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# Isoliquiritigenin protects against blood-brain barrier damage and inhibits the secretion of pro-inflammatory cytokines in mice after traumatic brain injury

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ABSTRACT

Traumatic brain injury (TBI) caused by an external mechanical force acting on the brain is a serious neurological condition. Inflammation plays an important role in prolonging secondary tissue injury after TBI, leading to neuronal cell death and dysfunction. Isoliquiritigenin (ILG) is a flavonoid monomer with anti-inflammatory characteristic. Thus, we had investigated the potential protective effects of ILG on TBI-induced injuries and identified the mechanisms underlying it. Here, we have demonstrated that ILG preserves blood brain barrier (BBB) integrity in vivo, suppresses the activation of microglia and inflammatory responses in mice after TBI, consequently leading to neurofunctional deficits, brain oedema, structural damage, and macrophage infiltration. In vitro, ILG exerts anti-inflammatory effect, and upregulates tight junction proteins 120-β-catenin and occludin in SH-SY5Y cells under oxygen glucose deprivation/reoxygenation (OGD/D) condition. Additionally, we found that PI3K/AKT/GSK-3β signalling pathway is involved in ILG treatment for TBI. To further confirm it, we had used SC79 (ethyl 2-amino-6-chloro-4-(1-cyano-2-ethoxy-2-oxoethyl)-4H-chromene-3-carboxylate), an Akt specific activator, to activate Akt, we found that SC79 partially reduces the protective effect of ILG for TBI. Overall, our current study reveals the neuroprotective role of ILG on TBI-induced BBB damage, downregulated tight junction proteins via PI3K/AKT/GSK-3β signalling pathway. Furthermore, ILG suppresses the secretion of pro-inflammatory cytokines after TBI through inhibiting the PI3K/AKT/GSK-3β/NF-κB signalling pathway. Our findings suggest that GSK-3β is a key regulatory factor during TBI-induced secretion of inflammatory cytokines, neuronal apoptosis and destruction of BBB.

## 1. Introduction

Traumatic brain injury (TBI) is a severe traumatic nervous system condition and can trigger other neurological complications such as depression, epilepsy, and dementia [1–3]. The condition can also result in secondary sequelae involving glutamate excitotoxicity, loss of ionic homeostasis, stress, and inflammatory responses [4–6]. Destruction of blood-brain barrier (BBB) integrity is the key mechanism that triggers these complex molecular events [7] and inflammatory responses can lead to the induction of neurodegeneration and delayed neurologic function repair, which aggravates nerve cell injury and neurologic dysfunction [9]. Thus, regulating BBB permeability along with anti-inflammatory treatment is predicted to be an effective therapeutic strategy for improving outcomes after TBI.

Glycogen synthase kinase-3 β (GSK-3β) is a serine/threonine kinase that exists in all eukaryotes and many signalling pathways are regulated by GSK-3β, which is involved in glycogen metabolism, cell survival, and neuronal polarity [10–11]. Previous studies have demonstrated that inhibition of GSK-3β can induce $Ca^{2+}$-independent deposition of tight junction (TJ) components at the plasma membrane [12], and additional research has shown that GSK-3β is regulated by Akt signal pathway [13]. In other words, regulating BBB permeability is likely to be achieved by regulating the AKT/GSK-3β pathway. However, it remains unclear whether GSK-3β can in fact regulate the BBB after TBI.

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Inflammatory response is another important adverse pathological event that occurs after TBI. Some studies point to the inflammatory response being regulated by GSK-3β [14,15]. Whether GSK-3β is related to inflammatory response after TBI remains unknown, however, it is widely accepted that the NF-κB signalling pathway plays an essential role in innate immune responses and inflammation. Recent reports have shown that the expression of NF-κB, regulated by IκBα, and p50/p65, is also regulated by GSK3-β [16]. However, the protective effects of the Akt/GSK-3β/NF-κB pathway after TBI have yet to be demonstrated. Akt activation is initiated by membrane recruitment via interacting with some specific protein, and then being phosphorylated by its activating kinase, the mammalian target of rapamycin complex 2 (at serine473) and phosphoinositide dependent kinase 1 (at threonine308) [17]. Due to phosphorylation, Akt is transferred from plasma membrane to cytoplasm and nucleus [18]. SC79 is a unique specific Akt activator that inhibits Akt membrane translocation and eccentrically activates Akt in cytosol [19]. Therefore, we use SC79 to activate AKT signalling pathway.

Isoliquiritigenin (ILG) is a natural flavonoid with a chalcone structure (Fig. 5A), and exhibits a variety of biological and pharmacological activities. Some studies have demonstrated the anti-diabetic potential as well as the anti-tumour and anti-oxidative stress activities of the compound [20–22]. More recently, a study highlighted the ability of ILG to enhance BBB integrity in septic mice via attenuation of NF-κB [23]. However, it is unknown whether ILG is effective in maintaining BBB integrity after TBI. Certain studies have shown that ILG treatment can prevent macrophage activation, suppress NF-κB activation, and reduce inflammatory responses in mice [22,24]. Results of these studies suggest the chalcone compound ILG may be a new anti-inflammatory treatment candidate. However, it is still unclear whether ILG can preserve neurological function and reduce inflammatory responses after TBI. Many studies have shown that ILG is associated with the AKT/GSK-3β pathway [25,26]. However, it is still unknown if ILG is involved in the role of regulating AKT/GSK-3β signalling after TBI.

The goal of this study was to explore whether ILG has a protective effect against the inflammatory response and destruction of the BBB, and the possible signalling pathways that mediate the beneficial effects of ILG after TBI. We demonstrated that ILG suppresses the PI3K/AKT/GSK-3β signalling pathway and consequently maintains BBB integrity and inhibits inflammatory responses. Collectively, our findings suggest that ILG may be an effective new treatment for TBI.

## 2. Materials and methods

### 2.1. Animals

Male C57BL/6 mice (20–25 g) were purchased from the Animal Center of Wenzhou Medical University (Wenzhou, China). The animal study protocols were approved by the Animal Care and Use Committee of Wenzhou Medical University. The animals were housed under standard conditions, including adequate temperature and humidity control with a 12:12 h light-dark cycle and free access to water and food. All the animals were acclimatized for a minimum of 7 days in the animal care facility before any experiment. The animals were randomly divided into the following four experimental groups: sham, TBI, TBI + ILG (20 mg/kg) TBI, TBI + ILG + SC79 (0.04 mg/g) (this dose of ILG administration was based on a study of neuroprotection by ILG in an ICH mice model) (Zeng et al., 2017). All the mice were returned to separate cages under standard conditions after the surgery.

### 2.2. Reagents and chemicals

ILG was obtained from the Aladdin Company (Shanghai, China). SC79 was purchased from Beyotime Biotech Inc. (Jiangsu, China). Anti-β-catenin antibody, anti-Akt and anti-p-Akt anti-β-catenin antibody, anti-NF-κB antibody, anti-p-NFκB antibody, anti-GSK3β antibody, and anti-IL-6 antibody were purchased from Cell Signalling Technology (Danvers, MA, USA). Anti-β-catenin, anti-p120-catenin, anti-p-GSK3β antibody, anti-CD68 antibody, anti-Iba1 and anti-TNFα antibody were purchased from Abcam (Cambridge, MA, USA). Anti-Mouse secondary antibodies and anti-rabbit secondary antibodies were purchased from MultiSciences Biotech Co. (Hangzhou, China). IL-6 and TNF-α enzyme-linked immunosorbent assay (ELISA) kits were purchased from eBioscience (San Diego, CA, USA).

### 2.3. Development of TBI mouse model

The animal model of TBI was used as previously described [24]. First, the mice were anaesthetized with 4% choral hydrate (10 ml/kg, ip), positioned in a stereotaxic system (David Kopf Instruments, Tujunga, California) under aseptic conditions, a right craniotomy was performed using a portable drill, and a 3-mm diameter manual trephine (Roboz Surgical Instrument Co., Gaithersburg, MD) was used to penetrate the right parieto-temporal cortex for removal of the bone flap. The pneumatic cylinder was used to control the cortical impact. The impact velocity was set at 4 m/s with a 1.5-mm flat-tip impounder, and the impact duration was 150 ms, after which the scalp was sutured closed, and the mice were returned to their cages to recover for 24 h. The animals in the sham group underwent the surgical procedure without cortical impact. SC79, 0.04 mg/g, ip was administered 30 min after the TBI. ILG (20 mg/kg, this dose of ILG was based on studies of ILG treatment for ICH mouse model [22]) was intraperitoneal (ip) injected 30 min into mouse after TBI, which was dissolved in PEG400 (20%). PEG400 is a commonly used as non-toxic solvent, and 20% concentration of PEG400 has been shown to be safe and has no effect on its inflammation and other indicators of mice [44–46].

### 2.4. Neurological evaluation

The sensorimotor Garcia Test [28], was administered in mice at 24 h and 72 h following the TBI. There were 7 individual tests to be performed by every mouse that represented spontaneous activity (1), axial sensation (2), vibrissae proprioception (3), and limb symmetry (4), as well as the animal's ability to perform lateral turning (5), forelimb outstretching (6), and climbing (7). One point was given for each sub-test as follows: 0 (worst performance) to 3 (best performance), and the total score was taken as the sum of all the sub-tests (maximum score of 21). The sequence of tests was randomized and performed by an investigator who was blinded to the experimental groups.

### 2.5. Brain water content

The left cerebral hemispheres of the mice were separated and placed on ice 24 h after TBI. The brain cortical samples were harvested and immediately weighed to evaluate the wet weight (WW), then dried in an oven for 48 h at 80 °C and weighed again to investigate the dry weight (DW). Brain water content was calculated as $([WW - DW]) \div WW \times 100\%$.

### 2.6. Cell culture

SH-SY5Y cells were obtained from the China Center for Type Culture Collection (Wuhan University, China, 22-4-2015, http://www.cctcc.org) and maintained at 37 °C in a humidified atmosphere containing 5% CO2. The cells were cultured in DMEM/F12 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (FBS, Invitrogen) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). SH-SY5Y cells have small, round cell bodies, scant cytoplasm and neurite-like cytoplasmic processes, and form dense mounding aggregates (pseudoganglia).