neuron viability (Hua et al., 2010), which provides opportunities for understanding the relationship between neurons and their microvasculature and for medical intervention.

Abbreviations: CM, condition medium; BMECs, brain microvascular endothelial cells; TLJN, TongLuoJiuNao; LDH, lactate dehydrogenase; PI, propidium iodide; GFP, green fluorescent protein.

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Herbal medicine has a long history of success in treating stroke (Kim et al., 1998; Wu et al., 2007). TongluojiuNaO (TLJN) is an herbal injection extracted from a traditional Chinese herbal medicine recipe that has been used clinically in the treatment of vascular diseases, like stroke, for decades (Hua et al., 2008). However, the molecular mechanism underlying its efficacy is unknown yet. Recent studies demonstrated that the two major components of TLJN are ginsenoside Rg1 and geniposide. Both ginsenoside Rg1 and geniposide have neuroprotective effects in culture cell of mouse (Ran et al., 2006). Ginsenoside improves performance in a passive avoidance-learning paradigm and enhances cholinergic metabolism, significantly stimulates neurite outgrowth in the absence of Nerve growth factor. Geniposide induced PI3K signaling pathway which is involved in the neuroprotection in PC12 cells against the oxidative damage induced by H2O2 (Liu et al., 2001; Liu et al., 2006; Leung et al., 2007; Zhang et al., 2008). Ginsenoside Rg1 protected brain from ischemic and reperfusion injuries in rat focal cerebral ischemia (Zhang and Liu, 1996). Geniposide showed a greater protective effect from damage in oxygen and glucose deprivation-exposed in hippocampal slice culture (Lee et al., 2006). Although in the clinical treatments by vaso-injection, TLJN could not directly act on neurons in brain at normal conditions, it is still possible for the components of TLJN that pass through the brain-blood barrier in pathological condition when the cerebral microvasculature is damaged. Therefore it is interesting to check if TLJN could protect neuron by direct treatment or not. Moreover, as BMECs are the first direct target of TLJN and condition medium from BMECs could affect neuron viability, it will be more interesting to see if TLJN could play some role on neuron by its effect on BMECs. In this study, we were wondering whether TLJN, which including ginsenoside Rg1 and geniposide, has neuroprotection via BMECs in either normal or pathological condition, whether it can affect on neuronal survival and neurite outgrowth in cultured rat hippocampal neurons by either direct treatments or indirect treatments with condition medium from BMECs.

2. Materials and methods

2.1. Primary rat hippocampal neuronal culture

Sprague–Dawley rats used in this study were provided by the Experimental Animal Center of Institution Biophysics, Beijing, China. The female Sprague–Dawley rats were individually housed. The rats had free access to water and food and were kept under a 12:12-h light–dark cycle, lights on at 7:00 a.m. All procedures concerning care and treatment of the rats were in accordance with the regulations of the ethical committee for the use of experimental animals of the Chinese Academy of Sciences (Beijing, China). Hippocampal cultures were prepared as described previously (Zhang et al., 2003) with some modifications. Briefly, whole brains were isolated from embryonic 16–18 days rats, and the hippocampi were dissected out and treated with 0.25% trypsin (Gibco, USA) at 37 °C for 10 min. Twenty-four hours after plating, the medium was replaced by serum-free neurobasal medium (Invitrogen) with 2% B27 supplement (Invitrogen) and 0.25% glucose (Gibco). Afterwards, half of the medium was changed twice a week. When they had developed a rich network, the hippocampal neurons were used for the experiments described below.

2.2. Primary brain microvascular cells culture

Brain capillary fragments were isolated, and endothelial cells were cultured, as described by Abbott et al. (Abbott et al., 1992; Nobles and Abbott, 1998) with minor modifications. Briefly, cerebrocortices were dissected from 3 male Sprague–Dawley rats, 225–250 g, and the meninges and choroid plexus were peeled off. Tissue was first cut by bistoury and treated with 10 ml 0.1% collagenase II solution (containing 50 μg/ml gentamycin and 2 mM l-glutamine) at 37 °C for 2 h on a theromixer with gentle shaking every 10 min. The cells were grown in a humidified atmosphere of 5% CO2 in air at 37 °C and pH 7.3, and the medium was changed three times a week.

2.3. TLJN and its composition analysis

TLJN was supplied by company (Pharmacal Manufactory of Beijing University of Chinese Traditional Medicine). To confirm its chemical contents, we analyzed the TLJN by using high performance liquid chromatography, as described previously (Ran et al., 2006). The major chemical components of the 5 mg/ml TLJN used in the present study were ginsenoside Rg1 (1.25 μM) and its intermediates geniposide (12.36 μM) as previously reported (Hua et al., 2010). In brief, the active components of TLJN are extracted from Panax notoginseng and Gardenia jasminoides. TLJN processing is carried out according to the protocol of the National Medical Dictionary of China and the amounts of Panax notoginseng (5 g) and Gardenia jasminoides (8.5 g) used were based on knowledge gained from clinical practice. A clear paste of Gardenia jasminoides is obtained by grounding and percolating with ethanol. The paste was diluted with ethanol and filtered and evaporated to the extract. Geniposide is obtained from liquid chromatography of the extract. Panax notoginseng extract is obtained from grounding and percolating. The extract is passed through a macroreticular resin chromatographic column and washed with distilled water, then diluted with ethanol. The ethanol is evaporated off and the residue is dried to obtain total saponins of Panax notoginseng extract. Ginsenoside Rg1 is obtained by liquid chromatography of the extract. To ensure the quality and stability of the TLJN solution, we used high performance liquid chromatography to test the components and confirm the final concentration of this solution. The protocol conditions were as reported previously (Liu et al., 2011). The concentrations of the three components: geniposide (4.95 mg/ml), ginsenoside (1.02 mg/ml) and geniposidic acid (1.73 mg/ml).

2.4. TLJN treatments and oxygen and glucose deprivation (OGD) induced cultured cells

Experimental design was shown in Fig. 1A.

Cell cultures were first treated with TLJN for 6 h, and then washed in Krebs buffer (pH 7.4). Then cultured cells were divided into three groups. Group I (Normal, N): cells were given hyperglucose DMEM medium equilibrated with 74% N2 + 21% O2 + 5% CO2 for 6 h. Group II (Ischemia, I): cells were administered with glucose-free DMEM medium incubated in 95% N2 + 5% CO2 for 6 h. Group III (Re-oxygenation, R): cells were given hyperglucose DMEM medium equilibrated with 74% N2 + 21% O2 + 5% CO2 for 6 h.

In this experiment of rat hippocampal neurons, one group was maintained along the entire experiment in Neurobasal medium consistent of B27 supplement solution equilibrated with 95% O2/5% CO2 (N group); the other was subjected to a 6-h OGD period (I group), followed by re-oxygenation (R group). Before OGD, the neurons were incubated for 6 h in drug treatment and then equilibrated OGD (NT, IT, KT group) to allow their equilibration to the experimental conditions. The hippocampal neurons were exposed for 6 days with 20% condition medium with neurobasal.

2.5. Analyses of cell viability

The viability of hippocampal neurons was determined by measuring the activity of the lactate dehydrogenase (LDH) released into the culture media with the use of the LDH assay kit (Roche).
Cell death was determined by propidium iodide (PI, Sigma, St. Louis, MO) and Hoechst 33258 (Sigma) double fluorescent staining. In these cases, hippocampal neurons were cultured at a density of $10^6$ cells/mm$^2$ on 8 mm glass coverslips in 24 well plates. After the indicated treatments, the cells were stained with PI (10 μg/ml) and Hoechst 33258 (10 μg/ml) for 30 min, and then fixed by 4% paraformaldehyde. For each coverslip, ten visual fields were selected randomly using the ANDOR image analysis system (Cold Spring Corporation, NY, USA). The results were expressed as the percentages of death cells.

2.6. Analyses of neurite outgrowth

Cultured rat hippocampal neurons were plated at a density of $2 \times 10^5$ cells/35-mm dish and transfected with 2.5 μg of DNA/dish, which encoded recombinant green fluorescent protein (GFP). Transfection was carried out by using Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen). After transfection for 3 days, the length and number of processes of GFP-transfected neurons were measured by using a Nikon fluorescence microscope with Image-Pro-Plus 5.0 software. A total of 12 wells were measured for each group at each time point. Five sample locations from each well were examined.

2.7. Statistic analysis

All data were expressed as mean ± S.E., and were analyzed by two-way analysis of variance (ANOVA). The Scheffe’s post hoc test was used for pair-wise comparisons. Differences were considered statistically significant at $p<0.05$.

3. Results and discussion

3.1. TLJN decreases cell death in rat hippocampal neurons

As the two major components of TLJN, ginsenoside Rg1 and geniposide, have been shown to have a role of neuroprotection. We firstly investigate whether TLJN could protect neurons against ischemia by direct treatment or not. Rat hippocampal neurons were cultured at the normal condition (N), ischemia simulation/OGD (I) and OGD plus following re-oxygenation (R), respectively. Neuron pretreated with TLJN was cultured parallel at these three conditions and marked correspondingly as groups NT, IT and RT (Fig. 1A). Death of neuron was measured by LDH release from the cultured neurons.

Our results showed that in control cells without any treatments, the LDH level is 50.41 ± 2.25%; in the cells cultured without glucose, the LDH level increase to 67.45 ± 2.21%, indicating the increase of cell death by glucose deprivation. In glucose-deprived neurons with following re-oxygenation treatment, the LDH level decreased to 43.67 ± 1.36%, indicating neuroprotective effects of re-oxygenation under the condition of glucose-deprivation. When neurons were pre-treated by TLJN, the LDH level significant decreases to 26.83 ± 1.41% ($p<0.05$), 44.96 ± 2.77% ($p<0.01$) and 16.36 ± 0.55% ($p<0.01$) in NT, IT and RT group versus the non-TLJN treated group, respectively, indicating increase in neuronal viability in all three cases (Fig. 1B). These findings suggest a neuroprotective role of TLJN, no matter at normal or pathological condition.

Moreover, the ratio of LDH decrease between R and RT groups is 27.31%, much higher than those between the N-NT (23.58%) and I-IT (22.49%) groups (Fig. 1B), suggesting a synergetic role of re-oxygenation and TLJN on neuroprotection. According to previous study, Geniposide can inhibit reactive oxygen species overproduction and NF-κB signaling pathway in human endothelial cells.
(Wang et al., 2010). Ginsenoside Rg1 suppressed the intracellular 
[Ca^{2+}] level during hypoxia/reoxygenation condition (Zhu et al., 2009). TLJN can have the neuroprotection through endothelial cells to neurons.

3.2. TLJN protects brain microvascular endothelial cells from cell death induced by OGD

Brain microvascular endothelial cells (BMECs) have recently been implicated as targets of hypoxia injury (Nagy et al., 2005). Thus, we studied the effects of TLJN on BMECs during OGD and re-oxygenation with similar condition as those for the rat hippocampal neurons (strategy as Fig. 1A). The morphological observations indicated marked morphological alterations of the BMECs after both 6 h OGD and 6 h re-oxygenation following OGD. It is showed that BMECs displayed less adhesion. And the OGD treatment causes cellular injury (Fig. 2). When the cells were pre-treated with TLJN (5 mg/ml) for 6 h before OGD, significant less cell damage and cell death was observed. The protective effect of TLJN was much more obvious in cells after re-oxygenation, and the morphology of neurons was quite similar to that of the normal control (N), suggesting combined treatments of TLJN with re-oxygenation could effectively antagonize the ischemic damages. These results suggest that TLJN treatment can prevent BMECs from OGD-induced cell death.

In addition, in contrast to the protective effect of TLJN on normal neuron cells (Fig. 1B), TLJN did not appear to have any effect on BMECs cultured under normal conditions (Fig. 2, NT), which implies TLJN has a specific role on protection of neurons under the burden of OGD.

3.3. TLJN-condition medium increases viabilities of rat hippocampal neurons

Microvascular apoptotic and neuronal damage of hypoxia are pathological features of ischemic (Zong et al., 2008). Neurons are affected by any changes of the surrounding glial cells and BMECs. In order to study whether the treatment of BMECs with TLJN induces their neuroprotective or neurotoxic secretion under both normal and ischemic conditions, we treated rat hippocampal primary neuronal cultures for 6 days with condition medium from BMECs cultured under normal or OGD conditions with or without pretreatment of TLJN (Fig. 3A).

We observed that more neurons survived when they were cultured in the condition medium (CM) from BMECs that were treated with TLJN as compared with those that were not treated with the drug (Fig. 3B). To further assess neuronal viabilities, the hippocampal neurons were double stained with Hoechst 333258 and PI to distinguish the alive and dead cells (Fig. 3C). The results of cell counting showed that the percentages of alive cells are similar in neurons cultured with N-CM or I-CM, while that in neuron cultured with R-CM increased significantly (Fig. 3D). This result suggests that condition medium from ischemic damaged BMECs do not affect cell viability, while re-oxygenation induces the ischemia-treated BMECs releasing some factor(s) which could increase neuron viability.

When condition mediums from TLJN treated BMECs were applied, significant higher percentages of alive neurons were found at conditions cultured with condition medium from normal or re-oxygenation-treated BMECs than those cultured with corresponding condition mediums from the TLJN-untreated BMECs (Fig. 3D). These results suggest that under both normal and re-oxygenation conditions, TLJN can stimulate BMECs to secrete some neuroprotective factors and also possibly enhance the reaction of OGD/ischemia-exposed BMECs to re-oxygenation. However, TLJN treatment showed no significant difference in cell viability to the none-TLJN treated group when neurons were cultured in condition mediums from BMECs exposed to OGD without re-oxygenation (Fig. 3D, IT-CM compare to I-CM). This indicates OGD/ischemic damaged BMECs have lost this secreting ability even the cells have been pretreated/protected by TLJN, and re-oxygenation is
necessary for OGD/ischemia-treated BMECs to keep the ability to responding to TLJN, therefore implies that TLJN might share the same pathway to that of re-oxygenation to stimulate BMECs. Ginsenoside-Rg1 (Rg1) is involved in PI3K, Akt, and p38 MAPK signaling pathways in myocardial rats study (Yin et al., 2011). Ginsenoside can reduce endothelial nitric oxide synthase (eNOS) in rat (Lu et al., 2010).

3.4. The TLJN-condition medium promotes neurite outgrowth of rat hippocampal neurons

As in the above experiments we also observed the neurons treated by TLJN-condition medium show more neurites/branches than those cultured with condition medium. To investigate whether TLJN stimulates BMECs to secrete any factors that facilitate neurite outgrowth and branching, especially under ischemic conditions, we analyzed the neurite growth in rat hippocampal neurons cultured under the same conditions mentioned above. The neurons were also transfected with GFP to facilitate measurements of neurites, and the length and number of processes of GFP-transfected neurons were measured with Image-Pro-Plus 5.0 software on the pictures taken by a fluorescent microscope (Fig. 4A–D).

As shown in Fig. 4E and F, in neurons cultured with condition medium from non-TLJN treated BMECs, the means of neurite length per neuron cultured with condition medium from normal (N-CM), ischemic (I-CM) or re-oxygenated (R-CM) cells showed no remarkable difference. The same result was obtained by comparison of the means of dendrite number per neuron, indicating no factor facilitating neurite outgrowth or branching is included in condition medium obtained in either of the above conditions, and the neuroprotective factor that BMECs secret by re-oxygenation stimulation has no role on neurite growth.

At the case of cell culture with condition medium from TLJN treated BMECs, the means of neurite length per neuron showed significant increase of 40.43%, 21.55% and 26.73% in NT-CM, IT-CM, and RT-CM groups, respectively (Fig. 4E). Similar increase is found on means of dendrite number per neuron in NT-CM (46.87%) and RT-CM (43.65%) groups, while CM from BMECs under OGD did not show any effect after TLJN treatment (Fig. 4F). The condition medium from ischemic BMECs after TLJN treatment appeared to increase the length (p<0.01), but not the number, of neurites. The condition medium from TLJN-treated BMECs cultured under all three conditions increased the length of neurites markedly, as compared with the condition medium from TLJN-untreated cells (Fig. 4E). These results suggest that TLJN may stimulate BMECs to secrete neurotrophic factors that promote neurite outgrowth, especially under normal condition and during re-oxygenation phase after OGD insult.

In addition, as the increases of neurite length (40.4%) and dendrite number (46.3%) are similar in NT-CM treated neurons, the promotion of neurite growth might be mainly due to induction of new branches/neurites. In IT-CM treated neuron, as no significant increase of dendrite number (6.09%) were observed but the neurite length still increase (21.6%), indicating no branching-promoting factor(s) or something against it present in NT-CM, however, some neurite extension-promoting factor(s) have been secreted in this medium. In the case of RT-CM treatment, the increase of dendrite number went back to 43.7%, while the increase of neurite length maintained as 26.8%, indicating that the TLJN mainly protect BMECs to secret branching-promoting instead of
neurite-extension factor(s) into condition medium. These results show that the neurite-growth factors secreted by TLJN-pretreated BMECs are mainly branching-promoting factor(s) in normal or re-oxygenation rescued conditions; while when re-oxygenation protection was applied, TLJN-pretreated BMECs mainly secret neurite extension-promoting factor(s).

Our study provided for the first time evidence that TLJN increases both outgrowth number and length of neurites in cultured hippocampal neurons in OGD-induced BMECs. Besides, our findings suggest that besides to its neuroprotective functions, TLJN may be used as a neurotrophic factor in nerve diseases. The present study clearly demonstrates the involvement of the crosstalk between cerebral microvascular and hippocampal neurons. However, little is known about the molecular mechanisms and physiological settings in which TLJN exerts its activities on primary neurons by BMECs. Accumulating evidence support the fact that ginsenoside plays a major role/ in modulating neurotransmission and prevent scopolamine-induced memory deficits by increasing cholinergic activity (Yamaguchi et al., 1996). Recent in vivo and in vitro experiments also showed that ginsenoside Rg1 which has neurotropic and neuroprotective effects in brain tissue might be mediated through the activation of crosstalk between ERα- and IGF-IR-mediated pathways (Meiri et al., 1998; Chen et al., 2003; Radad et al., 2004). Further study will be needed to determine the role of IGF-IR-mediated pathway in mediating the molecular actions of TLJN in brain tissue.

In conclusion, TLJN could protect both cultured primary rat hippocampal neurons and BMECs from cell death under both normal and oxygen/glucose-deprivation (OGD) conditions. Our findings, therefore, are important in that they help to summarize the current literature and provide insight into the potential outcomes as a function of the choice of pharmacologic prophylaxis.

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