Protective effect of *Houttuynia cordata* extract on propofol-induced injury of rat hippocampal neurons by regulating PI3K/Akt and Toll-like receptor 4/NF-κB signaling pathway

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**Objective**  This study was to detect the protective effects of *Houttuynia cordata* extract on the damage induced by propofol in hippocampal neuron of rats.

**Methods**  Propofol-induced neuron injury model and *H. cordata* extract administration were conducted. Immunofluorescence and immunoblot were conducted for the effect of *H. cordata* extract on neuronal activity and inflammation were detected in this model.

**Results**  *H. cordata* extracts increased neuronal activity, and reduced propofol-induced neuronal inflammation levels. *H. cordata* extract also reduced propofol-induced neuronal apoptosis. Mechanically, we noticed *H. cordata* extract activated phosphoinositide 3-kinase/AKT pathway and suppressed Toll-like receptor 4/nuclear factor kappaB pathway, therefore protected propofol-induced injury of rat hippocampal neurons.

**Conclusion**  Our findings provide references for anesthetic use in infants and young children. *NeuroReport* 32: 577–582 Copyright © 2021 The Author(s). Published by Wolters Kluwer Health, Inc.

**Keywords**: *Houttuynia cordata* extract, hippocampal neuron, propofol, PI3K/AKT pathway, Toll-like receptor 4/NF-κB pathway

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**Introduction**

General anesthesia refers to the temporary suppression of the central nervous system when anesthetics are inhaled through the respiratory tract, injected intravenously or intramuscularly into the body [1]. Studies have shown that general anesthesia affects brain development in young animals, and the potential neurotoxicity of anesthetics on infants and young children has aroused widespread concern [2]. Propofol, an intravenous general anesthetic, is a commonly used sedative for induction, maintenance and severe anesthesia in children [3]. Notably, there is growing evidence that propofol induces developmental neurotoxicity that can impair brain development in newborn rats and extend into adulthood [4]. Therefore, it is necessary to understand the mechanism of propofol inducing neuronal developmental toxicity in pediatric anesthesia, so as to provide ideas for developing prevention and treatment strategies.

*Houttuynia cordata* is a perennial herb that grows wild in moist, cool places in Asian countries, including China, and has a wide range of biological activities, such as anti-inflammatory, anti-cancer, antioxidant and immunomodulatory [5,6]. *H. cordata* induces the expression of brain derived neurotrophic factor, phosphorylated cAMP (Adenosine 3’5’ Cyclic Monophosphate)-Response Element Binding protein and phosphorylated AKT Serine/Threonine Kinase 1, has antidepressant effects in mice, and is used in the treatment of mast cell-derived allergic inflammatory diseases by inhibiting the nuclear translocation of nuclear factor kappaB (NF-κB) in HMC-1 cells and preventing the phosphorylation of inhibitor of κB alpha [7,8]. Interestingly, *H. cordata* can reverse the Th17/Treg balance by regulating the phosphoinositide 3-kinase (PI3K)/Akt/mammalian/mechanistic target of rapamycin signaling pathway, and alleviate the neuropathic pain induced by oxaliplatin in rats [9]. We, therefore, hypothesized that *H. cordata* may affect propofol-induced hippocampal neuron apoptosis and inflammation in rats.

The PI3K/AKT pathway is closely related to neuronal survival because many neurotrophic factors play a protective role in the brain by activating the pathway, thus inhibiting apoptosis [9,10]. Dex can reduce propofol-mediated neuronal apoptosis by enhancing the PI3K/Akt signaling pathway, and reduce the long-term neurotoxicity of propofol on rat brain development [11]. Previous studies showed circRNA00137 inhibited propofol-induced neurotoxicity and neuroinflammation through the PI3K/Akt signaling pathway and played a role in rat brain and nerve cells [12]. Therefore, activation of PI3K/Akt signaling pathway plays an important role in inhibiting propofol-induced neuronal cell injury. This study was objected to investigate the protective effect of *H. cordata* extracts on neuronal activity, and has a wide range of biological activities, such as anti-inflammatory, anticancer, antioxidant and immuno-modulatory [5,6].

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**Houttuynia cordata** extract; PPF, propofol.

Presented as means ± SD. aaa groups. Three independent experiments were performed. Data were cordata extract (50 μg/mL), propofol + **H. cordata** extract (25 μg/mL), propofol + **H. cordata** extract (50 μg/mL) groups. Cells were treated with the indicated drugs for 24 h.

**Immunofluorescence**

Primary hippocampal neurons were fixed with 4% paraformaldehyde for 10 min and then permeated with PBST (0.1 % Triton X-100 in PBS) followed by blocking with 2% BSA in PBST for 1 h. Then, cells were incubated with primary antibodies against NeuN (1:500; Santa Cruz, Dallas, Texas, USA). Then, fluorescein (FITC)-conjugated secondary antibody (Molecular Probes, Tokyo, Japan) was used at room temperature for 1 h. After washing with PBS, cells were imaged using Leica microscope (Leica, Vista, California, USA, CTR 5000).

**Cell Counting Kit-8 assay**

Cells at a density of 1000 cells were seeded into 96-well plates and cultured for 48 h upon the indicated treatment. Then, Cell Counting Kit-8 (CCK-8) was added to the wells for treatment of about 3 h. Then, the absorbance value in different groups was assessed at 490 nm wavelength.

**ELISA**

Cell lysates were obtained for tumor necrosis factor-alpha (TNF-α), (interleukin-6) IL-6 and IL-1β concentration measurement, in accordance with manufacturer’s instructions (Dakewei, Beijing, China). Cells were treated with the indicated drugs for 24 h, and then the ELISA assays were performed for assessing the proinflammatory marker levels. Briefly, samples were added into detection wells. Then, removed extra unbound solutions, biotin-conjugated specific antibodies were pipetted into each well followed by the addition of avidin conjugated horseradish peroxidase. After removal of any unbound avidin-enzyme reagent, substrate of the enzyme was added to the wells for color reaction. Stopped the reaction with a correspondent buffer and measured the intensity of the color.

**Flow cytometry**

Cells were treated with the indicated drugs for 24 h, and then the flow cytometry assays were performed. For cell apoptosis detection, cells were fixed using 70% alcohol for 24 h at −20°C and stained with FITC and propidium iodide at 37°C for 30 min. Then, cells were detected with a fluorescence-activated cell sorting Calibur flow cytometer. The number of cells in each phase was measured and recorded.

**Immunoblot analysis**

Cells were treated with the drugs for 24 h and lysed using radioimmunoprecipitation buffer (Beyotime, Shanghai, China) and with protein concentration detected with BCA

**Materials and methods**

**Isolation of primary hippocampal neurons**

Hippocampal neurons were isolated from Wistar rat embryos on embryonic day 18 utilized for primary neurons isolation. Briefly, about 20 hippocampi tissues were harvested, dissected. The attached meninges were removed carefully. Then, the hippocampus was rinsed with PBS. Digest the hippocampus with a mixture of papain (0.5 units/g, Wako, Richmond, Virginia, USA) and deoxyribonuclease I (DNase I) and maintained the mixture at 32°C for 12 min. After digestion, pipetted the mixture with a glass Pasteur pipette several times and filtered the digested solution through a cell strainer (40-µm mesh, BD Biosciences, San Jose, California, USA) to remove debris. To inactivate the enzymes, the solution was shaken gently and centrifuged for 10 min at 180g. The centrifuged supernatants were discarded and resuspended the pellet at a density of 3 x 10⁶ cells/mL. Subsequently, the cell was seeded on 12-well plates. Rat embryonic hippocampal neurons were cultured in Neurobasal medium (Gibco, Grand Island, Nebraska, USA) containing 2% B27 supplement (50x, Invitrogen, Carlsbad, California, USA) and 0.5 mM l-glutamine (Wako) at 37°C in a humidified atmosphere with 5% CO₂/95% air. The **H. cordata** extract or propofol was resolubilized in PBS before use and then added directly into the media. Cells were divided into control, propofol (100 μmol/L), propofol + **H. cordata** extract (25 μg/mL), propofol + **H. cordata** extract (50 μg/mL) and propofol + **H. cordata** extract (100 μg/mL) groups. Cells were treated with the indicated drugs for 24 h.

**Houttuynia cordata** extract elevates cell viability of primary hippocampal neurons. (a) Immunofluorescence of NeuN in rat primary hippocampal neurons. (b) CCK-8 assay revealed cell viability in control, propofol and 0.5 mM l-glutamine (Wako) at 37°C in a humidified atmosphere with 5% CO₂/95% air. The **H. cordata** extract or propofol was resolubilized in PBS before use and then added directly into the media. Cells were divided into control, propofol (100 μmol/L), propofol + **H. cordata** extract (25 μg/mL), propofol + **H. cordata** extract (50 μg/mL) and propofol + **H. cordata** extract (100 μg/mL) groups. Cells were treated with the indicated drugs for 24 h.
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Kit. Proteins with different sizes were separated using SDS-PAGE and transferred onto polyvinylidene fluoride membrane (Millipore, Burlington, Massachusetts, USA). Subsequently, the membranes were treated with 5% BSA in 0.1% Tween in TBS buffer for 2h and then dipped in the following antibody: Bax antibody (1:800 dilution, MAB4601, Millipore), Bcl-2 antibody (1:200 dilution, ab182858; Abcam, Cambridge, UK), cleaved caspase-3 (1:1000 dilution, ab32042; Abcam), glyceraldehyde 3-phosphate dehydrogenase (1:10000 dilution, ab9485; Abcam), p-PI3K (1:2000 dilution, ab141307; Abcam), PI3K (1:1000 dilution, ab32085; Abcam), p-Akt (1:1000 dilution, ab38449; Abcam) and Akt (1:1000 dilution, ab8805; Abcam). Then, the membranes were cultured...
with corresponding secondary antibodies. The bands were visualized with an enhanced chemiluminescent kit. Quantification of the targeted protein was analyzed by ImageJ software.

Statistical analysis
The data were analyzed by Graphpad. All data were expressed as means ± SD. One-way analysis of variance test was utilized to determine the difference among different groups. Significant differences were recognized at $P < 0.05$.

Results
Houttuynia cordata extract elevates cell viability of primary hippocampal neurons
Rat primary hippocampal neurons were derived from hippocampus of newborn rats. The primary neurons attached after culturing of 24 h and started to extend neurites. Then extended neurites as well as neurites with branches and spines (dendrites). After several days of culture, the cell body of neurons aggregated to form a complex and condensed neuron network. Immunofluorescence assay revealed the clear expression of NeuN, verified the nature of cells (Fig. 1a). Then, cells were treated with propofol and *H. cordata* extracts at indicated concentration and measured cell viability in these groups. As known, propofol treatment remarkably reduced the cell viability of neurons. Then, *H. cordata* extract addition, neuron viability recovered to some extent suggesting the function of *H. cordata* extract in the elevation of primary hippocampal neuron viability (Fig. 1b).

Houttuynia cordata extract reduces neuron cell inflammation induced by propofol
As propofol treatment led to obvious cell inflammation in neurons. We wondered whether the treatment of *H. cordata* extract brought about relief in inflammation. We detected the concentration of TNF-α, IL-6 and IL-1β in neurons treated with propofol of propofol and *H. cordata* extract. Obviously, propofol induction increased the accumulation of TNF-α, IL-6 and IL-1β in neurons. Then, *H. cordata* extract treatment at different concentrations dramatically reduced the level of TNF-α, IL-6 and IL-1β (Fig. 2a–c). In all, *H. cordata* extracts reduced neuron cell inflammation induced by propofol treatment.

Houttuynia cordata extract suppresses neuron cell apoptosis induced by propofol
Next, we evaluated the effect of *H. cordata* extracts in cell apoptosis in neuron cells. Flow cytometry assay depicted that propofol addition led to obvious cell apoptosis. *H. cordata* extract treatment could significant ameliorate the apoptotic cell number in neuron cells (Fig. 3a). Moreover, the protein level change of Bcl-2 elevation, Bax and cleaved caspase-2 decrease further validated the suppression of cell apoptosis caused by propofol (Fig. 3b).

Houttuynia cordata extract promotes activation of PI3K/Akt signaling pathway
The PI3K/AKT pathway was believed to be closely related to neuronal survival since many neurotrophic factors provide beneficial effects in the brain through activating this pathway, to inhibit apoptosis. We noticed the pro-apoptosis effect of *H. cordata* extract before. We attempted to test the effect of *H. cordata* extract on PI3K/AKT pathway. Consistent with our assumption, propofol-treated cells exhibited inhibited p-PI3K and p-Akt levels in neurons; however, *H. cordata* extract supplementation reversed the effect of propofol on PI3K/AKT pathway (Fig. 4a and b). Our results demonstrated the activation effect of *H. cordata* extract on PI3K/AKT pathway.

Houttuynia cordata extract inhibits Toll-like receptor-4/NF-κB signaling pathway
Toll-like receptor-4 (TLR4)/NF-κB signaling mediates many neuroinflammatory diseases. We investigated the potential involvement of TLR4/NF-κB pathway in *H. cordata* extract-mediated propofol-induced neuron
injury. In this study, we evaluate the activation state of TLR4, p-IκB and p-p65 in propofol stimulated neurons. As shown in Fig. 5a, propofol treatment effectively enhanced p-IκB, p-p65 and TLR4 expression. However, H. cordata extract treatment significantly inhibited p-IκB, p-p65 and TLR4 expression (Fig. 5a and b). Thus, these findings suggest that H. cordata extract inhibits TLR4 and NF-κB signaling pathways.

Discussion
General anesthesia has certain effects on the brain of children whose nervous system is not fully developed, such as short-term memory loss and slow reaction [13]. Though narcotic drugs used at present are generally well tolerated, with short action time and fast metabolism, general anesthetic drugs commonly used clinically can also produce neurotoxic effects on the brain of children during peak periods of development, leading to subsequent persistent and progressive cognitive dysfunction [14]. Interestingly, we found H. cordata, a type of perennial herb, had a protective effect on propofol-induced injury of rat hippocampal neurons. Our data, therefore, provide references for anesthetic use in infants and young children.

In this study, we successfully constructed a propofol-induced neuronal cell inflammation model. Propofol was known to lead to the growth cone collapse, loss of synaptic connectivity, axonal transport impairment, and the behavioral deficits of mice [15]. The model has been used in several studies. Previous study showed RTA-408 protected against propofol-induced cognitive impairment in neonatal mice [4]. Additionally, repeated propofol exposure could induce neuronal damage and cognitive impairment in aged rats [16]. Similarly, propofol was successfully used to induce inflammation and apoptosis of hippocampal neurons in rats [17]. The multiple biological activities of H. cordata have been widely revealed [18]. H. cordata could ameliorate bladder damage and improve bladder symptoms via anti-inflammatory effects in interstitial cystitis rats [8]. H. cordata could also attenuate H1N1-induced acute lung injury in mice via inhibition of influenza virus and TLR pathway [19]. H. cordata polysaccharide also alleviated intestinal injury and modulated intestinal microbiota in H1N1 virus-infected mice [19,20]. H. cordata also has a wide range of other biological activities, such as anticancer, antioxidant and immunomodulatory [19]. In this study, we found H. cordata extract had important effects on hippocampal neurons. H. cordata extract could increase neuronal activity and reduce neuronal apoptosis in hippocampal neurons. Our data suggest that H. cordata could serve as a promising drug for neuropathic disease and provide references for anesthetic use.

PI3K is an important cell signal transduction molecule, which can be activated by stimulation of extracellular signals such as growth factors, cytokines and hormones, to regulate cell proliferation, differentiation, survival and other processes [21]. AKT, also known as protein kinase B, is an important targeted kinase downstream of PI3K. This pathway is closely related to neuronal survival because many neurotrophic factors play a protective role in the brain by activating the pathway, thus inhibiting apoptosis [22]. Similar to our findings, a previous study showed that dexmedetomidine improved propofol-induced neuronal injury in rat hippocampus through the PI3K/Akt pathway [23]. Also, this pathway had protective effects of propofol on intestinal and lung injury induced by intestinal ischemia [24]. The PI3K/Akt pathway also affected propofol-induced postconditioning against focal cerebral ischemia-reperfusion injury in rats [25]. Based on these studies, our data provided evidence that PI3K/
Akt pathway affected the propofol-induced injury of rat hippocampal neurons.

Collectively, we noticed *H. cordata* extract increased neuronal activity, and reduced propofol-induced neuronal inflammation level and neuronal apoptosis. Our data further confirmed *H. cordata* extracts activated PI3K/AKT pathway and suppressed TLR4/NF-κB pathway, thereby protected rat hippocampal neuron injury induced by propofol. Our findings, therefore, provide references for anesthetic use in infants and young children.

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All data generated or analyzed during this study are included in this published article.

H.F designed the study and supervised the data collection. Y.Z analyzed the data and interpreted the data. L.Y prepare the article for publication and reviewed the draft of the article. All authors have read and approved the article.

**Conflicts of interest**

There are no conflicts of interest.

**Reference**

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