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ORIGINAL RESEARCH ARTICLE

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Anti-inflammatory effect of octyl gallate in alveolar macrophages cells and mice with acute lung injury

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Abstract

Acute lung injury (ALI) is an inflammatory process, and has high incidence and mortality. ALI and the acute respiratory distress syndrome are two common complications worldwide that result in acute lung failure, sepsis, and death. Pro-inflammatory substances, such as cytokines and chemokines, are responsible for activating the body's defense mechanisms and usually mediate inflammatory processes. Therefore, the research of substances that decrease the uncontrolled response of organism is seen as potential for patients with ALI. Octyl gallate (OG) is a phenolic compound with therapeutic actions namely antimicrobial, antiviral, and antifungal. In this study, we evaluated its action on lipopolysaccharide (LPS)-activated alveolar macrophages RAW 264.7 cells and ALI in male mice. Our results demonstrated protective effects of OG in alveolar macrophages activated with LPS and mice with ALI. The OG treatment significantly decreased the inflammatory markers in both studies in vitro and in vivo. The data suggested that OG can act as an anti-inflammatory agent for ALI.

KEYWORDS
acute lung injury, inflammation, macrophages, octyl gallate

1 | INTRODUCTION

Acute lung injury (ALI) is an inflammatory process characterized mainly by a severely damages the organ and, has a high incidence and mortality, around 35-40%. (Ferguson et al., 2012; Gotts & Matthay, 2014). ALI is a prevalent disease worldwide that result in acute lung failure, pneumonia, interstitial edema, sepsis, and death. The inflammatory cells' predominance in the lungs can cause changes in its morphological features and functions, and this is extremely harmful to the patient (Caser, Zandonade, Pereira, Gama, & Barbas, 2014; Fei et al., 2019; Grommes & Soehnlein, 2011; Jiang et al., 2017; Xu, Li, Gong, Zheng, & Zhao, 2018).

Animal models have been using lipopolysaccharide (LPS) as the intranasal (IN) inductor of ALI (Zhang, Wang, & Zhou, 2018). LPS, also known as endotoxin, is a substance found in the cell membrane of gram-negative bacteria and it actives an inflammatory cascade in response to Toll-like receptor 4 (TLR-4) activation. With the receptor-linker binding, the animals develop an inflammatory process after the release of inflammatory markers, such as cytokines and chemokines (Zhang et al., 2018). TLR-4 activation leads to an increase of nuclear factor κB (NF-κB) signaling pathway and mitogen-activated protein kinase (MAPK). These inflammatory factors act directly in LPS-induced ALI (Bosmann et al., 2013; Gharib et al., 2006; Jiang et al., 2017; Park, Lee, Kim, & Yang, 2016).

Studies suggest that the mammalian cells exposed to LPS lead to pro-inflammatory cytokines release and in turn activate a second level of inflammatory cascades. Macrophages are important cells in inflammatory process and have a critical role in several inflammatory diseases, including ALI. LPS-activated macrophages, as second level of inflammatory cascade, release inflammatory mediators, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), reactive oxygen species (ROS), inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2; Kharitonov & Sjobring, 2007; Lee et al., 2011; Zong et al., 2012).

Pro-inflammatory substances, such as cytokines and chemokines, are responsible for activating the body's defense mechanisms and usually mediate inflammatory processes. When the host's response is exacerbated, it causes severe damage to the organs leading the patient to death. Therefore, research of substances that decrease the uncontrolled response of an organism is seen as a benefit toward the treatment of patients with ALI.

There is no effective treatment for ALI, therefore the search for alternative drugs was the reason for the origin of this study. We used the octyl gallate (OG) compound, an ester derived from gallic acid. OG is a phenolic compound with therapeutic actions namely antimicrobial, antiviral, and antifungal (Hsu, Chang, & Chang, 2007; Kubo, Xiao, & Fujita, 2001; Latha & Daisy, 2013; Uozaki et al., 2007; Wolf, Bonacorsi, Raddi, da Fonseca, & Ximenes, 2017). In this study, we evaluated it action on LPS-induced alveolar macrophages RAW 264.7 and ALI in male mice.

2 | METHODS

2.1 | Ethics statement

The experimental protocol was approved by the Ethics Research Committee of Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS; protocol number 16/00495).

2.2 | Cell culture

The RAW 264.7 alveolar macrophage murine cell line was acquired from ATCC Bank (VA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Life Technologies), containing 10% fetal bovine serum (Gibco, Life Technologies) and 1% penicillin/streptomycin antibiotics, (ATB; Gibco, Life Technologies) at 37°C and 5% CO_2 humidified incubator.

2.2.1 | OG solution preparation and treatment

The OG (Sigma-Aldrich) stock solution was dissolved in dimethylsulfoxide (DMSO; Neon, Brazil) and then diluted in DMEM at 0.3–2.5 μM. All reagents used were filtered through a disposable sterile filter unit 0.22 μM (Kasvi, Brazil) before adding to the cells. RAW 264.7 cells were seeded into 6- to 24-well plates according to each experiment. After 24 hr, the cells were pretreated with different concentrations of OG (0.3–2.5 μM) for 1 hr, and then the cells were activated with 1 μg/ml LPS (*Escherichia coli* 026: B6; Sigma-Aldrich) and further cultured for 24 hr. The cells were used for viability assay, RNA isolation, and preparation of protein lysates.

2.3 | Cell viability assay

To evaluate the OG effect on cell viability, we performed 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Life Technologies). Cells were seeded in 24-well plates at a density of 2×10^4 cells/well in 0.6 ml of DMEM. The control group received DMEM plus DMSO (vehicle); the LPS group was composed of DMSO and 1.0 μg/ml LPS, and in the LPS + OG group, the cells were pretreated with OG (0.3–2.5 μM) for 1 hr, and then activated with 1.0 μg/ml LPS. After 24 hr of treatment time, the cell viability was analyzed. In brief, the medium was removed and then the MTT solution was added directly to all assay wells and incubated for 3 hr. After that, the solution was discarded and the formazan crystals were dissolved with DMSO. Finally, the optical density (OD) was read in a microplate read (EZ Read 400; Biochrom), using a test wavelength of 570 nm and a reference wavelength of 620 nm.

2.4 | Quantitative polymerase chain reaction (PCR) analysis

The cells were seeded in six-well plates at a density of 2.5×10^5 cells/well in 2 ml of DMEM, and were pretreated with OG in different concentrations (0.3 and 0.6 μM) for 1 hr and stimulated with 1.0 μg/ml LPS (Sigma-Aldrich) for 24 hr. The RNA was obtained from RAW cells culture using TRIzol (Invitrogen), according to the manufacturer's instructions. Later, complementary DNA (cDNA) was synthetized using a reverse transcription kit—GoScript™ Reverse Transcriptase (Promega). Real-time PCR reactions were performed using SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific) and the products were verified by melting curve analysis. The target genes expression were normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels and the primers used are listed in Table 1. All PCR reactions were performed in duplicate and water was used as a negative control.

TABLE 1 Primer sequences for real-time PCR analysis

Primers	Forward	Reverse
GAPDH	5-GGGGAGCCAAAAGGGTCATC-3	5-GACGCCTGCTTCACCACCTTCTTG-3
TLR-4	5-TTCAGAGCCGTTGGTGTATC-3	5-CTCCCATTCCAGGTAGGTGT-3
iNOS	5-CCTCCTCCACCCTACCAAGT-3	5-CACCCAAAGTGCTTCAGTCA-3
COX-2	5-TTGAAGACCAGGAGTACAG C-3	5-GGTACAGTTCCATGACATCG-3
TNF-α	5-ATAGCTCCCAGAAAAGCAAGC-3	5-CACCCCGAAGTTCAGTAGACA-3
IL-1β	5-GCCCATCCTCTG TGACTCAT-3	5-AGGCCACAGGTATTTTGTCG-3
IL-6	5-TGGAGTCACAGAAGGAGTGGCTAAG-3	5-CTGACCACAGTGAGGAATGTCCAC-3

Abbreviations: COX-2, cyclooxygenase-2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL-1ß, interleukin-1β; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; TLR-4, Toll-like receptor 4; TNF-α, tumor necrosis factor-α.

2.5 | Western blot analysis

Approximately 3×10^5 cells were seeded in six-well plates. The cells were cultured and treated as aforementioned. The RAW 264.7 cells were washed twice with phosphate buffered saline (PBS) and then lysed with CHAPS (Amresco) plus protease inhibitors. Aliquots containing 30 μg proteins from each sample were run on a 10% sodium dodecylsulphate polyacrylamide gel. After running the gel, the proteins were transferred to a nitrocellulose membrane (Sartorius, Germany) and then, the blots were blocked with Tris buffered salinea plus 5% nonfat dry milk and 0.05% Tween-20 for 1 hr. Subsequently, the membranes were incubated overnight at 4°C with primary antibodies (iNOS and COX-2; Cell Signaling) and α-tubulin (Cell Signaling) at 1:500 and 1:1,000, respectively. Later, the blots were incubated with secondary antibodies containing peroxidase-conjugated (1:2,000) for 1 hr at room temperature. The blots were analyzed by chemiluminescence, and digital images were caught using a luminescent image analyzer, Carestream Gel Logic 2200 PRO Imaging System. The total expression was evaluated in Image Studio Lite software (LI-COR) and, α-tubulin was used for normalization of quantitative densitometry values (de Mesquita et al., 2017; Tripathi et al., 2018).

2.6 | Animals

Male C57BL/6 mice (8–12-week old and 23–30 g) were acquired in the Center for experimental biological models of PUCRS (CeMBE, PUCRS). The animals were kept free access to water and ration on shelves with ventilated cages, on a 12:12 hr light–dark cycle and temperature of 22 ± 2°C. The animals were maintained in CeMBE and accordance with the Guiding Principles in the Care and Use of Animals.

2.6.1 | ALI induction and experimental groups

Eighteen mice were separated into three groups: control, LPS, and LPS + OG. The animals were slightly anesthetized with isoflurane and then ALI was induced by LPS inhalation: 50 μl (2 mg/kg) of LPS (*Escherichia coli* 026:B6; Sigma-Aldrich). Control group: the animals received 50 μl of saline solution and 30 min after, was administrated saline plus DMSO solution (vehicle). The LPS group: the animals received 50 μl (2 mg/kg) of LPS and then treated with 50 μl of saline plus DMSO (vehicle). The LPS + OG group: the animals received 50 μl (2 mg/kg) of LPS and 30 min after LPS inhalation, were treated with OG (0.75 mg/kg). After 12 hr of LPS inhalation, the animals were anesthetized and broncho-alveolar lavage fluid (BALF) was removed. The BALF and lung samples were stored at − 80°C until analyses. The protocol for LPS-induced ALI model was accomplished as described by Park et al. (2016) with minor modifications. Representative design is described in Figure 1.

2.7 | Total and differential cell analysis in BALF

The BALF was centrifuged at 1,000*g* for 10 min and the cell pellet was resuspended in PBS (350 μl). The total cell count (TCC) was performed using a Neubauer counting chamber. The slides containing BALF supernatants were marked with hematoxylin and eosin (H&E; Panótico Rápido; Laborclin, Brazil); for this analysis, 400 cells were observed by optic microscopy BMX 43 (Olympus, Japan), magnification ×200, for two independent and blinded research.

2.8 | Total protein and neutrophil extracellular traps (NETs) quantification in BALF

The BALF total protein was quantified using Nano Drop Lite (Thermo Fisher Scientific). The total DNA extracellular was quantified in BALF

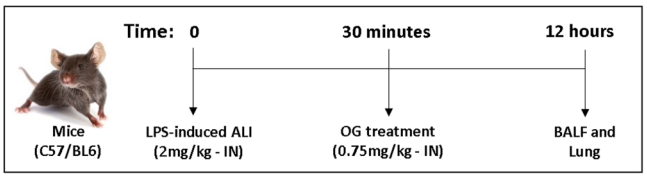

FIGURE 1 Experimental design of the study. The timeline is expressed in minutes or hours. ALI, acute lung injury; BALF, broncho-alveolar lavage fluid; IN, intranasal; LPS, lipopolysaccharide; OG, octyl gallate

of different groups using Quant-iT dsDNA HS kit (Invitrogen, Brazil) following the instructions of the manufacturer. The samples were measured in Qubit fluorimeter (Invitrogen, Brazil).

2.9 | Histological analysis

To evaluate the histological alterations, after euthanasia the lungs were perfused with 10% buffered formalin on a gravity column (20 mmHg), the superior lobe of the right lung was prepared for morphological and histological analysis. The lungs were perfused; the tissue was firmed in paraffin, and cut into pieces at 4 μm thickness. After deparaffinization and dehydration process, the lung sections were marked with H&E and lung tissues observed under a light microscope (Olympus, Japan) with magnification ×400 (Jiang et al., 2017).

2.10 | Cytokines quantification in lung tissue

The lung tissue was homogenized in PBS (1:2, wt/vol). The homogenate was centrifuged at 13,000*g* for 10 min at 4°C. The pellet was removed, and the supernatant was immediately separated and used for cytokines measurements. To determine TNF-α and IL-6 levels in the samples we used the MagPix according the manufacturer's instruction (Milliplex, Millipore, Germany) and the results were analyzed using the software xPONENT 4.2 (Milliplex, Millipore, Germany).

2.11 | Lung weight

After euthanasia, the lungs were removed and weighed. We evaluated the presence of edema caused by the cells accumulation in tissue.

2.12 | Oxidative stress evaluation in lung tissue

The lung tissue was homogenized in PBS (1:10, wt/vol). The homogenate was centrifuged at 13,000*g* for 10 min at 4°C. The pellet was discarded, and the supernatant was immediately separated and used for the measurements of oxidative stress. Total protein was measured using Nano Drop Lite (Thermo Fisher Scientific) for all techniques (Pedrazza et al., 2017).

2.12.1 | Thiobarbituric acid-reactive substances (TBARS) in lung tissue

The supernatant of lung lysates was used to determine the concentrations of TBARS. The compound resulting from the reaction was measured at fluorimeter; VICTOR microplate reader (PerkinElmer) at 485 nm (excitation) and 520 nm (emission) of wavelength. Results were normalized by protein and expressed as TBARS nM/mg protein (Draper & Hadley, 1990).

2.12.2 | Catalase (CAT) assay in lung tissue

The supernatant of lung lysates were used to determine the CAT unit. Catalase activity was performed conforming to the Aebi (1984) protocol with minor modifications. The product of the reaction was measured after 1 min using Spectrophotometer Genesys 10uv (Thermo Fisher Scientific) at 240 nm. The results were normalized by protein and expressed as UCAT/mg protein.

2.12.3 | Glutathione (GSH) in lung tissue

The supernatant of lung lysates were used to determine the GSH concentration. In this method, the products of the reaction, thiols react with 2-dinitrobenzoicacid (DTNB) and form thiolate measurable at 412 nm. Therefore, the lung supernatant was added to a precipitating solution and then, was added to other solution containing 300 mM Na_2HPO_4 and 0.05% DTNB. Later, this solution was measured using a Spectrophotometer Genesys 10uv (Thermo Fisher Scientific). The results obtained were corrected by protein and expressed as OD/mg protein (Beutler, Duron, & Kelly, 1963).

2.12.4 | ROS activity in lung tissue

ROS production was determined according to the method of 2′,7′-dichlorofluorescein diacetate (DCFH-DA). The lung supernatant was added to a medium containing 100 μM DCFH-DA solution. The dichlorofluorescein DCF fluorescence intensity was measured in a fluorimeter, VICTOR® microplate reader (PerkinElmer), at 485 nm (excitation) and 520 nm (emission) wavelength. The results obtained were corrected by protein, and DCF fluorescence was represented as DCF fluorescence/mg protein (LeBel, Ischiropoulos, & Bondy, 1992).

2.13 | Real-time PCR quantification in lung tissue

After euthanasia, the lung slices (approximately 80 mg) were separated and stored in ultra-freezer −80°C to RNA extraction. Total RNA was extracted from all samples using TRIzol (Invitrogen), following the manufacturer's instructions. Subsequently, cDNA was synthetized using a reverse transcription kit—Goscript™ Reverse Transcriptase (Promega) and real-time PCR reactions were performed using SYBR Green PCR Master Mix (Applied Biosystems–Thermo Fisher Scientific, Brazil). The reaction products were verified by melting curve analysis and all PCR reactions were performed in duplicate. The expression levels of target genes were normalized by GAPDH levels and the primers used are listed in

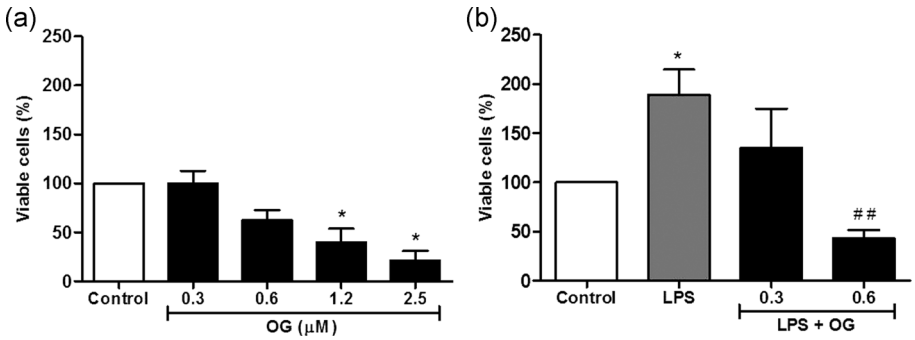

FIGURE 2 OG and OG plus LPS effect on the cell viability of alveolar macrophages RAW 264.7 cell viability. The cells were treated with the indicated concentrations of OG for 24 hr or the cells were pretreated with OG for 1 hr and then exposed to LPS (1 μg/ml) for 24 hr. The cell viability was assessed using an MTT assay and all experiments were performed in triplicate. (a) Response dose curve of OG (0.6–2.5 μM), the 1.2 and 2.5 μM OG concentrations decreased significantly the cell viability (*$p < .05$ vs control group). (b) The LPS group increased the cell viability in 88% vs control group and 0.6 μM OG treatment decreased significantly this effect ($^{\#\#}p < .01$ vs LPS group). LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OG, octyl gallate. Results are expressed as viable cells (%) and the data represent the mean ± *SEM* of five independent experiments ($n = 5$)

Table 1. All PCR reactions were performed in duplicate and water was used as a negative control.

2.14 | Statistical analysis

The data were analyzed by one-way analysis of variance. For comparison of significance, Tukey's test was used as a post hoc test according to the statistical program GraphPad Prism. Quantitative data are presented as means ± standard error of the mean (*SEM*). Differences were considered significant at * or $^{\#}p < .05$, ** or $^{\#\#}p < .01$, and *** or $^{\#\#\#}p < .001$. * vs control and # vs LPS.

3 | RESULTS

3.1 | Effect of OG pretreatment on macrophages RAW 264.7 viability

OG treatment caused a significant decrease in the cell viability at concentrations of 1.2 and 2.5 μM (Figure 2a). The group that received 0.6 μM OG as treatment before the LPS induction decreased the cell viability when compared with LPS group (Figure 2b).

3.2 | Effect of OG treatment on inflammatory gene expression (TLR-4, COX-2, iNOS, TNF-α, IL-1β, and IL-6) in LPS-activated macrophages RAW 264.7

The results demonstrate no significant differences in TLR-4 and TNF-α genes between the groups analyzed (Figure 3a,d). The LPS group increased significantly the iNOS mRNA expression and the concentration 0.6 μM OG decreased the LPS effect. We observed the difference in COX-2 expression after LPS induction, but the treatment did not block this process (Figure 3c). In addition, when we evaluated the IL-1β cytokine, the LPS group increased significantly the mRNA expression, and the OG group alleviated the LPS effect, but was not significant ($p = .087$; Figure 3e). All OG concentrations tested (0.3 and 0.6 μM) decreased significantly the IL-6 overexpression caused by LPS induction (Figure 3f).

3.3 | Effect of OG treatment on inflammatory protein expression (iNOS and COX-2) in LPS-activated alveolar macrophages RAW 264.7

LPS group induced iNOS and COX-2 protein expression, however, the treatment with OG did not block this process (Figure 4a–d).

3.4 | OG effects on the lung of mice with ALI

The LPS treatment induced inflammatory cell migration to BALF, and OG treatment decreased this effect (Figure 5a). We did not detect significant differences in the macrophage differential count (Figure 5b). In the neutrophils differential count, the LPS group increased significantly this cell's predominance and OG treatment inhibited the same (Figure 5c). In Figure 5d–f we have representative images of the differential counts performed in the different groups evaluated. No significant difference was found in the total protein levels of different groups (Figure 5g). The LPS group increased significantly the NETs release in BALF, however, the treatment does not reverse the LPS effect.

3.5 | OG decrease inflammatory histopathologic alterations, cytokines, and lung weight changes in an LPS-induced ALI model

The protective effects of OG on LPS-induced ALI were assessed by H&E staining. The results showed that lung tissue of LPS group

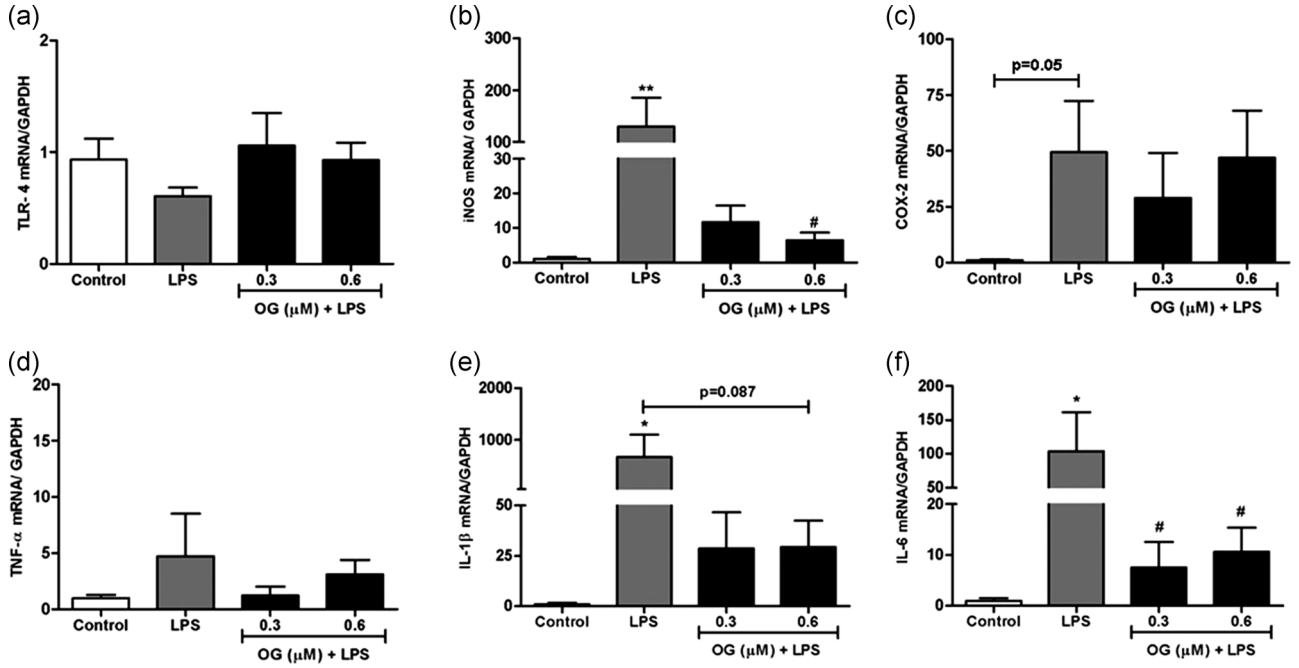

FIGURE 3 OG effect on TLR-4, iNOS, COX-2, TNF-α, IL-1β, and IL-6 mRNA expression in LPS-stimulated RAW 264.7 macrophages. The cells were pretreated with OG for 1 hr and then exposed to LPS (1 μg/ml) for 24 hr. The mRNA expression was assessed by real-time PCR and all experiments were performed in duplicate. (a) No significant differences were observed in TLR-4 expression in the different groups analyzed. (b) iNOS expression were increased in the LPS group (***$p < .001$ vs control group) and decreased significantly in the 0.6 μM OG + LPS group ($^{\#}p < .05$ vs LPS group). (e) IL-1β gene expression were increased in the LPS group (*$p < .05$ vs control group). (f) Significant differences were found in the different groups analyzed. IL-6 gene expression increased in the LPS group (*$p < .05$ vs control group) and all OG concentrations (0.6 and 1.2 μM) decreased the LPS effect ($^{\#}p < .05$ vs LPS group). All experiments were performed in duplicate and GAPDH was used as an internal control. Results are expressed as target genes/GAPDH and the data represent the mean ± *SEM* of five independent experiments ($n = 5$). COX-2, cyclooxygenase-2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL-1ß, interleukin-1β; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; mRNA, messenger RNA; OG, octyl gallate; PCR, polymerase chain reaction; TLR-4, Toll-like receptor 4; TNF-α, tumor necrosis factor-α

exhibited pathologic changes including thickening of alveolar wall and inflammatory cell infiltration (Figure 6a,b) and the OG treatment decreased this inflammatory process (Figure 6c). The results showed that the LPS group significantly increased the TNF-α and IL-6 cytokines and the OG treatment significantly decreased the TNF-α protein expression (Figure 6d) but not the IL-6 levels (Figure 6e). Animals that received LPS demonstrated a significant increase in the lung weight, suggesting edema and inflammatory cells presence, and the OG treatment did not decrease the LPS effect (Figure 6f).

3.6 | The effects of OG on oxidative stress in mice with ALI

The results did not demonstrate differences in the TBARS and CAT levels between the groups (Figure 7a,b). However, the group that received LPS reduced significantly the GSH activity and the OG treatment reversed this effect (Figure 7c). In addition, the LPS group demonstrated an increase in reactive species production that was inhibited by OG treatment (Figure 7d).

3.7 | Effects of OG on TLR-4, iNOS, COX-2, IL-1β, and IL-6 mRNA expression in mice with ALI

The results demonstrate no significant differences in TLR-4, iNOS, COX-2, and IL-6 mRNA expression between the groups analyzed (Figure 8a–c and e). The LPS group increased significantly the IL-1β levels and the OG treatment inhibited the LPS effect (Figure 8d).

4 | DISCUSSION

Identifying novel anti-inflammatory molecules that prevent the pro-inflammatory diseases is important for research as an alternative for debilitated patients. Plant-based molecules used to prevent diseases are also well regarded by the researchers. In this study, we explored the role of OG treatment on TLR-4 pathway in LPS-activated murine alveolar macrophages cell line RAW 264.7 and the ALI model in male mice. Our study showed through new evidence that OG can decrease RAW 264.7 cell activation, lung inflammation, and can have protective role in ALI.

The pathophysiology of different inflammatory diseases is mediated by immune and inflammatory cells, such as monocytes

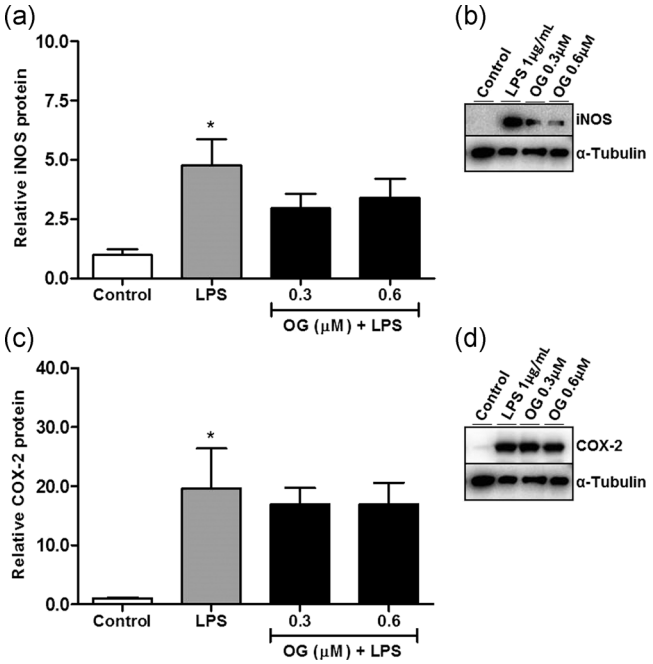

FIGURE 4 OG effect on iNOS and COX-2 protein expression in LPS-stimulated RAW 264.7 macrophages. The cells were pretreated with OG for 1 hr and then exposed to LPS (1 μg/ml) for 24 hr. The protein expression was assessed by western blot and all experiments were performed in duplicate. (a,b) LPS group induced significantly the iNOS expression and the treatment did not inhibit the LPS effect (*$p < .05$ vs control group). (b) Western blot analysis of RAW 264.7 cultured extracts for iNOS and α-tubulin proteins. Representative experiments were depicted. (c,d) LPS group induced the COX-2 expression ($p < .05$ vs control group). (d) Western blot analysis of RAW 264.7 cultured extracts for COX-2 and α-tubulin proteins. Representative experiments were depicted. All experiments were performed in duplicate and α-tubulin was used as an internal control. Results are expressed as target genes/α-tubulin and the data represent the mean ± *SEM* of four independent experiments ($n = 4$). COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; OG, octyl gallate

and macrophages. RAW 264.7 alveolar murine macrophages cell line have been utilized in in vitro studies to reproduce the inflammatory response as observed in ALI.

Therefore, our group first observed the OG effects on LPS-stimulated RAW 264.7 cells, and found that OG significantly downregulated the inflammatory mediators after LPS induction. We observed that the LPS provoked the release of cytokines (pro-inflammatory cytokines), such as IL-6 and IL-1β. The OG treatment significantly decreased the cell activation, thereby downregulating the levels of IL-1β and IL-6 cytokines (Shinbori, Walczak, & Krammer, 2004). In addition, Fu PK and collaborates related that upregulation of iNOS, that increased nitric oxide (NO) production, plays an important role in mediating lung inflammation (Kristof, Goldberg, Laubach, & Hussain, 1998; Razavi et al., 2002; Shinbori et al., 2004), and OG treatment decrease the iNOS mRNA expression. All results suggested that OG decreased LPS-induced inflammation by suppressing the production of inflammatory mediators.

To determine as OG protective the mice model of ALI, we first observed the presence of neutrophils in lung tissue after LPS administration. Neutrophil migration to alveolar space and septum is characteristic on ALI and acute respiratory syndrome (Martin, 2002). These cells are activated and lodge primarily in the capillaries of the lungs, and it is believed that neutrophil activation is a result of bacterial shedding of LPS, and can cause bacteremia or endotoxemia (Liu et al., 2019; Welbourn & Young, 1992). In our study, we observed the inflammatory cells infiltration and a predominance of neutrophil cells in lung tissues of mice with LPS-induced ALI. The LPS demonstrated abundance of neutrophils in BALF, interstitial, and alveolar spaces and all characters of ALI were decreased after OG administration. The OG treatment significantly decreased the total cells count and the neutrophils differential cell accumulation and recruitment in BALF. This action indicates that OG could decrease inflammation through suppression of neutrophil migration and accumulation in interstitial and alveolar spaces in the lung tissue.

Additionally, Brinkmann V and collaborates described a novel antibacterial strategy of neutrophils, that result in localizes and eliminates pathogens, called neutrophil extracellular traps (NETs; Brinkmann et al., 2004). NETs are released by activated neutrophils with an objective to trap microorganisms from the infection site. A recent study reported that the excessive production of NETs during LPS-induced ALI can cause organ damage and initiate the inflammatory response (Liu et al., 2016). Our results showed that LPS increase the NETs formation and release but the OG treatment not decrease this process, demonstrated that OG alleviates the ALI by other pathway.

To determine whether OG treatment contributed to the protective effects in LPS-induced ALI, we observed the histopathological changes in the lung. The histological results showed that LPS caused serious pathological lesions, and OG alleviate the histological evidence of lung injury and reduced the disruption of alveolar capillary barrier.

The cytokines presence in lung tissue, such as TNF-α, IL-β, and IL-6 play a vital part in LPS-induced ALI and promotes the severity of lung damage (Jiang et al., 2017). These chemokines promote the massive recruitment of neutrophils. Our results demonstrate that OG treatment suppressed the expression of TNF-α, IL-1β in the lung of mice with LPS-induced ALI. These cytokines are the most important pro-inflammatory cytokines and our results indicate that OG has an anti-inflammatory activity. The TLR-4 pathway induces the MAPK signaling pathway, which is activated during ALI. These pathways have a pivotal role in regulating the neutrophil chemotaxis, TNF-α, IL-1β, and IL-6 production in vivo and in vitro. Our results indicate that OG downregulated the cytokines expression as well in addition to reducing the neutrophil infiltration (Liu et al., 2012; Schnyder-Candrian et al., 2005; Xing et al., 2019).

ALI is directly associated with the ROS formation, which damage nucleic acids, lipids, cellular proteins, and extracellular matrix elements (Matthay, Geiser, Matalon, & Ischiropoulos,

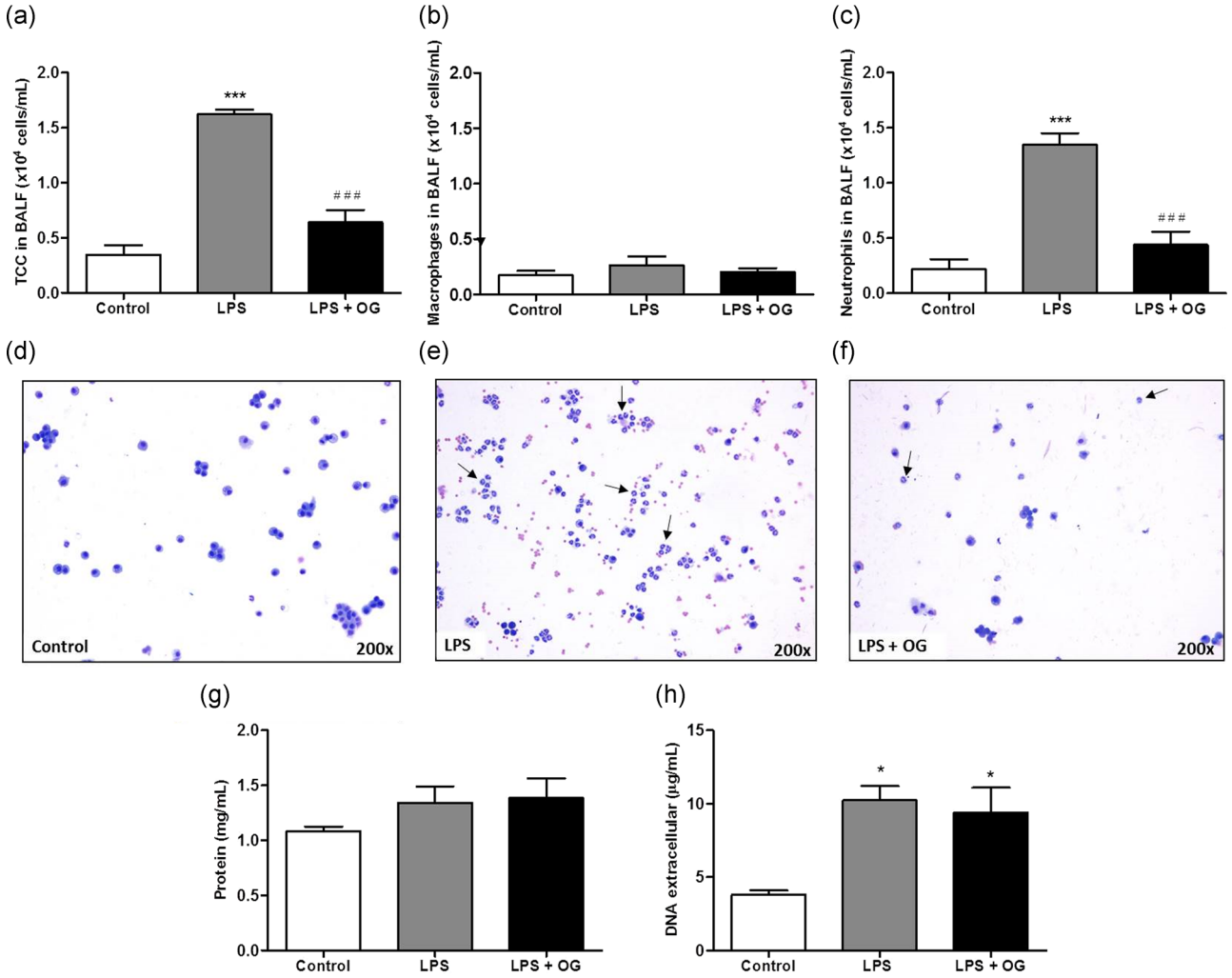

FIGURE 5 Effects of OG on inflammatory cell count, total proteins, and neutrophil extracellular traps in the BALF of LPS-induced ALI mice. (a) TCC, (b,c) differential cells counts (macrophages and neutrophils, respectively) quantified after 12 hr of ALI induction. (a) The LPS group showed a significant increase in the TCC compared with the levels in the control and the treatment with OG reversed this effect. (b) No significant changes in differential cells count of macrophages between groups were observed. (c) The differential neutrophil cells count had a significant increase in the LPS group compared with the levels in the control group and the OG treatment decreased significantly the LPS effect. (d–f) Slides representative of inflammatory cells presence in the different groups analyzed (H&E staining, magnification, ×200). The arrows indicate an increase of neutrophils cells in the lung tissue when compared with the LPS group with control and LPS + OG groups. (g) BALF levels protein were measured after 12 hr of ALI induction. No significant changes in protein levels between groups were found. (h) The LPS group increased DNA extracellular release when compared with control and the treatment did not have effect even after 12 hr of ALI induction. ALI, acute lung injury; BALF, broncho-alveolar lavage fluid; H&E, hematoxylin and eosin; LPS, lipopolysaccharide; OG, octyl gallate; TCC, total cells count. Significant difference $^{*}p < .05$ and $^{***}p < .001$ when compared with control group and $^{###}p < .001$ when compared with LPS group. Data represent the mean ± *SEM* ($n = 6$ animals per group)

1999; Terada, 2002; Zhang, Slutsky, & Vincent, 2000). The lung epithelial lining fluid (ELF) is rich in low molecular weight antioxidants such as GSH (van der Vliet et al., 1999). Depressed GSH levels in ELF has been shown to be a risk factor for developing the acute respiratory syndrome (Bowler et al., 2003; Moss et al., 2000). Our results indicate that LPS group decrease significantly the GSH, and the OG treatment reverts this effect. Searches suggest that treatments with antioxidants molecules may modulate the ALI development. The reactive species number, as ROS, observed through DCF increase in the group of animals that receive LPS, and these levels decrease significantly after OG administration.

In conclusion, our study has demonstrated the protective effects of OG in alveolar macrophages activated with LPS and on LPS-induced lung injury. OG treatment significantly inhibited the alveolar macrophages activated by decrease in iNOS and IL-6 expression and alleviated the IL-1β expression in vitro. The in vivo results demonstrated a similar effect; OG treatment

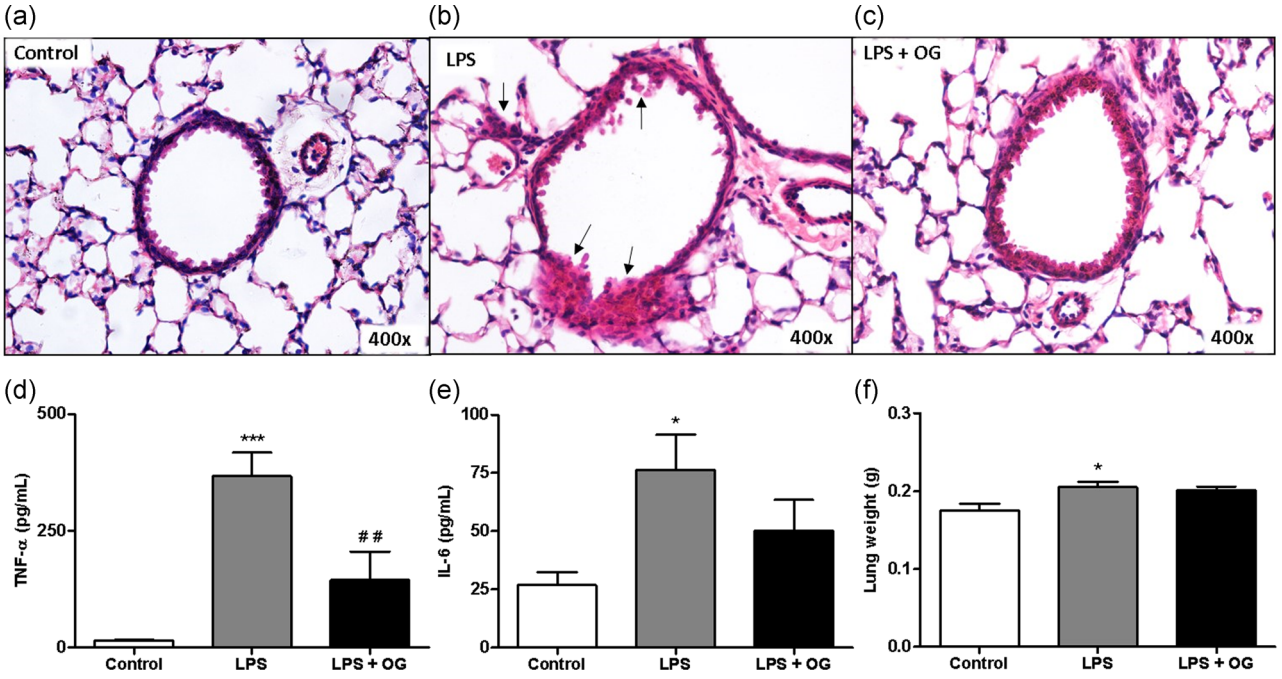

FIGURE 6 Effects OG on histopathological changes, pro-inflammatory cytokines, and lung weight changes in mice with ALI. The pro-inflammatory markers were analyzed after 12 hr of LPS-induced ALI. (a–c) Representative histological changes in the lung obtained from mice of different groups (H&E staining magnification, ×400). The arrows indicate an increase of inflammatory cells in the lung tissue when compared with the LPS group with control and LPS + OG groups. (d) The TNF-α production increased in the LPS group and decreased after OG administration. The results are expressed as TNF-α (pg/ml). (e) The LPS increased IL-6 pro-inflammatory cytokine and the OG treatment modulated the LPS effect. The results are expressed as IL-6 (pg/ml). (f) The lung weight (g) increased in the LPS group and the treatment did not reverse this effect. ALI, acute lung injury; H&E, hematoxylin and eosin; IL-6, interleukin-6; LPS, lipopolysaccharide; OG, octyl gallate; TNF-α, tumor necrosis factor-α. Significant difference $^{*}p < .05$ and $^{***}p < .001$ when compared with control group and $^{\#\#}p < .01$ when compared with LPS group. The data are expressed as mean ± *SEM*. ($n = 6$ animals per group)

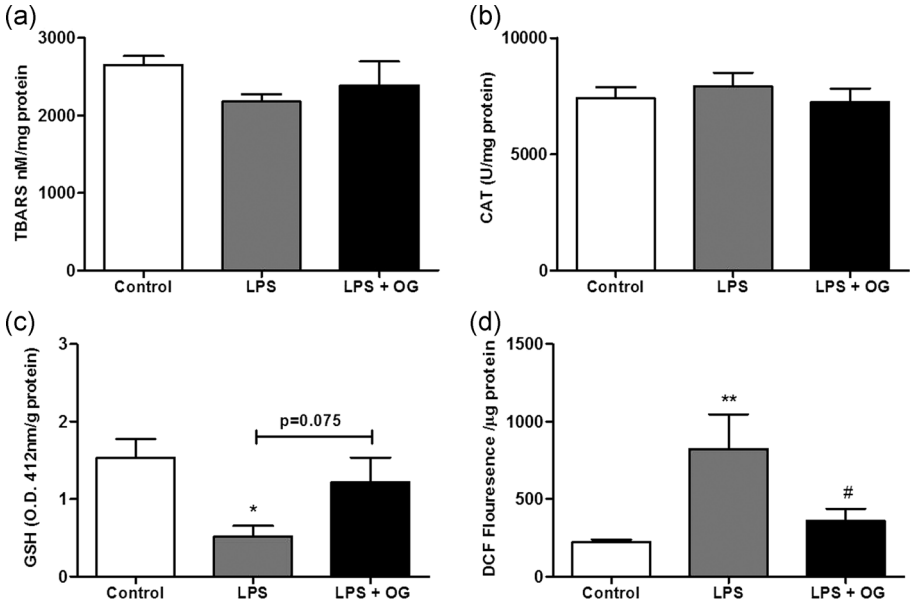

FIGURE 7 OG treatment reduced oxidative stress in the lung during ALI. Lung sections were collected 12 hr after ALI induction and all the measurements were performed as previously described using a spectrophotometer or fluorimeter. We evaluated the lipid peroxidation by TBARS, GSH activity, CAT activity, and reactive species production by DCFH-DA assay. (a,b) No significant changes in TBARS and CAT activity between groups were found. (c) LPS group decreased the GSH activity and OG treatment reverted the LPS effect ($p = .075$). (d) DCF level was increased in the LPS group and decreased after OG administration. ALI, acute lung injury; CAT, catalase; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; GSH, glutathione; LPS, lipopolysaccharide; OG, octyl gallate; TBARS, thiobarbituric acid-reactive substances. Significant difference $^{*}p < .05$ and $^{**}p < .01$ when compared with control group and $^{\#}p < .05$ when compared with LPS group. The data are expressed as mean ± *SEM*. ($n = 6$ animals per group)

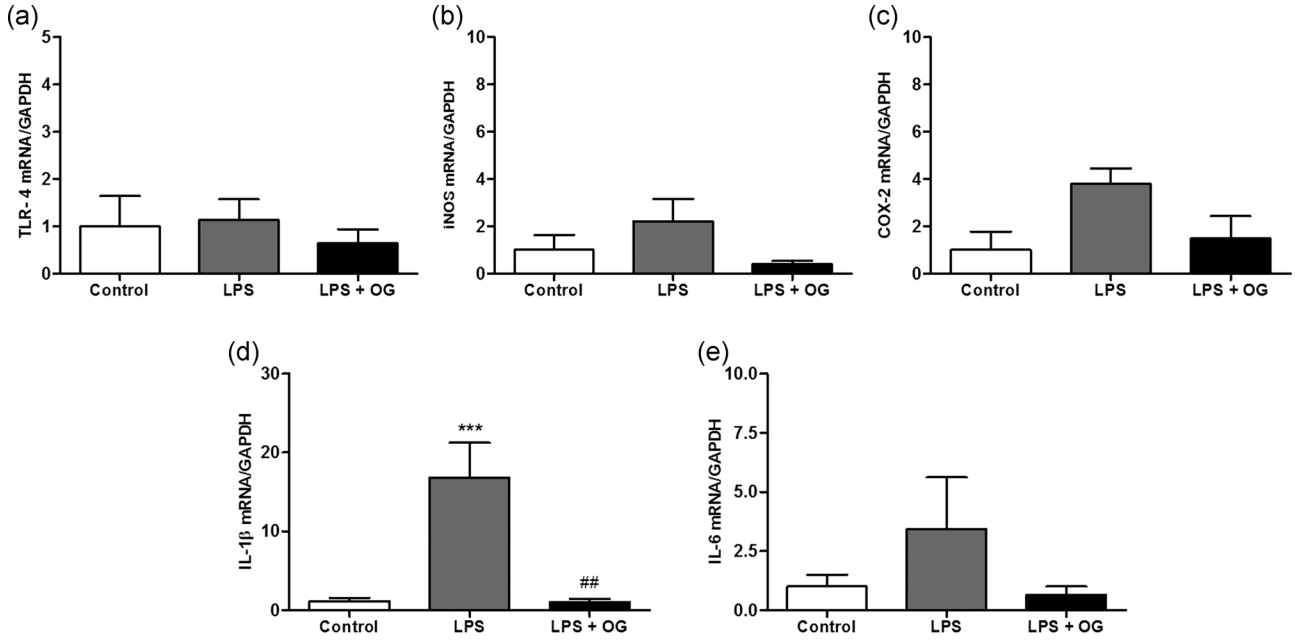

FIGURE 8 OG effect on TLR-4, iNOS, COX-2, IL-1β, and IL-6 mRNA expression in the lung during ALI. All lungs sections were collected after 12 hr of ALI induction and then the pro-inflammatory genes were assessed. (a–c) No significant changes in TLR-4, iNOS, and COX-2 between groups were found. (d) LPS group increased significantly the IL-1β levels and OG treatment decreased the LPS effect. (e) No significant differences were observed in IL-6 expression between the groups. All experiments were performed in duplicate and GAPDH was used as an internal control. ALI, acute lung injury; COX-2, cyclooxygenase-2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL-1ß, interleukin-1β; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; mRNA, messenger RNA; OG, octyl gallate; TLR-4, Toll-like receptor 4. Significant difference $^{***}p < .001$ when compared with control group and $^{\#\#}p < .01$ when compared with LPS group. Results are expressed as target genes/GAPDH and the data represent the mean ± *SEM*. ($n = 6$ animals per group)

decreased lung injury, inflammatory cells migration, and cytokines and protected the tissue from oxidative stress. These data suggest that OG can act as an anti-inflammatory agent for lung injury.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

GVH conceived the work, acquired data, drafted the paper, and approved the final version. CL, GLA, JS, BSB, and MSC acquired data, revised the article, and approved the final version. MVFD, JGS, and JRO conceived the work, revised the paper, and approved the final version.

DATA AVAILABILITY STATEMENT

The data used to support the findings of this study are available from the corresponding author upon request.

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