Effects of Flower Buds Extract of *Tussilago farfara* on Focal Cerebral Ischemia in Rats and Inflammatory Response in BV2 Microglia

Ji Hye Hwang, Vinoth Kumar R, Seok Yong Kang, Hyo Won Jung, and Yong-Ki Park

**ABSTRACT**

Objective: To investigate the effects of the flower buds extract of *Tussilago farfara* Linné (Farfarae Flos; FF) on focal cerebral ischemia through regulation of the inflammatory responses in activated microglia. Methods: Brain ischemia was induced in Sprague-Dawley rats by a transient middle cerebral artery occlusion (tMCAO) for 90 min and reperfusion for 24 h. Twenty rats were randomly divided into 4 groups (n=5 per group): normal, tMCAO-induced ischemic control, tMCAO plus FF extract 300 mg/kg-treated, and tMCAO plus MK-801 1 mg/kg-treated as reference drug. FF extract (300 mg/kg, p.o.) or MK-801 (1 mg/kg, i.p.) was administered after reperfusion. Brain infarction was measured by 2,3,5-triphenyltetrazolium chloride staining. Neuronal damage was observed by haematoxylin/eosin, Nissl staining and immunohistochemistry using anti-neuronal nuclei (NeuN), anti-glia fibrillary acidic protein (GFAP), and anti-CD11b/c (OX42) antibodies in ischemic brain. The expressions of inducible nitric oxide synthase (iNOS), tumor necrosis factor (TNF-α), and hypoxia-inducible factor-1α (HIF-1α) were determined by Western blot. BV2 microglial cells were treated with FF extract or its main bioactive compound, tussilagone with or without lipopolysaccharide (LPS). Nitric oxide (NO) production was measured in culture medium by Griess assay. The expressions of iNOS, COX-2 and pro-inflammatory cytokines mRNA were analyzed by reverse transcription-polymerase chain reaction. The expression of iNOS, and COX-2 proteins, the phosphorylation of ERK1/2, JNK, and p38 MAPK and the nuclear expression of NF-κB p65 in BV2 cells were determined by Western blot. Results: FF extract significantly decreased brain infarctions in ischemic rats (P<0.01). The neuronal death and the microglia/astrocytes activation in ischemic brains were inhibited by FF extract. FF extract also suppressed iNOS, TNF-α, and HIF-1α expression in ischemic brains. FF extract (0.2 and 0.5 mg/mL, P<0.01) and tussilagone 20 and 50 μmol/L, P<0.01) significantly decreased LPS-induced NO production in BV2 microglia through downregulation of iNOS mRNA and protein expression. FF extract and tussilagone significantly inhibited LPS-induced expression of TNF-α, IL-1β, and IL-6 mRNA, and also suppressed the phosphorylation of ERK1/2, JNK and p38 MAPK and the nuclear expression of NF-κB in a dose-dependent manner. Conclusion: FF extract has a neuroprotective effect in ischemic stroke by the decrease of brain infarction, and the inhibition of neuronal death and microglial activation-mediated inflammatory responses.

**KEYWORDS**

*Tussilago farfara*, focal cerebral ischemia, inflammation, microglia, tussilagone

Ischemic stroke in the brain (cerebral ischemia) occurs when there is insufficient blood flow to the brain for normal function, and leads to neuronal apoptosis, resulting in a hypoxic brain injury. A drug is used under some circumstances to reopen the artery and allow blood to flow to the brain again. Currently, recombinant tissue-type plasminogen activator (tPA) is the only approved drug by the Food Drug Administration for the treatment of ischemic stroke, but it increases risk of brain bleeding, so new strategies for stroke treatment are needed.

Brain inflammation is important in the pathology of ischemic stroke. Clinically and experimentally, the brain responds to ischemic damage with acute and systemic inflammatory processes that are characterized by rapid activation of resident cells, mainly microglia, production of inflammatory mediators such as nitric oxide (NO), and proinflammatory cytokines, and infiltration of various inflammatory cells including neutrophils, different subtypes of T cells, monocyte/macrophages, ...
and other cells into the ischemic brain tissue. Therefore, inhibition of this inflammatory process can decrease the infarct size and improve neurological deficit in ischemic stroke. Although anti-inflammatory approaches have proven successful in animal models, attempts to translate this into clinical application have been unsuccessful because of the heterogeneity in pathological mechanisms underlying post-ischemic inflammation and the uncertain time window at which inflammation could be targeted in human disease. However, there is great interest in controlling microglial activation-mediated inflammatory response for potential therapy of ischemic stroke.

Medicinal plants have long been used in traditional medical practice to treat brain diseases and to maintain the normal cerebral blood flow. MUCH many recent researches are focused on finding neuroprotective medicinal plants, because they are thought to be better treatment for ischemic stroke than current drugs. The flower buds of 
Tussilago farfara Linné (Compositae; Farfarae Flos; FF) are traditionally used to treat cough, wheezing, bronchitis, and asthmatic disorders through a humidoterapeutic effect in lung and clearance of phlegm. Experimentally, FF has biological properties including neuroprotective and antioxidant, antimicrobial, and anti-inflammatory effects, and inhibits arachidonic acid metabolism, which serves as a substrate to generate inflammatory mediators such as prostaglandins, thromboxanes, leukotrienes, and reactive oxygen radicals and inhibits NO synthesis in macrophages activation.

In this study, we investigated the effects of FF extract on focal cerebral ischemia in rats and inflammatory responses in microglial activation with the bioactive compound, tussilagone (TG).

**METHODS**

**Preparation of FF Extract**

The flower buds of FF and TG (14B1001-04) were from Korean Food and Drug Safety Administration (KFDA) and A plant voucher specimen was deposited in the Herbarium of KFDA under registration number EAB333 (standard compound in CP: TG 0.07%). TG was previously analyzed as a bioactive marker compound of FF. FF extract was prepared by the following standard procedure. The dried flower buds (100 g) were ground into small pieces, then extracted in 1 L of 70% ethanol at 95 °C for 3 h, filtered through a two-layer mesh, and concentrated under vacuum pressure till the solvent was completely removed. The final yield of FF extract from the whole procedure was 48% of the dried powder, and it was stored at 4 °C until use.

**Animals**

Twenty male Sprague-Dawley (SD) rats weighing 280 ± 10 g (specific pathogen free grade, Orient Bio Inc., Gyeonggi-do, Korea) were used in all experiments. The animals were housed under controlled environmental conditions with ambient temperature of 23 ± 1 °C, relative humidity of 50% ± 10%, 12 h light/dark cycle and free access to food and water. All animal care and use followed the animal welfare guidelines issued by the Korean National Institute of Health and the Institutional Animal Care and Use Committee at the Dongguk University (IACUC-2016-018).

**Preparation of Ischemic Stroke In Vivo Model and Drug Treatment**

Ischemic stroke was induced in rats by standard procedure. The rats were anesthetized with 4% enflurane and maintained using 1% enflurane in a mixture of 30% oxygen and 70% nitrous oxide during the experiment. Rectal temperature was measured with a rectal probe and maintained at 37 °C with a heating pad (FHC Inc., ME, USA) during the surgical procedure. The left common carotid artery (CCA) was exposed, separated carefully from the vagus nerve, and then ligated at the more proximal side through a right paramedian incision. The external carotid artery (ECA) was ligated. The occipital artery and the pterygopalatine artery were coagulated. Ischemia was produced by advancing the tip of a rounded 3-0 nylon suture into the CCA through the ECA. After placement, the intraluminal suture was secured with a suture tied around the ECA. Reperfusion was produced by withdrawal of the intraluminal suture. In the sham group, the ECA was surgically prepared for the insertion of the filament, but the filament was not inserted. Twenty animals were randomly divided into 4 groups (n=5 per group): normal; transient middle cerebral artery occlusion (tMCAO) with water treatment (vehicle); tMCAO with FF extract at 300 mg/kg body weight; and tMCAO with MK-801 (Dizocilpine; Sigma-Aldrich, St. Louis, MO, USA) at 1 mg/kg as a reference drug, which is an uncompetitive antagonist of the N-methyl-D-aspartate acid receptor (NMDA) receptor. FF extract (p.o.) and MK-801 (i.p.) were administered once as soon as reperfusion started after MCAO for 90 min.

**Measurement of Infarct Volume**

After the neurological deficit score was determined,