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Protective effects of *Lingguizhugan* decoction on amyloid-beta peptide (25–35)-induced cell injury

Anti-inflammatory effects☆

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Abstract

In the present study, a human neuroblastoma cell line (SH-SY5Y) and BV-2 microglia were treated with amyloid-β peptide (25–35), as a model of Alzheimer's disease, to evaluate the protective effects of 10^{-3}–10^{-8} g/mL *Lingguizhugan* decoction and to examine the underlying anti-inflammatory mechanism. *Lingguizhugan* decoction significantly enhanced the viability of SH-SY5Y cells with amyloid-β peptide-induced injury, and lowered levels of interleukin-1β, interleukin-6, tumor necrosis factor-α and nitric oxide in the culture supernatant of activated BV-2 microglia. The effects of 10^{-3} g/mL *Lingguizhugan* decoction were more significant. These results suggest that *Lingguizhugan* decoction can protect SH-SY5Y cells against amyloid-β peptide (25–35)-induced injury in a dose-dependent manner by inhibiting overexpression of inflammatory factors by activated microglia.

Key Words

Alzheimer's disease; *Lingguizhugan* decoction; phlegm-warming and fluid-dispersing; inflammatory reaction; SH-SY5Y; BV-2

Research Highlights

(1) *Lingguizhugan* decoction inhibits overexpression of interleukin-1β, interleukin-6, tumor necrosis factor-α and nitric oxide in activated BV-2 microglia in a dose-dependent manner.
(2) *Lingguizhugan* decoction protects a human neuroblastoma cell line (SH-SY5Y) against amyloid-β peptide (25–35)-induced injury.

Abbreviations

Aβ, amyloid-β; AD, Alzheimer's disease; IL, interleukin; TNF, tumor necrosis factor; NO, nitric oxide

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INTRODUCTION

Microglial activation caused by amyloid-β (Aβ) proteinosis and the resulting inflammatory reaction underlie the pathology of Alzheimer's disease (AD)[1]. Based on theories of traditional Chinese medicine, retention of phlegm and fluid plays an important role in the occurrence and development of AD[2-4].

Treatment with phlegm-warming and fluid-dispersing formulations shows remarkable efficacy in clinical practice[5-7]. Pharmacological studies show that phlegm and retained fluid play critical roles in the pathogenesis of inflammation[8].

Lingguizhugan decoction (LG) has its origins in *Treatise on Cold Pathogenic and Miscellaneous Diseases*[9]. As the basic prescription for phlegm and fluid retention, it has been widely applied in the clinical treatment of multiple diseases related to retention of phlegm and fluid[10]. In addition, pharmacological research has demonstrated that LG and its main components have good anti-inflammatory activities[11-16].

In the present study, we used a human neuroblastoma cell line, SH-SY5Y cells, to establish an AD cell culture model (induced by $Aβ_{25-35}$), and we investigated the protective effects of different LG concentrations on $Aβ_{25-35}$-induced damage in these cells.

Considerable evidence indicates that microglia (BV-2) participate in the inflammatory pathological process in neurodegenerative diseases[17]. Excessively activated microglia can produce neurotoxic factors, such as interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, nitric oxide (NO), transforming growth factor-β and free radicals, as well as complement protein and other immune molecules[18]. These toxic substances further activate microglia, worsening injury and promoting neuronal death[19]. Thus, in the present study, we also explored the anti-inflammatory effects and mechanisms of action of LG on activated microglia.

RESULTS

Changes in SH-SY5Y cell morphology

Under the inverted phase-contrast microscope, cells in the normal group (SH-SY5Y cells) had spread and adhered without obvious changes in appearance.

After 24 hours, cells in the model group (25 μM $Aβ_{25-35}$-treated SH-SY5Y cells) had shrunk, the intercellular spaces widened, and the number of round cells increased.

At 48 hours, a large number of cells in the model group were round and floating, with enhanced refractivity. The spreading and adherence of cells in each LG group increased, while the number of shrunken, round and floating cells was significantly less than in the model group.

LG enhances the viability of $Aβ_{25-35}$-treated SH-SY5Y cells

MTT results showed that the viability of SH-SY5Y cells significantly decreased after 25 μM $Aβ_{25-35}$ treatment (P < 0.01), but LG (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} g/mL) significantly improved the viability of these cells (P < 0.05; Table 1, supplementary Figure 1 online).

Table 1 Effect of *Lingguizhugan* decoction (LG) on the survival of SH-SY5Y cells

Group	Absorbance (mean±SD)	Survival rate (%)
Normal	0.738±0.033	100
Model	0.533±0.035[a]	72.2[a]
LG1	0.641±0.022[b]	86.8[b]
LG2	0.649±0.034[b]	87.9[b]
LG3	0.623±0.021[b]	84.4[b]
LG4	0.599±0.032[b]	81.1[b]
LG5	0.542±0.025	73.4
LG6	0.543±0.036	73.5

The experiment was performed in triplicate of six wells in each group. [a]P < 0.01, *vs.* normal group; [b]P < 0.05, *vs.* model group (one-way analysis of variance followed by Student's *t*-test).

Normal: SH-SY5Y cells; model: 25 μM $Aβ_{25-35}$-treated SH-SY5Y cells; LG1, LG2, LG3, LG4, LG5 and LG6: 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} g/mL LG, respectively, in combination with 25 μM $Aβ_{25-35}$ treatment. $Aβ_{25-35}$: Amyloid-β peptide (25–35).

LG inhibits IL-1β expression in Aβ-activated microglia (BV-2)

A standard curve was drawn and sample concentration was determined based on the standard curve. The IL-1β content in the cell culture supernatant in the model group (25 μM $Aβ_{25-35}$-treated BV-2 cells) was significantly increased compared with the normal group (BV-2 cells), but was significantly reduced in all LG groups compared with the model group.

In particular, 10^{-3} and 10^{-4} g/mL LG significantly decreased IL-1β content (P < 0.01), followed by 10^{-5} and 10^{-6} g/mL LG (P < 0.05), while 10^{-7} and 10^{-8} g/mL LG slightly lowered IL-1β content (Table 2, supplementary Figure 2 online).

Table 2 Effect of *Lingguizhugan* decoction (LG) on the release of interleukin-1β in BV-2 cells

Group	Absorbance (mean±SD)	Concentration (pg/mL)
Normal	0.025±0.003	15.56
Model	0.712±0.039[a]	427.58[a]
LG1	0.358±0.018[c]	205.70[c]
LG2	0.407±0.026[c]	243.67[c]
LG3	0.498±0.012[b]	301.96[b]
LG4	0.589±0.027[b]	358.32[b]
LG5	0.663±0.020	396.53
LG6	0.674±0.017	403.58

The experiment was performed in triplicate of six wells in each group. [a]P < 0.01, vs. normal group; [b]P < 0.05, [c]P < 0.01, *vs.* model group (one-way analysis of variance followed by Student's *t*-test).

Normal: BV-2 cells; model: 25 μM $Aβ_{25-35}$-treated BV-2 cells; LG1, LG2, LG3, LG4, LG5 and LG6: 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} g/mL LG, respectively, in combination with 25 μM $Aβ_{25-35}$ treatment. $Aβ_{25-35}$: Amyloid-β peptide (25–35).

LG inhibits IL-6 expression in Aβ-activated BV-2 cells

A standard curve was drawn and sample concentration was determined based on the standard curve. The IL-6 content in the cell culture supernatant in the model group was significantly elevated compared with the normal group, but was significantly reduced in all LG groups compared with the model group.

In particular, 10^{-3} and 10^{-4} g/mL LG significantly decreased IL-6 content (P < 0.01), followed by 10^{-5} and 10^{-6} g/mL LG (P < 0.05), while 10^{-7} and 10^{-8} g/mL LG slightly lowered IL-6 content (Table 3, supplementary Figure 3 online).

Table 3 Effect of *Lingguizhugan* decoction (LG) on release of interleukin-6 in BV-2 cells

Group	Absorbance (mean±SD)	Concentration (pg/mL)
Normal	0.046±0.004	21.43
Model	0.775±0.014[a]	346.23[a]
LG1	0.342±0.009[c]	148.60[c]
LG2	0.389±0.011[c]	178.37[c]
LG3	0.478±0.016[b]	211.21[b]
LG4	0.601±0.022[b]	277.68[b]
LG5	0.695±0.015	317.54
LG6	0.684±0.020	312.38

The experiment was performed in triplicate of six wells in each group. [a]P < 0.01, *vs.* normal group; [b]P < 0.05, [c]P < 0.01, *vs.* model group (one-way analysis of variance followed by Student's *t*-test).

Normal: BV-2 cells; model: 25 μM $A\beta_{25-35}$-treated BV-2 cells; LG1, LG2, LG3, LG4, LG5 and LG6: 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} g/mL LG, respectively, in combination with 25 μM $A\beta_{25-35}$ treatment. $A\beta_{25-35}$: Amyloid-β peptide (25–35).

LG inhibits TNF-α expression in Aβ-activated BV-2 cells

A standard curve was drawn and sample concentration was determined based on the standard curve. The TNF-α content in the cell culture supernatant in the model group was significantly increased compared with the normal group, but was significantly reduced in all LG groups compared with the model group.

In particular, 10^{-3}, 10^{-4} and 10^{-5} g/mL LG significantly decreased TNF-α content (P < 0.01), followed by 10^{-6} and 10^{-7} g/mL LG (P < 0.05), while 10^{-8} g/mL LG slightly lowered TNF-α content (Table 4, supplementary Figure 4 online).

LG inhibits NO expression in Aβ-activated BV-2 cells

The volume of NO secreted by BV-2 cells in each group was quantified based on the standard curve. NO secretion by BV-2 cells in the model group was significantly increased compared with the normal group (P < 0.01), but was significantly reduced in the 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} g/mL LG groups compared with the model group (P < 0.05). However, the effects of 10^{-7} and 10^{-8} g/mL LG were not obvious (Table 5, supplementary Figure 5 online).

Table 4 Effect of *Lingguizhugan* decoction (LG) on release of tumor necrosis factor-α in BV-2 cells

Group	Absorbance (mean±SD)	Concentration (pg/mL)
Normal	0.056±0.008	14.23
Model	0.403±0.011[a]	115.99[a]
LG1	0.215±0.016[c]	57.67[c]
LG2	0.220±0.009[c]	60.60[c]
LG3	0.228±0.012[c]	66.64[c]
LG4	0.266±0.015[b]	78.83[b]
LG5	0.321±0.011[b]	90.29[b]
LG6	0.357±0.018	105.21

The experiment was performed in triplicate of six wells in each group. [a]P < 0.01, vs. normal group; [b]P < 0.05, [c]P < 0.01, *vs.* model group (one-way analysis of variance followed by Student's *t*-test).

Normal: BV-2 cells; model: 25 μM $A\beta_{25-35}$-treated BV-2 cells; LG1, LG2, LG3, LG4, LG5 and LG6: 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} g/mL LG, respectively, in combination with 25 μM $A\beta_{25-35}$ treatment. $A\beta_{25-35}$: Amyloid-β peptide (25–35).

Table 5 Effect of *Lingguizhugan* decoction (LG) on release of nitric oxide in BV-2 cells

Group	Absorbance (mean±SD)	Concentration (pg/mL)
Normal	0.056±0.008	56.76
Model	0.403±0.011[a]	126.82[a]
LG1	0.215±0.016[b]	87.24[b]
LG2	0.220±0.009[b]	90.36[b]
LG3	0.228±0.012[b]	94.38[b]
LG4	0.266±0.015[b]	99.25[b]
LG5	0.321±0.011	115.30
LG6	0.357±0.018	118.84

The experiment was performed in triplicate of six wells in each group. [a]P < 0.01, *vs.* normal group; [b]P < 0.05, *vs.* model group (one-way analysis of variance followed by Student's *t*-test).

Normal: BV-2 cells; model: 25 μM $A\beta_{25-35}$-treated BV-2 cells; LG1, LG2, LG3, LG4, LG5 and LG6: 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} g/mL LG, respectively, in combination with 25 μM $A\beta_{25-35}$ treatment. $A\beta_{25-35}$: Amyloid-β peptide (25–35).

DISCUSSION

Modern pharmacological research has shown that LG and its major components have good anti-inflammatory effects[20]. The main chemical components of Poria cocos are polysaccharide and three terpenoids. Studies have shown that pachyman and tuckahoe triterpene have good anti-inflammatory effects[21]. Liu[22] found that periapical inflammation was effectively alleviated by cinnamaldehyde. Largehead atractylodes rhizome has diuretic, anti-tumor, anti-diabetes and anti-aging effects. Huang *et al* [23] found that largehead atractylodes

rhizome decoction can reduce serum levels of phlogogenic TNF-α in mouse. These observations indicate that largehead atractylodes rhizome decoction has anti-inflammatory effects.

The chief constituents of Radix Glycyrrhizae are triterpenoids (glycyrrihza glabra, *i.e.* glycyrrhizic acid salt, glycyrrhetic acid), flavonoids (licoflavone, isolicoflavone, glycyrrhizin) and licorice polysaccharide compounds. Qu *et al*[24] found that licorice decoction can significantly inhibit ear swelling in mice induced by dimethylbenzene, diminish egg white-induced foot swelling in rats, reduce peritoneal permeability and inhibit the proliferation of granuloma. Thus, licorice decoction has a good anti-inflammatory effect as well.

MTT assay is a routine method of analyzing the viability of mammalian cells, and is an index of metabolic activity. Results from the present study showed that after treatment with LG for 48 hours, the cell viability of each group was significantly enhanced compared with the model group, especially by LG at 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} g/mL. However, the effects of 10^{-7} and 10^{-8} g/mL LG were minimal.

In the present study, after Aβ induction, the levels of IL-1, IL-6 and TNF-α in the BV-2 supernatant were significantly increased. However, after treatment with different concentrations of LG, the secreted amounts of these three cytokines were reduced, most strongly by the higher concentration of the decoction. This shows that LG protects against cell injury induced by Aβ. Furthermore, the protection correlated positively with drug concentration.

In the pathological process of AD, activated microglia trigger and accelerate the immunological inflammatory reaction by releasing mediators of inflammation. Excessive release of IL-1β is the initial step in the AD inflammatory reaction. IL-6 also plays an important role in this process. It may induce protein aggregation in amyloid plaques. TNF-α is produced primarily by activated microglia. Excessive TNF-α release is toxic to neurons and neuroglial cells, and the factor cooperates with IL-1 to increase the production of IL-6[25-29].

In addition, activated microglia release NO[30], which is neurotoxic[31-33]. NO mediates and amplifies oxidative stress and the inflammatory reaction. This selectively damages neurons related to learning and memory, and promotes the formation of senile plaques, which further exacerbates AD pathology.

In the present study, we assessed changes in microglia-derived inflammatory factors, including IL-1β, IL-6, TNF-α and NO, induced by LG treatment. This was performed to evaluate the anti-inflammatory action of LG in AD. We found that LG inhibits the release by activated microglia of IL-1β, IL-6, TNF-α and NO. This indicates that LG is an anti-inflammatory agent and inhibits the release of inflammatory factors by activated microglia.

In Chinese medicine, the pathogenesis of AD is closely related to phlegm-fluid, and LG is the basic decoction to treat phlegm and fluid disease. In modern medicine, inflammatory mechanisms, triggered by Aβ-activated microglia, are considered crucial in AD etiology and pathogenesis. Pharmacological studies have shown that LG and its major components have good anti-inflammatory effects. Therefore, both traditional Chinese medicine and modern pharmacological research show that LG may have therapeutic potential in AD. However, few data are available. Therefore, in the present study, we propose the hypothesis that LG may have good therapeutic effect in AD, and its mechanism may be related to its anti-inflammatory effect. Using cell biology and molecular biology techniques, we examined the protective effect of LG in an AD cell culture model *in vitro*, and we investigated the immunological mechanisms to further the study of LG in AD prevention and treatment. Our study provides insight into the efficacy and mechanisms of action of traditional Chinese treatments for AD.

In conclusion, as a typical phlegm-warming and fluid-dispersing decoction, LG significantly protects neuronal cells from injury. This effect is mediated by its ability to inhibit the overexpression of IL-1β, TNF-α, IL-6, NO and other inflammatory factors released by activated microglia. Our research provides a new therapeutic strategy for AD. Further studies are needed to more completely clarify the effects and mechanisms of action of LG decoction in the treatment of AD.

MATERIALS AND METHODS

Design

A randomized, controlled cell biology study.

Time and setting

This experiment was conducted at the Experimental Center of Preclinical Medical College, Nanjing University of Traditional Chinese Medicine, China from March to July 2010.

Materials

Preparation of Aβ25-35

$Aβ_{25-35}$ was purchased from Sigma (St. Louis, MO, USA). $Aβ_{25-35}$, 1 mg, was added to 9 431 μL serum-free medium to prepare the 100 μM solution, and was filtered using a 0.22-μm filter membrane and incubated at 37°C for 3 days. The solution was aliquoted into 1-mL EP tubes, sealed with parafilm wrap, cryopreserved at –20°C, and thawed for subsequent use.

Preparation of LG

LG was composed of Poria cocos, Cassia Twig, largehead atractylodes rhizome and prepared Radix glycyrrhizae. All medicinal materials were purchased from Chinese Medicine Hall, Nanjing University of Traditional Chinese Medicine and appraised by the Drug Identification Department of the School of Traditional Chinese Materia Medica, Nanjing University of Traditional Chinese Medicine. Poria cocos was the dry sclerotium of one kind of epiphyte of Polyporaceae. Cassia Twig was the dry burgeon of Cinnamomum cassia of Lauraceae. Largehead atractylodes rhizome was the dry rhizome of Atractylodes Macroce Phala Koidzthe of the composite family. Prepared Radix Glycyrrhizae was the dry, processed root of Glycyrrhiza uralensis Fisch, a leguminous plant. According to the proportion in *Treatise on Febrile Caused by Cold* [9], Poria cocos:Cassia Twig:Bighead Atractylodes Rhizome: prepared Radix Glycyrrhizae = 4:3:2:2. Thus, 40 g Poria cocos, 30 g Cassia Twig, 20 g Bighead Atractylodes Rhizome and 20 g prepared Radix Glycyrrhizae were combined (110 g in total). The herbs were soaked in cold water for 30 minutes, 2–3 cm from the water surface. The medicine was decocted three times using a conventional method, filtered three times, and concentrated using a rotating evaporator at 60°C to obtain a 1 g/mL solution. After autoclave sterilization, the mixture was stored at 4°C.

Methods

Cell grouping and treatment

SH-SY5Y cells (China Center for Type Culture Collection, Wuhan, China) were cultured in high-glucose Dulbecco’s modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Hangzhou Sijiqing Biotechnology, Ltd., Hangzhou, China) in an incubator at 37°C, 5% CO_2 and saturated humidity. The medium was changed every 2 days and the cells were passaged every 3–4 days. BV-2 cells (Cell Center of Basic Medicine, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Beijing, China) were cultured in high-glucose DMEM containing 10% FBS in a 37°C incubator at 5% CO_2 and saturated humidity. The cells were passaged every 2–3 days. The starting culture was cultured for 24 hours, and the tissue culture plate was cleaned twice using PBS.

Cells were divided into normal, model and LG groups. Each group had 6 parallel wells (cell density of each type of cell was 1×10^5/mL, 100 μL per well). In the normal group, the DMEM culture medium was changed; in the model group, 25 μM $Aβ_{25-35}$ was used, based on a previous study[34]; in the LG groups, 25 μM Aβ and LG at the final experimental concentration were added. The final concentration of LG ranged from 10^{-3}–10^{-8} g/mL. This range was determined in a preliminary experiment. 1 mL LG (1 g/mL) was diluted in 100 mL high-glucose serum-free DMEM, and filtered and sterilized following centrifugation. Serum-free DMEM was used to dilute LG to concentrations of 1×10^{-3}, 1×10^{-4}, 1×10^{-5}, 1×10^{-6}, 1×10^{-7} and 1×10^{-8} g/mL. The six concentrations were designated LG1, LG2, LG3, LG4, LG5 and LG6, respectively.

MTT detection of SH-SY5Y cell viability

MTT assay was used to detect cell viability[35]. After 48 hours of administration, 20 μL MTT (Sigma) was added to every well and cultured for 4 hours. Then, 150 μL dimethyl sulfoxide was added to each well after discarding the supernatant, and the plate was shaken carefully to completely dissolve the formazan product (the bluish violet crystal of the MTT reaction). The absorbance value (A) at 490 nm was measured. Cell viability = $A_{\text{experimental group}}/A_{\text{normal group}} \times 100\%$. Every experiment was performed in triplicate.

Enzyme linked immunosorbent assay (ELISA) detection of inflammatory factor expression in BV-2 cells

BV-2 cells in the logarithmic growth phase were cultured in 96-well microtiter plates (1×10^5 cells/mL), with 100 μL in each well. When the cells adhered, they were treated with LG at different concentrations (LG1, LG2, LG3, LG4, LG5, LG6), in addition to Aβ. The upper portion of the medium was collected to determine the concentration of IL-1β, IL-6 and TNF-α by ELISA (ELISA kit was purchased from Beijing Sizhengbai Biotech Co., Ltd.)[36].

ELISA detection of NO secretion in BV-2 cells

The cells, cultured for 24 hours, were treated with drugs, and 100 μL supernatant was collected from each well and transferred to another 96-well microtiter plate, mixed with 100 μL Griess[37] reagent (Sigma) for 10 minutes, and A_{545} was measured for ELISA (Beijing Sizhengbai Biotech Co., Ltd.). The concentration of NO was

calculated based on the standard curve[38].

Statistical analysis

Data were expressed as mean ± SD and analyzed with SPSS 16.0 for windows (SPSS, Chicago, IL, USA). Comparison among groups was performed with one-way analysis of variance, and comparison between two groups was performed with Student’s *t*-test. A value of $P < 0.05$ was considered statistically significant.

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Supplementary information: Supplementary data associated with this article can be found, in the online version, by visiting www.nrronline.org.

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