Molecular Biology Reports
https://doi.org/10.1007/s11033-021-06937-2

SHORT COMMUNICATION

Octyl gallate decrease lymphocyte activation and regulates neutrophil extracellular traps release

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Received: 2 July 2021 / Accepted: 5 November 2021

Abstract

Background Inflammation is a complex mechanism with an objective to destroy and eliminate the invading microorganisms. During acute inflammation, the neutrophils are the major cells involved in this process and, although they defend the organism, must die to not generate damage. The two major mechanisms that drive neutrophils to death are: apoptosis and a novel mechanism recently discovered denominated NETosis. This process is a "suicidal mechanism", in which the cells release "neutrophil extracellular traps" (NETs) during the inflammatory response. Octyl gallate (OG) is one of the gallic acid derivates, with several protective effects, such as antioxidant and anti-inflammatory in cancer models. Thus, this study aimed to investigate the action of OG on the proliferation of lymphocytes, neutrophils activation, and its effectiveness in an experimental sepsis model.

Methods Lymphocytes and neutrophils were obtained from healthy donors. Cell viability, apoptosis, NETs release and antioxidant capacity of OG were observed. In addition, survival was evaluated in an experimental model of sepsis in C57BL/6 mice.

Results Our study demonstrated, for the first time, that the OG can act as an inhibitor of reactive oxygen species (ROS) release, NETs formation in primary human neutrophils and, modulates the lipopolysaccharide (LPS) effect in neutrophil apoptosis. The OG also inhibited peripheral blood mononuclear cells (PBMCs) proliferation in vitro. Despite the positive results, we did not observe an increase in the survival of septic animals.

Conclusions The pharmacological potential of OG, modulating activation of neutrophils and lymphocytes, suggests the use as an adjuvant therapeutic strategy in inflammatory diseases.

Keywords Octyl gallate · Neutrophils · NETosis · Apoptosis · Immunomodulation · Sepsis

Introduction

Inflammation is a complex mechanism that aims to destroy and eliminates microorganism invaders in the organism. Two main defense mechanisms are involved in this response, denominated acute and adaptive inflammation. When an acute inflammation occurs has an accumulation of fibrin, fluid and leukocytes, and a predominance of polymorphonuclear cells (PMNs), like neutrophils. These cells migrate to the infection site by chemotaxis. The peripheral blood mononuclear cells (PBMCs), like lymphocytes, represent cells of a late response or adaptive immune response, which are activated when the acute response mechanism fails and does not eliminate the microorganism invaders [1]. Neutrophils are the most abundant cells in the blood and, although they defend the organism, must die to not cause damage. They

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Published online: 16 November 2021

could die for different mechanisms: apoptosis, necrosis and a novel mechanism recently discovered denominated NETosis. Normally, neutrophils die by apoptosis, playing an important role in the physiological control of the immune response and in the resolution of inflammation. During physiologic conditions, neutrophils die in a few hours, however, microbial components such as lipopolysaccharide (LPS) delay the neutrophil-apoptosis [2, 3].

Several studies have shown that neutrophils have another antimicrobial mechanism called NETosis, which can be induced by infection, inflammation or trauma and represents an innate immune activation mechanism. The NETosis process is mechanism, which the cells release "neutrophil extracellular traps" (NETs) for inflamed space. NETs are composed of decondensed chromatin and antimicrobial factors, including granular compounds such as neutrophil elastase (NE) and myeloperoxidase (MPO). These structures capture and kill bacterias, fungi and parasites [4–7].

Over the last few years, many studies have been conducted to find new anti-inflammatory molecules to modulate the immune response. The development of new therapies may represent a significant advance in the treatment and prevention of several inflammatory diseases that are highly prevalent—such as sepsis. Therefore, anti-inflammatory molecules originated from plants can be an alternative. Octyl gallate (OG) is one of the gallic acid derivates, widely contained in natural sources, such as green tea, grapes, pineapple, and strawberries [8]. Several studies indicate that OG has antiviral, antifungal and antibacterial action [9–13]. Besides that, an antioxidant and anti-inflammatory effect in cancer models treated with OG, were described previously [14, 15]. Thus, the objective of the present study was to investigate the action of OG on the proliferation of lymphocytes, neutrophils activation and its effectiveness in an experimental sepsis model.

Materials and methods

Reagents

Octyl gallate (Sigma-Aldrich), LPS—Escherichia coli 026:B6 (Sigma-Aldrich), Ficoll-Paque TM PLUS (GE healthcare), NH 4 Cl (Nuclear), Heparin sodic (Hepamax-S), RPMI 1640 medium (Gibco-Life Technologies), Quanti-it TM PicoGreen ® dsDNA assay kit (Invitrogen), Trypan blue dye (Gibco-Life Technologies), 2,2-diphenyl-1-picrylhydrazyl—DPPH (Sigma-Aldrich), 96-well microtiter bottomed flat plates (Nunc TM—Immuno Modules), Garamycin (Schering—Plough), Phytohemagglutinin (PHA) (Invitrogen), Vitamin C (Sigma-Aldrich), Phosphate buffer saline—PBS (Laborclin), 2′,7′-Dichlorofluorescin diacetate—DCFH-DA (Sigma-Aldrich), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide—MTT (Gibco-Life Technologies).

Cell culture

PBMCs (lymphocytes) and PMNs (neutrophils) were isolated from healthy donors using gradient separation, as previously described (17). The cellular viability was performed by trypan blue dye exclusion, which was uniformly greater than or equal to 90%. The PMNs purity was ≥ 95% of neutrophils in all experiments. All human subjects read and signed an informed consent [16].

PBMCs and neutrophil viability assay

PBMCs (1.6×10^5 cell/well) or PMNs (2.0×10^5 cells/well) were seeded in 96-well microtiter bottomed flat plates in a 5% CO_2 -humidified incubator. The cellular viability of OG was performed by trypan blue dye exclusion assay after 96 h of treatment for PBMCs and 16 h for PMNs. The control group received RPMI-1640 medium (Gibco, Life Technologies) plus 0.1% DMSO (vehicle of OG treatment), and the OG group was composed of different concentrations of OG (0.6 to 5 µM). The OG was primarily dissolved in DMSO and diluted in RPMI-1640 medium, and after was added to the cells at stated concentration. All groups were made in triplicate.

Proliferation of PBMCs

PBMCs (1.6×10^5 cells/well) were plated in 96-well microtiter bottomed flat plates in a 5% CO_2 -humidified incubator for 96 h. The control group was composed of RPMI-1640 medium plus 0.1% DMSO. A group with phytohemagglutinin (PHA) was used to induce lymphocyte proliferation. The PHA was diluted in medium and added directly to the cells in the concentration of 10 µg/mL plus 0.1% DMSO. The OG was primarily dissolved in DMSO and diluted in RPMI-1640 medium, after was added to the cells at a stated concentration (0.6 to 5 µM). In the OG + PHA group, the two drugs were diluted in RPMI-1640 medium and then added to the cells. Lymphoproliferation was determined by MTT (3-(4–5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. In brief, MTT solution was added to all assay wells. Plates were incubated at 37ºC for 3 h. After, they were read in a microplate reader (EZ Read 400—Biochrom), using a test wavelength of 570 nm and a reference wavelength of 620 nm.

Neutrophil apoptosis assay

Neutrophil apoptosis was evaluated by light microscopy (BMX 43-Olympus, Japan) analysis of 300 cells stained

with May-Grunwald-Giemsa. Nuclear morphological (condensation of chromatin and simplification of nuclear structure) changes were used to determine the number of apoptotic cells. The control group was composed of RPMI-1640 medium and 0.1% DMSO. In the LPS group, the drug was diluted in medium and added directly to the cells at the stated concentration (50 ng/mL) plus 0.1% DMSO. The OG was primarily dissolved in DMSO and diluted in RPMI-1640 medium, after was added to the cells at a stated concentration (2.5 and 5 µM). In the OG + LPS group, the two drugs were diluted in medium and then added to the cells. We used 96-well microtiter bottomed flat plates. All groups were made in triplicate. Quantification was performed by two independent examiners in a blind manner on three different experiments.

Neutrophil extracellular traps induction and detection

The quantification of cell-free DNA (cfDNA)/NETs was performed in the supernatants of PMNs (2.0×10^5 cells/200 µL). Briefly, cells were incubated with LPS (inductor) for 3 h at 37 °C in a 5% CO^2 humidified incubator. To quantify cfDNA/NETs release, we used Quant-iT™ PicoGreen® dsDNA kit according to the manufacturer′s instructions. The fluorescence intensity reflects the amount of cfDNA was measured by a microplate reader (Victor 3, PerkinElmer).

Reactive oxidative species in neutrophils

The generation of intracellular reactive oxidative species (ROS) of PMNs (2.0×10^5 cells/200 µL) was evaluated based on the intracellular peroxide-dependent oxidation of 2′,7′-dichlorodihydrofluorescein di-acetate (DCFH-DA) which forms a fluorescent compound, 2′,7′-dichlorofluorescein (DCF), as previously described [17].

Antioxidant activity

The DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) method was used to verify the percentage of antioxidant action of OG. The DPPH method is based on the capture of DPPH radical (2′,2′-diphenyl-1-picrylhydrazyl) by antioxidants, producing a decreased absorbance at 515 nm (17). Ascorbic acid (Vitamin C) was used as a positive control (reference standard) in this experiment. The concentrations of OG tested were 2.5 and 5 µM and of Vitamin C was 5.681 µM.

Animals

Male C57BL/6 mice (8—12 weeks old) were kept on shelves with ventilated cages that provide 60 air cycles per hour, relative humidity ranging between 55 and 65%, a 12-h light–dark cycle, temperature of 22 ± 2 °C with free access to food and water. The animals were maintained following the Guiding Principles in the Care and Use of Animals approved by the Council of the American Physiological Society. The experimental protocol was approved by the Bioethics Research Committee of Pontifícia Universidade Católica do Rio Grande do Sul (Protocol Number 16/00495).

Experimental sepsis induction

This experimental model was performed according to the technique developed by our group [18, 19]. The animals were divided into three groups: control group: the animals received 50 µl of saline plus DMSO (vehicle) by intranasal (IN) application and 30 min after, the mice were anesthetized and implanted an empty capsule. Sepsis group: the animals receive 50 µl of saline + DMSO by IN application and 30 min after, the mice were anesthetized and implanted a capsule with *Escherichia coli* suspension and a non-sterile fecal content. Sepsis + OG group: the animals received 50 µL of OG (0.75 mg/kg) by IN application and 30 min after, the animals were anesthetized and implanted a capsule with *Escherichia coli* suspension and a non-sterile fecal content. A survival curve for different experimental groups was performed. After 7 days, animals that continued alive were anesthetized with an IP solution of ketamine (100 mg/kg) and xylazine (50 mg/kg) and euthanized. Figure 3A represents the experimental design.

Statistical analysis

The normality of the data was verified by the Shapiro–Wilk test. For comparison between groups was applied analysis of variance (ANOVA) and post hoc Tukey′s test for multiple comparisons. Differences were considered significant at * or # $p < 0.05$, ** or ## $p < 0.01$, and *** or ### $p < 0.001$. The statistical analysis was performed with the statistical program GraphPad Prism (version 5.0, GraphPad Software Inc, San Diego, California). Quantitative data were presented as mean ± standard error of the mean (SEM).

Results

OG modulates lymphocyte activation and does not affect cell viability

Firstly, we evaluated the PBMCs cellular viability after 96 h of treatment with OG (0.6 to 5 µM). None concentration decreased cell viability, which demonstrates low cytotoxicity of OG (Fig. 1A). After that, we evaluated the OG's ability to modulate the activation and proliferation of lymphocytes using the PHA-stimulation. PHA showed higher affinity for

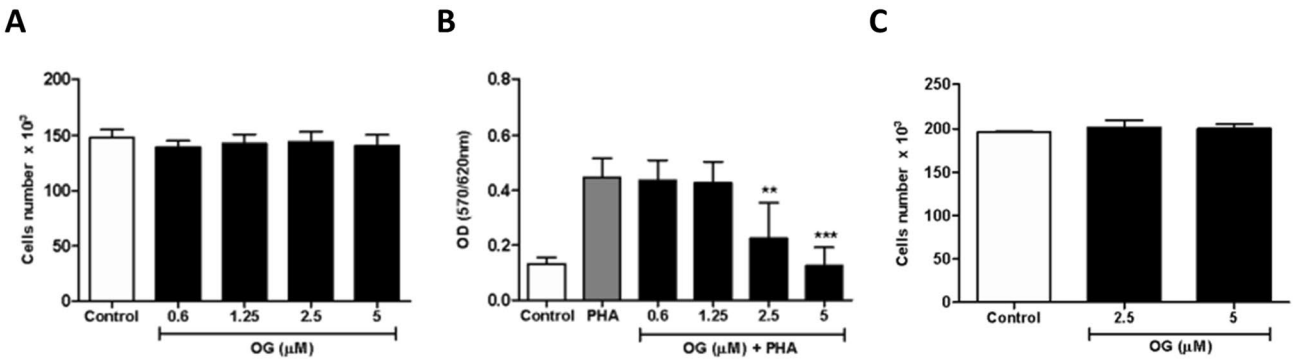

Fig. 1 Effect of OG on the viability and proliferation of PBMCs and PMNs. **A** Cells were exposed to different concentrations of OG for 96 h. Data are expressed as cells number $\times 10^3$. **B** Cells were stimulated with PHA (10 μg/mL) and treated with different concentrations of OG. Data are expressed as OD 570/620 nm. ***$p < 0.001$, and **$p < 0.01$ vs PHA group. **C** Neutrophils were exposed to two concentrations of GO (2,5 and 5 μM) for 16 h. Results are expressed as cells number $\times 10^3$. The data represent the mean ± SEM (n = 5)

lymphocytes and leads to lymphocyte activation and proliferation. The OG treatment reduced significantly the PBMCs proliferation induced by PHA (positive control) in the concentrations of 2.5 and 5 μM (Fig. 1B). We also evaluated whether OG could show any cytotoxicity specifically in the neutrophil population (Fig. 1C). Both doses tested did not decrease the cell viability, corroborating with the results showing low drug cytotoxicity.

OG affects the apoptosis and DNA release

Previous studies in cancer models have shown that OG can induce cellular apoptosis [20–22]. In this way, we investigated if the reduction in cell proliferation was due to an increase in apoptosis. PMNs were treated with OG and stained with May-Grunwald-Giemsa. Nuclear morphological changes (condensation of chromatin and simplification of nuclear structure) were used to determine the number of apoptotic cells. Our results indicated that OG is not capable of inducing apoptosis (Fig. 2A). In addition, we also stimulated the cells with LPS and evaluated if OG could have any influence on apoptosis. LPS is known to prolong the functional lifespan of neutrophils [23, 24] by preventing apoptosis and can be used as an experimental model of endotoxemia. The LPS group showed a significant anti-apoptotic effect and the OG decreased this response (Fig. 2B). Morphological cellular alterations after LPS stimulation (treated or not treated with OG) were visualized by optical microscopy (Fig. 3C). In this representative image, we could verify the morphological differences of cells during apoptosis in the different groups. Arrows shows morphological details of apoptotic cells, with loss of its original shape—the cells lose their chromatin fine granularity (condensation). The neutrophils LPS-stimulated group showed a predominance of normal neutrophils with fine granularity of chromatin and normal lobulated nucleus.

This surprising effect of OG on neutrophils led us to investigate if it could be acting on other pathways affected by the LPS stimulus. LPS is responsible for inducing the formation NETs, characterized by the DNA release. To analyze the possible effect of OG on the NETs formation, we decided to measure the free DNA in the supernatant from the PMNs. LPS group demonstrated an increase in the NETs formation when the group stimulated and treated with OG (5 μM) had a decrease in the concentration of free DNA (Fig. 2D).

OG reduce the oxidative stress

OG has antioxidant properties described [25] and probably reduced the release of NETs by modulating oxidative stress. Higher ROS concentrations are responsible for the activation and release of neutrophil-derived proteases and the formation of NETs [26]. In order to confirm the antioxidant capacity of OG, we performed the DPPH method. The OG and Vitamin C (positive control) demonstrated decreased free radical DPPH (Fig. 2E). Based on this information, we decided to investigate whether OG could inhibit the LPS-induced ROS generation. To evaluate this effect, the cells were stimulated with LPS and treated with different concentrations of OG (2.5 and 5 μM). The LPS group demonstrated increased ROS release and, when associated with OG (2.5 and 5 μM), there was a significant decrease in this effect (Fig. 2F).

OG does not increase the survival in the experimental model of sepsis

Based on these positive effects found in effective cells from the immune response, we hypothesized that OG could modulate this response in inflammatory diseases and have some beneficial effects. Using a model of sepsis previously described by our laboratory, we investigated the

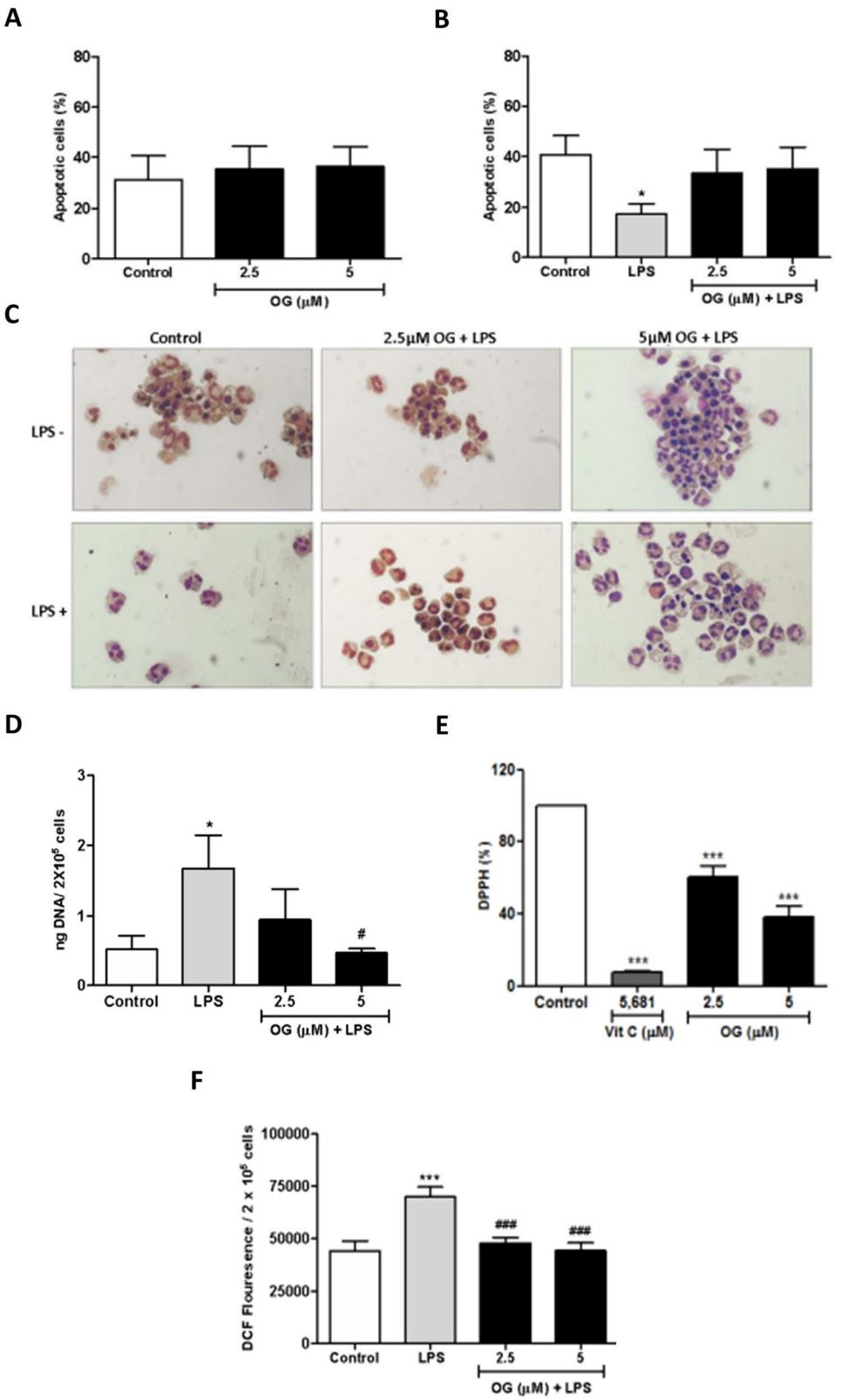

Fig. 2 OG has an antioxidant effect and regulates the NETs release. **A** Cells were treated with 2,5 or 5 µM of OG and 300 cells stained with May-Grunwald-Giemsa were counted to evaluate the spontaneous neutrophil apoptosis. Data are expressed by the percentage (%) of apoptotic cells. **B** Neutrophils were stimulated with LPS (50 ng/mL) and LPS + OG and analysed like described before. *$p < 0.05$ vs control group. **C** Representative microscopic images of Figs. 2A and 2B. Magnification × 400. **D** Cells were stimulated with LPS (50 µM) and treated with or without different concentrations of OG (2,5 and 5 µM) for 3 h. Cell free DNA (cfDNA)/NETs was performed in the supernatants and the results are expressed as ngDNA/2×10^5 cells. *$p < 0.05$ vs control group, # $p < 0.05$ vs LPS group. **E** DPPH method was used to verify the percentage of antioxidant action of OG (2,5 and 5 µM). Vitamin C (5,681 µM) was used like positive control of antioxidant effect. Results are expressed as DPPH (%). ***$p < 0.001$ vs control group. **F** Cells were stimulated with LPS (50 µM) and treated with or without different concentrations of OG (2,5 and 5 µM) for 3 h. ROS release by DCF fluorescence. Results are expressed as DCF Fluorescence/2×10^5 cells. ***$p < 0.001$ vs control group and ### $p < 0.001$ vs LPS group. The data represent the mean ± SEM (n = 5)

effectiveness of the treatment. The animals were pre-treated with OG and subsequently sepsis was induced (Fig. 3A). Despite partially delaying the death of the animals, the OG was not able to increase survival (Fig. 3B).

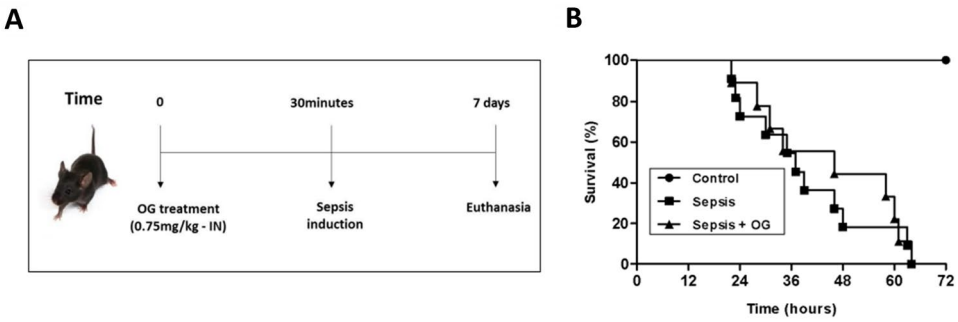

Fig. 3 OG can be helpful in inflammatory disorders. **A** Experimental design of the study. The timeline is expressed in hours. OG: Octyl gallate. **B** Kaplan–Meier survival curve was performed. The animals were pre-treated with octyl gallate and subsequently the procedure for septic induction was performed. *$p < 0.05$ vs sepsis group. Data represent the mean ± SEM ($n = 10$)

Discussion

Our first objective was to evaluate the action of OG in the PBMCs viability and activation. Our findings demonstrated that the concentrations tested did not decrease the PBMCs viability. In parallel, we verified the possible immunomodulation of OG. Uncontrolled proliferation of these cells causes tissue damage by the release of inflammatory cytokines [27]. Our results indicated that the concentrations 2.5 and 5 µM OG decrease the PBMCs proliferation.

In addition to these positive results found in PBMCs we identified an OG modulating capacity in neutrophils. Neutrophils are the first line of defense of our organism and the first cells to reach the focus of inflammation. In response to inflammatory stimuli, they migrate to infected tissues, where efficiently bind, engulf, and inactivate bacteria [4, 16], however, their permanence in the inflamed site has a harmful effect. OG decreased the anti-apoptotic effect of LPS, which indicates that OG could have a protective role against infection-induced tissue damage.

Studies describe that neutrophils, when activated by chemicals agents (PMA and LPS) or pathogens, suffer NETosis and release DNA extracellular medium. NETs are important to control and kill bacteria [4, 7], however, the formation of NETs may have deleterious effects on the host inducing local cytotoxicity [28, 29]. In this way, OG can be an important modulator of this response. Our results were able to show that OG significantly decreased in vitro LPS-induced NETs release. This reduction is linked to the antioxidant properties of OG which decreased ROS levels released by LPS-treated neutrophils. Although the mechanisms are not yet well established about the relationship between OG and ROS production, a previous study demonstrated that OG has antioxidant activity, decreasing the production of the radical anion superoxide [13]. Similarly, our group also demonstrated that gallic acid promotes a decrease in ROS production in human neutrophils incubated with LPS [16]. The antioxidant potential of gallic acid has already been demonstrated on the inhibition of MAPK/NF-κB and on the increase of the AKT/AMPK/Nrf2 complex [30]. A study has also shown that gallic acid increases the expression of nuclear factor E2-related factor 2 (Nrf2) and promotes a decrease in the production of mitochondrial ROS (mtROS). These findings suggest that OG may have an activity on the intracellular regulation of the antioxidant response [31].

Despite these effects found in vitro, when we tried to challenge the OG's ability to immunomodulate the response in vivo we did not find a significant survival improvement in a mice sepsis model. The OG treatment delayed the mortality during the experiment, but the results were not significant. It′s worth mentioning that our sepsis model is extremely severe and possibly octyl gallate alone is not able to reverse this condition, however, as an adjunct in future studies using antimicrobials or another anti-inflammatory drug it could be interesting.

In conclusion, our group demonstrated for the first time that the OG can act as an inhibitor of ROS release, NETs formation in primary human neutrophils and modulates the LPS effect under neutrophils apoptosis. The OG also acts as an inhibitor of PBMCs proliferation. The effect of OG showed in this research suggests their use as an adjuvant therapeutic strategy in inflammatory diseases mediated by activation of neutrophils and lymphocytes. These results demonstrate that OG can be used in both the primary response (neutrophils) and in the secondary response (lymphocytes).

Acknowledgements This study was supported by CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, 142335/2015-0). G.V.H. received a fellowship from CNPq and C.L. received a fellowship from CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Financial Code 001).

Author′s contributions GVH conceived the work, acquired data, drafted the paper, and approved the final version. CL acquired data, revised the article, and approved the final version. LP drafted the paper, revised the article, and approved the final version. MVFD and JRO conceived the work, revised the paper, and approved the final version.

Declarations

Conflict of interest The authors declare they have no competing interests.

Ethical approval The experimental protocol was approved by the Ethics Research Commitee of Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Protocol Number: 2.066.902.

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