Brilliant blue G attenuates lipopolysaccharide-mediated microglial activation and inflammation

Kui Lu1, Jue Wang2, Bin Hu3, Xiaolei Shi1, Junyi Zhou4, Yamei Tang1, Ying Peng1

1 Department of Neurology, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou 510120, Guangdong Province, China
2 Department of Gynaecology and Obstetrics, First Affiliated Hospital of Kunming Medical University, Kunming 650032, Yunnan Province, China
3 Department of Neurosurgery, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University, Guangzhou 510120, Guangdong province, China
4 Department of Biochemistry and Molecular Biology, Medical school of Sun Yat-sen University, Guangzhou 510080, Guangdong Province, China

Abstract

Previous studies have confirmed that oxidized adenosine triphosphate, a P2X7 receptor antagonist, attenuates lipopolysaccharide-mediated microglial activation and inflammatory expression following neuronal damage in rat brain. NaCl and temperature may affect the potency of oxidized adenosine triphosphate. Brilliant blue G is a derivative of a widely used food additive and has little toxicity. This study explored the effects of brilliant blue G, a selective P2X7 receptor antagonist, on microglial activation and inflammation. Results demonstrated that brilliant blue G inhibited the release of cyclooxygenase-2 and interleukin-6 in BV2 cells. Immunofluorescence displayed that brilliant blue G could suppress lipopolysaccharide-induced microglial activation. This study used RNA interference to block P2X7 receptor expression and found that small interfering RNA also suppressed the release of cyclooxygenase-2 and interleukin-6 in BV2 cells. These results suggested that downregulation of the P2X7 receptor by brilliant blue G was involved in the inhibition of microglial activation and inflammation.

Key Words

neural regeneration; neurodegenerative disease; brilliant blue G; P2X7 receptor; lipopolysaccharide; microglia; inflammatory cytokines; RNA interference; cyclooxygenase-2; interleukin-6; grants-supported paper; photographs-containing paper; neuroregeneration

Research Highlights

(1) This study established the relative specificity of P2X7 receptor expression on microglia from murine brain tissue, providing evidence for the selective regulation of microglia via this receptor.
(2) RNA interference and brilliant blue G intervention could inhibit microglial activation and inflammatory reactions by regulating the P2X7 receptor, and provided experimental evidence of a novel way for the prevention and treatment of inflammatory-mediated neurodegenerative disease.
(3) Brilliant blue G is characterized by low toxicity and high selectivity, and can be used as an appropriate drug for blocking microglia-mediated inflammation and degeneration.
INTRODUCTION

Microglia are resident immune cells of the brain parenchyma that play important roles in the developing and adult central nervous system of mammals. In normal conditions, resting microglia act as supportive glial cells with their ramified processes dynamically monitoring the physiological homeostasis of the extracellular space\(^1\). When presented with specific stimuli stemming from pathologies, such as infection or trauma, microglia become activated, change their morphology to an amoeboid shape and release proinflammatory cytokines, such as cyclooxygenase-2 and interleukin-6\(^2\). A growing body of research supports involvement of activated microglia in brain pathologies caused by infectious diseases, trauma, tumors, ischemia, Alzheimer’s disease, Parkinson’s disease, Down’s syndrome, multiple sclerosis and neuro-AIDS\(^3\).

Brilliant blue G is a polysulfonate dye and a very potent noncompetitive selective antagonist of the P2X7 receptor. This compound has a nanomolar affinity for this receptor, and produces an action by inhibiting the initial cationic influx currents through P2X7 receptors. The downstream events associated with the activation of the P2X7 receptor can also be blocked by brilliant blue G\(^4\). Activation of the P2X7 receptor involves upregulated levels of extracellular adenosine triphosphate and participates in a diversity of microglia-mediated inflammatory responses\(^5,6\). To observe the role of the P2X7 receptor in microglial activation in the present study, lipopolysaccharide (a traditional microglial activator) was used to induce activation of microglia and to trigger a series of inflammatory cascade reactions. Lipopolysaccharide is known to induce nitric oxide and tumor necrosis factor-α production in microglia through various extracellular signal-regulated kinases, p38 mitogen-activated protein kinase, and c-Jun N-terminal kinase pathways\(^7,8\). Previous studies have proven that oxidized adenosine triphosphate, a P2X7 receptor antagonist, attenuates lipopolysaccharide-mediated microglial activation and inflammation following neuronal damage in rat brain\(^9\). Given that NaCl and temperature may affect the potency of oxidized adenosine triphosphate\(^10\), we confirmed that brilliant blue G will exert an inhibitory effect on lipopolysaccharide-induced encephalitis caused by microglial activation.

Currently, no report has been published confirming our hypothesis that the application of brilliant blue G can regulate microglia-mediated neuroinflammation. The current study investigated the microglial inflammatory response in the presence of brilliant blue G both in vitro and in vivo. Furthermore, this study examined the effect of brilliant blue G in lipopolysaccharide-induced microglial activation and used the findings to characterize the anti-inflammatory effects of brilliant blue G and other P2X7 receptor inhibitors to provide experimental evidence for the prevention and treatment of inflammatory-mediated neurodegenerative disease.

RESULTS

Lipopolysaccharide-induced microglial activation and upregulation of P2X7 receptor expression

Normal microglia were generally in a resting state, with the presence of branch-like forms and with the absence of CD11b expression in immunofluorescence staining (Figure 1A). After stimulation with lipopolysaccharide for 24 hours, microglia were transformed into an early activated condition characterized by large soma and amoeba-like shapes with intense CD11b staining (Figure 1B). P2X7 receptor protein expression in BV2 cells increased in a dose-dependent manner 24 hours after cells were stimulated with lipopolysaccharide, peaking at a lipopolysaccharide concentration of 100 ng/mL (Figure 2).

![Figure 1](image-url)  
**Figure 1.** Effect of lipopolysaccharide on the morphology of microglia (immunofluorescence staining, Cy3 labeled, scale bar: 20 μm).

(A) Control group: BV2 cells in a resting state.

(B) Lipopolysaccharide group: the morphological transformation of activated microglia with an amoeba-like shape.
Brilliant blue G inhibited lipopolysaccharide-induced microglial activation in vitro

This study examined morphological changes of lipopolysaccharide-stimulated BV2 cells in the presence of brilliant blue G, a P2X7 receptor inhibitor. Brilliant blue G was added to culture medium of BV2 cells 30 minutes before lipopolysaccharide treatment. Immunofluorescence staining was performed at 24 hours after lipopolysaccharide stimulation. More activated microglia, characterized by round cells with abundant cytoplasm and CD11b-positive staining, were present in the lipopolysaccharide group (Figure 3B) compared with the control group (Figure 3A). The trend was reversed in the lipopolysaccharide plus brilliant blue G group (Figure 3C). After brilliant blue G treatment, the expression of CD11b decreased and the morphology of the BV2 cells approached the “resting” ramified state. Fluorescence intensity of the control group and lipopolysaccharide plus brilliant blue G group was similarly weaker than the lipopolysaccharide group (Figure 3).

Brilliant blue G inhibited lipopolysaccharide-induced microglial activation and downregulated P2X7 receptor expression in mouse brain

Fifteen BALB/c mice were randomly divided into control, lipopolysaccharide, and lipopolysaccharide plus brilliant blue G groups. After 3 consecutive days of intervention, immunofluorescence staining was used to observe CD11b and P2X7 receptor expression. Results showed that the number of microglia (CD11b-positive cells) in the cerebral cortex of the lipopolysaccharide-stimulated brain at day 3 was more than that seen in the control and lipopolysaccharide plus brilliant blue G groups (Figure 4A). Double immunostaining for CD11b and P2X7 receptor revealed a predominant colocalization of CD11b-positive cells with the P2X7 receptor in each group. Moreover, the number of P2X7 receptor/CD11b-positive cells in the cortex of mice was significantly greater in the lipopolysaccharide group than in the control and lipopolysaccharide plus brilliant blue G groups (Figure 4B; P < 0.05).

Figure 2 Effects of different concentrations of lipopolysaccharide on the expression of the P2X7 receptor (P2X7R) in BV2 cells.

(A) Western blot demonstrated the trend in P2X7R expression in BV2 cells after 24 hours following increasing concentrations of lipopolysaccharide stimulation (0, 50, 100, 200 ng/mL equate to Control, LPS50, LPS100, LPS200, respectively).

(B) Relative absorbance ratios of P2X7R compared with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) calculated by densitometric quantification of the bands. Data are expressed as mean ± SD, n = 3. *P < 0.05, vs. control group one-way analysis of variance followed Student’s t-test.

Figure 3 Effect of brilliant blue G on lipopolysaccharide-induced morphologic changes of BV2 cells (fluorescence microscope, scale bar: 20 μm).

The immunofluorescence staining of CD11b (red, Cy3 labeled) showed that activated microglia after lipopolysaccharide stimulation (B) became amoeba-like compared with the control group (A), while lipopolysaccharide plus brilliant blue G group (C) took on a greater number of branch-like cells compared with the lipopolysaccharide group. Nuclei of BV2 cells were counterstained with 4',6-diamidino-2-phenylindole (blue).
Inhibition of the P2X7 receptor by brilliant blue G or small interfering RNA (siRNA) alleviated the inflammatory response induced by lipopolysaccharide

The expression of P2X7 receptor and relevant inflammatory mediators was analyzed at 24 hours post lipopolysaccharide stimulation. Brilliant blue G had a distinct inhibitory effect on P2X7 receptor expression in BV2 cells, both at the protein and mRNA levels (Figure 5; \( P < 0.05 \)). Western blot assay demonstrated that the expression of cyclooxygenase-2 protein was significantly elevated after 24 hours of lipopolysaccharide stimulation and this upregulation was distinctly reversed by brilliant blue G intervention (Figure 5C; \( P < 0.05 \)). Real-time PCR displayed that brilliant blue G decreased the expression of proinflammatory cytokines, including interleukin-6 and cyclooxygenase-2 (Figure 5D; \( P < 0.05 \)).

To further confirm the role of P2X7 receptors in the production of proinflammatory factors, siRNA was delivered to BV2 cells to confirm the specific block of P2X7 receptor expression. Negative control siRNA had no effects on the level of P2X7 receptor, cyclooxygenase-2 and interleukin-6 mRNA (Figure 6; \( P > 0.05 \)). BV2 cells pretreated with specific siRNA for P2X7 receptors were stimulated with lipopolysaccharide, whereupon the levels of proinflammatory cytokines were estimated. As seen with brilliant blue G, genetic silencing of P2X7 receptor with a Kv1.3-specific siRNA inhibited lipopolysaccharide-induced increase in cyclooxygenase-2 and interleukin-6 (Figure 6; \( P < 0.05 \)).
DISCUSSION

Proinflammatory cytokines released by activated microglia are contributing factors to pathological processes seen in many neurological disorders\[^{[1]}\]. It is commonly accepted that chronic and progressive neurodegeneration is associated with neurogenic inflammation, mainly mediated by microglia, the resident macrophage population in the brain. Control of inflammation via regulation of microglial activation has become an area of interest in the study of neurodegenerative disease. Specifically, a recent focus has turned to the involvement of ion channels in microglial activation\[^{[12]}\]. Various microglial ion channels have been shown to be involved in modulating membrane potential, cell volume, and intracellular ion concentrations to change proliferation, cell morphology, migration, and cytokine production\[^{[13]}\]. More than five probable paths provoke microglial activation with relevant signaling mediators and/or receptors, including fractalkine, interferon-g, monocyte chemoattractant protein-1, P2X4 and Toll-like receptor 4\[^{[14]}\].

Delayed-rectifier potassium voltage-gated channel kv1.3 and a21-activated K1 channel KCNN4/KCa3.1, for instance, play a vital role in mediating microglial activation\[^{[15]-[16]}\]. Additionally, microglial inflammatory activities are induced by P2X7 receptor activation via its downstream p38 mitogen-activated protein kinase-early growth response pathway\[^{[17]}\].

The P2X7 receptor is a member of the family of nonselective cationic channels gated by extracellular adenosine triphosphate. This receptor has unique properties with respect to other P2X members, such as its low affinity for adenosine triphosphate and its ability to be activated in murine cells by ADP-riboseylation through ADP-ribosyltransferase and nicotinamide adenine dinucleotide\[^{[18]-[19]}\]. Unlike other P2X receptors, the P2X7 receptor forms macropores when exposed to continuous or high-level applications of adenosine triphosphate. These macropores allow the passage of 900-Da molecules (as Lucifer Yellow) to induce cell death\[^{[20]-[21]}\]. This process seems to involve both dilation of the channel pore and the opening of additional channels, such as pannexin-1\[^{[22]}\]. Although P2X7 receptor expression has been detected in various tissues, including neurons, glial cells, fibroblasts, smooth muscle, endothelial and epithelial cells\[^{[21]}\], it is also in immune cells such as monocytes, macrophages, dendritic cells and T cells, where P2X7 receptor function has been extensively studied\[^{[23]}\]. Past studies have shown that the P2X7 receptor drives microglial activation and proliferation, therefore identification of extracellular adenosine triphosphate and the P2X7 receptor is a crucial factor in amyloid-β-dependent microglia activation\[^{[24]}\]. Abundant evidence reinforces this receptor as a crucial player for induction of inflammatory responses caused by microglial activation. In accordance with previous studies, our findings demonstrated that lipopolysaccharide-induced microglial activation had an apparent effect on upregulation of the P2X7 receptor.

This study demonstrated an anti-inflammatory effect for...
brilliant blue G on lipopolysaccharide-stimulated microglia in vitro and in vivo. Brilliant blue G exhibited potent inhibitory activity on P2X7 receptor, cyclooxygenase-2 and interleukin-6 production in lipopolysaccharide-stimulated microglia. This study confirmed that direct inhibition of P2X7 receptor expression by P2X7 receptor siRNA in lipopolysaccharide-stimulated microglia led to the same downregulation of cyclooxygenase-2 and interleukin-6 production, which indicated that the P2X7 receptor is a key regulatory point in the microglial inflammatory response. Double-immunofluorescence staining for CD11b and P2X7 receptor in this study, and a previous study of immunofluorescence colocalization,[9] confirmed that the P2X7 receptor was mainly present in lipopolysaccharide-stimulated microglia but less so in astrocytes and neurons. This observation increases the feasibility of P2X7 receptor-mediated microglial regulation.

It is known that microglia play a key role in mediating inflammatory processes in the central nervous system, which are associated with various neurodegenerative diseases.[25-26] Lipopolysaccharide, a glycolipid derived from the membrane surface of gram-negative bacteria (an endotoxin), has been used extensively in inflammatory studies.[27-28] Lipopolysaccharide activates microglia and exerts neurotoxic effects in both in vitro and in vivo systems.[29-30] This study demonstrated that lipopolysaccharide pretreatment activated microglia, which was indicated by morphological changes and a dramatic increase in levels of CD11b, a microglial cell marker, in lipopolysaccharide-treated BV2 cells. Lipopolysaccharide-treated microglia exhibited an increase in levels of interleukin-6 and cyclooxygenase-2, indicating that brain microglia release these proinflammatory cytokines when activated. Therefore, lipopolysaccharide-treated microglia in culture provide a feasible model for studying the roles of microglia in brain inflammation.

Brilliant blue G, an inhibitor of the P2X7 receptor, is a derivative of a commonly used blue food coloring, which can traverse the blood-brain barrier. Systemic administration of brilliant blue G may comprise a readily feasible approach for inflammatory disease in human brains. This study inferred that administration of brilliant blue G reduced the severity of brain inflammation without evident toxicity. Currently, more than 1 million pounds of blue food color are consumed yearly in the United States, corresponding to a daily intake of 16 mg per person[31-32] and has no known toxicity[33], except for the potential of metabolic acidosis in septic patients. This favorable toxicity profile, accompanied with its potent P2X7 receptor inhibition,[34], prompted us to test the neuroprotective actions of brilliant blue G in brain inflammation. This analysis showed that brilliant blue G significantly reduced brain inflammation, with its only notable side effect being the transient acquisition of a blue tint to the skin. Brilliant blue G targeted resident microglial cells because they express high levels of P2X7 receptors. Brilliant blue G strongly inhibited microglial activation at 24 hours after lipopolysaccharide treatment. As such, the suppression of reactive gliosis in mice treated with brilliant blue G might be secondary to the inhibition of the P2X7 receptor on microglia.

A critical difference in the anti-inflammatory mechanism of brilliant blue G compared with both minocycline and methylprednisolone is that P2X7 receptors are activated within seconds when cytosolic adenosine triphosphate flows out of traumatized cells, whereas both steroids and minocycline target inflammatory processes downstream to P2X7 receptor activation[39]. Furthermore, the expression of the P2X7 receptor was chiefly increased in lipopolysaccharide-stimulated microglia and brilliant blue G only has a single cellular target with few effects on neurons and astrocytes. Brilliant blue G may have a unique therapeutic profile, capable of suppressing the earliest steps in microglia-mediated inflammatory cascades. Since the deleterious effect of activated microglia on neurogenesis is probably mediated through the action of proinflammatory cytokines, such as interleukin-1, interleukin-6, and tumor necrosis factor-α[36], brilliant blue G may be inferred to restore neurogenesis in inflammation, though this has not yet been confirmed by scientific evidence.

In summary, brilliant blue G has indirect potency exerting substantial anti-inflammatory effects on activated microglial cells, although the exact mechanisms responsible for these actions are not well understood. Results suggest that anti-inflammatory activity of brilliant blue G may be mediated, at least in part, by inhibition of P2X7 receptor expression in microglia. Thus, brilliant blue G may serve as a potential alternative drug for microglial inflammatory-mediated neurodegenerative disorders. However, the detailed anti-inflammatory mechanisms of brilliant blue G in microglial inflammation will require further investigations.

**MATERIALS AND METHODS**

**Design**
A randomized, controlled experiment.
Immunohistochemistry: After administration for 3 consecutive days, animals were anesthetized with 10% chloral hydrate (4 mL/kg) and then received intracardiac perfusion with PBS and 4% paraformaldehyde. After perfusion, brains were quickly removed, post-fixed overnight with 4% paraformaldehyde and then dehydrated in gradient sucrose solution (10%–20%–30%) at 4°C. Serial sections of mouse brain (10 μm) were cut using a cryostat microtome and mounted onto microscope slides. These slides were stored temporarily in cryoprotectant solution at ~30°C. Frozen sections were blocked in solution containing 5% normal goat serum for 1 hour after rinsing in 0.3% Triton X-100 for 30 minutes at 37°C. After several washes, sections were simultaneously incubated overnight at 4°C with the following primary antibodies: monoclonal rat anti-mouse CD11b (1:100; AbD Serotec, Kidlington, Oxford, UK) and polyclonal rabbit anti-P2X7 receptor (1:200; Alomone Labs, Jerusalem, Israel). Primary antibodies were detected with Alexa-488-conjugated goat anti-rabbit and Alexa-594-conjugated goat anti-rat antibodies (1:500; Jackson ImmunoResearch, Jacksonville, PA, USA) for 1 hour at room temperature. Sections were coverslipped with glycerol and fluorescent signals were then detected using an Olympus BX5 microscope (Tokyo, Japan). P2X7 receptor+/CD11b+ cells were counted in five random 200 × fields in the cortex for each repeated experiment. Five coronal slices per animal, including the hippocampus (five mice in each group), were counted. The average of all fields for one mouse was included in statistical analysis.

Immunocytochemistry: Prior to staining, BV2 cultures were washed twice with PBS. Cells were fixed with 4% paraformaldehyde for 20 minutes, and then blocked with 5% goat serum albumin for 1 hour. Afterwards, cells were incubated with monoclonal rat anti-mouse CD11b (1:100; AbD Serotec) at 4°C overnight. After repeated washing with PBS three times, cells were incubated with rabbit anti-rat Cy3-conjugated secondary antibodies (1:200; Millipore, Billerica, MA, USA) for 1 hour at 37°C in a thermostat incubator and coverslipped in anti-fluorescence-quenching reagent (Boster, Wuhan, Hubei Province, China). After 4',6-diamidino-2-phenylindole (Sigma) staining, fluorescent images were detected and analyzed, as mentioned above.

Inhibiting expression of P2X7 receptor by siRNA
BV2 microglia cells were maintained in proliferating medium in 6-well plates for 2 days, at which time they reached 30–50% confluence and were used for transfection of siRNA. The siRNAs targeting mouse P2X7 receptor sequence were chemically synthesized by GenePharma: forward and reverse RNA strands (5'-CCG UAC UCA UCA AGA AUA ATT-3'; 5'-UUA UUC UUG
AUG AGU ACG GTT-3'). Control non-specific sequences were as follows: 5'-UUC GAA CGU GUC AGC UTT-3'; 5'-AGC UGA CAC GUU CGG AGA ATT-3'. Briefly, 5 μL of transfectamine (Invitrogen, Carlsbad, CA, USA) was added to Opti-MEM I (Gibco) reduced serum medium to a final volume of 250 μL. A volume of 10 μL of siRNA was added to Opti-MEM I to a final volume of 250 μL and incubated for 5 minutes. The serine and transfectamine solutions were combined and mixed by gentle pipetting and incubated at room temperature for 20 minutes. Cells were washed with Opti-MEM I, and 1 500 μL of fresh Opti-MEM I was added to each well. The transfection agent/siRNA complex was added dropwise into the cells and incubated at 37°C. BV2 cells were treated with P2X7 receptor siRNA or a negative control siRNA for 6 hours, followed by an addition of lipopolysaccharide (100 ng/mL) for 24 hours. Gene and protein expression were detected by real-time PCR and western blot assay, respectively.

**Expression of P2X7 receptor, interleukin-6 and cyclooxygenase-2 mRNA in BV2 cells**

P2X7 receptor, interleukin-6 and cyclooxygenase-2 mRNA expression was determined by real-time PCR. BV2 cells were treated and total RNA was extracted using a RNeasy mini kit [TaKaRa Biotechnology (Dalian) Co., Ltd., Dalian, Liaoning Province, China]. RNA samples were used for first-strand cDNA synthesis using a SuperScript cDNA kit (TaKaRa). Real-time PCR was performed using a Roche Lightcyle 480 (Roche, Welwyn Garden, Hertfordshire, UK) and a SYBR green real-time kit (TaKaRa). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The ratios of P2X7 receptor/GAPDH, interleukin-6/GAPDH and cyclooxygenase-2/GAPDH for every sample were first calculated, and results were expressed as fold changes compared with control. Primer sequences are shown in Table 1.

**Western blot analysis for P2X7 receptor and cyclooxygenase-2 expression in BV2 cells**

Protein expression of P2X7 receptor and cyclooxygenase-2 was monitored in microglia by western blot assay. Total protein extracts were prepared and quantified from BV2 cells in radioimmunoprecipitation assay lysates. In brief, 60 μg of total proteins for each lane were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane using an electrophoretic transfer apparatus. The membrane was then incubated with primary antibodies, including polyclonal rabbit anti-P2X7 receptor (1:400; Alomone Labs), polyclonal rabbit anti-cyclooxygenase-2 (1:300; Cell Signaling Technology, Beverly, MA, USA), and polyclonal rabbit anti-GAPDH (1:500; Cell Signaling Technology) at 4°C overnight. Subsequently the membrane was incubated with the secondary antibody: goat anti-rabbit IgG-horseradish peroxidase (1:5 000; Cell Signaling Technology) for 1 hour at room temperature. After washing in Tris-buffered saline Tween-20, blots were developed with enhanced chemiluminescence detection reagents (Millipore). The relative absorbance values of bands were analyzed using a gel imaging analysis system (Genetics, Campbell, CA, USA).

**Statistical analysis**

Statistical analysis was performed using SPSS 16.0 software (SPSS, Chicago, IL, USA). At least three independent experiments were performed for each experimental procedure. Measurement data are expressed as mean ± SD. Differences were assessed using one-way analysis of variance for multiple comparisons followed by the Student-Newman-Keuls test for intergroup comparisons or Student’s t-test for intergroup comparisons. Two-tailed values of P < 0.05 were considered statistically significant.

**Funding:** This study was supported by the National Natural Science Foundation of China, No. 81072242, and the Excellent Supervisor & Yat-Sen Creative Talent Development Program of Sun Yat-sen University.

**Author contributions:** Kui Lu, Jue Wang, Junyi Zhou, Ying Peng and Yamei Tang designed the study. Kui Lu, Jue Wang, Bin Hu and Xiaolei Shi conducted the experiments and analyzed the data. Kui Lu wrote the paper. All authors approved the final version of the paper.

**Conflicts of interest:** None declared.

**Ethical approval:** All procedures followed the guideline of the Chinese National Institute of Health for Humane Care and protocols were approved by the committee on animal research

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### Table 1  Primer sequences used for quantitative real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer (5’–3’)</th>
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<tbody>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>Forward: ATC TTC TGG TGG AGC AGC AGC</td>
</tr>
<tr>
<td>Cyclooxygenase-2</td>
<td>Forward: GCC CGA CAC CTT CAA CAT T</td>
</tr>
<tr>
<td>Interleukin-6</td>
<td>Forward: GTC ACA GAA GGA GTG GCT AAG GA</td>
</tr>
<tr>
<td>P2X7 receptor</td>
<td>Forward: TTA TGG CAC CGT CAA GTG G</td>
</tr>
</tbody>
</table>
at Sun Yat-sen University in China.

**Author statements:** The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

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(Edited by Xie SY, Yuan DF/Qiu Y/Song LP)