

Penahyclidine hydrochloride alleviates LPS-induced inflammatory responses and oxidative stress via ROS/Nrf2/HO-1 activation in RAW264.7 cells

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Abstract

Background. Inflammation is a biological response of the immune system to harmful stimuli. Penahyclidine hydrochloride (PCH) can alleviate inflammation and oxidative stress by activating reactive oxygen species (ROS), nuclear factor erythroid 2-related factor (Nrf2) and heme oxygenase 1 (HO-1) in animal models, but there is a lack of cellular evidence.

Objectives. This study investigated the effects of PCH on lipopolysaccharide (LPS)-induced inflammation response and oxidative stress in RAW264.7 cells.

Materials and methods. RAW264.7 cells were treated with 1 µg/mL or 5 µg/mL of PCH, with interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), IL-1β, and prostaglandin E2 (PGE2) levels measured with enzyme-linked immunosorbent assay (ELISA) and nitric oxide (NO) measured using the Griess test. Reactive oxygen species were examined with flow cytometry and immunofluorescence, and b-related factor 2 (BRF-2) and NAD(P)H-quinone oxidoreductase 1 (NQO1) using western blot.

Results. Penahyclidine hydrochloride partly, but substantially, reversed LPS-related NO and PGE2 production by RAW264.7 cells in a dose-dependent manner and suppressed LPS-induced expression of IL-6, TNF-α and IL-1β messenger ribonucleic acid (mRNA), secretion of IL-6, TNF-α and IL-1β, and ROS production. Lipopolysaccharide stimulation did not affect Nrf2, heme oxygenase 1 (HO-1) or NQO1 protein expression in RAW264.7 cells not treated with PCH. However, PCH treatment significantly elevated Nrf2, HO-1 and NQO1 protein in LPS-treated RAW264.7 cells, an effect that was dose-dependent. The ROS scavenging using N-acetyl-L-cysteine abolished the PCH-induced upregulation of Nrf2 and HO-1.

Conclusions. Penahyclidine hydrochloride may alleviate LPS-induced inflammation and oxidative stress by activating Nrf2 signaling in RAW264.7 macrophages. These findings suggest that PCH could alleviate inflammation by targeting activated macrophages.

Key words: macrophages, inflammation, lipopolysaccharide, oxidative stress, penahyclidine hydrochloride

Background

Inflammation represents a critical biological response of the immune system to harmful stimuli such as pathogenic organisms, injured cells and tissues, toxic molecules, and irradiation.¹ Although inflammation is necessary to the normal healing process by promoting the disposal of injured cells and pathogenic organisms,^{2,3} uncontrolled or excessive inflammation can trigger acute and/or chronic damage in multiple organs, leading to disease.^{1,4} Inflammation can even promote the growth of tumor cells and cancers.⁵ The cytokine storm resulting from acute excessive immune and inflammatory reactions is also involved in the morbidity and mortality of several conditions like sepsis and coronavirus disease-19 (COVID-19).^{6,7} Low-grade chronic inflammation is involved in conditions such as type 2 diabetes, obesity and hypertension, and contributes to the development of complications and associated diseases.^{8,9}

Uncontrolled inflammation is characterized by the overproduction of pro-inflammatory modulators, including nitric oxide (NO), prostaglandin E2 (PGE2) and cytokines,¹⁰ playing a key role in the pathogenetic mechanisms of various chronic disorders, e.g., cardiovascular diseases, type 2 diabetes, rheumatoid arthritis, and cancers.¹¹ Therefore, targeting inflammation is a promising avenue for chronic disease prevention and treatment.^{11–13}

Inflammation is also involved in neurological and psychiatric disease development and progression.^{14–18} There is a functional interplay between the central and autonomic nervous systems, and prefrontal cortex disruption may contribute to irregular behavioral responses, leading to many cognitive dysfunctions common in neurological diseases.¹⁹ In this regard, some anti-inflammatory drugs (e.g., celecoxib and aspirin) or drugs with pleiotropic anti-inflammatory effects (e.g., pioglitazone, statins and minocycline) function in bipolar disorder, major depressive disorder and schizophrenia, given that baseline inflammation is present.²⁰

Inflammation is a complex process involving several cell types and mechanisms,^{1,21} with macrophages critical in its initiation, maintenance and resolution. Indeed, they can be activated and deactivated by different cytokines produced during inflammatory processes. Activating cytokines and signals include interferon gamma (IFN γ) granulocyte-monocyte colony-stimulating factor, tumor necrosis factor alpha (TNF- α), bacterial lipopolysaccharide (LPS), extracellular matrix proteins, and chemical mediators. On the other hand, deactivating cytokines and signals include interleukin (IL)-10, transforming growth factor beta (TGF- β), and the removal or deactivation of mediators.^{22,23}

Oxidative stress contributes significantly to inflammatory reactions observed in chronic disorders,²⁴ involves cellular damage that can trigger immune cells and processes,^{25,26} and occurs when the reactive oxygen species (ROS) exceed antioxidant capacity.²⁶ Reactive oxygen species are normal by-products of adenosine triphosphate

(ATP) production by the mitochondria, but can increase under pathogenic conditions.²⁷ In addition, some immune cells can produce ROS to destroy pathogens and abnormal cells.²⁸ Besides consuming antioxidant compounds, several innate antioxidant mechanisms exist in mammals.^{29,30}

Nuclear factor erythroid 2-related factor (Nrf2) is a pleiotropic transcription factor that induces defense mechanisms counteracting oxidative stress and inflammation. During oxidative stress, Nrf2 upregulates multiple genes responsible for antioxidant activity, including heme oxygenase 1 (HO-1), which reduces oxidative stress by clearing heme, and NAD(P)H-quinone oxidoreductase 1 (NQO1), that prevents hydroquinone conversion to ROS.³¹ Besides its role in oxidative stress, the Nrf2 activation mediates the anti-inflammatory features of some commonly used nonsteroidal anti-inflammatory drugs,³² suggesting a contribution of Nrf2 in the crosstalk between inflammation and oxidative stress. It can also decrease the transcription of pro-inflammatory cytokines in activated macrophages.³³

Penhyclidine hydrochloride (C₂₀H₂₉NO₂·HCl; PHC) is an anticholinergic drug designed by the Chinese Academy of Military Sciences that exhibits substantial peripheral and central anticholinergic activities by simultaneously binding to the M and N cholinergic receptors.³⁴ It is widely used in China as a reversal drug for treating organophosphorus poisoning and a pre-anesthetic medication to reduce respiratory secretion and vagus nerve reflex.³⁵ Penhyclidine hydrochloride also possesses anti-inflammatory and antioxidant properties, demonstrating protective effects on inflammation and oxidative stress-related disorders such as heart ischemia/reperfusion, acute kidney injury and septic shock.^{36–38} Furthermore, PHC can attenuate the Toll-like receptors in inflammatory chronic lung diseases³⁹ and acute lung injury,⁴⁰ and has been shown to decrease the postoperative expression of pro-inflammatory cytokines⁴¹ and reduce cerebral injury in models of cardiopulmonary bypass by decreasing inflammation and neuronal apoptosis.⁴² Toxicity and adverse events associated with PHC are dry mouth, flushing and dry skin. High doses can cause dizziness, urinary retention, delirium, and elevated body temperature.³⁵

Studies have shown that PHC induces Nrf2/HO-1 signaling in acute lung/kidney damage triggered by ischemia/reperfusion or rhabdomyolysis in animal models, along with suppressing ROS and pro-inflammatory cytokine production, e.g., TNF- α , IL-6 and IL-1 β .^{36,43,44} However, there is still a lack of cellular evidence of Nrf2/HO-1 pathway involvement in the anti-inflammatory and antioxidant activities of PHC.

Activated macrophages are a major source of pro-inflammatory mediators in the development of inflammation,⁴⁵ and LPS-stimulated murine RAW264.7 macrophages are widely used in inflammation research.⁴⁶

In this study, we performed dose–response experiments on the anti-inflammatory and antioxidant activities of PHC and the PHC-induced alterations in Nrf2 signaling

in LPS-treated RAW264.7 cells. The study provides cellular evidence of Nrf2 pathway involvement in the anti-inflammatory and antioxidant activities of PHC, serving as a potential therapeutic target for managing inflammatory diseases.

Objectives

This study aimed to investigate the effects of PHC on LPS-induced inflammatory responses and oxidative stress in RAW264.7 cells.

Methods

Cell culture

RAW264.7 cells, provided by The Central Laboratory of Peking University Binhai Hospital (Beijing, China), underwent culture in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Waltham, USA) containing high glucose, 10% fetal bovine serum (Thermo Fisher Scientific), 100 IU/mL penicillin and 100 IU/mL streptomycin (Solarbio Life Science, Beijing, China), at 37°C with 5% CO₂.

Cell Counting Kit-8 (CCK-8) assay

Cell viability was assessed using Cell Counting Kit-8 (CCK-8; Solarbio Life Science, Beijing, China) as directed by the manufacturer.⁴⁷ RAW264.7 cells were cultured in 96-well microplates (Corning Company, Corning, USA) at a density of 5×10^3 /well. Cells were serum starved overnight and administered various PHC doses (0, 1, 5, 10, 20, and 40 µg/mL; Jinzhou Aohong Pharmaceutical Industry Co., Ltd., Jinzhou, China) for 24 h. Doses as high as 40 µg/mL have not been reported in the literature previously, and such a wide range of increasing doses was chosen for this study to verify the toxicity of PHC on RAW264.7 cells and provide a basis for dose selection for future studies. The results of cell viability showed that PHC concentrations ranging from 1 µg/mL to 10 µg/mL had no significant effect on RAW264.7 cell viability. Thus, we treated RAW264.7 cells with 1 µg/mL or 5 µg/mL PHC in the subsequent experiments. Cell Counting Kit-8 solution (10 µL) was added to each well, and absorbance (A) was measured at 450 nm after incubation for 1 h at 37°C using an Epoch microplate spectrophotometer (BioTek, Winooski, USA). Cell viability was calculated as $(A - A_0)/A_0 \times 100\%$.

Enzyme-linked immunosorbent assay

RAW264.7 cells underwent culture in 6-well plates at 2.5×10^6 /well. After overnight serum starvation, cells were administered 1 µg/mL LPS (O111:B4; #L4391; Sigma-Aldrich, St. Louis, USA) with or without 1 µg/mL or 5 µg/mL PHC

in serum-deprived DMEM for 24 h.⁴⁷ Serum-free medium was used as a negative control. The culture medium was collected for measuring IL-6, TNF-α, IL-1β, and PGE2 using specific enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Shanghai, China) as directed by the manufacturer.

Nitric oxide measurement using the Griess reagent

RAW264.7 cells underwent the treatments outlined above. Secreted NO concentration was measured using an NO assay kit containing Griess reagent (Beyotime Biotechnology, Shanghai, China) as directed by the manufacturer.⁴⁸

Flow cytometry

Intracellular ROS was assessed using flow cytometry.⁴⁹ Cells washed with phosphate-buffered saline (PBS) underwent a 20-min incubation with dichlorofluorescein diacetate (DCFH-DA; 10 µM) at 37°C. Fluorescence intensity was obtained with a Dx FLEX flow cytometer (Beckman Coulter, Brea, USA), with FlowJo 10.8.1 (FlowJo LLC, Ashland, USA) used for analysis.

Immunofluorescence staining

Intracellular ROS was detected using DCFH-DA staining.⁴⁹ Briefly, RAW264.7 cells underwent a 20-min incubation with DCFH-DA (10 µM) (Reactive Oxygen Species Assay Kit, S0033S; Beyotime Biotechnology) at 37°C, fluorescence was visualized, and images were acquired under a Zeiss LSM 800 confocal laser scanning microscope (Carl Zeiss AG, Jena, Germany).

Quantitative reverse transcription polymerase chain reaction

Total ribonucleic acid (RNA) was isolated with an Eastep® Super RNA extraction kit (Shanghai Promega Biological Products, Shanghai, China) as instructed by the manufacturer.⁵⁰ After complementary DNA (cDNA) synthesis, quantitative reverse transcription polymerase chain reaction (qRT-PCR) was carried out with SYBR green and the following primers (Genewitz, South Plainfield, USA):

β-actin,
5'-GTGACGTTGACATCCGTAAAGA-3' (sense) and
5'-GCCGGACTCATCGTACTCC-3' (antisense);
IL-6,
5'-TGATGGATGCTACCAAACTGGA-3' (sense) and
5'-TGTGACTCCAGCTTATCTCTTGG-3' (antisense);
inducible NO synthase (iNOS),
5'-GCGCTCTAGTGAAGCAAAGC-3' (sense) and
5'-GGGATTCTGGAACATTCTGTGC-3' (antisense);
IL-1β,
5'-TGCCACCTTTTGACAGTGATG-3' (sense) and
5'-ATGTGCTGCTGCGAGATTTG-3' (antisense);

TNF- α ,
 5'-CCCTCACAACCAACCAC-3' (sense) and
 5'-ACAAGGTACAACCCATCGGC-3' (antisense).
 The 2- $\Delta\Delta CT$ method was utilized for data analysis.

Western blot

RAW264.7 cell lysis used radioimmunoprecipitation assay (RIPA) buffer, with supernatants collected after a 10-min centrifugation at 4°C (12,000 r/min). A bicinchoninic acid assay (BCA) (P0010S; Beyotime Biotechnology) was performed for protein quantitation. Identical quantities of total protein (40 μ g) were resolved using 12% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. The specimens were blocked with 5% nonfat milk for 1 h at ambient, followed by overnight incubation with primary antibodies against Nrf2 (1:1,000; #12721S; Cell Signaling Technology (CST), Danvers, USA), HO-1 (1:1,000; #43966S; CST), NQO1 (1:1000; #ab28947; Abcam, Cambridge, UK), GAPDH (1:1,000; #5174S; CST), or β -tubulin (1:1000; #ab18207; Abcam) at 4°C. Next, washing with tris-buffered saline containing 0.1% Tween 20 (TBST) was performed, with subsequent incubation with secondary antibodies for 1 h at ambient temperature. Protein bands were visualized with the enhanced chemiluminescence kit (Advansta, Menlo Park, USA) and analyzed using ImageJ (National Institutes of Health (NIH), Bethesda, USA).

Statistical analyses

The analysis employed GraphPad Prism 9 (GraphPad Software, San Diego, USA), with Fig. 1,2 using 6 data points and Fig. 3,4 using 3 data points. Differences in characteristics between groups were analyzed using the Kruskal–Wallis test with Dunn's post hoc tests, with $p < 0.05$ indicating statistical significance.

Results

PHC suppressed LPS-associated NO and PGE2 production in RAW264.7 cells

In order to select the appropriate PHC treatment doses, we examined RAW264.7 cell viability after administering diverse PHC concentrations and found that PHC ranging from 1 μ g/mL to 10 μ g/mL had no marked effects (Fig. 1A and Supplementary Tables 1,2). Thus, the subsequent assays used 1 μ g/mL or 5 μ g/mL PHC to remain below the toxicity threshold. For evaluating PHC's effect on LPS-related inflammation, RAW264.7 cells were treated with various PHC concentrations in the presence of LPS. Lipopolysaccharide dramatically enhanced NO and PGE2 production by RAW264.7 cells (Fig. 1B,C and Supplementary Tables 3–6). Penicillamine hydrochloride partly but

substantially reversed LPS-related NO and PGE2 production in a dose-dependent manner, with the same trend observed for iNOS mRNA expression (Fig. 1D and Supplementary Tables 7,8). In addition, compared to untreated round-shaped RAW264.7 cells, LPS-stimulated cells became rod-shaped, bearing pseudopodia. Of note, the morphological alterations were alleviated by PHC treatment dose-dependently (Fig. 1E). The above findings indicated that PHC inhibited LPS-related inflammation in macrophages.

PHC suppressed LPS-induced expression and secretion of pro-inflammatory cytokines in RAW264.7 cells

To assess PHC's effect on LPS-associated inflammation, we determined the expression and secretion of pro-inflammatory cytokines in RAW264.7 cells administered LPS and PHC. As depicted in Fig. 2A–F and Supplementary Tables 9–20, LPS dramatically elevated IL-6, TNF- α and IL-1 β mRNA amounts and secretion, and these effects were partially but significantly attenuated by PHC dose-dependently. The above data suggested that PHC suppressed LPS-related inflammation in macrophages.

PHC abrogated LPS-induced ROS production in RAW264.7 cells

In order to examine PHC's effect on LPS-induced oxidative stress, we determined ROS production in RAW264.7 cells administered with LPS and PHC. DCFH-DA-based flow cytometry analysis (Fig. 3A,B and Supplementary Tables 21–23) and immunofluorescence (Fig. 3C,D and Supplementary Tables 24–26) consistently showed that PHC dose-dependently and remarkably abrogated LPS-stimulated ROS production. These data suggested that PHC alleviated LPS-induced oxidative stress in macrophages.

PHC activated Nrf2 signaling in RAW264.7 cells

In order to investigate Nrf2 pathway involvement in PHC's effect in RAW264.7 cells, we determined the protein amounts of Nrf2 and its target genes HO-1 and NQO1 in RAW264.7 cells. As depicted in Fig. 4A,B and Supplementary Tables 27–34, LPS stimulation did not affect the protein expression of Nrf2, HO-1 and NQO1 in cells without PHC. However, PHC treatment significantly elevated Nrf2, HO-1 and NQO1 protein in LPS-treated cells dose-dependently.

We also focused on the effects of the ROS scavenger n-acetyl-l-cysteine (NAC) on PHC-induced Nrf2 and HO-1. Penicillamine hydrochloride at a concentration of 5 μ g/mL significantly upregulated Nrf2 expression, but this was significantly inhibited by NAC. Moreover, PHC at a concentration of 5 μ g/mL showed a similar trend in nuclear HO-1 expression, although the difference was not statistically significant, possibly due to limitations

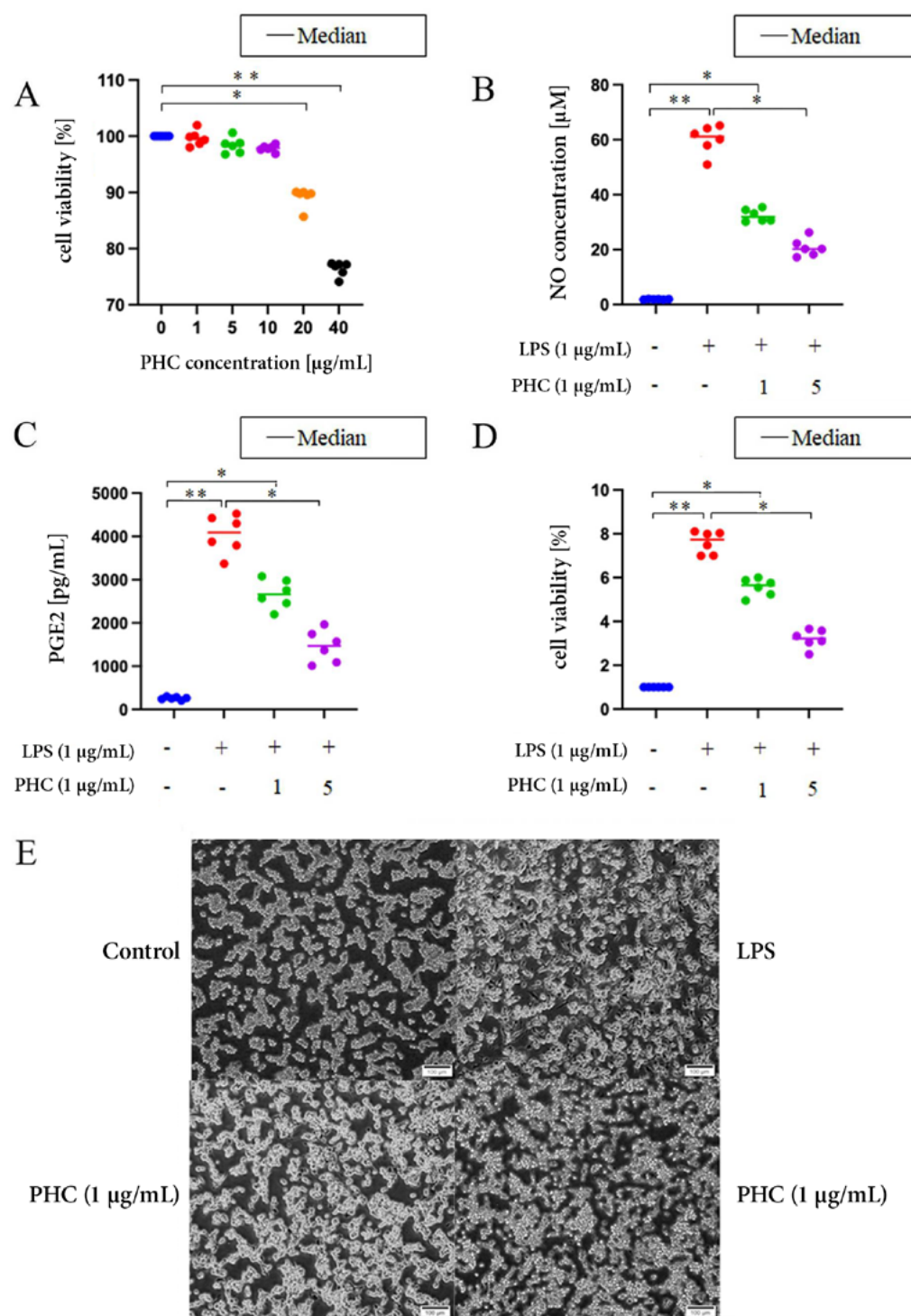


Fig. 1. Penehyclidine hydrochloride (PHC) inhibited lipopolysaccharide (LPS)-induced nitric oxide (NO) and prostaglandin E2 (PGE2) production in RAW264.7 cells. **A.** RAW264.7 cells were serum starved overnight and administered PHC at various concentrations (0, 1, 5, 10, 20, and 40 μg/mL) for 24 h. Untreated cells were used as a negative control. The Cell Counting Kit-8 (CCK-8) assay was used to determine cell viability. **B–E.** RAW264.7 cells were administered 1 μg/mL LPS alone or in combination with 1 μg/mL or 5 μg/mL PHC for 24 h. Nitric oxide (**B**) and PGE2 (**C**) were measured in the cell culture medium. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was conducted to determine the messenger ribonucleic acid (mRNA) expression of inducible nitric oxide synthase (iNOS) (**D**) in RAW264.7 cells. Cell morphology (**E**) was observed under a bright-field microscope at ×10 magnification. Data are mean ± standard deviation (mean ± SD).

* $p < 0.05$; ** $p < 0.01$ ($n = 6$). Differences in characteristics between groups were analyzed using the Kruskal–Wallis test with Dunn's post hoc test.

in sample size. These findings suggest that the activation of Nrf2/HO-1 in macrophages by PHC is associated with ROS (Fig. 4C and Supplementary Tables 35–40).

Discussion

This study showed that PHC could reverse LPS-related NO and PGE2 production in RAW264.7 cells. PHC also suppressed LPS-induced IL-6, TNF-α and IL-1β mRNA

expression, IL-6, TNF-α and IL-1β secretion, and ROS production. LPS stimulation did not affect Nrf2, HO-1 and NQO1 protein expression in RAW264.7 cells without PHC, but PHC treatment elevated Nrf2, HO-1 and NQO1 protein after LPS treatment. Reactive oxygen species scavenging using NAC abolished the PHC-induced up-regulation of Nrf2 and HO-1, highlighting the role of ROS in the process. Therefore, PHC partially abrogated LPS-related overproduction of pro-inflammatory mediators and ROS in RAW264.7 cells, and, in the presence of LPS,

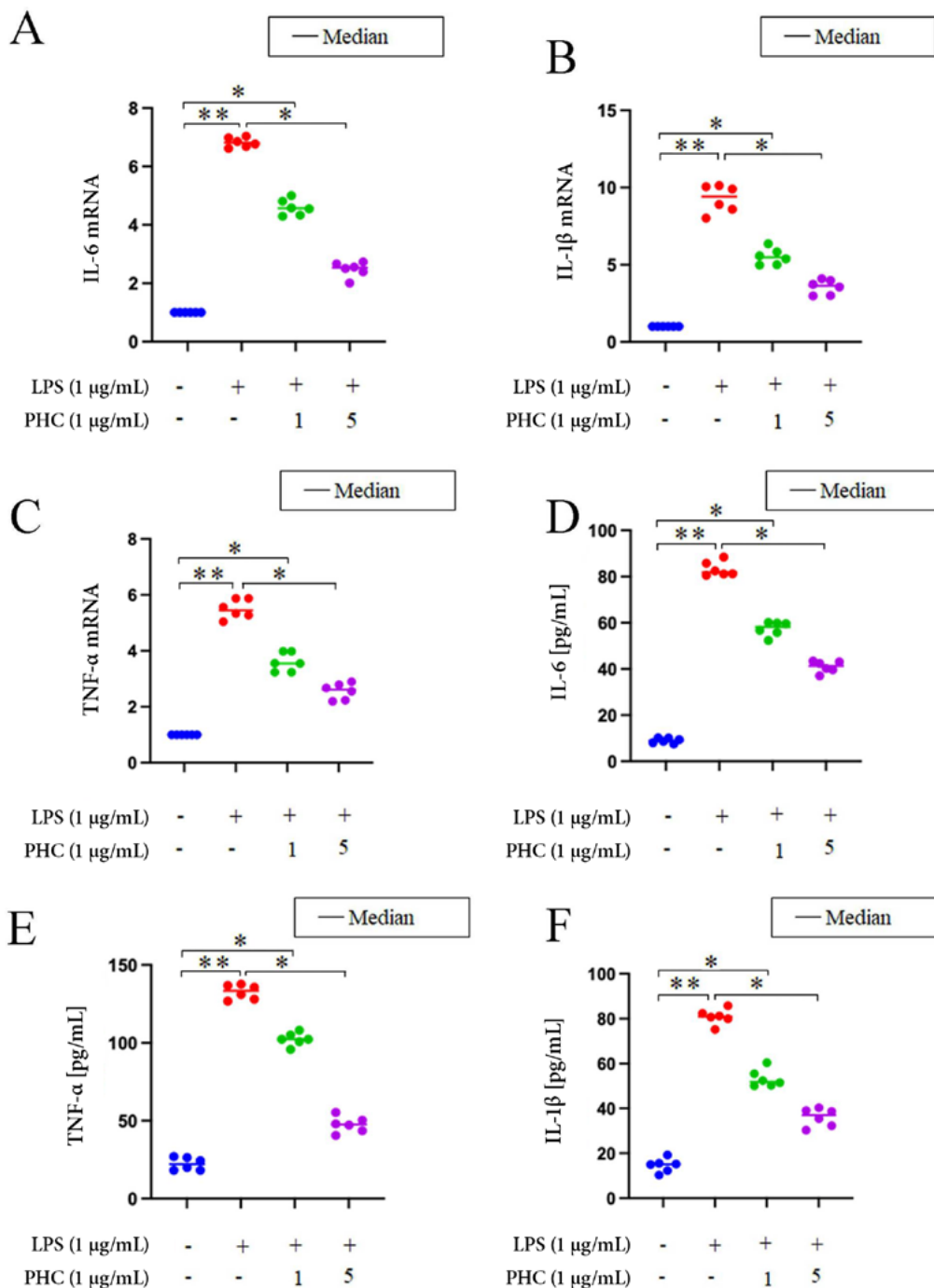


Fig. 2. Penehyclidine hydrochloride (PHC) suppressed lipopolysaccharide (LPS)-induced expression and secretion of pro-inflammatory cytokines of RAW264.7 cells. RAW264.7 cells were administered 1 µg/mL LPS alone or in combination with 1 µg/mL or 5 µg/mL PHC for 24 h. A–C. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed to determine messenger ribonucleic acid (mRNA) levels of interleukin (IL)-6, IL-1 β and tumor necrosis factor alpha (TNF- α) in cells; D,E. Enzyme-linked immunosorbent assay (ELISA) was conducted to measure IL-6, IL-1 β and TNF- α in the cell culture medium

* $p < 0.05$; ** $p < 0.01$ ($n = 6$). Differences in characteristics between groups were analyzed using the Kruskal–Wallis test with Dunn's post hoc test.

PHC enhanced Nrf2, HO-1 and NQO1 protein expression. These findings suggest that PHC may enhance Nrf2 signaling in activated macrophages, and could serve as an anti-inflammatory and antioxidant compound for managing inflammatory disorders.

Penehyclidine hydrochloride toxicity on RAW264.7 cells was examined. Weng et al. demonstrated that PHC ranging from 1 µg/mL to 5 µg/mL did not change the activity of cultured alveolar macrophages but dose-dependently reduced LPS-mediated apoptosis.⁵¹ Penehyclidine hydrochloride can also decrease neuronal apoptosis in the context of inflammation caused by cardiopulmonary bypass.⁴² Our results showed

that PHC concentrations ranging from 1 µg/mL to 10 µg/mL had no significant effect on RAW264.7 cell viability. Thus, we treated RAW264.7 cells with 1 µg/mL or 5 µg/mL PHC in the subsequent experiments. Selecting the lowest doses could ensure the avoidance of possible PHC toxicity.

Lipopolysaccharide promoted the release of pro-inflammatory factors such as NO, PGE2, IL-6, IL-1 β , and TNF- α , consistent with the known effects of LPS.^{10,52,53} Meanwhile, LPS-induced ROS production by RAW264.7 cells was similar to previous reports.^{54,55} These data suggest that an in vitro inflammation and oxidative stress model was successfully established.

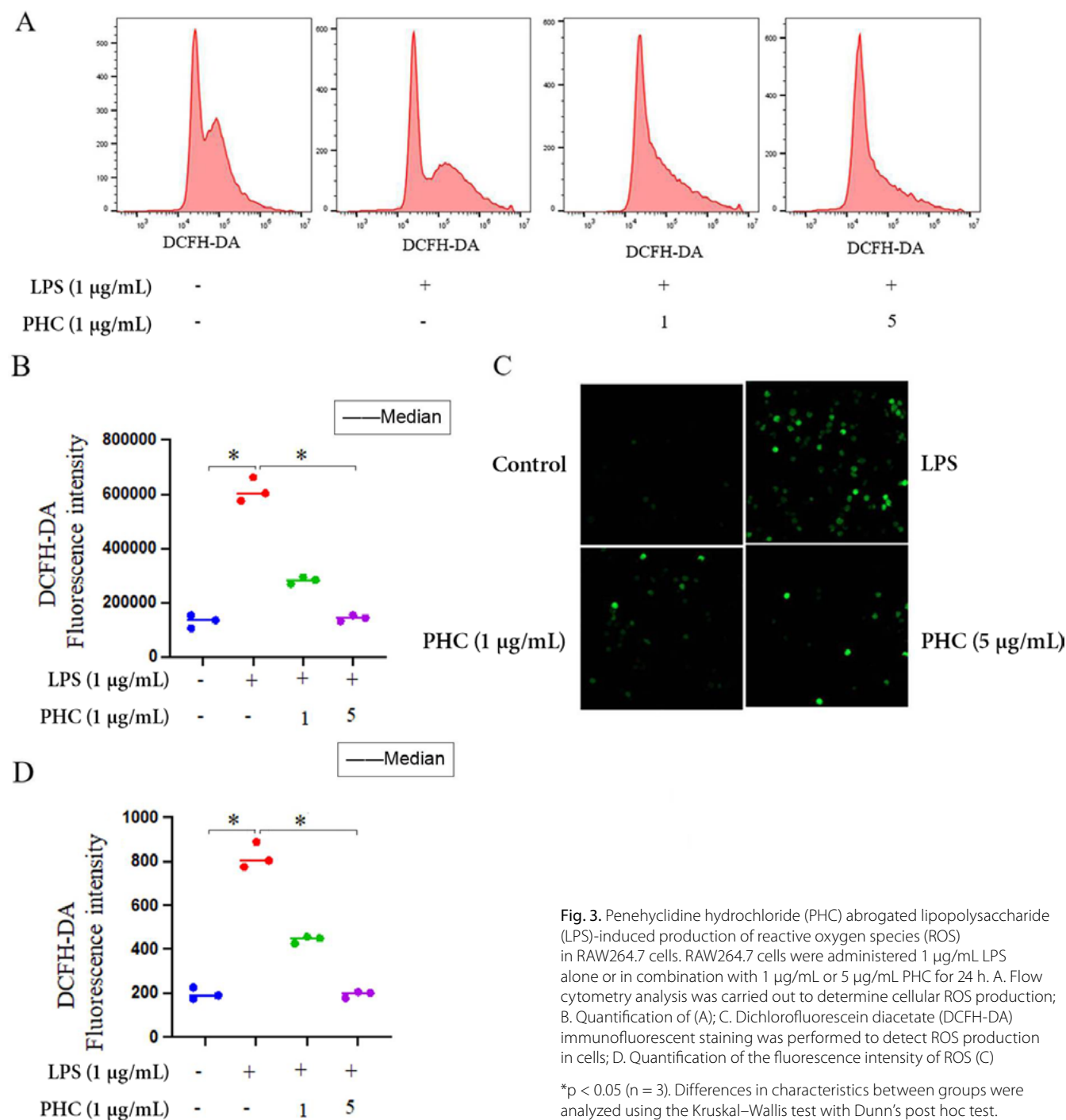


Fig. 3. Pennehydridine hydrochloride (PHC) abrogated lipopolysaccharide (LPS)-induced production of reactive oxygen species (ROS) in RAW264.7 cells. RAW264.7 cells were administered 1 µg/mL LPS alone or in combination with 1 µg/mL or 5 µg/mL PHC for 24 h. A. Flow cytometry analysis was carried out to determine cellular ROS production; B. Quantification of (A); C. Dichlorofluorescein diacetate (DCFH-DA) immunofluorescent staining was performed to detect ROS production in cells; D. Quantification of the fluorescence intensity of ROS (C)

* $p < 0.05$ ($n = 3$). Differences in characteristics between groups were analyzed using the Kruskal–Wallis test with Dunn's post hoc test.

Macrophages are among the first immune cells to encounter pathogenic organisms and other stimuli, and activated macrophages are important sources of pro-inflammatory cytokines.^{22,23} Polarized macrophages are important in the pathogenetic mechanisms of inflammatory diseases by inducing the production of pro-inflammatory mediators.⁵⁶ As shown above, PHC effectively reversed LPS-associated excessive synthesis of pro-inflammatory factors and ROS, suggesting that PHC may alleviate the inflammatory response of macrophages.

Oxidative stress is critical in the etiology and perpetuation of inflammation.⁵⁷ Recently, Liu et al. reported that

PHC administration enhanced Nrf2 expression while suppressing mouse lung inflammation resulting from renal ischemia/reperfusion.⁴³ Similarly, Yang et al. found that PHC upregulated Nrf2 and HO-1 while suppressing the production of pro-inflammatory cytokines and ROS in renal ischemia/reperfusion-induced injured lungs. Furthermore, PHC-induced protection is lost in Nrf2^{-/-} mice, suggesting that Nrf2 is essential for the protective role of PHC against inflammation and oxidative stress.⁴⁴ However, whether PHC affects Nrf2 signaling in macrophages remains unknown.

A protective role for PHC in LPS-associated inflammation and oxidative stress has been extensively demonstrated

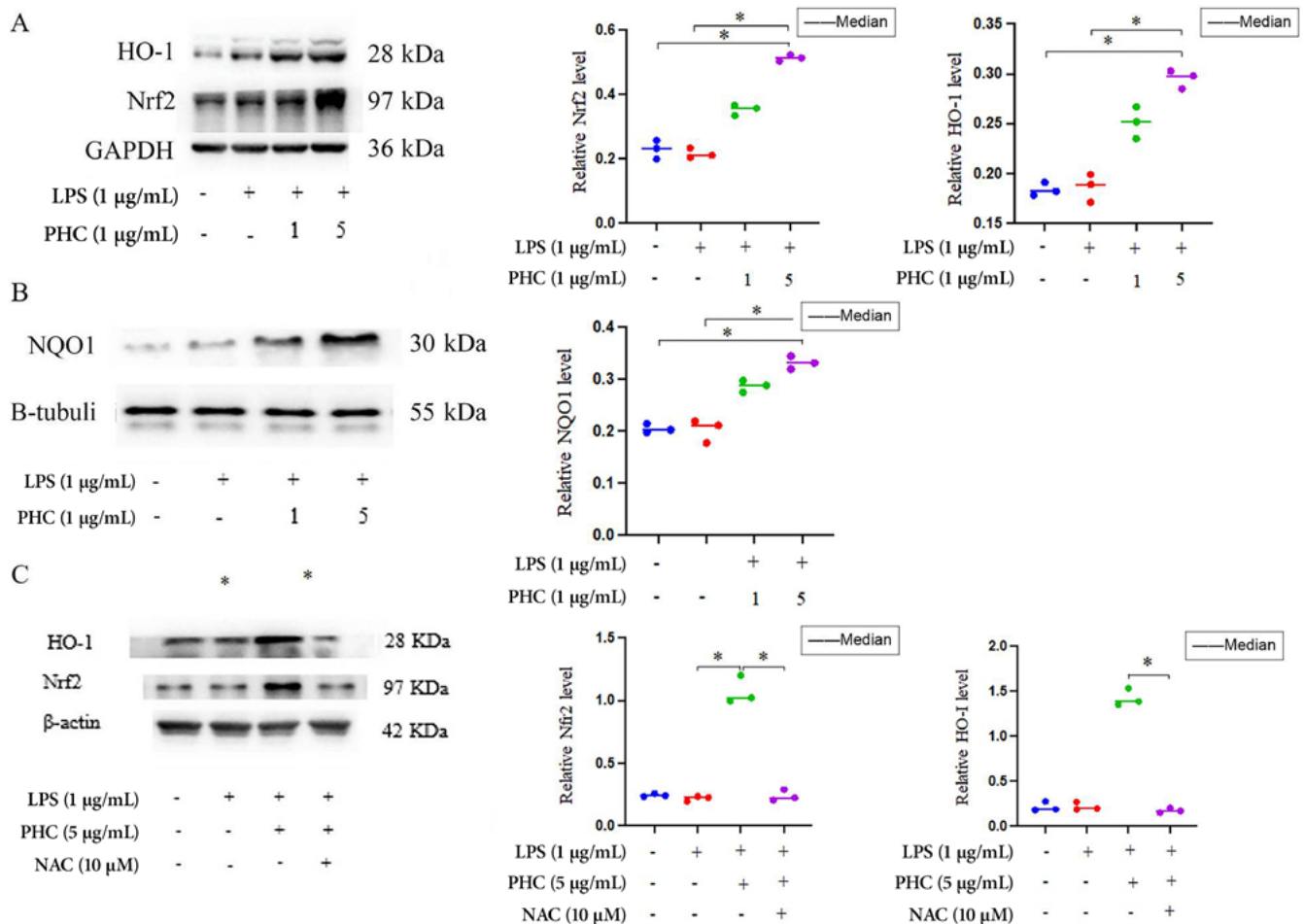


Fig. 4. Penhyclidine hydrochloride (PHC) induced nuclear factor erythroid 2-related factor (Nrf2) signaling in RAW264.7 cells. RAW264.7 cells were administered 1 µg/mL lipopolysaccharide (LPS) alone or in combination with 1 µg/mL or 5 µg/mL PHC for 24 h. Immunoblot was conducted to determine the protein expression of Nrf2 (A, left panel), heme oxygenase (HO)-1 (A, right panel) and NAD(P)H-quinone oxidoreductase 1 (NQO1) (B); C. RAW264.7 cells were administered LPS (1 µg/mL), LPS (1 µg/mL) + PHC (5 µg/mL) or LPS (1 µg/mL) + PHC (5 µg/mL) + N-acetyl-L-cysteine (NAC; 10 µM). The left panel shows Nrf2, and the right panel shows HO-1. Immunoblot was carried out to determine the protein expression of HO-1 and Nrf2, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or β-actin as an internal reference

* $p < 0.05$ ($n = 3$). Differences in characteristics between groups were analyzed using the Kruskal–Wallis test with Dunn's post hoc test.

in animal models. Indeed, Guo et al. found that PHC alleviated LPS-associated acute lung injury and lung inflammation in a rat model, accompanied by considerable reductions in TNF- α , IL-8 and IL-6 in bronchoalveolar lavage fluid and downregulation of iNOS expression in the lung tissue.⁵⁸ Ye et al. revealed that PHC inhibits LPS-associated lung myeloperoxidase expression and ROS production in rats.⁵⁹ Furthermore, PHC can attenuate the impact of Toll-like receptors in models of inflammatory chronic lung diseases³⁹ and acute lung injury.⁴⁰ Similarly, PHC decreases serum TNF- α and IL-1 β while improving renal function indicators in rats with LPS-induced acute kidney injury.⁶⁰ In humans, PHC decreased the expression of pro-inflammatory cytokines after surgery.⁴¹ At the cellular level, PHC inhibited NO, PGE2, IL-1 β , and TNF- α release in LPS-activated microglia,⁶¹ and reduced the inflammatory response in cultured human pulmonary microvascular endothelial cells after LPS stimulation, reflected by lower secretion of lactate dehydrogenase, TNF- α and IL-6, and attenuated

expression of vascular cell adhesion molecule 1 (VCAM-1) and intercellular cell adhesion molecule-1 (ICAM-1).⁶²

N-acetyl-L-cysteine has been shown to downregulate Nrf2/HO-1 signaling in the presence of hydrogen peroxide (H_2O_2) in MC3T3-E1 preosteoblasts⁶³ and attenuate kaempferol-induced Nrf2 interaction with the HO-1 promoter and subsequent HO-1 expression.⁶⁴ Our findings suggest that Nrf2/HO-1 signaling induction by PHC in macrophages is ROS-dependent. The findings are consistent with Zhu et al., who demonstrated that Nrf2 decreased the transcription of pro-inflammatory cytokines in activated macrophages.³³ Therefore, it is our opinion that PHC could have a role in managing disorders involving inflammation and oxidative stress. Of course, such management is outside the approved indications, and studies are necessary to examine the possibility, efficacy and safety of using PHC for acute or chronic inflammatory diseases. Acute and chronic inflammatory diseases carry high morbidity and mortality worldwide, and the available

treatments have limited efficacy. As such, exploring novel strategies warrants attention.

Several points require further elucidation. For example, in Fig. 3C, only a select number of cells showed increased ROS levels instead of a universal increase across all treated cells. Unfortunately, the present study cannot provide the answer. In order to understand what is happening in those cells, microdissection and single-cell omics will be necessary but were not performed in the present study. Compared with the control group, the cells in the LPS group showed a general increase in ROS levels, and this difference was dose-dependent. In addition, the western blots appeared to show secondary bands, and since there are different post-translational modifications and different forms of proteins, multiple bands can be recognized by the same antibody. Still, the target bands were analyzed according to the molecular weight of the corresponding marker, and the trend of the bands was consistent. Nonetheless, the nature of the protein modifications and isoforms could be investigated. Furthermore, LPS stimulation did not affect Nrf2, HO-1 and NQO1 protein expression in RWA264.7 cells without PHC. It could be hypothesized that the effect of PHC is specific to Nrf2, HO-1 and NQO1 elevation instead of a reversal of the effects of LPS, but the present study was not designed to examine such mechanisms, and additional studies are necessary.

Limitations

This study failed to verify that PHC can relieve LPS-stimulated macrophage inflammation and oxidative stress through the Nrf2 signaling pathway by silencing the expression of Nrf2. Inflammation is a complex process involving several effectors, pathways and cells. Future studies should use agonists, antagonists, and knockout/silenced cell and animal models to examine the involvement of these effectors, pathways and cells in the mechanisms involved in PHC modulation of inflammation and oxidative stress.

Only 1 μ g and 5 μ g doses were explored and were selected because they were far below the toxic dose, avoiding any risk of interference from PHC toxicity. In addition, starting with a small dose that works is consistent with clinical dosing principles, where anticholinergics have been found to increase the incidence of postoperative cognitive impairment in the elderly.⁴¹ Still, future studies should include doses up to 20 μ g to determine the exact dose-dependency of the effects of PHC. Therefore, the results of this study need further verification.

Conclusions

In conclusion, PHC may alleviate LPS-induced inflammation and oxidative stress while activating Nrf2 signaling in RAW264.7 macrophages. These findings suggest that PHC could be used for inflammation therapy by targeting activated macrophages.

Supplementary data

The Supplementary materials are available at <https://doi.org/10.5281/zenodo.10622908>. The package contains the following files:

Supplementary Table 1. Descriptive statistics for CCK-8 for Fig. 1A.

Supplementary Table 2. Kruskal–Wallis test results ($H = 29.44$, $p < 0.0001$) for CCK-8 for Fig. 1A.

Supplementary Table 3. Descriptive statistics for NO concentrations for Fig. 1B.

Supplementary Table 4. Kruskal–Wallis test results for NO concentrations for Fig. 1B ($H = 21.62$, $p < 0.0001$).

Supplementary Table 5. Descriptive statistics for PGE2 for Fig. 1C.

Supplementary Table 6. Kruskal–Wallis test results ($H = 21.60$, $p < 0.0001$) for PGE2 for Fig. 1C.

Supplementary Table 7. Descriptive statistics for iNOS mRNA for Fig. 1D.

Supplementary Table 8. Kruskal–Wallis test results ($H = 21.93$, $p < 0.0001$) for iNOS mRNA for Fig. 1D.

Supplementary Table 9. Descriptive statistics for IL-6 mRNA for Fig. 2A.

Supplementary Table 10. Kruskal–Wallis test results ($H = 21.93$, $p < 0.0001$) for IL-6 mRNA for Fig. 2A.

Supplementary Table 11. Descriptive statistics for IL-1 mRNA for Fig. 2B.

Supplementary Table 12. Kruskal–Wallis test results ($H = 21.93$, $p < 0.0001$) for IL-1mRNA for Fig. 2B.

Supplementary Table 13. Descriptive statistics for TNF- α mRNA for Fig. 2C.

Supplementary Table 14. Kruskal–Wallis test results ($H = 21.93$, $p < 0.0001$) for TNF- α mRNA for Fig. 2C.

Supplementary Table 15. Descriptive statistics for IL-6 for Fig. 2D.

Supplementary Table 16. Kruskal–Wallis test results ($H = 21.60$, $p < 0.0001$) for IL-6 for Fig. 2D.

Supplementary Table 17. Descriptive statistics for TNF- α for Fig. 2E.

Supplementary Table 18. Kruskal–Wallis test results ($H = 21.62$, $p < 0.0001$) for TNF- α for Fig. 2E.

Supplementary Table 19. Descriptive statistics for IL-1 α for Fig. 2F.

Supplementary Table 20. Kruskal–Wallis test results ($H = 21.60$, $p < 0.0001$) for IL-1 for Fig. 2F.

Supplementary Table 21. Raw data for ROS for Fig. 3B.

Supplementary Table 22. ROS descriptive statistics for Fig. 3B.

Supplementary Table 23. Kruskal–Wallis test results ($H = 9.359$, $p = 0.0020$) for ROS for Fig. 3B.

Supplementary Table 24. Raw data for ROS for Fig. 3D.

Supplementary Table 25. Descriptive statistics for ROS for Fig. 3D.

Supplementary Table 26. Kruskal–Wallis test results ($H = 9.359$, $p = 0.0020$) for ROS for Fig. 3D.

Supplementary Table 27. Raw data for Nrf2 for Fig. 4A.
Supplementary Table 28. Descriptive statistics for Nrf2 for Fig. 4A.

Supplementary Table 29. Kruskal–Wallis test results ($H = 9.359$, $p = 0.0020$) for Nrf2 for Fig. 4A.

Supplementary Table 30. Raw data for HO-1 for Fig. 4A.

Supplementary Table 31. Descriptive statistics for HO-1 for Fig. 4A.

Supplementary Table 32. Kruskal–Wallis test results ($H = 9.359$, $p = 0.0020$) for HO-1 for Fig. 4A.

Supplementary Table 33. Descriptive statistics for NQO1 for Fig. 4B.

Supplementary Table 34. Kruskal–Wallis test results ($H = 9.359$, $p = 0.0020$) for NQO1 for Fig. 4B.

Supplementary Table 35. Raw data for Nrf2 for Fig. 4C.

Supplementary Table 36. Descriptive statistics for Nrf2 for Fig. 4C.

Supplementary Table 37. Kruskal–Wallis test results ($h = 7.308$, $p = 0.0328$) for Nrf2 for Fig. 4C.

Supplementary Table 38. Raw data for HO-1 for Fig. 4C.

Supplementary Table 39. Descriptive statistics for HO-1 for Fig. 4C.

Supplementary Table 40. Kruskal–Wallis test results ($H = 7.205$, $p = 0.0328$) for HO-1 for Fig. 4C.

Note: In Supplementary Tables 3–40: A – control group, B – LPS group, C – LPS+PHC (1 $\mu\text{g/mL}$) group, D – LPS+PHC (5 $\mu\text{g/mL}$) group.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

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