Geniposide protects PC12 cells from lipopolysaccharide-evoked inflammatory injury via up-regulation of miR-145-5p

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\section*{ABSTRACT}

Geniposide is an active ingredient with anti-apoptotic and anti-inflammatory properties. This study was to examine the effects of geniposide on a cell model of spinal cord injury (SCI). PC12 cells were administrated with geniposide before subjected to LPS. The effects of geniposide were analyzed by utilizing CCK-8 assay, apoptosis assay, ELISA, RT-qPCR and Western blot. We found that PC12 cells viability was unchanged by treating with geniposide. However, geniposide with concentrations of 200 or 300 $\mu$g/mL significantly mitigated LPS-evoked viability loss. Meanwhile, apoptosis driven by LPS was mitigated by geniposide, which accompanied with p53, Bax and cleaved caspase-3 down-regulation, and Bcl-2 up-regulation. Besides this, the expression and release of IL-1$\beta$, IL-6, IL-8 and TNF-$\alpha$ evoked by LPS were mitigated by geniposide. miR-145-5p was a target of geniposide. miR-145-5p expression was up-regulated by geniposide, and geniposide did not protect PC12 cells against LPS injury when miR-145-5p was silenced. Moreover, geniposide inhibited NF-$\kappa$B and JNK pathways via up-regulating miR-145-5p. In short, the present work described the neuroprotective effects of geniposide by targeting miR-145-5p. Further mechanisms involved in geniposide’s beneficial effects are correlated with the inhibited NF-$\kappa$B and JNK pathways.

\section*{HIGHLIGHTS}

1. Geniposide prevents LPS-induced injury in PC12 cells;
2. Geniposide up-regulates miR-145-5p;
3. Geniposide protects PC12 cells via up-regulation of miR-145-5p;
4. Geniposide inhibits NF-$\kappa$B and JNK pathways via up-regulation of miR-145-5p.

\section*{Introduction}

Spinal cord injury (SCI) is a serious crippling disease of central nervous system that mainly caused by physical trauma, degenerative disease and injection such as HIV and bacteria [1,2]. The pathology of SCI is divided into primary and secondary injury. The primary injury refers to damage caused by direct or indirect external forces on spinal cords. The secondary injury is a complex series of response including local oedema, ischemia, focal haemorrhage, oxidative stress and inflammatory responses, which occurs on the basis of the primary injury. Due to the non-regeneration of the neurons, patients often suffer from sensory disturbance as well as loss of sensation, sphincter or muscle function, which significantly declines the quality of life of patients [3]. Therefore, preventing spinal cord against traumatic or nontraumatic injury may be a hopeful method for reducing the morbidity of SCI.

Recently, increasing amount of literature evidenced that traditional Chinese medicines have benefits in preventing SCI and recovery of injured spinal cord [4]. Geniposide is an active ingredient of traditional Chinese herbal medicine \textit{Gardenia jasminoides} Ellis, which is extensively applied in clinic to treat stroke in China. Geniposide has various pharmacological properties, such as anti-inflammatory, anti-diabetic, anti-fibrotic, antioxidative, anti-apoptotic and anti-tumour activities [5–9]. Besides this, geniposide was revealed to be beneficial in suppressing \textit{Helicobacter pylori} infections [10]. Moreover, the neuroprotective effects of geniposide were observed in diverse cells like SH-SY5Y and PC12 to against oxygen and glucose deprivation-evoked damage [11,12]. That interests us to conduct an \textit{in vitro} study to explore the effects of geniposide on SCI.

microRNAs (miRNAs) are a sort of non-protein coding RNAs that play momentous roles in the pathology of almost all human diseases including SCI [13]. Besides this, multiple literature recognized miRNAs as major targets of traditional Chinese medicines in exerting their beneficial functions [14,15]. miR-145-5p has long been known as a tumour suppressor that its expression is frequently down-regulated in human cancers [16,17]. Apart from the anti-tumour role,
miR-145-5p was also found to be implicated in other diseases including SCI. As reported by Wang et al., miR-145-5p was enriched in rat spinal neurons and astrocytes and was low expressed following SCI [18]. A later literature confirmed the down-regulated miR-145-5p in SCI rats and further revealed its down-regulation altered myostatin signalling paralyzed by motor neuron injuries [19]. Therefore, whether miR-145-5p was an effector miRNA of geniposide was explored in this study, to provide a possible explanation of geniposide's beneficial effect. To this end, an in vitro cell model of nontraumatic SCI was constructed by stimulating PC12 cells with LPS in the present work. The effect of geniposide pre-treatment on this cell model was investigated. Besides this, whether miR-145-5p was involved in the experimental system was studied. This study will provide preliminary evidence for identification geniposide as a potential treatment for SCI.

Materials and methods

**PC12 cells**

PC12 cells (ATCC, Manassas, VA) were utilized throughout this paper. The cells were routinely in RPMI-1640 medium (ATCC). The complete growth medium was made by adding 10% horse serum and 5% fetal bovine serum (both from Gibco, Grand Island, NY) into RPMI-1640 medium. The cells were maintained at an atmosphere with 5% CO₂.

To stimulate inflammatory injury, cells were treated by 5 µg/mL LPS (Sigma-Aldrich, St. Louis, MO) for 12 h, as described elsewhere [20].

Geniposide (HPLC ≥ 98%, Sigma-Aldrich) was dissolved in PBS to a concentration of 5 mg/mL for storage. The storage solution was diluted with culture medium to 0–300 µg/mL and used for treating cells for 6 h after LPS stimulation.

**miRNAs transfection**

mo-miR-145-5p inhibitor (5'-AGGGAUUCCUGGGAAAACUUGAC-3') and its scramble negative control (NC) were from GenePharma (Shanghai, China). The inhibitor and NC with concentration of 200 nM were transfected into cell with Lipofectamine 3000 (Invitrogen, Carlsbad, CA). 48 h later, the transfection was stopped and transfection efficiency was tested using RT-qPCR.

**Cell viability**

Cells in 96-well plates (5000 cells/well) were treated by LPS alone or in combination with geniposide. CCK-8 kit (Dojindo Molecular Technologies, Kyushu) was utilized for cell viability assessment. The OD-values was read at 450 nm by a Microplate Reader (Bio-Rad, Hercules, CA).

**Apoptosis assay**

Cells in 6-well plates (5 x 10⁵ cells/well) were treated by LPS alone or in combination with geniposide. The cells were collected and stained by FITC-Annexin V and PI using a commercial Apoptosis Detection kit (Beutime, Shanghai, China). The percentage of apoptotic cells was analyzed by a FACS can (Beckman Coulter, Fullerton, CA).

**Elisa**

The cells in 24-well plates (5 x 10⁴ cells/well) were treated by LPS alone or in combination with geniposide. The culture supernatant was collected and concentrations of IL-1β, IL-6, IL-8 and TNF-α were analyzed using ELISA kits (Cusabio, Wuhan, China).

**RT-qPCR**

Total RNA was extracted using RNAiso Plus extraction reagent (Takara, Dalian, China). cDNA synthesis and qPCR were respectively carried out using PrimeScript™ RT Kit and TB Green Premix Ex Taq (both from Takara), based on the description of specification. The level of miR-145-5p was tested using Mir-XTM miRNA First-Strand Synthesis Kit and Mir-XTM miRNA qRT-PCR SYBR® Kit (both from Takara) were used. β-Actin and U6 used as reference controls. Data were calculated by 2^(-ΔΔCT) method.

**Western blot**

RIPA buffer (Beutime) was utilized for protein extraction. Immunoblotting was carried out using primary antibodies specific against p53 (ab131442), Bcl-2 (ab196495), Bax (ab53154), pro-caspase-3 (ab32499), cleaved-caspase-3 (ab2302), IL-1β (ab2105), IL-6 (ab208113), IL-8 (ab18672), JNK (ab199380), p-JNK (ab47337), all from Abcam, Cambridge, MA), TNF-α (#3707), IκBα (#9242), p-IκBα (#2859), p65 (#8242), and p-p65 (#3033, all from Cell Signaling Technology, Danvers, MA). Anti-β-actin (ab8227) antibody was used as a reference control. After probing with secondary antibody, the target bands were developed by Immobilon Western Chemiluminescent HRP Substrate (Millipore, Bedford, MA). The intensity of bands was quantified by Image Lab™ Software (Bio-Rad, Hercules, CA).

**Statistics**

Data presented as mean±SD. SPSS 19.0 software (SPSS Inc., Chicago, IL) was utilized for statistical analysis. ANOVA was used for comparison of significant difference between groups. A p value of < 0.05 was considered to indicate statistical significance.

**Results**

**Geniposide attenuates PC12 cells viability loss and apoptosis induced by LPS**

Various doses of geniposide were utilized to treat PC12 cells for 6 h and PC12 cells viability was examined to see the cytotoxicity of geniposide. As seen in Figure 1(A), PC12 cells viability was unchanged by treating with geniposide as relative to the untreated control (p > .05). However, pre-treating cells
with geniposide attenuated LPS-evoked viability loss ($p < .05$ in 200 $\mu$g/mL group and $p < .001$ in 300 $\mu$g/mL group, Figure 1(B)). 300 $\mu$g/mL was selected as geniposide-treating condition for use in the follow-up experiments. Figure 1(C) showed that apoptosis rate was declined by pretreating with geniposide as relative to LPS treated alone ($p < .05$). This observation was coupled with the repressed accumulation of p53, Bax and cleaved-caspase-3, as well as the elevated accumulation of Bcl-2 in geniposide-pretreated group (all $p < .01$, Figure 1(D,E)).

Geniposide elevates miR-145-5p expression

miR-145-5p has been recognized to be a sensitive miRNA in response to LPS stimulation, as its expression was repressed by LPS in various cell types, like MH7A [21] and H9c2 cells [22]. The present work discovered that miR-145-5p expression was also declined by LPS in PC12 cells, as relative to control ($p < .05$, Figure 3). Besides this, treating cells with geniposide remarkably elevated miR-145-5p expression even under LPS-stimulated condition ($p < .01$).

Geniposide protects PC12 cells against LPS-evoked cell damage via up-regulating miR-145-5p

Next, whether the elevated expression of miR-145-5p involved in geniposide’s cytoprotective effects was studied. To this end, miR-145-5p expression was silenced by inhibitor transfection. RT-qPCR Data in Figure 4(A) displayed that miR-145-5p expression was clearly suppressed by inhibitor transfection as compared to NC ($p < .001$). Following experiments...
displayed that the cytoprotective effects of geniposide on PC12 cells were all impeded by miR-145-5p silence. As relative to NC-transfected cells, the cells transfected with miR-145-5p inhibitor that were treated by LPS and geniposide exhibited lower cell viability \( (p < .01, \text{Figure 4(B)}) \), higher apoptosis (Figure 4(C–E)) and greater release of pro-inflammatory cytokines (Figure 4(F–H)).

**Geniposide blocks NF-κB and JNK pathways via up-regulating miR-145-5p**

Finally, the effects of geniposide on NF-κB and JNK pathways were explored. These two pathways are crucial in mediating immune response to infection. As seen in Figure 5(A–D), the phosphorylation of IκBα, p65 and JNK was accelerated by LPS \( (p < .001) \). Nonetheless, the levels of phosphorylated IκBα, p65 and JNK were declined by pre-treating with geniposide \( (p < .01 \text{ or } p < .001) \). More interestingly, the geniposide did not decline the levels of phosphorylation when miR-145-5p was silenced \( (p < .05 \text{, } p < .01 \text{ or } p < .001) \).

**Discussion**

Since 1976, PC12 cell line has been considered as a useful model system for neurobiological and neurochemical studies [23], as its versatility, ease of culture, and the large information of their proliferation and differentiation [24]. In the present study, PC12 cells were utilized to study the effect of geniposide on SCI. To this end, an in vitro cell model of nontraumatic SCI was made by stimulating PC12 cells with LPS which is always found in the membrane of Gram-negative bacteria. By testing cell viability, apoptosis and the release of pro-inflammatory cytokines, we confirmed the cell model of SCI was constructed successfully. More importantly, geniposide with concentrations ranged from 100–300 \( \mu \text{g/mL} \) had no impact on normal PC12 cells, but 200 and 300 \( \mu \text{g/mL} \) geniposide was able to eliminate LPS-induced cell viability loss, apoptosis and inflammatory response. miR-145-5p was identified as one of the effector genes of geniposide. That is, the beneficial function and the inhibitory effects on NF-κB and JNK pathways of geniposide were both flattened when miR-145-5p was silenced.

The treating strategy of SCI is mainly focused on two aspects, i.e., neuroprotection and neuroregeneration. Herein, we studied the neuroprotective effects of geniposide and found that geniposide targeted secondary injury effect including control of apoptosis and inflammation. Actually, previous studies have reported the neuroprotective effects of geniposide in various experimental systems, such as a mouse model of Alzheimer’s disease [25] and oxygen/glucose deprivation-induced cell damage [12]. However, we, for the first time, demonstrated the neuroprotective effects of geniposide against LPS injury. Besides this, the neuroprotective effects of geniposide may attribute to its anti-apoptotic and anti-inflammatory properties, which were in line with findings reported elsewhere [26,27]. More interestingly, in vitro data displayed that geniposide was able to increase the viability of LPS-injured cells. But, no such increase was observed in normal (untreated) cells. This phenomenon hinted us that

![Figure 2. Geniposide ameliorates the release of pro-inflammatory cytokines evoked by LPS. PC12 cells were treated by LPS or LPS plus geniposide. (A) The mRNA and (B) protein levels of pro-inflammatory cytokines, as well as (C) their concentrations in the culture supernant were respectively measured by RT-qPCR, Western blot and ELISA. * \( p < .05 \); ** \( p < .01 \); *** \( p < .001 \).](image)

![Figure 3. Geniposide elevates miR-145-5p expression. PC12 cells were treated by LPS or LPS plus geniposide. miR-145-5p expression was examined by RT-qPCR. * \( p < .05 \); *** \( p < .001 \).](image)
geniposide could protect PC12 cells against LPS injury without direct functions to cells growth.

In neuroscience, more and more attention has been paid on the roles of miRNAs [28,29]. miRNAs have been considered as promising biomarkers of SCI and drug targets. miR-145-5p is a widely expressed miRNA in rat spinal neurons and astrocytes [18]. Herein, we focused on investigating miR-145-5p as its expression was significantly down-regulated...
following SCI [18,19]. In line with these previous findings, in vitro assays confirmed the down-regulation of miR-145-5p in the cell model of SCI made by stimulating PC12 cells with LPS. Besides this, miR-145-5p was found to be a target of geniposide as its expression was elevated by geniposide and the neuroprotective effects of geniposide were eliminated by miR-145-5p silence. Similarly, Su et al. demonstrated that geniposide exerted protective effects on H9c2 cells in challenging LPS-induced injury via elevating the expression of miR-145 [22].

Inflammation is a main contributor of the secondary injury of SCI, in which lots of signalling pathways are involved. In central nervous system, several stimuli such as LPS can induce the activation of NF-kB and JNK pathways [30,31]. The activated signalling triggers the production of pro-inflammatory cytokines and thereby induces inflammatory response and increases vulnerability to apoptosis [32,33]. Apart from inflammation, these two signalling pathways are also critical in regulating neuron survival and death [33,34]. This here, the activated NF-kB and JNK signalling made by LPS was found to be attenuated by pre-treating PC12 cells with geniposide. Based on these evidence, we preliminary draw a conclusion that geniposide protected PC12 cells against LPS injury via blocking NF-kB and JNK pathways. The results were consistence with previous studies, in which the anti-inflammatory effects of geniposide were observed to be correlated with NF-kB and JNK pathways [35,36]. Moreover, the effects of geniposide on these two signalings were flattened when miR-145-5p was silenced, indicating geniposide inhibited NF-kB and JNK pathways via a miR-145-5p-dependent way.

To conclude, our work demonstrated the neuroprotective effects of geniposide by targeting miR-145-5p. Further mechanisms involved in geniposide’s beneficial effects are associated with the deactivated NF-kB and JNK pathways. This study enlarged our understanding of geniposide and evidenced geniposide as an adjuvant drug for preventing SCI.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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