Original Research Article

Inhibition of Lipopolysaccharide-Induced Neuroinflammatory Events in Bv-2 Microglia by Chestnut Peel Extract

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Abstract

Purpose: To evaluate the protective effects of chestnut (Castenea cranata Siebold & Zucc., Fagaceae) peel extract on stimulated BV-2 microglial cells as well as its anti-oxidant properties.
Methods: The ethyl acetate fraction of C.cranata peel (CCP) extract was used in the study to evaluate the anti-neuroinflammatory effects in BV-2 microglial cells. Cell viability was performed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyl-tetrazolium bromide (MTT) assay. Lipopolysaccharide (LPS) is used to activate BV-2 microglia. Nitric oxide (NO) levels were measured using Griess assay. Inducible NO synthase (iNOS) expressional levels were measured by Western blot analysis. Tumor necrosis factor-alpha (TNF-α) production was evaluated by enzyme-linked immunosorbent assay (ELISA). Anti-oxidant properties were evaluated by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging assay.
Results: LPS-activated excessive release of NO in BV-2 cells was significantly inhibited (p < 0.001 at 100 µg/mL) by CCP extract. LPS-induced excessive production of inflammatory mediator such as iNOS was also significantly attenuated by CCP extract. Further, CCP extract significantly and dose dependently inhibited the TNF-α levels in LPS-induced BV-2 microglial cells (p < 0.05 at 20 µg/mL, p < 0.01 at 40 µg/mL and p < 0.001 at 80 and 100 µg/mL). CCP extract also scavenged DPPH radicals in a dose-dependent fashion (p < 0.05 at 0.01 mg/mL and p < 0.001 at 0.1 and 1 mg/mL) with an IC50 value of 0.08 µg/mL.
Conclusion: Data from this study indicate that CCP extract attenuates neuroinflammatory responses in LPS-activated BV-2 microglia by inhibiting excessive production of pro-inflammatory mediators such as NO, iNOS and TNF-α. The strong anti-oxidant effect of CCP extract suggests that it possesses anti-neuroinflammatory properties.

Keywords: Castenea cranata, Chestnut peel extract, DPPH radicals, Anti-oxidant, Neuroinflammation, BV-2 microglia

INTRODUCTION

Neuroinflammation mediated by microglial activation appears to play an essential role in the pathogenesis of neurodegenerative diseases [1]. It was well documented that activated microglia releases proinflammatory mediators including nitric oxide (NO), inducible NO synthase (iNOS), interleukins (IL), tumor necrosis factor-alpha (TNF-α), toxic free radicals [1,2] and may lead to progressive damage in a number of neurodegenerative disorders including Alzheimer's and Parkinson's disease [2,3]. It is well known that microglia can be activated by

lipopolysaccharide (LPS) and is recognized to be a useful *in vitro* tool for studying neuro-inflammatory mechanisms [4]. LPS-activated BV-2 microglia cells enhances the production of immune-related cytotoxic factors and pro-inflammatory cytokines [4,5]. Thus, agents that reduce microglial activation and their pro-inflammatory responses might be considered as an important therapeutic strategy for treating neuroinflammatory disorders.

Chestnut (*Castenea cranata* Siebold & Zucc.) from the family Fagaceae has been used in folk medicine in many countries for centuries and several studies have reported antioxidant, antiallergic, antidiabetic and anti-amnesic properties [6,7]. The Chestnut peel has also been used as a cosmetic material for a long time in Korea and other Asian countries. It was described as an anti-wrinkle and anti-aging agent when mixed with honey [8]. It was well documented that agents that scavenge free radicals might be beneficial in delaying the process of neurodegeneration and neuro-inflammation via the antioxidant defense mechanisms [9-11]. In view of the published works that CCP possessed antioxidant and anti-aging properties, in the present study we aimed to investigate whether CCP extract exhibits protective effects on LPS-activated neuro-inflammatory processes in BV-2 microglial cells.

EXPERIMENTAL

Plant material and extraction

Chestnut inner skin was purchased from local market, Korea in August 2013. The material was authenticated by Prof. Jong-Bo Kim, a taxonomist at Konkuk University, Korea and a voucher specimen (CC-KU2013) was kept in laboratory herbarium, Konkuk University, Korea for future reference. To obtain the CCP extract the material was washed with running tap water and chopped into smaller pieces. It was then oven-dried at 40 °C for 48 h, and ground to a powder using conventional mixer. The ethyl acetate fraction from the water extract of chestnut inner skin was obtained as described previously [7]. CCP powder (200 g) was suspended and extracted with 1L of water at 70 °C for 2h in a heating mantle. Water extracts were filtered and evaporated to dryness using a rotary vacuum evaporator. The dried material was re-dissolved in 500 mL of double distilled water and the solution was partitioned using a separation funnel with equal volumes of ethyl acetate. The ethyl acetate fraction obtained was concentrated in a rotary evaporator and stored at -20 °C until use.

DPPH radical scavenging activity

The anti-oxidant activity of the CCP extract was determined using the stable radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich, St. Louis, MO, USA). The radical scavenging capacity was evaluated by employing a reaction mixture constituted by aliquots of the CCP extract and a DPPH methanolic solution as described previously [12]. A sample solution of 60 µL of each concentration of CCP extract (0.01, 0.1 and 1 mg/mL), was added to 60 µL of DPPH (60 µM) in methanol. After mixing vigorously for 10 sec, the mixture was then transferred to a 100 µL Teflon capillary tube and the scavenging activity of each sample was measured using a JES-FA ESR spectrometer (Jeol Ltd, Tokyo, Japan). A spin adduct was measured on an ESR spectrometer exactly after 2 min. Experimental conditions were as follows: central field, 3,475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 5 mW; gain, 6.3 x 105, and temperature, 298 K.

Cell culture and viability assay

BV-2 microglia cells were cultured at 37 °C in 5 % CO_2 in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 5 % FBS (Hyclone, Logan, UT, USA) and antibiotics (Invitrogen). In all experiments, cells were pre-treated with CCP extract at indicated concentrations (10 - 100 µg/mL) for 1 h before the addition of LPS (5 µg/mL, Sigma-Aldrich, St Louis, MO, USA) in serum- free DMEM. An equal volume of sterile water was added to all control treatments.

For viability assay, 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyl-tetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO, USA) assay was used as described previously [10]. BV-2 cells were plated onto 96- well plates and exposed to CCP extract. MTT was added to each well then incubated for additional 2 h in dark at 37 °C. The medium was then aspirated from the wells and the blue formazan product obtained was dissolved in DMSO. The plates were analyzed at 570 nm using a microplate reader (Tecan Trading AG, Switzerland). Each experiment was conducted in triplicate. Percentage of cell viability was calculated using Eq 1.

Cell viability (%) = (At/Au)100 ……………… (1)

Where At and Au are the absorbance of extract-treated and control (untreated) samples, respectively.

Immunoblot analysis and antibodies

Cells were washed in cold PBS three times and lysed in a buffer containing 50 mM Tris-HCl, pH 7.4, 1 % (v/v) NP-40, 0.25 % sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 25 mM NaF, 2 mM Na_3VO_4 and protease inhibitor cocktail (Complete MiniTM, Roche, Mannheim, Germany) at 4 °C. The lysate was clarified by centrifugation at 10,000 g for 20 min at 4 °C to remove insoluble components. Cell lysates were normalized for protein content using BCA reagent (Pierce, Rockford, IL, USA). Equal amounts of protein were loaded onto 10 % PAGE gels and separated by standard SDS-PAGE procedure. Proteins were transferred to an NC membrane (S&S, Dassel, Germany) and blocked with 5 % non-fat dry milk in TBS. To detect protein expression, the blots were probed with the specific antibodies against iNOS followed by the secondary antibodies coupled to horseradish peroxidase (Bio-Rad, Herculus, CA, USA). The detection of β-actin with a specific antibody was used for an internal control. The immunoreactive proteins on the membrane were detected by chemiluminescence using the West-Save substrate (Lab-Frontier, Seoul, Korea) on X-ray film. The antibodies against iNOS and β-actin were purchased from Cell Signaling Technology INC. (Beverly, MA, USA).

Nitric oxide assay

Production of NO was assayed by measuring the levels of nitrite in the culture supernatant using colorimetric assay with Griess reagent [13]. BV-2 cells (2×10^5 cells/ml) were seeded in 6-well plates in 500 μl complete culture medium and treated with the CCP extract at indicated concentrations for 1 h prior stimulation with LPS (5 μg/ml) for 2 h. Culture supernatant (50 μl) was reacted with an equal volume of Griess reagent (0.1 % naphthylethylenediamine and 1% sulfanilamide in 5 % H_3PO_4) in 96-well plates at room temperature in the dark. Absorbance was determined at 540 nm using a microplate reader (Tecan). Nitrite concentrations were determined by extrapolation from a sodium nitrite standard curve.

TNF-α assay

BV-2 microglia cells (1×10^5 cells/well) were cultured on 96-well plates and treated with the CCP extract at indicated concentrations for 1 h and stimulated with LPS (5 μg/mL). At 4 h post LPS treatment, the cells were collected and the supernatants were evaluated for TNF-α level using a murine TNF-α ELISA kit from BD

Biosciences (San Jose, CA, USA) according to the manufacturer's instructions.

Statistical analysis

All data are represented as the mean ± S.E.M of at least three independent experiments.. Statistical analyses were performed using SAS statistical software (SAS Institute, Cray, NC, USA) using one-way analysis of variance, followed by Dunnett's multiple range tests. *P* values <0.05 was considered statistically significant.

RESULTS

Effect of CCP extract on DPPH radical scavenging activity

As shown in Fig. 1A, CCP extract exhibited significant DPPH radical- scavenging activity in a concentration-dependent manner showing a maximum effect at 1 mg/mL ($p < 0.001$). The ESR spectroscopy data is represented in Fig 1B. The concentration required for 50 percent inhibition of DPPH free radicals was found to be at 0.08 μg/mL.

Effect of CCP extract on BV-2 cell viability

Treatment with CCP extract at indicated concentrations (10-100 mg/mL) did not affect the overall cell viability nor did they exhibit any cytotoxicity on BV-2 microglia cells (Fig 2). This data indicate that the concentrations used in the study were safe to BV-2 microglial cells.

CCP extract attenuates NO production in LPS-stimulated BV-2 cells

Cells treated with LPS (5 μg/ml) alone significantly increased in NO levels ($p < 0.001$) as shown in Fig 3. Pre-treatment with CCP extract at indicated concentrations significantly and dose-dependently suppressed the excessive release of NO in BV-2 cells (Fig. 3). The significant effect was observed at 10 μg/ml ($p < 0.05$) and maximum effect was observed at a concentration of 100 μg/ml ($p < 0.001$).

CCP extract attenuates iNOS expression in LPS-stimulated BV-2 cells

Western blot analysis revealed that LPS-stimulation to BV-2 microglia increased the protein expressional levels of iNOS (Fig 4). However, the increased expression of iNOS in LPS-stimulated BV-2 cells was suppressed when these cells were treated with CCP extract (40 and 80 μg/mL).

Effect of CCP extract on TNF-α production in LPS-stimulated BV-2 cells

As shown in Fig. 5, TNF-α levels increased significantly after LPS treatment (5 µg/mL) when compared to those in untreated cells ($p < 0.001$). However, CCP extract significantly inhibited TNF-α production in a concentration-dependent manner showing $p < 0.05$ at 20 µg/mL, $p < 0.01$ at 40 µg/mL and $p < 0.001$ at 80 and 100 µg/mL concentrations, respectively in LPS-stimulated BV-2 cells.
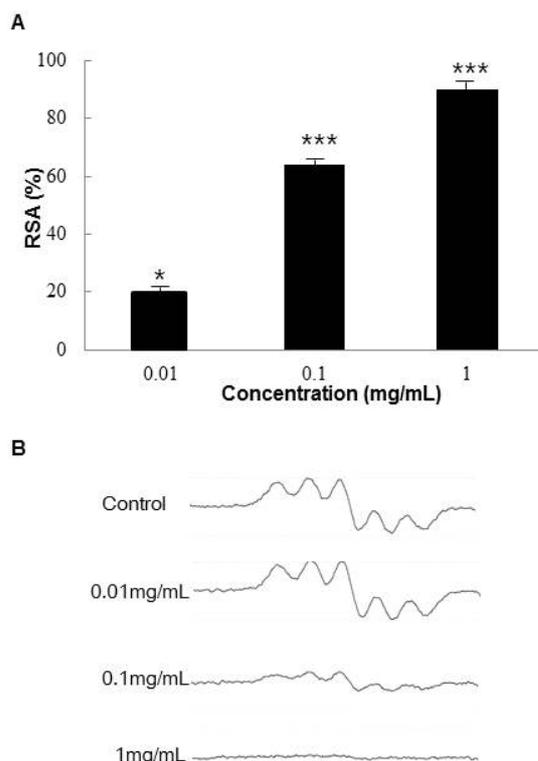

Fig 1: Effect of CCP extract on DPPH radical-scavenging activity (RSA). A: The capacity of CCP extract to scavenge DPPH radicals at various concentrations (0.01, 0.1 and 1 mg/mL) was shown. B: ESR spectral data. BV-2 cells were treated with or without CCP extract at the various concentrations (0.01, 0.1 and 1 mg/mL). The scavenging activity of each sample on DPPH radical was measured using a JES-FA ESR spectrometer. A spin adduct was measured on an ESR spectrometer exactly 2 min later. Data are presented as mean ± SEM (n = 3) for three independent experiments; *$p < 0.05$, and ***$p < 0.001$, compared with control group

DISCUSSION

This report demonstrates that CCP extract markedly inhibits LPS-induced inflammatory responses in BV-2 microglial cell line. Neuro-inflammation is characterized by the activation of

microglia and expression of major inflammatory mediators in the CNS. Excessive production of inflammatory mediators, NO and proinflammatory cytokines from activated microglia has been implicated in inflammation mediated neuro-degeneration. Evidence suggest that iNOS is the most important contributor to NO production in the brain after inflammatory assault [3]. Therefore inhibition of cytokine production in activated-microglia might serve as a key mechanism in the control of neuroinflammatory responses in neurodegeneration. In our study,
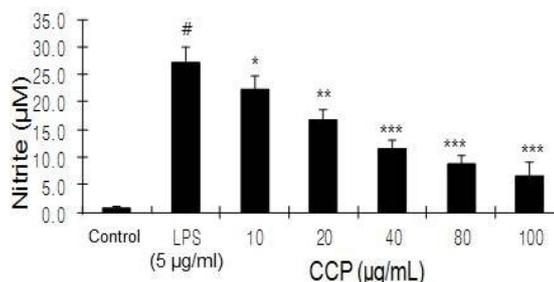

Fig 3: Effect of CCP extract on NO Production in LPS-stimulated BV-2 microglial cells. BV-2 cells were treated with CCP extract at various concentrations (10, 20, 40, 80 and 100 µg/mL) with or without LPS (5 µg/mL) for 4 h. The nitrite in the culture supernatant was evaluated using Griess reagent. Data are presented as the mean ± SEM (n = 3) for three independent experiments. #$p < 0.001$, when compared with control group; *$p < 0.05$, ** $p < 0.01$ and ***$p < 0.001$, when compared with only LPS-treated group (***Note:*** CCP = *Castenea cranata* peel extract; LPS = lipopolysaccharide)
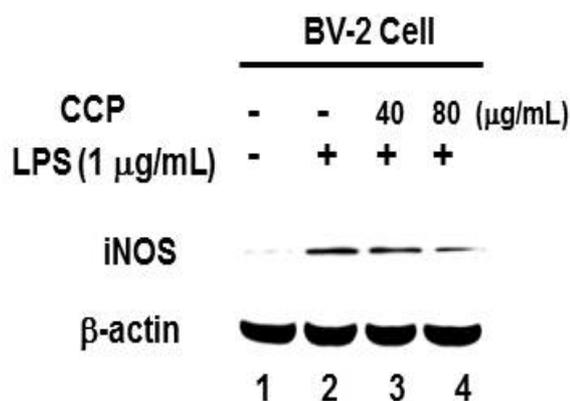

Fig 4: Effect of CCP extract on iNOS expressional levels in LPS-stimulated BV-2 microglial cells. The expression levels of iNOS production in the LPS (5 µg/mL) - stimulated BV-2 cells at two concentrations of the CCP extract (40 and 80 µg/mL) was monitored by immunoblot analyses with the specific antibody against iNOS. The internal control used was β-actin. (***Note:*** CCP = *Castenea cranata;* LPS = lipopolysaccharide; iNOS: Inducible nitric oxide)
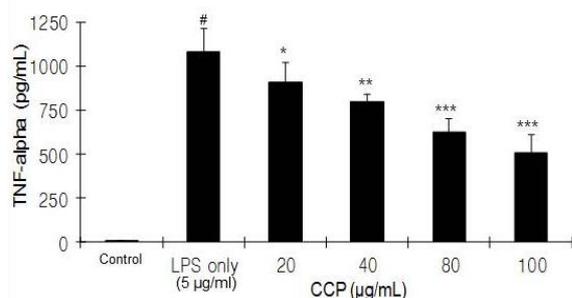
Fig 5: Effect of CCP extract on TNF-α production in LPS-stimulated BV-2 microglial cells. Suppression of pro-inflammatory cytokine TNF-α expression by CCP extract was measured with ELISA test. BV-2 cells were treated with CCP extract at 20, 40, 80 and 100 μg/mL with or without LPS (5 μg/mL) for 4 h. The TNF-α in the culture supernatant was evaluated using a murine TNF-α ELISA kit. Data are presented as the mean ± S.E.M. (n = 3) for three independent experiments. [#]$p < 0.001$, when compared with control group; [*]$p < 0.05$, [**]$p < 0.01$ and [***]$p < 0.001$, when compared with LPS alone group (***Note:*** CCP = TNF-α = tumor necrosis factor-alpha; LPS = lipopolysaccharide)

CCP extract significantly inhibited the LPS-stimulated increase in NO production and suppressed the iNOS protein expression in LPS-stimulated BV-2 cells.

Activated microglial cells are known to release several proinflammatory cytokines including TNF-α which may not only amplify the inflammatory cascade, but also cause inflammatory injury [14,15]. Our results indicate that CCP extract significantly suppressed the production of TNF-α in the LPS-activated BV-2 cells. Considering the data obtained we suggest that CCP extract might have a potent anti-neuroinflammatory activity via the inhibition of LPS-stimulated production of TNF-α, NO, as well as iNOS protein in microglia.

The mechanism of microglia-mediated neuro-inflammation is partly attributed to release of reactive oxygen substances (ROS). Excessive production of ROS results in tissue injury by damaging macromolecules and lipid peroxidation of membranes. In addition, it propagates the release of inflammatory mediators responsible for the recruitment of additional neutrophils and macrophages in the CNS thereby hastening neuro-inflammation [16]. Thus free radicals are important mediators that provoke or sustain inflammatory responses and their neutralization by antioxidants and radical scavengers can reduce brain inflammation. It was well known that DPPH radical assay as one of the widely used methods for evaluating the free radical scavenging activities of several antioxidants [9]. Earlier reports revealed that CCP possessed strong antioxidant compounds [17,18]. It has been demonstrated that chestnut fruits contain several phenolic compounds [19]. The antioxidant compounds such as scoparone and scopoletin isolated from CCP are known to possess anti-oxidant properties and anti-inflammatory activities [20-22]. CCP was reported to suppress the liver cytotoxicity induced by chronic ethanol administration due to its potent antioxidant effects [17,18]. Reports also revealed that CCP possess neuroprotective activity and anti-amnesic activities by attenuating the ROS and oxidative stress in neuronal cells [8]. In our present study, the CCP extract also exhibited significant free radical-scavenging effects indicating that CCP extract might contain potential antioxidant agents.

CONCLUSION

The present study reveals for the first time that CCP extract attenuates neuroinflammatory processes in LPS-induced BV-2 microglial cells. The anti-neuroinflammatory effects of CCP extract may be attributed to its regulatory effect on the release of pro-inflammatory cytokines, such as TNF-α, and its strong antioxidant effect. Therefore, CCP extract should be explored as a therapeutic agent in the treatment of microglia-mediated neuro-inflammatory disease.

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