

# Callistephin enhances the protective effects of isoflurane on microglial injury through downregulation of inflammation and apoptosis

LILI ZHAO, SHIBIAO CHEN, TIANYIN LIU, XIUHONG WANG, HAIJIN HUANG and WEICHENG LIU

Department of Anesthesiology, The First Affiliated Hospital of Nanchang University, Nanchang, Jiangxi 330006, P.R. China

Received June 18, 2018; Accepted March 15, 2019

DOI: 10.3892/mmr.2019.10282

**Abstract.** Microglia are the major immune cells in the central nervous system. Microglial activation can be beneficial or detrimental depending on the stimuli and the physiopathological environment. Microglial activation is involved in a variety of neurodegenerative disorders. Different anesthetic agents have exhibited diverse effects on microglial activation and the engulfment process. The anthocyanin callistephin has been demonstrated to have antioxidant and anti-inflammatory properties, and these were assessed in the present study, with a focus on its effect on microglial activation. Mouse microglial cells C8-4B were treated with 100 ng/ $\mu$ l lipopolysaccharide (LPS) and 1 ng/ $\mu$ l interferon- $\gamma$ . Cells were subsequently treated with 2% isoflurane, 100  $\mu$ M callistephin or both. LPS promoted apoptosis in C8-B4 cells, and this was reduced following treatment with isoflurane and callistephin. LPS-treated C8-B4 cells also exhibited enhanced production of reactive oxygen species and nitric oxide, excessive engulfment and increased caspase 3/7 activity. These detrimental alterations were suppressed following co-treatment with isoflurane and callistephin. LPS-induced apoptosis was facilitated via the expression of B-cell lymphoma-2 like 1 and poly (ADP-ribose) polymerase, which were subsequently restored following treatment with isoflurane and callistephin. Callistephin was demonstrated to be involved in the modulation of inducible nitric oxide synthase, cytochrome c oxidase subunit 2, tumor necrosis factor- $\alpha$  and nuclear factor- $\kappa$  B. Callistephin enhanced the protective effects of isoflurane by modulating engulfment and apoptosis in C8-B4 cells. The potential underlying mechanism was identified to be the suppression of p38 phosphorylation. The present study thus suggested that the negative effects on microglial activity induced by LPS were ameliorated following treatment with callistephin, which also

enhanced the effects of isoflurane. Callistephin may therefore constitute a candidate drug agent that may target inflammatory and growth regulatory signaling pathways, thus ameliorating certain aspects of neurodegenerative diseases.

## Introduction

Microglial cells are the major immune cell in the central nervous system (CNS), responding against types of endogenous and exogenous stimuli, including infection by bacteria, viruses, prions and  $\beta$ -amyloid plaques (1). Microglia are activated upon exposure to different stimuli and, depending on the environmental context, this may be beneficial or detrimental to the functionality and physiology of the CNS (2). The effects of microglial phagocytosis are primarily beneficial, as it eliminates dead cells and induces cellular anti-inflammatory response. Removing dead or decaying cellular components may also prevent proinflammatory intracellular responses, thus contributing to a reduction in inflammatory burden (3). However, microglial activation and subsequent phagocytosis may exacerbate cell cytotoxicity and neuroinflammation (4). This may in turn cause a respiratory burst, producing toxic reactive oxygen/nitrogen species (ROS/RNS), which lead to oxidative damage (5). Cellular systems are constantly exposed to various toxic and mutagenic substances that cause the generation of reactive metabolites. These free radicals collectively induce oxidative stress, which may cause damage to the cellular components or the cells themselves, thus disturbing physiological functions. The protective strategies against oxidative stress primarily include the initiation of cellular detoxification systems, which, in the CNS, involves the recruitment of antioxidant enzymes in microglial cells.

Volatile anesthetics like isoflurane have been reported to reduce lipopolysaccharide (LPS) and interferon (IFN)- $\gamma$  induced neuronal inflammation and apoptosis (6,7). However, the effect of volatile anesthetics on microglial phagocytosis/engulfment requires further elucidation, particularly regarding its potential detrimental consequences. The anesthetic propofol has been reported to reduce arterial blood flow in the brain, reducing intracranial pressure and maintaining the metabolic blood-oxygen supply ratio even during hypoxia, suggesting that it may have protective effects against hypoxic brain damage (8-10). Propofol may also serve a protective role via the modulation of  $\text{Ca}^{2+}$  and the reduction of free radicals in a  $\gamma$ -aminobutyric acid (GABA)

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*Correspondence to:* Dr Lili Zhao, Department of Anesthesiology, The First Affiliated Hospital of Nanchang University, 18 Yongwaizheng Road, Nanchang, Jiangxi 330006, P.R. China  
E-mail: zoedoddatt@yahoo.com

**Key words:** anthocyanin, callistephin, isoflurane, microglia, neurodegenerative diseases, apoptosis

and N-methyl-D-aspartate (NMDA) receptor dependent manner (11-14). All volatile anesthetics differ in potency, function and adverse effects when used in different physiological conditions (15,16). Isoflurane (2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane;  $\text{CHF}_2\text{-O-CHCl-CF}_3$ ) is a halogenated ether used as inhalational anesthetic, and has been reported to exhibit preventative roles in neonatal hypoxia-ischemia induced brain injury (17) and neurodegenerative diseases (18). Isoflurane has also been reported to have beneficial effects in animal models of various other diseases, including LPS-induced acute lung inflammation and injury (19,20), glucose-induced oxidative stress (21), renal ischemia/reperfusion injury (22) and cardiac injury models (23).

Anthocyanins, glycosides of anthocyanidins, are the compounds responsible for colors observed in plants (24). Anthocyanins have recently attracted consideration due to their wide range of potential bio-pharmacological benefits, including anti-inflammatory, antioxidant, antiproliferative, antitumor and cardioprotective properties (24-28). Anthocyanin-rich extract administration to vitamin E-depleted rats has been demonstrated to have protective effects against tert-butyl hydroperoxide-induced hepatic toxicity by decreasing lipid peroxidation and DNA damage (29,30). Anthocyanins, being glucosides, have high water solubility, which allows them to be directly absorbed and dissolved in the blood (31), and may be incorporated into cellular membranous compartments, including the cytosol (32). Anthocyanins have also been reported to have free radical scavenging and antioxidant activity (33). Therefore, these glycosides may aid in the prevention of chronic pathophysiological conditions, particularly considering that oxidative stress is a key factor in the development of numerous pathologies, including neurodegenerative diseases (34,35). For instance, anthocyanin metabolites have been reported to protect PC12 cells and SH-SY5Y neuroblastoma cells from oxidative stress induced by hydrogen peroxide (36-38); and treatment of retinal ganglion cells with anthocyanidin components from bilberries has been reported to rescue cells from oxidative stress induced by peroxynitrite and to protect cells from excitotoxicity (39). Callistephin (pelargonidin-3-O-glucoside) is a glucoside consisting about 60-80% of the total anthocyanin content in strawberries (40). Callistephin is found in various berries, and has been prospectively used in a number of preventive studies (40). Callistephin has been reported to have high activity equivalence to Trolox, suggesting that it may be a potent intrinsic antioxidant and may suppress free radical generation (41).

Therefore, the present study was designed to evaluate the effects of callistephin and isoflurane on microglial engulfment induced by LPS and IFN- $\gamma$  in mouse microglial cells. The study evaluated the association of callistephin with isoflurane in exerting anti-inflammatory and antioxidant effects in cells exposed to LPS.

## Materials and methods

**Cell culture.** Mouse microglial cloned C8-B4 cells (CRL-2540™), isolated from the cerebellum of an 8-day old mouse, were procured from the American Type Culture Collection (Manassas, VA, USA). The C8-B4 microglial cells were cultured in Dulbecco's modified Eagle's medium

(DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 4 mM L-glutamine, 4,500 mg/l glucose, 1 mM sodium pyruvate, 1,500 mg/l sodium bicarbonate, and supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Inc.), penicillin and streptomycin (100 U/ml), in an incubator (New Brunswick; Eppendorf, Hamburg, Germany) with a humidified atmosphere with 5%  $\text{CO}_2$ , at 37°C. The culture medium was changed every 48-72 h to maintain the cell cultures.

**LPS/IFN- $\gamma$  treatment and isoflurane exposure.** Mouse microglial C8-B4 cells were challenged with LPS and IFN- $\gamma$  at 100 and 1 ng/ $\mu\text{l}$  concentrations, respectively, for 24 h at 37°C, as previously reported (41). Cells were exposed to isoflurane after 2 h of LPS/INF- $\gamma$  administration, in an airtight chamber at the aforementioned temperature and atmospheric conditions. Isoflurane was supplemented via a vaporizer for 1 h. The isoflurane concentration was measured from the outlet of the chambers using an Infrared Gas Analyzer (GE Healthcare, Chicago, IL, USA). This was performed to ensure that the isoflurane was kept at a constant concentration for the duration of the treatment. The isoflurane-exposed cells were transferred from the airtight sealed chamber to normal cell culture conditions for further incubation at 37°C for 24 h. Following optimization and the establishment of anesthetic effect, a concentration of 2% isoflurane was used for further experimental procedures. Callistephin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was dissolved in dimethyl sulfoxide (DMSO), ensuring a final concentration of 0.1% DMSO in the cell cultures.

**Cell viability analysis.** Mouse microglial C8-B4 cells were plated at a density of 5,000 cells/well in 96-well plates for 24 h. The medium was removed and replaced with fresh medium. Cells were treated with LPS and INF- $\gamma$ , followed by exposure to isoflurane as described earlier. Isoflurane was tested at concentrations of 1, 2, 4, 6, 8 and 10% in an independent viability optimization assay. Callistephin was tested at 50, 100, 150, 200, 300 and 400  $\mu\text{M}$  concentrations for 24 h in an independent viability optimization assay. Following the previous tests for optimization of cell viability, 2% isoflurane and 200  $\mu\text{M}$  callistephin were chosen for further experimental procedures. Microglial cell viability was determined by the Cell Titer Glo (CTG) 2.0 assay kit (Promega Corporation, Madison, WI, USA), according to a modified version of the manufacturer's protocol, since luminescence was stabilized for 15 min instead of 10 min. The CTG 2.0 assay is a consistent method of determining the number of viable cells in cultures. This is achieved via quantification of the amount of ATP present, which serves as an indicator of metabolically active cells. This method is a luminescent cell viability assay system ideal for automated high-throughput screening of cell proliferation and cytotoxicity assays (42). C8-B4 cells in 96-well plates were treated with CTG reagent, and luminescence was measured using an ELISA microplate spectrophotometer at 490 nm. Cell viability is presented as the percentage of viable cells as compared with the vehicle control (100%).

**Cell engulfment analysis.** Cell engulfment was assessed using Nile red polystyrene microspheres (1.0  $\mu\text{m}$ ; Sigma-Aldrich;

Merck KGaA) at a concentration of 10,000 particles/ml cell culture. Microglial C8-B4 cells were exposed to different treatment conditions in 6-well culture plates for 24 h. Subsequently, Nile red polystyrene microspheres were added to the culture wells and incubated for 30 min at 37°C in a CO<sub>2</sub> incubator with 5% CO<sub>2</sub>. Engulfment was facilitated by bovine serum albumin (BSA; Thermo Fisher Scientific, Inc.) supplementation as an opsonizing agent. Cells were washed with PBS and fixed with 100% methanol for 15 min at 4°C. Cells were permeabilized with 0.1% Triton X-100 diluted in PBS for 15 min, and blocked with 1% BSA for 30 min at room temperature. Following blocking, cells were incubated with rabbit monoclonal anti-Iba1 antibody (cat. no. ab178847; Abcam, Cambridge, UK; dilution, 1:1,000) in the dark at 4°C overnight, and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (cat. no. ab6702; Abcam; dilution, 1:5,000) for 1 h at room temperature the following day. The cells were washed, and the nuclei were counterstained with Hoechst 33342 staining reagent for 10 min at room temperature. Fixed and stained cells were imaged under a fluorescence microscope (Zeiss AG, Oberkochen, Germany; magnification, x40) equipped with a digital microscopic camera system. The percentage of engulfed microglial cells was calculated as: No. of microglial cells with fluorescent microspheres/total no. microglial cells x100, and compared with the control.

**Measurement of ROS in microglial cells.** The antioxidant activity of callistephin in C8-B4 cells was determined by measuring the production of ROS using a fluorescent indicator kit, namely the dichlorofluorescein diacetate (DCFDA) assay kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Briefly, mouse microglial C8-B4 cells were plated at a density of 5,000 cells/well in 96-well plates for 24 h. The medium was removed and replaced with fresh medium. Cells were treated with LPS and IFN- $\gamma$ , followed by exposure to isoflurane as described earlier. Cells were treated with 100  $\mu$ M callistephin for 24 h. Subsequently, medium from the culture wells was removed and replaced with fresh medium containing DCFDA (20  $\mu$ M). The plates were incubated at 37°C for 30 min, the DCFDA-containing medium was removed and the cells were washed. The amount of ROS production in cells was measured using a fluorescence ELISA microplate spectrophotometer at excitation and emission wavelengths of 495 and 529 nm, respectively. The relative fluorescence units (RFU) were counted for each treatment groups.

**Measurement of total nitric oxide (NO) generation.** The production of total NO in microglial C8-B4 cells was determined via the Griess reagent assay kit (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Briefly, mouse microglial C8-B4 cells were plated at a density of 5,000 cells/well in 96-well plates for 24 h. Following the addition of fresh medium, cells were treated with LPS and IFN- $\gamma$ , exposed to isoflurane and treated with 100  $\mu$ M callistephin for 24 h. Following incubation, culture medium from the treated plates was transferred to a 96-well plate for determination of total NO. The Griess reagent mixture was prepared by mixing equal volumes of Component A [N-(1-naphthyl)ethylenediamine] and Component B (sulfanilic acid). A total volume of 20  $\mu$ l freshly prepared Griess reagent

was added to each well of the 96-well plate, and incubated at room temperature for 30 min. The absorbance in each well was measured using an ELISA microplate spectrophotometer at 548 nm.

**Measurement of caspase-3/7 activity in microglial cells.** The apoptosis of microglial cells was further elucidated by analyzing the activation of caspases. Upon activation of apoptotic processes, executioner caspases-3 and 7 are frequently activated. Therefore, the levels of caspase-3/7 activity were verified using a Caspase-Glo 3/7 assay kit (Promega Corporation). The Caspase-Glo 3/7 assay is a pro-luminescent caspase-3/7 with the tetrapeptide DEVD bound to aminoluciferin. When caspase-3/7 are active, the DEVD-aminoluciferin is cleaved, releasing aminoluciferin, which has thermostable luciferase activity (43). The mouse microglial C8-B4 cells were plated at a density of 1x10<sup>6</sup> cells/well in 6-well plates. After 24 h, the medium was removed and replaced with fresh medium containing LPS and IFN- $\gamma$ , followed by exposure to isoflurane, as described earlier, and treatment with 100  $\mu$ M callistephin for 24 h. Following incubation, caspase-Glo 3/7 assay was performed according to the manufacturer's instructions. The assay kit results in cell lysis which, upon the addition of caspase 3/7 assay reagent, produces a luminescent signal which is considered proportional to caspase 3/7 activity. The relative luminescence units (RLU) were counted in different treatment groups of cells using a spectrophotometer at 490 nm.

**RNA isolation, cDNA synthesis, and semi-quantitative polymerase chain reaction (sqPCR).** C8-B4 cells were treated with LPS (100 ng/ $\mu$ l) and IFN- $\gamma$  (1 ng/ $\mu$ l), isoflurane (2%), callistephin (100  $\mu$ M) or a combination of the two for 24 h. Cells were collected, and RNA was isolated using TRIzol<sup>®</sup> reagent (ThermoFisher Scientific, Inc.), according to the manufacturer's instructions. RNA was quantified, and a total of 1  $\mu$ g RNA per sample was subjected to cDNA synthesis using the Reverse Transcriptase SuperScript III kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The reaction was performed at 65°C for 5 min and then cooled on ice. Subsequently, the mixture containing the SuperScript III reverse transcriptase was incubated at 50°C for 1 h and at 70°C for 15 min. The obtained cDNA was subjected to PCR amplification with the iTaq DNA Polymerase (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and dNTPs mix (Thermo Fisher Scientific, Inc.), using a Veriti<sup>™</sup> Thermal Cycler (Applied Biosystems; Thermo Fisher Scientific, Inc.). The specific primers and thermocycling conditions used in the analysis were: Inducible nitric oxide synthase (iNOS) forward, 5'-GCAAACCCAAGGTCTACGTT-3'; iNOS reverse, 5'-GGA AAAGACTGCACCGAAGA-3' (initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 58°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 10 min); tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) forward, 5'-AGC ACAGAAAGCATGATCCG-3'; TNF- $\alpha$  reverse, 5'-CTG ATGAGAGGGAGGCCATT-3' (initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 59°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 10 min); cytochrome c oxidase subunit 2 (COX-2) forward, 5'-CCTGTGCCTGATGATTGC-3'; COX-2 reverse, 5'-CGG TGAAACTCTGGCTAG-3' (initial denaturation at 95°C for

5 min, followed by 35 cycles of 95°C for 1 min, 58°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 10 min); nuclear factor- $\kappa$  B subunit/p65 (NF- $\kappa$ B-p65) forward, 5'-CCT CTGGCGAATGGCTTTAC-3'; NF- $\kappa$ B-p65 reverse, 5'-GCT ATGGATACTGCGGTCTGG-3' (initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 60°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 10 min);  $\beta$ -actin forward, 5'-ATCACTATTGGCAACGA GCG-3';  $\beta$ -actin reverse 5'-TCAGCAATGCCTGGGTACAT-3' (initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 54°C for 30 sec and 72°C for 1 min, with a final extension at 72°C for 10 min). PCR products were separated via agarose gel electrophoresis (1.5%), and visualized using ethidium bromide. The agarose gels were scanned under UV light, and images were captured. The agarose gel bands were analyzed for band densitometry using Image J Software (version 1.5; National Institutes of Health, Bethesda, MD, USA).

**Protein isolation and western blotting.** Microglial C8-B4 cells, exposed to different treatment conditions in 6-well plates, were harvested by scraping, washed with cold PBS and pelleted by centrifugation at 5,000  $\times$  g for 10 min at 4°C. Cells were lysed in 0.5 ml cold cell lysis buffer [20 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl, 2.5 mM sodium pyrophosphate, 1% Triton X-100, 1% sodium vanadate, 1 mM phenylmethylsulfonyl fluoride and a protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA)] for 1 h in ice with intermittent vortex mixing. The cell lysate was centrifuged at 12,000  $\times$  g for 20 min at 4°C. The supernatant containing the total cellular protein was collected and the cell pellet was discarded. Protein estimation was performed from protein isolates using the Pierce bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. A total of 20  $\mu$ g protein per sample was separated via 12% SDS-PAGE. Separated proteins were transferred onto polyvinylidene difluoride membranes using a western blot wet transfer system. Membranes with transferred proteins were blocked with 5% fat-free dried skimmed milk prepared in PBS with 0.1% Tween-20 (PBST) for 2 h at 4°C. Membranes were washed with PBST buffer and incubated with primary antibodies for different proteins overnight at 4°C. The following day, the membranes were washed and incubated with corresponding secondary antibodies for 1 h at room temperature. Protein bands were visualized using Pierce enhanced chemiluminescence detection reagent (Thermo Fisher Scientific, Inc.) on photographic film. The primary antibodies and respective dilutions used in the western blotting were: Anti-iNOS antibody (cat. no. ab3523; Abcam), 1:500 dilution; anti-Cox-2 antibody (cat. no. sc-1747; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), 1:500 dilution; anti-NF- $\kappa$ B p65 antibody (cat. no. NB100-2176; Novus Biologicals, LLC, Littleton, CO, USA), 1:500 dilution; anti-GAPDH antibody (cat. no. MAB374; Merck KGaA), 1:500 dilution; anti-B-cell lymphoma 2 like 1 (Bcl-xL) antibody (cat. no. 2762; Cell Signaling Technology Inc., Danvers, MA, USA), 1:1,000 dilution; and poly (ADP-ribose) polymerase 1 (PARP) antibody (cat. no. 9542; Cell Signaling Technology Inc.), 1:1,000 dilution. The secondary antibodies used were: Horseradish peroxidase (HRP)-conjugated anti-rabbit (cat. no. ab6702; Abcam; dilution, 1:5,000), and

HRP-conjugated anti-mouse (cat. no. ab97046; Abcam; dilution, 1:5,000).

**Statistical analysis.** Data are presented as the mean  $\pm$  standard deviation of at least three independent experimental repeats. Data were analyzed using SPSS version 17 software (SPSS, Inc., Chicago, IL, USA) and the Systat SigmaPlot 11.0 statistical program (Systat Software Inc., San Jose, CA, USA). Data were statistically compared between groups using a one-way analysis of variance and Tukey's post-hoc test for multiple comparisons.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Effects of isoflurane and callistephin on C8-B4 microglial cell viability.** The neuroprotective effects of callistephin and isoflurane were evaluated in C8-B4 microglia exposed to inflammatory LPS/IFN- $\gamma$ , using the CTG 2.0 method. Firstly, the effect of callistephin and isoflurane alone were assessed on C8-B4 cells in order to determine the effective cytotoxic concentration. The results presented in Fig. 1A indicated that callistephin did not affect cell viability at 50, 100, 150, 200 and 300  $\mu$ M concentrations. The viability of cultured cells was decreased by 9% at 200  $\mu$ M and 12% at 300  $\mu$ M, but not significantly. However, callistephin at higher concentrations (400  $\mu$ M) reduced cell viability by 18%, with statistical significance. This demonstrated that callistephin did not affect cell viability at moderate concentrations, but was slightly cytotoxic at concentrations  $>300$   $\mu$ M. Likewise, the effect of isoflurane exposure was also assessed on C8-B4 cells. The results presented in Fig. 1B indicated that cell viability was unaffected with 1, 2, 4, 6 and 8% isoflurane. However, isoflurane was cytotoxic at 10% with statistical significance. At 10% isoflurane, 78% of cells were viable, which is indicative of the notable cytotoxicity of isoflurane at higher concentrations. Therefore, 100  $\mu$ M callistephin and 2% isoflurane were used in the subsequent experiments, and similar concentrations have also been used in other studies (18,19,21,22).

Subsequently, the effect of an immune challenge with 100 ng/ $\mu$ l LPS and 1 ng/ $\mu$ l IFN- $\gamma$  on C8-B4 cells was assessed in the context of cell viability. Furthermore, the effect of treatment with isoflurane, callistephin or their combination on cell viability following the challenging step was also tested (Fig. 1C). The LPS/IFN- $\gamma$  challenge to C8-B4 cells caused significant cytotoxicity, with  $52 \pm 5\%$  survival. The treatment of LPS/IFN- $\gamma$ -challenged C8-B4 cells with 2% isoflurane had a preventative effect, with  $68 \pm 5\%$  survival ( $P < 0.05$  vs. control and vs. LPS/IFN- $\gamma$ ). Likewise, LPS/IFN- $\gamma$ -challenged C8-B4 cells treated with 100  $\mu$ M callistephin resulted in  $74 \pm 6\%$  survival ( $P < 0.05$  vs. control and vs. LPS/IFN- $\gamma$ ), meaning that callistephin exhibited a potent preventative effect. The combined treatment of LPS/IFN- $\gamma$ -challenged C8-B4 cells with isoflurane and callistephin resulted in a strong synergistic effect. This co-treatment maintained survival at  $91 \pm 6\%$  ( $P < 0.05$  vs. LPS/IFN- $\gamma$ ; not significant vs. control). Therefore, these results indicated that the combination of callistephin and isoflurane maintained the viability of C8-B4 cells following an immune challenge with LPS/IFN- $\gamma$ .

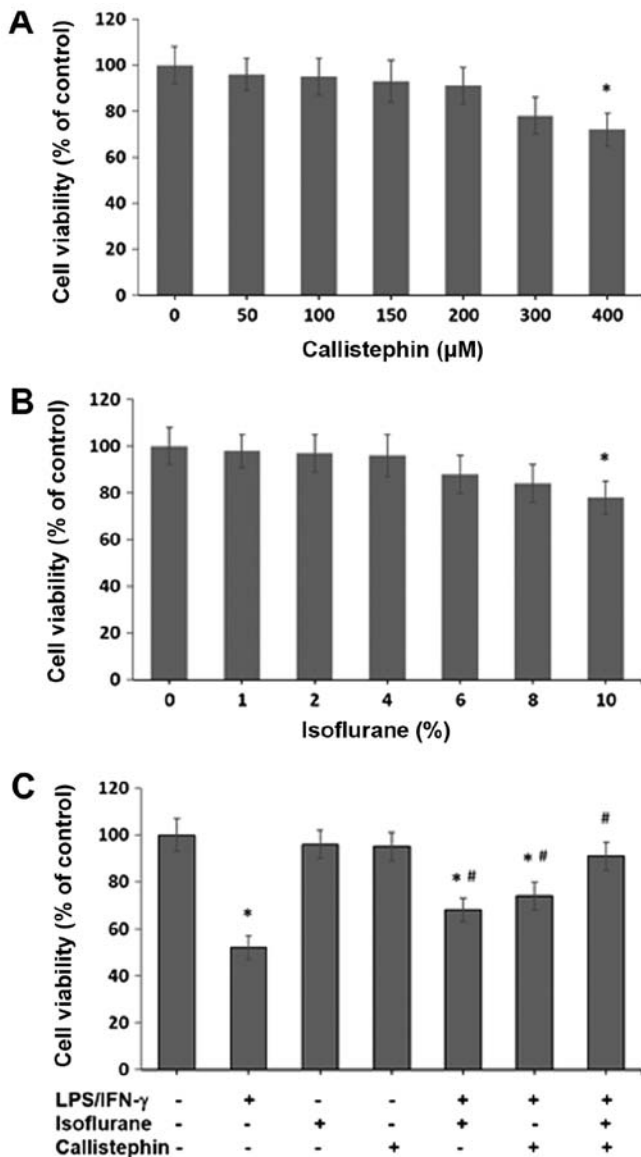


Figure 1. Effect of callistephin and isoflurane on the viability of LPS/IFN- $\gamma$ -challenged cells. The viability of C8-B4 cells was verified following treatment with various concentrations of (A) callistephin and (B) isoflurane to determine the levels that caused significant cytotoxicity. (C) Control cells and cells challenged with LPS (100 ng/ $\mu$ l) and IFN- $\gamma$  (1 ng/ $\mu$ l) were treated with 2% isoflurane, 100  $\mu$ M callistephin or both, and cell viability was assayed and compared. The results indicated that isoflurane and callistephin may act synergistically and maintain cultured cells viable following the immune challenge. \*P<0.05 vs. respective control; #P<0.05 vs. LPS/IFN- $\gamma$ -challenged cells. LPS, lipopolysaccharide; IFN- $\gamma$ , interferon- $\gamma$ .

**Effects of callistephin and isoflurane on microglial engulfment.** Microglial phagocytosis involves engulfment of cellular material or entire cells. The process is followed by digestion of the material through lysosomal degradation (3,4). Cell engulfment was assessed using Nile red polystyrene microspheres, and the percentage of engulfed microglial cells is presented and compared with the control in Fig. 2. The control levels of microglial engulfment were 38±3% in the vehicle control. The challenge with LPS and IFN- $\gamma$  increased the percentage of C8-B4 microglia engulfment to 82±6% (P<0.05 vs. control). Treatment with isoflurane, applied 2 h after the challenge with LPS and IFN- $\gamma$  stimulation, caused a decrease in microglial

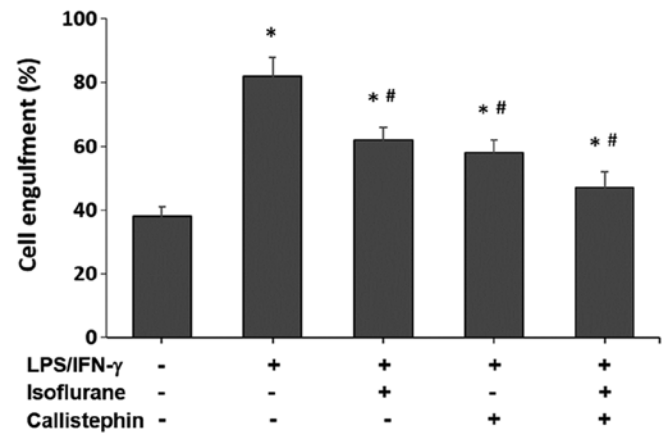


Figure 2. Effect of callistephin and isoflurane on LPS/IFN- $\gamma$ -induced engulfment. The phagocytic responses of control C8-B4 cells and cells challenged with LPS (100 ng/ $\mu$ l) and IFN- $\gamma$  (1 ng/ $\mu$ l) were verified. Subsequently, challenged cells were treated with isoflurane, callistephin or co-treated with isoflurane and callistephin. The percentage of cell engulfment was compared between the different treatment groups. \*P<0.05 vs. respective control; #P<0.05 vs. LPS/IFN- $\gamma$ -challenged cells. LPS, lipopolysaccharide; IFN- $\gamma$ , interferon- $\gamma$ .

engulfment to 62±4% (P<0.05 vs. LPS/IFN- $\gamma$  and control). Similarly, treatment with callistephin decreased microglial engulfment to 58±4% (P<0.05 vs. LPS/IFN- $\gamma$  and control). Lastly, co-treatment of LPS/IFN- $\gamma$ -challenged C8-B4 microglial cells with isoflurane and callistephin decreased microglial engulfment to 47±5% (P<0.05 vs. LPS/IFN- $\gamma$  and control). These results suggested that the combined effect of callistephin and isoflurane reduced the phagocytic responses of cultured microglia following an immune challenge with LPS/IFN- $\gamma$ , which was expected considering the results of the cytotoxicity assay.

*Callistephin prevents oxidative stress in microglia and enhances the effect of isoflurane.* Metabolism of anthocyanins in the gut by microflora results in the production of phenolic acids and aldehydes. The metabolized anthocyanins have unique chemical structures derived from the parent compounds, and may be metabolized into phloroglucinol aldehyde (44,45). The possible beneficial health effects of anthocyanins may be therefore due to their metabolites. These have reported neuroprotective capabilities as they are thought to regulate the levels of oxidative stress mediators (44,46). Consequentially, the antioxidant potential of callistephin and the combination of callistephin and isoflurane were evaluated in C8-B4 microglia challenged with LPS and IFN- $\gamma$ . A DCFDA assay was performed to assess the levels of ROS generated across the different treatment combinations (Fig. 3), and it demonstrated that ROS generation, (as indicated by RFU levels) drastically increased in LPS/IFN- $\gamma$ -challenged C8-B4 cells by 1.82±0.12-fold compared with the control. LPS/IFN- $\gamma$ -exposed C8-B4 cells treated with isoflurane alone produced 1.44-fold more ROS compared with control cells, while treatment with callistephin resulted in a 1.31-fold increase. The co-treatment of LPS/IFN- $\gamma$ -challenged C8-B4 cells with isoflurane and callistephin prevented the production of ROS, as the RFU levels of the co-treatment group were similar to the control (P>0.05 vs. control). These results collectively indicated that



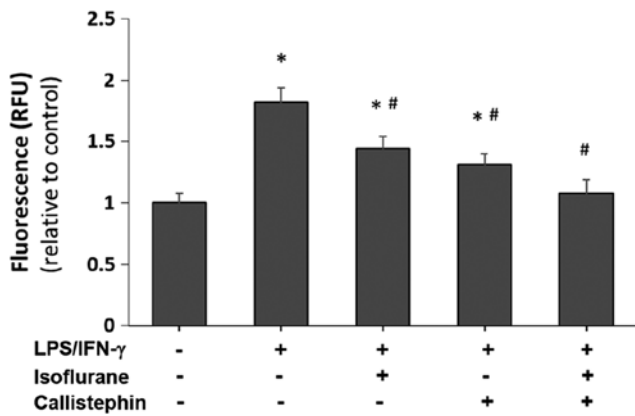


Figure 3. Effect of callistephin and isoflurane on ROS generation. The levels of ROS generated in control (untreated) C8-B4 cells were verified via dichlorofluorescein diacetate. Subsequently, the levels of ROS of cells challenged with LPS (100 ng/ $\mu$ l) and IFN- $\gamma$  (1 ng/ $\mu$ l) and treated with isoflurane, callistephin or in combination and ROS generation were determined as fold change relative to control, and compared between groups. \* $P$ <0.05 vs. control; # $P$ <0.05 vs. LPS/IFN- $\gamma$ . ROS, reactive oxygen species; LPS, lipopolysaccharide; IFN- $\gamma$ , interferon- $\gamma$ ; RFU, relative fluorescence units.

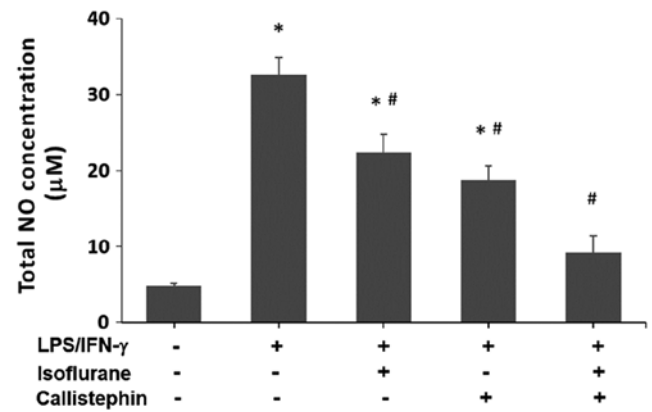


Figure 4. Effect of callistephin and isoflurane on NO concentration. The levels of NO were measured in control C8-B4 cells, cells challenged with LPS (100 ng/ $\mu$ l) and IFN- $\gamma$  (1 ng/ $\mu$ l), and challenged cells treated with isoflurane, callistephin or both. NO concentrations were determined using the Griess assay system, and absorbance was measured at 548 nm, with the NO concentration represented in  $\mu$ M. Overall, isoflurane, callistephin or co-treatment with both compounds resulted in a decrease in NO production. \* $P$ <0.05 vs. control; # $P$ <0.05 vs. LPS/IFN- $\gamma$ . NO, nitric oxide; LPS, lipopolysaccharide; IFN- $\gamma$ , interferon- $\gamma$ .

callistephin may have been significantly effective in combination with isoflurane in reducing the oxidative stress generated following exposure to LPS/IFN- $\gamma$ .

**Anti-inflammatory effects of callistephin and isoflurane on microglia.** Nitrosative stress, caused by the generation and accumulation of toxic RNS, including NO and peroxynitrite, is capable of causing damage to vital cellular components. The damage may be generated, for instance, by modifications to S-nitrosylation, involved in protein homeostasis and the mitochondrial respiratory chain system (47). The generation of RNS and ROS is associated with diseases, including amyotrophic lateral sclerosis and Parkinson's disease (48). Neuroinflammatory processes may generate excess NO and peroxynitrite via activation of microglia, which is involved in the etiology and progression of certain neurodegenerative diseases (47-49). Therefore, compounds that effectively scavenge RNS or prevent NO generation may markedly suppress inflammatory responses in neuronal cells. This may be of therapeutic significance as it may ameliorate the symptoms of certain neurodegenerative diseases. Thus, the capabilities of callistephin and isoflurane in reducing the inflammatory responses in microglia challenged with LPS/IFN- $\gamma$  were evaluated. Common indicators of the inflammatory response in microglia include enhanced production of the inflammatory cytokine TNF- $\alpha$  (50) and the production of the proinflammatory proteins Cox-2 (51) and iNOS, the latter being responsible for the production of NO (52).

Firstly, NO levels in control and LPS/IFN- $\gamma$ -challenged cells, treated with callistephin, isoflurane or both, were evaluated (Fig. 4). The results demonstrated that LPS/IFN- $\gamma$  induced a considerable increase in NO ( $32.6 \pm 2.3 \mu$ M) compared with the control ( $4.8 \pm 0.3 \mu$ M). The treatment of LPS-challenged C8-B4 cells with isoflurane led to a significant reduction in NO levels compared with the LPS/IFN- $\gamma$  treatment ( $22.4 \pm 2.4 \mu$ M). Treatment with callistephin also resulted in a significant decrease in the NO levels ( $18.7 \pm 1.9 \mu$ M) compared with the

LPS/IFN- $\gamma$  treatment. The combination of callistephin and isoflurane prevented an increase in the NO levels following the immune challenge with LPS/IFN- $\gamma$ , ( $9.2 \pm 2.2 \mu$ M;  $P > 0.05$  vs. control). Therefore, treatment with callistephin may have enhanced the protective effects of isoflurane in C8-B4 cells against nitrosative stress.

Secondly, the expression pattern of genes involved in inflammatory pathways were evaluated in the same treatment conditions. Thus, a semi-quantitative PCR analysis of iNOS, TNF- $\alpha$ , COX-2, and the p65 subunit of NF- $\kappa$ B was performed (Fig. 5A). The results revealed that LPS/IFN- $\gamma$  exposure increased the expression of each gene compared with the control. LPS/IFN- $\gamma$ -challenged C8-B4 cells treated with isoflurane exhibited lower expression levels for each gene, with the exception of NF- $\kappa$ B, whose expression was slightly increased. Challenged cells treated with callistephin exhibited lower expression levels of iNOS, TNF- $\alpha$  and COX-2, and slight increase in NF- $\kappa$ B levels. Lastly, the combination of callistephin with isoflurane reduced the expression level of iNOS, TNF- $\alpha$  and COX-2, while NF- $\kappa$ B expression was markedly reduced (Fig. 5A). The combined treatment was most effective on regulating the expression of iNOS gene, which is has been reported to be upregulated in certain neurodegenerative diseases (52,53). PCR also demonstrated that TNF- $\alpha$  and COX-2 expression were approximately equally suppressed by the combination of callistephin and isoflurane. However, NF- $\kappa$ B expression was only moderately reduced (Fig. 5A).

Lastly, the protein levels of iNOS, COX-2 and NF- $\kappa$ B were evaluated (Fig. 5B). The results indicated that C8-B4 cells challenged with LPS/IFN- $\gamma$  exhibited higher levels of iNOS, COX-2 and NF- $\kappa$ B. The treatment of challenged C8-B4 cells with isoflurane or callistephin caused a reduction in the level of iNOS and COX-2 compared with the challenged cells. The combination of callistephin with isoflurane further reduced the expression levels of iNOS, COX-2 and NF- $\kappa$ B protein compared with the LPS/IFN- $\gamma$ -challenged cells (Fig. 5B). The observations from gene and protein expression analysis

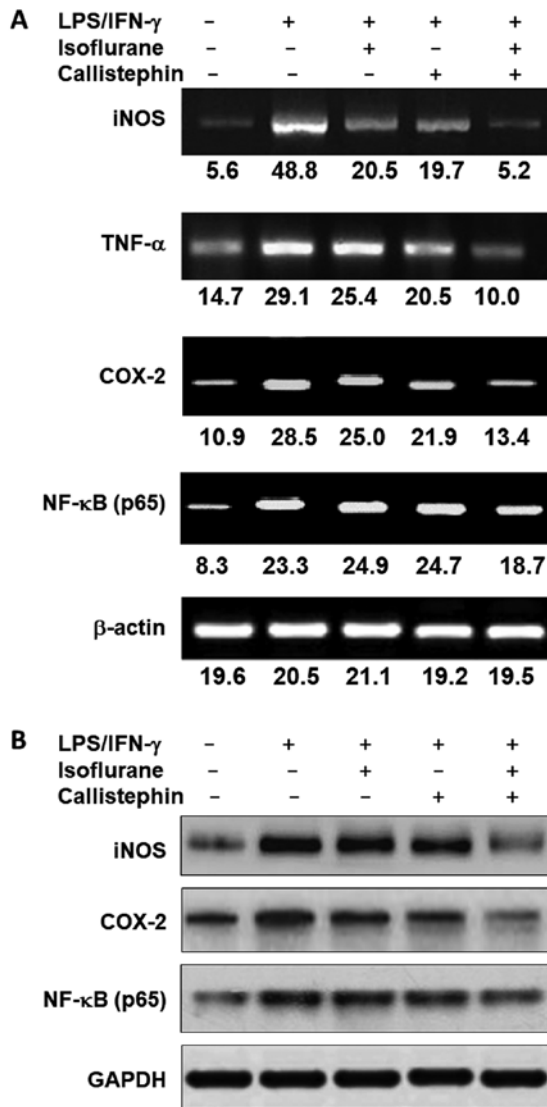


Figure 5. Effect of callistephin and isoflurane on the expression of inflammatory genes and proteins. C8-B4 cells were treated with LPS (100 ng/ $\mu$ l) and IFN- $\gamma$  (1 ng/ $\mu$ l), isoflurane (2%) and callistephin (100  $\mu$ M) in the indicated combinations. (A) Gene expression was assessed via semi-quantitative polymerase chain reaction, using  $\beta$ -actin as control. The respective relative ratios of cDNA bands are indicated under each band. (B) Protein levels were assessed via western blotting using GAPDH as loading control. LPS, lipopolysaccharide; IFN- $\gamma$ , interferon- $\gamma$ ; iNOS, inducible nitric oxide synthase; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; COX-2, cytochrome c oxidase subunit 2; NF- $\kappa$ B, nuclear factor- $\kappa$  B subunit.

suggested and strengthened the hypothesis that callistephin may synergistically interact with isoflurane to prevent microglial inflammation.

*Callistephin and isoflurane protect against microglial inflammation via p38.* Cell death induction in the context of inflammation is characterized by activation of caspase-3/7 (35), and this was also evaluated in the present study using a Caspase-Glo 3/7 assay. The results (Fig. 6) indicated that the activity of caspase-3/7 in C8-B4 cells challenged with LPS/IFN- $\gamma$  increased 3.2-fold compared with the control (1-fold RFU). Treatment with isoflurane significantly decreased the caspase-3/7 activity levels (2.41-fold RFU) compared with LPS/IFN- $\gamma$  challenged cells ( $P < 0.05$ ), yet

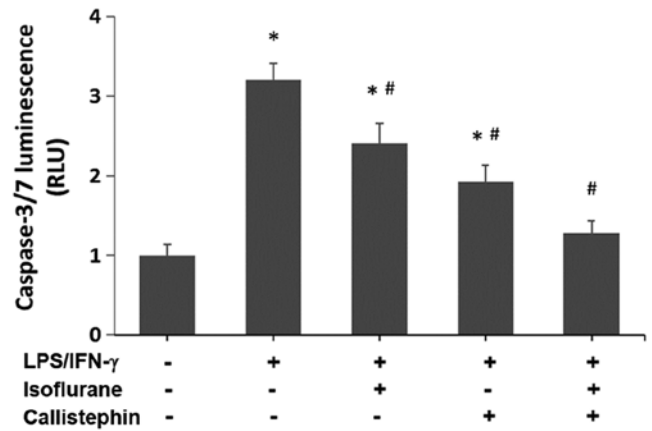


Figure 6. Effect of callistephin and isoflurane on microglial apoptosis. C8-B4 cells were treated with LPS (100 ng/ $\mu$ l) and IFN- $\gamma$  (1 ng/ $\mu$ l), isoflurane (2%) and callistephin (100  $\mu$ M) in the indicated combinations, and caspase activity was measured as an indicator of apoptosis using the Caspase 3/7-Glo. The luminescence was measured and calculated as the RLU change relative to the control. \* $P < 0.05$  vs. control; # $P < 0.05$  vs. LPS/IFN- $\gamma$ . LPS, lipopolysaccharide; IFN- $\gamma$ , interferon- $\gamma$ ; RLU, relative luminescence units.

they remained significantly higher than the control. Similarly, treatment with callistephin further decreased the caspase-3/7 activity (1.92-fold RFU) compared with LPS/IFN- $\gamma$  challenge alone ( $P < 0.05$ ). Lastly, the combination of callistephin with isoflurane prevented an increase in caspase-3/7 activity (1.28-fold RFU) following the immune challenge with LPS/IFN- $\gamma$ . The level of caspase activity observed with the co-treatment was significantly decreased compared with LPS/IFN- $\gamma$  ( $P < 0.05$ ) and not significantly different compared with the control. These observations demonstrate that while isoflurane and callistephin may have suppressed the apoptosis in microglial cells, with callistephin being slightly more efficient, their combination may have synergistically prevented apoptosis in microglial cells challenged with LPS/IFN- $\gamma$ .

Microglial activation following CNS injury promotes phagocytosis, a process regulated by the mitogen-activated protein kinase (MAPK) p38 (54). p38 is a group of important intracellular signaling proteins that respond to cellular stress and inflammatory cytokines, including LPS challenge (54). Thus, to understand the probable mechanism underlying the observed effects of callistephin, its effect on the expression of proteins associated with cell growth signaling was evaluated (Fig. 7). The western blot analysis revealed that challenging C8-B4 cells with LPS/IFN- $\gamma$  slightly downregulated the level of total p38, which was further recovered following treatment with isoflurane, callistephin or with their combination. The levels of phosphorylated p38 were upregulated in LPS/IFN- $\gamma$ -challenged C8-B4 cells compared with the control group. This upregulation was decreased following treatment with isoflurane or callistephin. The combination of callistephin and isoflurane may have further reduced the challenge-induced increase in phosphorylation levels of p38.

The relative levels of apoptosis-associated proteins Bcl-xL and PARP were further evaluated. The results (Fig. 7) suggested that LPS/IFN- $\gamma$ -challenged C8-B4 cells may have exhibited reduced levels of Bcl-xL. This was further confirmed by analyzing PARP, as the presence of its cleaved form (89 kDa fragment) confirmed higher caspase activity

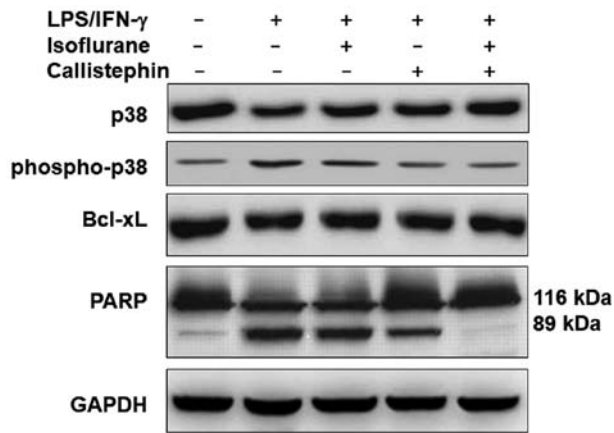


Figure 7. Effect of callistephin and isoflurane on p38 and apoptosis-associated proteins. C8-B4 cells were treated with LPS (100 ng/ $\mu$ l) and IFN- $\gamma$  (1 ng/ $\mu$ l), isoflurane (2%) and callistephin (100  $\mu$ M) in the indicated combinations. Protein expression was assessed via western blotting, using GAPDH as loading control. LPS, lipopolysaccharide; IFN- $\gamma$ , interferon- $\gamma$ ; phospho, phosphorylated; Bcl-xL, B-cell lymphoma 2 like 1; PARP, poly (ADP-ribose) polymerase 1.

following LPS/IFN- $\gamma$ -challenge. Isoflurane or callistephin treatment may have elevated the levels of Bcl-xL and the activity of caspase-3 in LPS/IFN- $\gamma$ -challenged C8-B4 cells. Likewise, compared with isoflurane, callistephin further reduced the level of cleaved-PARP, elevated following LPS/IFN- $\gamma$  challenge. Furthermore, the combination of callistephin may have prevented alterations to the levels of both proteins compared with the control. These results suggest that LPS/IFN- $\gamma$ -challenged microglial cells may have undergone inflammatory apoptotic cell death via p38 phosphorylation, and that the combination of callistephin and isoflurane may have prevented this apoptotic effect.

## Discussion

Anthocyanins are a unique family of compounds with natural antioxidant properties that have attracted considerable attention for their therapeutic potential (24-30). Various types of berries, including blueberries, cranberries, chokeberries and lingonberries, contain a variety of polyphenolics with protective properties (24,32,33). Anthocyanin rich fractions have been reported to reduce oxidative stress, and to protect neurons from inflammatory response in the CNS (30). Anthocyanins may protect neurons from various damaging chemicals and processes, including carbon tetrachloride, psychological stress, d-galactose, accelerated senescence, ischemia/reperfusion or middle cerebral artery occlusion (55). Isoflurane is a volatile substance commonly used for neurosurgical procedures. Isoflurane, at clinically relevant concentrations, has been reported to reduce the inflammation, oxidative stress and NO production in microglia following exposure to LPS and IFN- $\gamma$  in microglia (6,7). However, microglial overactivity may have detrimental consequences (2-4), as inflammation and phagocytosis may cause lasting harm to healthy neural tissue.

Thus, the present study aimed to understand the effects of isoflurane and callistephin following LPS/IFN- $\gamma$ -induced inflammatory responses in mouse C8-4B microglial cells, and assessed the impact of callistephin in combination with

isoflurane, a previously reported neuroprotective anesthetic agent (17-19). The results indicated that exposure to LPS/IFN- $\gamma$  increased microglial engulfment, which is required for the removal of cell debris or damaging agents from the neural tissue (9). LPS/IFN- $\gamma$  induced a strong immune response in microglia, which was verified by the increase in the expression inflammatory mediators like iNOS, TNF- $\alpha$  and COX-2, typical inflammation markers (47-49). The present results further suggested that treatment with isoflurane callistephin may have reduced microglial apoptosis by reducing p38 phosphorylation. Callistephin and isoflurane may have also reduced microglial engulfment responses and oxidative stress following an immune challenge. Notably, it is possible that callistephin may have enhanced the protective effects of isoflurane by suppressing inflammation and the consequent production of ROS and RNS.

The possible beneficial health effects of anthocyanin may be due to its metabolites, phenolic acids and poly-galacturonic acid, which have been shown to exhibit neuroprotective capabilities as they may regulate the levels of mediators of oxidative stress (44,45). Callistephin and kuromanin, anthocyanins derived from strawberries and black rice, were reported to exhibit neuroprotective effects against mitochondrial oxidative stress-induced death of cultured cerebellar granule neurons by reducing B-cell lymphoma-2 expression and reducing mitochondrial glutathione, and were also reported to reduce nitrosative stress (56,57). Two common phenolic acids, 4-hydroxybenzoic acid and protocatechuic acid, were observed to mitigate oxidative stress induced by hydrogen peroxide and may have suppressed neuronal cell death in cerebellar granule neurons (58). These reports seem to suggest that anthocyanins, including callistephin, may have neuroprotective and anti-inflammatory characteristics that may be of use in the treatment of certain aspects of neurodegenerative diseases.

Inflammatory immune responses in the CNS are primarily generated by microglia activation (4-6). The common markers of inflammatory responses in microglia include enhanced production of proinflammatory cytokines, including TNF- $\alpha$  (50), induction of proinflammatory proteins, including COX-2 (50) and promoting the activity of iNOS, which leads to the production of NO (52). In addition to oxidative stress, nitrosative stress, caused by the generation and accumulation of toxic RNS like NO and peroxynitrite, may damage various vital cellular components. The present study demonstrated that LPS/IFN- $\gamma$ -challenged microglia overproduced ROS and NO, which may have potentiated apoptosis. The ROS generated by the immune challenge were subsequently suppressed by independent treatment with isoflurane and callistephin, while the combination of both caused a synergistic suppression of ROS generation. Similarly, LPS/IFN- $\gamma$  exposure was also observed to increase NO, and treatment with isoflurane and callistephin reduced these levels, while the co-treatment further reduced these NO levels. This may have been achieved via reducing/suppressing the expression of TNF- $\alpha$  iNOS, COX-2 and NF- $\kappa$ B, which were seemingly overexpressed following exposure to LPS/IFN- $\gamma$ . Notably, the combination of callistephin with isoflurane caused the greatest reduction in the expression levels of these inflammation indicators. In addition, protein levels were also suggestive of the same results.



LPS and IFN- $\gamma$  are associated with injury in the CNS, and therefore promote microglial engulfment, phagocytosis and systemic inflammation (50-52). Injuries to the CNS cause activation of microglial cells, which migrate towards the injury site and eliminate decaying cells and other debris (59). Among various molecular mechanisms, MAPK p38 is an intracellular signaling pathway involved in cellular stress and inflammation responses (54). LPS is known to activate p38 by binding to Toll-like receptors, which are a class of pattern-recognition receptors in the innate immune system that induce inflammatory responses (54). A report has also indicated that p38 is involved in LPS-induced microglial phagocytosis of axonal debris (60). The present study further suggested that individual treatment with isoflurane and callistephin may have suppressed the phosphorylation of p38 caused by LPS/IFN- $\gamma$  exposure. Furthermore, these treatments may have also reduced caspase-3 activation and decreased the expression of the apoptosis markers Bcl-xL and cleaved PARP. Just as for the inflammation markers, callistephin may have also enhanced the protective effects of isoflurane by synergistically modulating the protein levels of these apoptosis associated proteins and the phosphorylation levels of p38. Therefore, the modulation of p38 phosphorylation may be the underlying mechanism by which callistephin and isoflurane exert neuroprotective effects.

As discussed, isoflurane has been reported to have numerous neuroprotective effects: The reduction of neuronal inflammation and apoptosis (6,7); suppression of neonatal hypoxia-ischemia induced neuronal injury (15); suppression of oxidative stress; and reduction of the levels of pro-inflammatory mediators (19-21). Phytochemicals, namely callistephin, have also been reported to exhibit anti-inflammatory, antioxidant, antiproliferative and antitumoral properties (24-30), and also to ameliorate certain aspects of neurodegenerative diseases (34-39). The present study suggested that the combined use of isoflurane and callistephin may therefore further help prevent neuronal apoptosis and degeneration.

In conclusion, the present study suggested that callistephin may have maintained the viability of C8-B4 cells against an inflammatory shock through downregulation of caspase-3/7 activity. These results further support previous studies reporting on the neuroprotective effects of phenols by reducing oxidative stress-induced cell death in neuronal cells (24-30). The observations from gene and protein expression analyses indicated that callistephin, like isoflurane, may have interacted with signaling pathways and regulated gene expression and protein levels. Moreover, the present study also revealed that callistephin may have strengthened the neuroprotective effects of isoflurane in maintaining the viability of microglia and reducing inflammatory responses. Finally, single treatment or co-treatment with callistephin and isoflurane may have attenuated the immune response and improved cell viability via phosphorylation of p38. Overall the present study suggested that the use of callistephin may be of help in the treatment of aspects of neurodegenerative diseases associated with microglial activation.

## Acknowledgements

Not applicable.

## Funding

No funding was received.

## Availability of data and materials

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

## Authors' contributions

LZ conceived and supervised the present study, analyzed data and wrote the manuscript. SC, TL and XW performed experiments, analyzed data and assisted in the preparation of the manuscript. HH and WL analyzed data, performed literature search and assisted in the preparation of the manuscript. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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