

Neochlorogenic Acid Inhibits Lipopolysaccharide-Induced Activation and Pro-inflammatory Responses in BV2 Microglial Cells

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Abstract Microglia is the resident innate immune cells that sense pathogens and tissue injury in the central nervous system. Microglia becomes activated in response to injury, infection, and other stimuli that threaten neuronal survival. Microglia activation plays an important role in neurodegenerative diseases. Neochlorogenic acid (NCA) is a natural polyphenolic compound found in dried fruits and other plants. Although previous studies have shown that phenolic acids including NCA have outstanding antioxidant, antibacterial, antiviral, and antipyretic activities, there has not yet been investigated for anti-inflammatory effects. Therefore, for the first time we have examined the potential of NCA to inhibit microglial activation and pro-inflammatory responses in the brain. We found that lipopolysaccharide-induced inducible nitric oxide synthase, and cyclooxygenase-2 expression, and nitric oxide formation was suppressed by NCA in a dose-dependent manner in BV2 microglia. NCA also inhibited the production of pro-inflammatory mediators, tumor necrosis factor- α and interleukin-1 beta. Furthermore, phosphorylated nuclear factor-kappa B p65 and p38 mitogen-activated protein kinase activation were blocked by NCA. Taken together, these results suggest that NCA exerts neuroprotective

effects through the inhibition of pro-inflammatory pathways in activated microglia.

Keywords BV2 · Neochlorogenic acid · Lipopolysaccharide · Neuroinflammation · Microglia activation

Introduction

Microglia is the resident macrophages of the central nervous system (CNS). They play a major role in the defense against neuronal injury and contribute to homeostasis in the brain [1–3]. However, activation of microglia can cause chronic neuroinflammation due to inflammatory and pathophysiological stimuli such as lipopolysaccharide (LPS). LPS is a bacterial endotoxin and is widely used to create inflammation in the brain [4]. Activated microglia releases pro-inflammatory mediators and cytokines, including nitric oxide (NO), cyclooxygenase-2 (COX-2), tumor necrosis factor- α (TNF- α), and interleukin-1 beta (IL-1 β), eventually leading to neuronal dysfunction and cell death [5–7]. Clinical evidence has shown that microglial activation and neuroinflammation are implicated in the pathogenesis of neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, brain ischemia, and Huntington's disease [8–10]. Thus, regulating microglial activation and pro-inflammatory responses may help patients with neurodegenerative diseases by reducing brain damage or neuronal cell death.

Neochlorogenic acid (NCA) (Fig. 1), an isomer of chlorogenic acid, is a natural polyphenolic compound found in some types of dried fruits and a variety of other plant sources such as peaches [11]. Previous studies have shown that phenolic acids have outstanding antioxidant,

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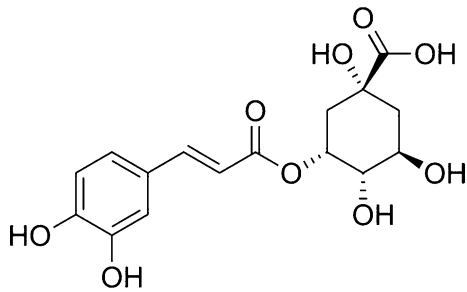


Fig. 1 The chemical structure of neochlorogenic acid (NCA)

antibacterial, antiviral, and antipyretic activities [12–14]. In addition, geniposide, another phenolic acid, has shown analgesic and anti-inflammatory effects [12, 15]. However, the effects of NCA on inflammation have not yet been investigated. Therefore, for the first time we examined the potential of NCA to inhibit microglial activation and pro-inflammatory responses in the brain.

Materials and Methods

Materials

Murine microglial BV2 cells were provided to us by Dr. Myungsook Oh from Kyunghee University (Seoul, Korea). LPS, NCA, dexamethasone, Griess reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (St. Louis, MO, USA). Cell culture media [Dulbecco's modified Eagle's minimal essential medium (DMEM)], antibiotic–antimycotic solution, fetal bovine serum (FBS) and other ingredients required to culture BV2 cells were purchased from Life Technologies (Grand Island, NY, USA). Protease and phosphatase inhibitor cocktail tablets were supplied by Thermo Scientific (Rockford, IL, USA). Primary and secondary antibodies used for western blot analysis were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Cell Signaling Technology (Danvers, MA, USA), and BD Biosciences (San Jose, CA, USA). ELISA kits were obtained from Enzo Life Sciences (Farmingdale, NY, USA).

Cell Culture and Treatment

Mouse BV2 microglial cells were maintained in DMEM, supplemented with 5 % FBS and 1 % antibiotic–antimycotic in a humidified incubator with 5 % CO₂ at 37 °C. NCA and dexamethasone as positive control were dissolved in DMSO to a final concentration of 10 mM for the stock solution. Treatments with LPS and/or NCA were carried out under serum-free conditions.

NO Production Assay

NO production of LPS-activated BV2 cells was measured by the Griess test. BV2 cells were plated into 96-well plates at a density of 3×10^5 and treated with LPS (4 µg/ml), Dexamethasone (10 µM) and NCA (0, 10, 50, and 100 µM) for 24 h. The culture supernatant from each sample (50 µl) was mixed with the same volume of the Griess reagent (1 % sulfanilamide, 0.1 % N-1-naphthylethylenediamine dihydrochloride in 5 % phosphoric acid) and then incubated for 10 min at room temperature. The absorbance values were measured at 540 nm using a microplate reader, and NaNO₂ was used as the standard to determine NO₂ concentrations.

MTT Cell Viability Assay

Cell viability was confirmed by the MTT assay. The medium was removed from the wells, MTT was added, and the samples were then incubated for 3 h at 37 °C. The formazan crystals were dissolved by adding DMSO, and the absorbance values were measured at 540 nm using a microplate reader.

Enzyme Immunoassay

The inhibitory effects of NCA on the production of the proinflammatory cytokines TNF-α and IL-1β were determined by an enzyme-linked immunosorbent assay (ELISA). Conditioned media were analyzed as per the manufacturer's instructions with a mouse enzyme immunoassay (EIA) kit (Enzo Life Sciences).

Western Blot Analysis

BV2 cells were seeded in 6-well plates. The cells were lysed with Protein Extraction Solution (Elpis biotech, Daejeon, Korea) and cell lysates were separated into the nuclear and cytosol fractions using a nuclear/cytosol fraction kit (Biovision Inc., Milpitas, CA, USA). The protein concentration was measured using a protein assay dye reagent with bovine serum albumin as the standard. The same amount of protein from each sample was separated by 10 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The membranes were blocked with 5 % skimmed milk and incubated with different primary antibodies inducible nitric oxide synthase (iNOS), COX-2, β-actin, p38 mitogen-activated protein kinase (p38 MAPK), phospho-p38 MAPK, nuclear factor kappa B p65 (NF-κB p65), and phospho-NF-κB p65 overnight at 4 °C. Then, the horseradish peroxidase-conjugated secondary antibodies were incubated for 1 h. Antibody detection was carried out using ECL reagent

(Thermo Scientific, USA) and visualized by ChemiDoc (Bio-Rad Laboratories, Hercules, CA, USA). The intensities of the bands were normalized to the β -actin band using Image Lab Software (Bio-Rad).

Statistics Analysis

All statistical parameters were calculated using GraphPad Prism 3.0 software (GraphPad Software Inc., San Diego, CA, USA). Values were expressed as the mean \pm standard error of the mean (SEM). The results were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Differences with a *P* value less than 0.05 were considered statistically significant.

Results

Inhibitory Effects of NCA on NO Production in LPS-Induced BV2 Microglia Cells

To determine the effects of NCA on the LPS-induced production of the inflammatory mediator NO in microglia, the NO levels in the cell culture medium were measured using the Griess assay (Fig. 2a). NCA or dexamethasone treated alone was not any affected to NO production. LPS-treated BV2 cells showed a marked increase of cellular nitrite levels compared to the control group. In the presence of NCA, NO production by LPS-induced BV2 cells was inhibited in a dose-dependent manner, with levels significantly inhibited after treatment with 50 and 100 μ M NCA. Especially 100 μ M NCA had similar inhibitory effects to dexamethasone. Dexamethasone is widely used as a positive control to anti-inflammatory

effect and used 10 μ M, which had a marked effective dose in previous studies [16].

No Effect of NCA on Cell Viability

The potential cytotoxicity of NCA on LPS-induced BV2 cells was evaluated using the MTT assay. Our results showed that cell viability was not affected at any concentration used (Fig. 2b), confirming that the inhibition of NO production in LPS-induced BV2 cells was not due to cytotoxic effects of NCA. Moreover, NCA was dissolved in DMSO to less than 0.01 % and it was not affected to cell death (data not shown).

Inhibitory Effects of NCA on iNOS and COX-2 Protein Expression in LPS-Induced BV2 Microglia Cells

In order to determine whether the inhibitory effect of NCA on NO production was associated with a downstream modulation of iNOS and COX-2 expression, we measured the protein levels using western blotting. The protein levels of these enzymes were analyzed in the whole cell lysates 24 h after treatment with LPS. As shown in Fig. 3, iNOS and COX-2 levels were increased in LPS-induced BV2 cells, but this increase was significantly inhibited after treatment with 50 and 100 μ M NCA.

Effects of NCA on the LPS-Induced Release of Pro-inflammatory Cytokines in BV2 Microglia Cells

The effects of NCA on the secretion of pro-inflammatory cytokines such as TNF- α and IL-1 β were investigated by ELISA in LPS-induced BV2 microglial cells (Fig. 4). The

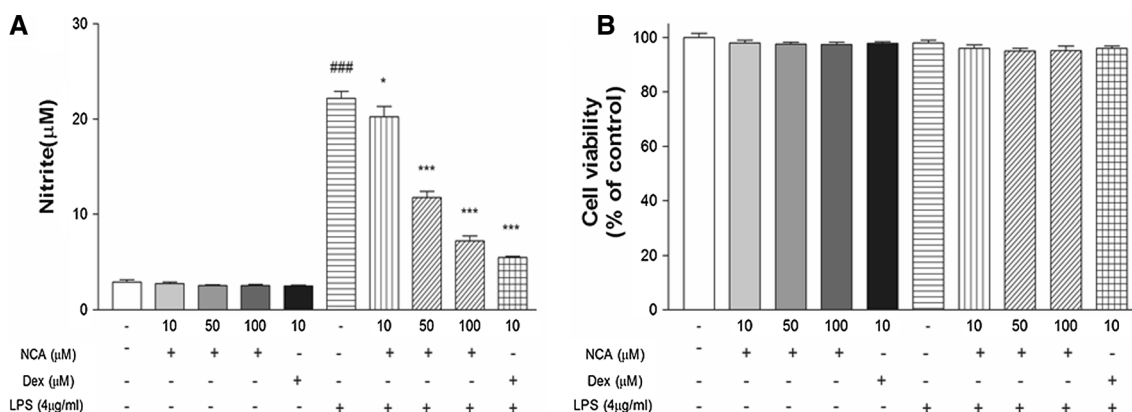


Fig. 2 Effects of neochlorogenic acid (NCA) on nitric oxide (NO) production and cell viability in lipopolysaccharide (LPS)-stimulated BV2 microglial cells. The cells were treated with or without LPS (4 μ g/ml) and NCA (10, 50, and 100 μ M) for 24 h. Dexamethasone (10 μ M) was used for positive control. Then, **a** nitrite concentrations in the medium were determined using the Griess reagent. **b** Cell

viability was confirmed by the MTT assay. Each value indicates the mean \pm SEM and represents results obtained from three independent experiments. ^{###}*P* < 0.001 indicates that the mean value was significantly different from the control group receiving no treatment. ^{*}*P* < 0.05 and ^{***}*P* < 0.001 indicate that the mean value was significantly different from the group receiving LPS alone

results showed that the expression of TNF- α and IL-1 β increased in LPS-induced BV2 cells, but these increases were blocked in the NCA-treated group. Specifically, treatments with 50 and 100 μ M NCA significantly inhibited the increase in TNF- α and IL-1 β expression relative to treatment with LPS alone.

Degradation Effects of NCA on Activation of p38 MAPK and Nuclear Translocation of NF- κ B p65 in LPS-Induced BV2 Microglia Cells

To clarify that the inhibition of the increases of iNOS and COX-2 expression was mediated by an NCA-induced

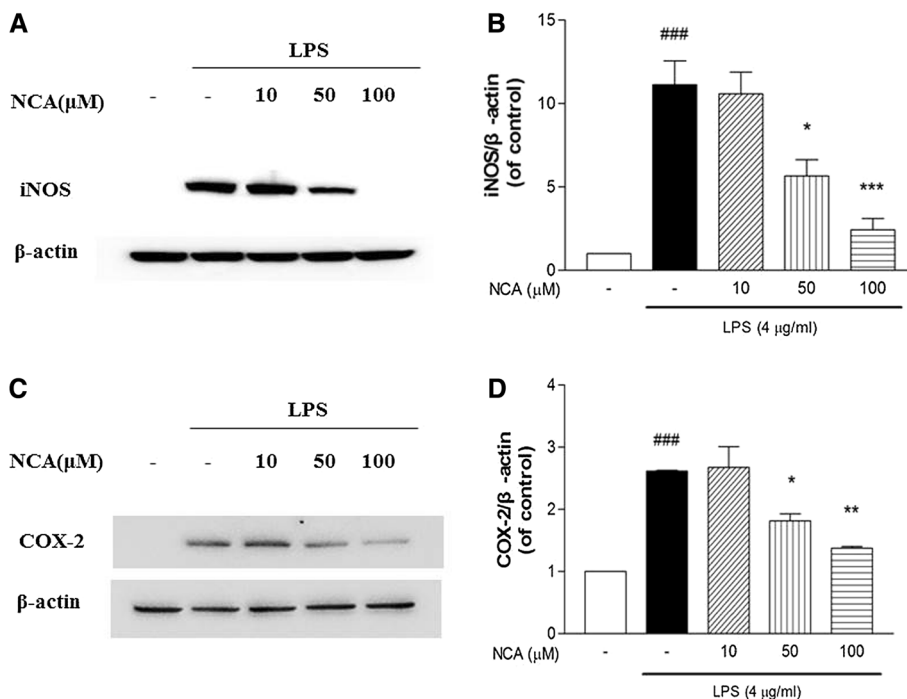


Fig. 3 Inhibitory effects of neochlorogenic acid (NCA) on inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) protein induction in lipopolysaccharide (LPS)-stimulated BV2 microglial cells. After treating with LPS (4 μ g/ml) and NCA (10, 50, and 100 μ M) for 24 h, the cell lysates were prepared and western blot analysis was performed using anti-iNOS and anti-COX-2 antibodies. Protein levels of iNOS (**b**) and COX-2 (**d**) were normalized to β -actin.

Representative band images from individual experiments are shown in **a** and **c**. Each value indicates the mean \pm SEM and represents results obtained from three independent experiments. ### P < 0.001 indicates that the mean value was significantly different from the control group receiving no treatment. * P < 0.05 and *** P < 0.001 indicate that the mean value was significantly different from the group receiving LPS alone

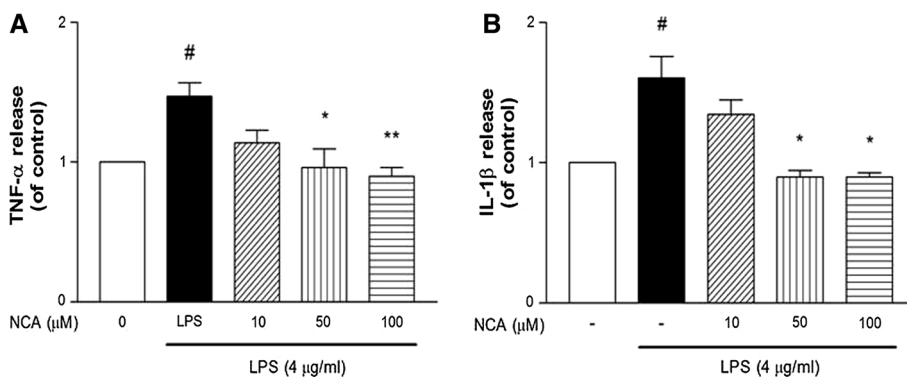


Fig. 4 Inhibitory effects of NCA on tumor necrosis factor- α (TNF- α) and interleukin-1 beta (IL-1 β) production in lipopolysaccharide (LPS)-stimulated BV2 microglial cells. The cells were treated with LPS (4 μ g/ml) and NCA (10, 50, and 100 μ M) for 24 h. The levels of **a** TNF- α and **b** IL-1 β present in the supernatants were measured by

ELISA. Results indicate the mean \pm SEM and represent results obtained from three independent experiments. # P < 0.05 indicates that mean value was significantly different from the control group receiving no treatment. * P < 0.05 and ** P < 0.01 indicate that mean value was significantly different from the group receiving LPS alone

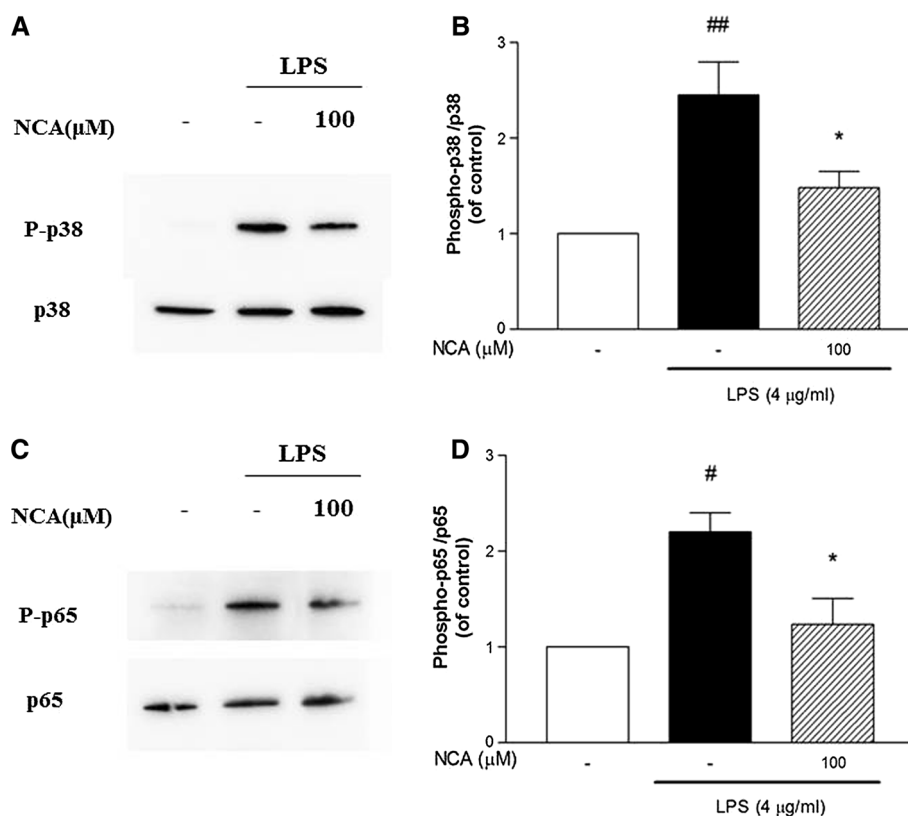


Fig. 5 Inhibitory effects of neochlorogenic acid (NCA) on activation of p38 mitogen-activated protein kinase (p38 MAPK) and nuclear translocation of NF- κ B p65 in liposaccharide (LPS)-stimulated BV2 microglia cells. The cells were treated with LPS (4 μ g/ml) and NCA (0 and 100 μ M) for 30 min. The effects of NCA on phosphorylation of p38 MAPK and NF- κ B p65 were investigated using western blotting analysis. For p38 MAPK detection, antibodies directed against phosphorylated p38 MAPK (p-p38) and total p38 MAPK were used in whole protein lysate. For NF- κ B p65 detection, antibodies directed against phosphorylated NF- κ B p65 (p-p65) and total NF- κ B

p65 were used in the nuclear fraction. Levels of p-p38 (**b**) and p-p65 (**d**) were normalized to total p38 MAPK and total NF- κ B p65. Representative band images from each experiment are shown in **a** and **c**. Each value indicates the mean \pm SEM and represents results obtained from three independent experiments. [#] $P < 0.05$ and ^{##} $P < 0.01$ indicate that mean value was significantly different from the control group receiving no treatment. ^{*} $P < 0.05$ indicates that mean value was significantly different from the group receiving LPS alone

blockade of LPS-induced microglial activation, the effects of NCA on activation of phosphorylated p38 MAPK and NF- κ B p65 were examined. Figure 5a shows that NCA at 100 μ M, the most effective concentration in other assays, also attenuated the LPS-induced increase of p38 MAPK phosphorylation compared with the group treated with LPS alone. NCA also markedly attenuated the LPS-induced increase of NF- κ B p65 phosphorylation compared with the group treated with LPS alone (Fig. 5b).

Discussion

NCA is reported to be a major bioactive component of dried fruits and other plants, and has been demonstrated to have various beneficial effects [12–14]. However, the effects of NCA on inflammatory processes are still unclear. Our present results provide, for the first time, evidence for the anti-inflammatory effects of NCA. In this study, NCA

showed a reduction of LPS-induced NO production by suppressing iNOS and COX-2 protein expression and production of pro-inflammatory cytokines, such as TNF- α and IL-1 β , in BV2 microglia cells. In addition, phosphorylated p38 MAPK and NF- κ B p65 were also inhibited by NCA in activated microglia. These results suggest that NCA exhibited anti-inflammatory effects in BV2 microglial cells.

Microglia is sensitive to changes in their microenvironment and is activated in response to infection or injury. Microglia-mediated neuroinflammation is a major component enhancing neurodegeneration. Overactivation and dysregulation of microglia cause neuronal death and brain injury, most likely due to the production of high levels of cytotoxic and pro-inflammatory factors [17–19]. Microglial activation is pivotal in the initiation of neuroinflammation and prolonged activation of microglial cells leads to excessive release of NO by iNOS in the brain [20]. NO plays an important role in cell survival and NO can induce

neuronal cell death [21]. COX-2 is upregulated in response to various inflammatory stimuli and NO functions to enhance its expression and activity [22]. A number of studies have shown that the expression of iNOS and COX-2, key enzymes for NO, is upregulated in activated glial cells. Pro-inflammatory cytokines such as TNF- α and IL-1 β are also initiators of the inflammatory response and mediators in the pathogenesis of neurodegenerative disorders [23, 24]. Previous evidence has demonstrated the damaging and inflammatory effects of these cytokines in neuropathology [25]. Thus, downregulation of these inflammatory molecules could be beneficial for alleviating the progression of neurodegeneration caused by microglial activation. In the present study, treating LPS-stimulated BV2 cells with NCA effectively decreased iNOS, COX-2, TNF- α , and IL-1 β levels and the release of their respective end-products, including NO. Further, the MTT assay results demonstrated that this effect was not due to any cytotoxicity of NCA.

The critical transcription factor, NF- κ B p65, is part of a well-known signaling cascade that contributes to the production of an array of inflammatory mediators including COX-2, iNOS, and cytokines. Generation and amplification of TNF- α and IL-1 β have been reported to be regulated by NF- κ B p65 [26–28]. In addition to NF- κ B p65 signaling, MAPKs are also important signaling molecules involved in the production of pro-inflammatory mediators and cytokines and the modulation of NF- κ B p65 in microglia [29, 30]. One of the MAPK families, p38, is related to LPS signaling in microglial cells, which respond to pro-inflammatory cytokines [31, 32]. In this study, we detected the inhibitory effects of NCA on LPS-induced activation of NF- κ B p65 and p38 MAPK. These results suggest that NCA-mediated inhibition of pro-inflammatory mediators and cytokines is associated with the downregulation of the MAPK signaling pathway.

In conclusion, the present study demonstrates that NCA inhibits LPS-induced NO production by iNOS and COX-2 protein expression in BV2 microglial cells. NCA also inhibits the production of the pro-inflammatory cytokines, TNF- α and IL-1 β , and inflammation mediated via phosphorylated NF- κ B p65 and p38 MAPK. Our findings suggest that NCA may provide a beneficial effect in the treatment of inflammatory brain damage induced by microglial activation, which should be verified through more precise in vivo model experiments. To our best knowledge, in vivo studies on permeability of NCA into brain and anti-inflammatory effect have not performed. We hope that these studies would contribute to the treatment of neurological disorder involved in microglial activation.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and animal rights This article does not contain any studies with human participants or animals performed by any of the authors.

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