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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.

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 stoke 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 H_2O_2 (Liaw and Chao, 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 direct 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% glutamine (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, cerebral cortices 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 thermomixer with gentle shaking every 10 min. The cells were grown in a humidified atmosphere of 5% CO_2 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% N_2 + 21% O_2 + 5% CO_2 for 6 h. Group II (Ischemia, I): cells were administered with glucose-free DMEM medium incubated in 95% N_2 + 5% CO_2 for 6 h. Group III (Re-oxygenation, R): cells were given hyperglucose DMEM medium equilibrated with 74% N_2 + 21% O_2 + 5% CO_2 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% O_2/5% CO_2 (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, RT 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).