Synergistic neuroprotective effect of microglial-conditioned media treated with geniposide and ginsenoside Rg1 on hypoxia injured neurons

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Abstract. The synergistic mechanism underlying the effects of multi-component combined drug use for complex diseases remains to be fully elucidated. Microglial activation following ischemia can either affect neural survival or cause neuronal injury. The aim of the present study was to determine the synergistic effect of geniposide and ginsenoside Rg1, based on microglial-neuronal communication. N2a neuronal cells were divided into the following seven groups: Control group; normal cultured microglial cells in conditioned medium (N-MG-CM) group; oxygen-glucose deprivation (OGD) model group; OGD-injured MG-CM (I-MG-CM) group; geniposide-treated MG-CM (G-MG-CM) group; ginsenoside Rg1-treated MG-CM (R-MG-CM) group; and combination-treated MG-CM (C-MG-CM) group. A series of assays were used to detect the effects of the different MG-CM on neurons in terms of: (i) cell viability, determined using a Cell Counting Kit-8; (ii) lactate dehydrogenase (LDH) leakage rate; (iii) expression of NMDAR1 and activated caspase-3, detected using western blotting; (iv) mitochondrial transmembrane potential, determined by JC-1; and (v) mitochondrial ultrastructural features, determined using electron microscopy. The experimental results demonstrated that MG-CM including the integrated use of geniposide and ginsenoside Rg1 significantly protected neuronal cell viability and inhibited LDH leakage, suppressed the expression of N-methyl-D-aspartate receptor subunit 1 and activated caspase-3, increased the mitochondrial transmembrane potential and improved the mitochondrial ultrastructure. MG-CM from separately used geniposide or ginsenoside Rg1 demonstrated differential neuroprotection at different levels. These findings revealed that the synergistic drug combination of geniposide and ginsenoside Rg1 in the treatment of stroke is a feasible approach for use.

Introduction

As the third leading cause of mortality worldwide, stroke is a major health issue in the elderly population as it leads not only to physical impairment, but also to a high risk of disability and social handicap (1). Evidence from preclinical studies (2) and randomized control trials (3) have demonstrated that combination therapy for ischemic stroke provides a survival advantage and increases the effectiveness of treatment without substantial side effects. Chinese herbs or their pharmacodynamic constituents have been widely used for the treatment of ischemic cerebral vascular disease through combination therapies (4). However, the complexity of the chemical ingredients has led to a substantial bottleneck in determining the mechanism of interaction among the ingredients for treating ischemic stroke (5).

Microglia, the resident immune cells of the central nervous system, have been implicated in triggering signaling cascades that lead to cell death in brain ischemic diseases (6). In physiological conditions, resting microglial processes make brief and direct contacts with neuronal synapses (7). In addition, microglial cells are restrained by numerous microenvironmental inhibitory effects, several of which are produced by neurons (8,9). Neuron-microglial-cell inhibitory signaling is mediated by interactions, including CD200-CD200 receptor and CD22-CD45, also termed PTPRC (10). In pathological conditions, neuronal degeneration and microglial activation following transient cerebral ischemia (11) can effect the survival of neural cells through several pathways or cause neuronal injury (12,13). Therefore, the inhibition of activated microglia may promote the survival of neurons.
Geniposide and ginsenoside Rg1 (Fig. 1) are bioactive compounds, which are extracted from Cape Jasmine Fruit (*Fructus Gardeniae*) and Sanchi (*Radix Notoginseng*), respectively (14), and are two Chinese medicines, which have been used for the treatment of stroke for thousands of years (15,16). Although the pharmacological mechanism of the individual use of geniposide or ginsenoside Rg1 on cerebral ischemia are well understood (17,18), current understanding of the effect of the combined use of geniposide and ginsenoside Rg1 on stroke remains limited. Our previous study demonstrated that the combination of geniposide and ginsenoside Rg1, prescribed as a Tongluo Jiunao injection, can reduce the expression of macrophage inflammatory protein (MIP)-1β and C-C chemokine receptor type 5 (CCR5) in oxygen-glucose deprivation (OGD)-injured microglial cells (MCs), as well as inhibit the proliferative activity of microglial cells, suggesting the therapeutic potential of the combination of geniposide and ginsenoside Rg1 on ischemic cerebral ischemia (19). However, the synergistic mechanism of multi-component combined use for complex diseases remains to be fully elucidated. Synergistic therapeutic effects can be obtained from combining effective components from Chinese herbs through cell-cell communication. The present study, based on microglia-neuron communication, aimed to determine the synergistic effect of geniposide and ginsenoside Rg1 on hypoxia-injured neurons through treatment in differently treated microglial cell-conditioned media (MC-CM).

Materials and methods

**BV2 microglia and N2a neuronal cells culture.** The murine BV2 microglia cells and N2a neuronal cells (obtained from the Cerebrovascular Diseases Research Institute, Xianwu Hospital of Capital Medical University, Beijing, China; third passage) were grown in T-25 tissue culture cell flasks at 5% CO2 and 37°C humidified atmosphere using Dulbecco's modified Eagle's medium (DMEM)/F12 (Invitrogen Life Technologies, Carlsbad, CA, USA) culture media, supplemented with 10% fetal bovine serum (FBS; GE Healthcare Life Sciences, Logan, UT, USA), 2 mM glutamine and 100 µg/ml penicillin-streptomycin. The BV2 microglial cells and N2a neuronal cells were maintained via two to three passages each week.

**Establishment of the OGD model, drug administration and preparation of different microglial cell-conditioned media.** An OGD model was used to mimic ischemia, as described in our previous study (20). For the microglial cells or N2a neuronal cells in OGD, culture medium was replaced with glucose-free DMEM, and the culture flasks (or plates) were placed into a sealed tank with a persistent low-flow (1.5 l/min) 95% N2 and 5% CO2 mixture to expire the oxygen for 20 min. The inlet and outlet ends of the tubes were then clipped, and the tank was placed into an incubator for 6 h to mimic ischemia.

Geniposide (purity>98%; batch. no. 110749-200714) and ginsenoside Rg1 (purity>95%, batch. no. 110703-201027) were chemically standardized products obtained from the National Institutes for Food and Drug Control (Beijing, China), which were validated using fingerprint chromatographic methodologies, according to the manufacturer’s instructions. The microglial cells were divided into the following five groups: Control group; model group; geniposide-treated group (geniposide; 25 µg/ml); ginsenoside Rg1-treated group (ginsenoside Rg1; 5 µg/ml); and combination-treated group (geniposide and ginsenoside Rg1 at a ratio of 1:1). The microglial cells (1x10^5 cells/ml) were preconditioned with the different drug treatments for 2 h and were maintained for another 6 h in hypoxia. The conditioned media from the five groups were collected and centrifuged at 1,000 x g for 10 min at 4°C to remove cell debris for the subsequent experiments.

The N2a neuronal cells were divided into seven groups: Control group (C), in which N2a neuronal cells were cultured in normal culture medium; model (M) group, in which N2a neuronal cells were challenged by OGD; N-MG-CM group, in which N2a neuronal cells were cultured in normal cultured microglial cell-conditioned medium; I-MG-CM group: N2a neuronal cells were OGD-injured and cultured in microglial cell-conditioned medium; C-MG-CM group, in which N2a neuronal cells were OGD-injured and cultured in geniposide and ginsenoside Rg1-treated microglial cell-conditioned medium; G-MG-CM group, in which N2a neuronal cells were OGD injured and cultured in geniposide-treated microglial cell-conditioned medium; R-MG-CM: N2a neuronal cells were OGD-injured and cultured in ginsenoside Rg1-treated microglial cell-conditioned medium. The proportion of conditioned media in each group was 100% and the incubation duration in the different conditioned media was 6 h, according to our previous study (21).

**CCK-8 assay.** N2a neuronal cells at 1x10^5 cells per well were seeded onto 96-well plates. Following incubation with the different microglial cell-conditioned media, the media in the 96-well culture plates were replaced with DMEM/F12 to avoid background interference. Subsequently, 10 µl CCK-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added to each well and incubated for 2 h at 37°C, followed by measurement using a microplate reader (Safire2; TecanGroup Ltd., Männedorf, Switzerland) with a test wavelength of 450 nm and a reference wavelength of 620 nm.

**Lactate dehydrogenase (LDH) assay.** N2a neuronal cells at 1x10^5 cells/well were seeded onto 96-well plates. A CytoTox assay kit (Promega Corporation, Madison, WI, USA) was used for the enzymatic assessment of LDH release in the N2a neuronal cells. Reagents (substrate mixture and analysis buffer) were added into the 96-wells, according to the manufacturer's instructions. A fluorescence emission of 590 nm was used for measurement using a microplate reader. The rate of LDH leakage was calculated, according to the optical density (OD) values, using the following equation: LLR = (OD value of the medium supernatant / OD value of the lysed cell supernatant) x 100%.

**Western blot analysis.** N2a neuronal cells at 1x10^5 cells/well were seeded onto 6-well plates. Western blot analysis was performed to quantify the protein expression levels of N-methyl-D-aspartate receptor subunit 1 (NMDAR1) and activated caspase-3 (Abcam, Cambridge, UK) in the N2a neuronal cells. In brief, the N2a neuronal cells were washed with ice-cold PBS and scraped in lysis buffer (Beyotime Institute...
of Biotechnology, Jiangsu, China) comprised of 50 mM Tris and 150 mM NaCl (TBS; pH 7.4), containing 1% Triton X-100, 1% Nonidet P-40, 0.5% sodium-deoxycholate, 0.1% sodium-dodecyl-sulfate, 1 mM phenylmethylsulfonyl fluoride, 15 µg/ml leupeptin, 71 µg/ml phenanthrolyne and 20 U/ml apro-
tine. The insoluble material was removed by centrifugation at
9,500 x g for 20 min at 4°C. The protein content was measured,
according to the bicinchoninic acid method (WellBiz Brands,
Inc., Highlands Ranch, CO, USA). Subsequently, 20 µg protein
was processed using SDS-PAGE separation on 12.5% poly-
acrylamide gel, and transferred onto a 0.45 µm nitrocellulose
membrane (Pierce Biotechnology, Inc., Rockford, IL, USA).
Non-specific binding sites were blocked with TBS, comprised
of 40 mM Tris, (pH 7.6) and 300 mM NaCl, containing 5%
nonfat dry milk, for 1 h at 37°C. The membrane was then
incubated with the following antibodies: Rabbit polyclonal anti-NMDAR1 (1:500; cat. no. ab17345; Abcam) and rabbit
polyclonal anti-activated caspase-3 (1:500; cat. no. ab2302;
Abcam) overnight at 4˚C, followed by incubation in a 1:5,000
dilution of horseradish-peroxidase-conjugated goat anti-rabbit
IgG (cat. no. ZB-2301; ZSGB-BIO, Beijing, China) for 1 h. Immune-
reactive proteins were detected by enhanced chemiluminescence
(Pierce Biotechnology, Inc.), according to the manufacturer's instructions. The membrane was then
incubated with stripping buffer (Applygen Technologies Inc, Beijing, China) for 0.5 h at room temperature, followed
by incubation with rabbit polyclonal anti-β-actin (1:5,000;
cat. no. ab19716; Abcam) and the corresponding secondary
antibody. The experiment was repeated in triplicate, and three
wells were used for each group.

Analysis of mitochondrial transmembrane potential. N2a
neuronal cells at 1x10^5 cells/well were seeded onto 24-well plates. The mitochondrial membrane potential was investigated
using a JC-1 probe (Beyotime Institute of Biotechnology),
which exists either as a green fluorescent monomer at depolar-
ized membrane potentials or as a red fluorescent J-aggregate
at hyperpolarized membrane potentials. The JC-1 was added
into the 24-wells, according to the manufacturer's instruc-
tions. Fluorescent images were captured and the ratios of the
mitochondrial aggregates (red) to the monomeric form of JC-1
(green) were analyzed using fluorescence microscopy (Nikon
Eclipse 80i; Nikon Corporation, Tokyo, Japan).

Transmission electron microscopy. N2a neuronal cells at
1x10^5 cells/well were seeded onto 24-well plates. The mito-
chondrial changes of the N2a neuronal cells were observed
using transmission electron microscopy. In brief, the N2a
neuronal cells were incubated with 4% glutaraldehyde and 1%
osmichydrate in sequence, and then dehydrated with acetone
(Sigma-Aldrich, St. Louis, MO, USA). Subsequently, 50-70 nm ultrathin sections were cut using an ultramicrotome
(LKB, Margate, FL, USA) and stained with 2% uranyl acetate
(Sigma-Aldrich). Transmission electron microscopy (H7650;
Hitachi, Ltd., Tokyo, Japan) was used to observe the autoph-
gosome in the cells.

Statistical analysis. All results are expressed as the
mean ± standard deviation. SPSS 13.0 software (SPSS, Inc.,
Chicago, IL, USA) was used for statistical analysis. One
way analysis of variance was used to determine statistically
significant differences among groups. P<0.05 was considered
to indicate a statistically significant difference.

Results

N2a neuronal cellular viability and LDH leakage. The
neuronal cells incubated with the different MG-CM were as-
sewed using a CCK-8 assay and LDH kits. Compared with the control
group, the viability of the N2a neuronal cells in the model
group was reduced significantly by OGD injury (P<0.001;
Fig. 2A), while LDH leakage in the N2a neuronal cells in
the model group was increased significantly following chal-
lenge by OGD injury (P<0.001; Fig. 2B), which indicated that
the N2a neuronal cells were injured by OGD. No significant
change in viability or LDH leakage were observed in the
N-MG-CM group, compared with control group, which indi-
cated that the microglial cells had no effect on the normal
cultured N2a neuronal cells. The viability was reduced and
LDH leakage was increased in the N2a neuronal cells incu-
ibrated with 1-MG-CM, with more severe injury, compared
with the group exposed to OGD injury alone. The viability
and LDH leakage of the N2a neuronal cells incubated with
C-MG-CM, G-MG-CM and R-MG-CM were recovered to
different extents. There were different effects of the MG-CM
of geniposide and ginsenoside Rg1 on the N2a neuronal

Figure 1. Chemical structure of (A) geniposide (C17H24O10; molecular weight, 300) and (B) ginsenoside Rg1 (C42H72O14; molecular weight, 800).
cells. In terms of cell viability, the effect of the ginsenoside Rg1-treated MG-CM was more marked than that of geniposide. For the LDH leakage improvement, the effect of the geniposide-treated MG-CM was more marked than that of ginsenoside Rg1. Incubation with MG-CM with the combined use of geniposide and ginsenoside Rg1 improve cell viability and suppressed LDH leakage.

Expression of NMDAR1 and activated caspase-3 in N2a neuronal cells. The present study subsequently investigated the expression levels of NMDAR1 and activated caspase-3 in the N2a neuronal cells in the different groups. As shown in Fig. 3, NMDAR1 and activated caspase-3 in the N2a neuronal cells exhibited a significant increase in expression in the model group (2.89-fold, P<0.05 and 3.73-fold, P<0.001, respectively) and I-MG-CM group (3.85-fold, P<0.01; 4.34-fold, P<0.001, respectively), compared with the control group, indicating that injury and apoptosis were induced by OGD and I-MG-CM. Compared with the model group, the use of MG-CM with ginsenoside Rg1 alone had no effect on the expression of NMDAR1. Marked suppression of the expression of NMDAR1 was observed in the N2a neuronal cells incubated in MG-CM with ginsenoside Rg1 alone, the effect of which was more marked than that of MG-CM with geniposide and ginsenoside combined (P<0.05). In terms of the expression of activated caspase-3, the effect of MG-CM with combined use of geniposide and ginsenoside was the same as that observed in the R-MG-CM group, with a clear reduction. The protein level of activated caspase-3 in the R-MG-CM group was unaffected relative to the model group.
Mitochondrial transmembrane potential of N2a neuronal cells. Almost all the cells visualized were well-spread and exhibited red or orange fluorescence in the untreated N2a neuronal cells (Fig. 4A) and cells in the N-MG-CM group (Fig. 4B). By contrast, in the OGD or I-MG-CM groups (Fig. 4C and D), the majority of fluoresced green exclusively, indicating loss of mitochondrial membrane potential. The N2a neuronal cells incubated with C-MG-CM, G-MG-CM or R-MG-CM exhibited red or orange fluorescence, which indicated an improvement relative to the model group, with C-MG-CM being the most similar to the control cells (Fig. 4E-G). Ratios of JC-1 aggregates/monomeric forms in the groups (Fig. 4H) indicated that the OGD- or I-MG-CM-induced loss of N2a neuronal cell mitochondrial membrane potential was prevented by MG-CM with geniposide and/or ginsenoside.

Mitochondrial changes in the N2a neuronal cells. The mitochondrial structure of the N2a neuronal cells in the control group was evident, with an intact mitochondrial membrane and mitochondrial cristae (Fig. 5A). There was no change to the mitochondrial structure of the cells treated with N-MG-CM (Fig. 5B). By contrast, OGD (Fig. 5C) or I-MG-CM (Fig. 5D) incubation damaged the N2a neuronal cells mitochondrial structure, characterized by disordered mitochondrial cristae arrangement or vacuolation. Following incubation with C-MG-CM, G-MG-CM or R-MG-CM, the mitochondrial structure of the N2a neuronal cells recovered to different extents, compared with the model group, with the G-MG-CM group and C-MG-CM group exhibiting more significant effects (Fig. 5E and F).

Discussion

Pharmacodynamic constituents from natural medicines have been investigated for the treatment of ischemic stroke. Multi-component treatments, characterized by two or more agents interacting with multiple targets simultaneously, are considered to be a rational and efficient form of therapy that is designed to control complex diseases (22,23), including stroke, which is a more complex disease than initially anticipated. Our previous study and those of others have already demonstrated that multi-component prescription (Tongluo Jiunao injection), composed of ginsenoside and ginsenoside Rg1 is effective for the treatment of stroke due to its anti-inflammatory, neuronprotective and neurotrophic roles (19,24). The present study aimed to elucidate the synergistic effects of microglial cell-conditioned media treated with geniposide and ginsenoside Rg1 on hypoxia-injured neurons.

According to the results obtained from the CCK-8 assay and LDH leakage assessment, certain proteins secreted from the OGD-activated microglial cells were involved in the neuronal cell damage, which indicated that the OGD-induced microglial cells generated neurotoxicity. An improvement in the I-MG-CM-induced increase of LDH was observed by using G-MG-CM, while cell viability enhancement was not significantly difference. R-MG-CM increased the neuronal cell viability, however had no effect on LDH leakage, suggesting that microglial cells treated by ginsenoside Rg1 alone improved neuron survival by secreting certain cytokines through pathways other than LDH. MG-CM with the
combined use of ginsenoside and ginsenoside Rg1 increased the cell viability and decreased LDH leakage, which demonstrated an integral effect of compatibility.

Activated microglia release glutamate, which is the major neurotoxic factor released into the extracellular space following neural injury and causes neuronal death at high concentrations (25). Glutamate activates ionotropic and metabotropic receptors, and NMDA is a type of ionotropic receptor. The action of glutamate causes Na\(^+\) and Ca\(^{2+}\) influx, which can lead to Ca\(^{2+}\) overload and subsequent Ca\(^{2+}\) dependent neural injury (26,27). Another important mechanism of OGD-injury in neural cells is apoptosis. Glutamate can also induce the loss of neurons and activation of caspase-3 (28), a key apoptotic protease-mediated cascade downstream. Several reports have demonstrated that cytochrome c-dependent caspase-3 activation is an important mechanism responsible for ischemia-induced apoptosis (29,30).

Consequently, the present study hypothesized that the secretion of microglia can be altered due to geniposide and ginsenoside Rg1 compatibility, to improve the survival of the neuronal microenvironment. The results demonstrated that microglial cells, following treatment with geniposide, down-regulated the expression of the NMDA receptor in neurons, which indicated that geniposide inhibited the expression of NMDA receptors by reducing the glutamate secretion of the ischemic microglia. The above effect was almost absent following treatment with ginsenoside Rg1, however down-regulated of caspase-3 was observed, which indicated an anti-apoptotic effect. By contrast, the MG-CM with geniposide alone had no effect on caspase-3, suggesting that other possible neuroprotective pathways were involved, including anti-inflammatory or caspase-independent pathways. Treatment with MG-CM with geniposide and ginsenoside Rg1 in combination suppressed the expression of NMDA receptor and caspase-3, which demonstrated integrated and synergistic modulation due to the compatibility of the effective components.

Mitochondria are one of the important pathways of neuronal cells apoptosis. Several studies have demonstrated that OGD induces apoptosis by favoring the release of cytochrome c and the consequent formation of the apoptotic complex (31,32). In the present study, when the neuronal cells were exposed to OGD or I-MG-CM, rapid depolarization of the mitochondrial membrane potential was observed, which is indicative of mitochondrial dysfunction. The above mitochondrial dysfunction was observed in the electron microscopy images, characterized by mitochondrial swelling and osmotic expansion, suggestive of typical mitochondrial pathological change (33). The observed mitochondrial dysfunction was alleviated by treatment with MG-CM with geniposide and ginsenoside Rg1 in combination, which indicated that the synergistic use of geniposide and ginsenoside Rg1 suppressed OGD-induced neuronal apoptosis through inhibition of the mitochondrial-mediated apoptotic pathway. Of note, it was observed that, compared with treatment with geniposide alone, ginsenoside Rg1 exhibited a more significant effect on the improvement of mitochondrial membrane potential and mitochondrial ultrastructure. Combined with the results obtained on the expression of caspase-3, it was hypothesized that, in the synergistic effect of geniposide and ginsenoside Rg1, ginsenoside Rg1 is the predominant effector in the inhibition of the mitochondrial-mediated apoptotic pathway.

From the above-mentioned results, it appears that paracrine signaling in differently treated microglia are involved in neuroprotection. However, which signal is elicited by geniposide and/or ginsenoside Rg1, and how the synergistic effect produces the observed results remains to be fully elucidated. Our previous study indicated that the synergistic use of geniposide and ginsenoside Rg1 can balance microglial TNF-α and TGF-β1 following ischemic injury (34). The balance of microglial neurotoxic factors and the neuroprotective factor, Rg1, may explain the neuroprotective effects observed following geniposide and ginsenoside synergistic use.
In conclusion, the present study demonstrated that MG-CM with ginsenoside and ginsenoside Rg1 in combination exerted a synergistic effect on the neuronal mitochondrial-mediated apoptotic pathway triggered by OGD, with ginsenoside and ginsenoside Rg1 exhibiting different regulatory effects.

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References