Toxicology in Vitro 30 (2015) 309–317

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/tiv

Gallic acid reduces the effect of LPS on apoptosis and inhibits the formation of neutrophil extracellular traps

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ARTICLE INFO

Article history:
Received 25 May 2015
Received in revised form 28 September 2015
Accepted 12 October 2015
Available online 23 October 2015

Keywords:
Inflammation
Gallic acid
Neutrophils
ROS
NETosis
Apoptosis

ABSTRACT

Apoptosis and NETosis of neutrophils are two major mechanisms of programmed cell death that differ in their morphological characteristics and effects on the immune system. Apoptosis can be delayed by the presence of pathogens or chemical components such as lipopolysaccharide (LPS). Neutrophils have other antimicrobial strategy, called neutrophil extracellular traps (NETs), which contributes to the elimination and control of the pathogen. NETosis is induced by infection, inflammation or trauma and represents an innate immune activation mechanism. The objective of this study was to evaluate the effect of gallic acid (GA) in the modulation of apoptosis and NETs release. The results show that GA decreased the anti-apoptotic effect of LPS, blocked the induction of NETs and prevented the formation of free radicals induced by LPS. These findings demonstrate that the GA is a novel therapeutic agent for decreasing the exacerbated response of the body against an infectious agent.

1. Introduction

Sepsis is a complex syndrome that results in an exaggerated systemic inflammatory response against an infectious agent (Bone et al., 1997; Matot and Sprung, 2001). This reaction aims to destroy, dilute or immobilize the infectious agent. (Teixeira et al., 2003) Inflammations are divided into acute and chronic. Acute inflammation is characterized by the accumulation of fluid, fibrin, leukocytes (especially neutrophils) and red blood cells in the aggression area. Upon arrival in the inflamed site, neutrophils are already equipped with the necessary proteins to destroy infectious agents (Brinkmann et al., 2004). The encounter with the pathogen causes the activation of the cells with the immersion of the microorganism in a phagosome (Brinkmann et al., 2004; Guimarães-Costa et al., 2012; Fuchs et al., 2007). In the phagosome two events occur: first, there is great generation of reactive oxygen species (ROS), and second, the granules of neutrophils merge the phagosome, and unload antimicrobial peptides and enzymes. Together these two events lead to microbial death (Vaughan, 2013; Liu et al., 2000). The inflammatory reaction is mediated endogenously by active substances, called "inflammatory mediators" and excessive production of these mediators leads to an increase in host response, causing a metabolic imbalance that can propagate the inflammatory response (Teixeira et al., 2003; Fuchs et al., 2007; Esmann et al., 2010; Mello, 2012). Chronic inflammation is the sum of the reactions of the organism as consequence of the offending agent residence, which was not eliminated by the mechanisms of acute inflammation (Mello, 2012).

Septic shock is an example of the increase of uncontrolled inflammatory response that results in a metabolic imbalance (Radic, 2014). The shock and the complications are mainly related to the release of components of the bacterial wall. The endotoxin of gram-negative bacteria, lipopolysaccharide (LPS), and teichoic acid of gram-positive bacteria indirectly trigger the inflammatory cascade *via* induction of cytokine production by activated macrophages and monocytes, and sequentially, produce tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6) and interleukin-8 (IL-8). These cytokines interact with other cells and cellular elements (polymorphonuclear cells, endothelial cells, fibroblast cells, platelets and monocytes), inducing production and release of secondary mediators, which contribute to a delayed inflammatory response. (Sulowska et al., 2005) The

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http://dx.doi.org/10.1016/j.tiv.2015.10.005
0887-2333/

overproduction or inappropriate expression of these factors can lead to a variety of pathological conditions, including septic shock and systemic toxicity (Mello, 2012; Radic, 2014; Kirchner et al., 2013).

NETosis and apoptosis of neutrophils are two major mechanisms of programmed cell death that differ in their morphological characteristics and their effects on the immune system (Brinkmann and Zychlinsky, 2012). Apoptosis is characterized by packaging of nuclear chromatin and nuclear fragments, subsequently occurring absorption of apoptotic cells by phagocytes, which generally suppress the immune response. Neutrophils under physiological conditions suffer apoptosis in 20 h. However, in infected tissues this can be delayed by microbial components such as LPS and pro-inflammatory stimuli (Teixeira et al., 2003; Esmann et al., 2010). Apoptosis of neutrophils is an important point in the physiological control of the immune response, playing an important role in the resolution of inflammation. In this context, apoptosis should be delayed until the essential functions of pathogen are completely phagocyted, then these cells must die to undo the inflammation and prevent tissue damage (Vaughan, 2013; Saffarzadeh and Preissner, 2013).

Recent 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 (Brinkmann and Zychlinsky, 2012). When neutrophils are activated by phorbol myristate acetate (PMA), IL-8, LPS or fungi, they release means for the chromatin that are associated with different proteins, forming a complex called neutrophil extracellular traps (NETs), which capture and kill pathogens (Fuchs et al., 2007; Meng et al., 2012). NETs are abundant in inflamed sites, as found in patients with appendicitis, preeclampsia and infection by *Streptococcus pneumoniae* (Fuchs et al., 2007). Some studies suggest a pathophysiological role of NETs and their components in autoimmune diseases such as small vessel vasculitis, lupus nephritis, systemic lupus erythematosus (SLE), psoriasis, and rheumatoid arthritis (Meng et al., 2012; Bone, 1991; Thijs and Hack, 1995). Recent studies suggest that this action may cause tissue damage and the control of NETs release can result in beneficial effects in autoimmune diseases (Meng et al., 2012; Vilcek and Lee, 1991).

Gallic acid (GA) is a phenolic compound found in various plants, fruit and food, and it has antioxidant, anti-carcinogenic and anti-viral properties (You et al., 2011; Chandramohan Reddy et al., 2012). Other studies report that GA also has antibacterial, antifungal, anti-inflammatory, anti-malarial and anti-herpetic effects and is present in some of the most consumed beverages in the world, such as green tea (You et al., 2011; Chandramohan Reddy et al., 2012; Eslami et al., 2010). Hence, the objective of this study was to evaluate the effect of GA controlling apoptosis and formation of NETs in primary cultures of human neutrophils.

2. Materials & methods

2.1. Ethics statement

Study experimental protocol (443.648) was approved by the Ethics Research Committee of Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS).

2.2. Peripheral blood polymorphonuclear cells preparation

The peripheral blood polymorphonuclear cells (PMNs) were isolated from whole blood obtained from healthy human donors by Ficoll-Paque™ PLUS (GE Healthcare) density gradient centrifugation. Briefly, 12 mL of blood samples were collected by venipuncture in heparin-containing tubes. Plasma was discarded and blood cells were diluted 1:2 with saline solution. After, 4 mL Ficoll-Paque™ PLUS were added to cell solution and centrifuged at 720 ×*g* at room temperature for 20 min. After centrifugation, the supernatant was removed and the cells were washed with hypotonic lysis buffer, containing 0.83% NH_4Cl, to lyse red blood cells (erythrocytes). The solution was centrifuged at 200 ×*g* at 4 °C for 10 min and this procedure was repeated twice. The resultant pellet was washed with phosphate-buffered saline (PBS). Following isolation, neutrophils (2.0×10^5 / 200 μL) were maintained in RPMI 1640 medium supplemented with 10% autologous serum and 0.15% garamycin (Schering-Plough) in 96-well flat-bottom plates (Nunc™-Immuno Modules) at 37 °C in a humidified atmosphere containing 5% CO_2 (Morita et al., 2014; Czerwinska et al., 2013). Cell viability was assessed by trypan blue exclusion assay. Purity of this preparation was ≥95% of neutrophils. All reagents used were filtered through a disposable sterile filter unit 0.22 μM (Millex). All human subjects read and signed an informed consent.

2.3. Peripheral blood mononuclear cells (PBMCs) preparation

PBMCs were isolated from whole blood of healthy human donors (12 mL of heparinized blood) using Ficoll-Paque™ PLUS density gradient centrifugation. Briefly, plasma was discarded and the blood cells were diluted 1:2 with saline solution. After, 4 mL Ficoll-Paque™ PLUS were added to cell solution and centrifuged at 720 ×*g* at room temperature for 20 min. PBMCs were removed from the interface formed by centrifugation and washed with PBS. Cells were maintained in RPMI 1640 medium supplemented with 10% autologous serum and 0.15% garamycin (Schering-Plough) in 96-well flat bottom plates at 37 °C in a humidified atmosphere containing 5% CO_2. Cell viability was assessed by trypan blue exclusion assay. All reagents used were filtered through a disposable sterile filter unit 0.22 μM (Millex). All human subjects read and signed an informed consent.

2.4. Cytotoxicity assay

1.088 mg GA (MW 170.12) (Sigma Aldrich) was weighed and dissolved directly into 1 mL serum-free RPMI 1640 medium. After vortexing for a couple of minutes, GA is totally dissolved in the medium. From a stock solution of 6400 μM, the serial dilution was prepared as follows: 3200, 1600, 800, 400, 200, 100, 50, 25, and 12.5 μM. 100 μL of each concentration was added to wells containing 100 μL of RPMI 1640 medium + cells. Thus, the final concentrations in the wells were 1600, 800, 400, 200, 100, 50, 25, 12.5 and 6.25 μM, respectively. Neutrophils (2.0×10^5 cells/200 μL) were incubated for 16 h. Control group was composed of neutrophils in RPMI 1640 medium. LPS group (*Escherichia coli* 026: B6), the drug was diluted in RPMI 1640 medium and added directly to cell culture at the stated concentration (25, 50 and 100 ng/mL). GA group was composed by a GA serial dilution (6.25 to 1600 μM) and GA + LPS group was composed by the same serial dilution of GA + LPS (50 ng/mL), the drugs were diluted in medium and then added to the cell culture. All groups were made in triplicate and the viability was performed by trypan blue exclusion assay.

PBMCs (1.6×10^5 cells/200 μL) were incubated for 96 h. Control group was composed of PBMCs in RPMI 1640. GA group was composed by a GA serial dilution (6.25 to 1600 μM). All groups were made in triplicate and the viability was performed by trypan blue exclusion assay.

2.5. Apoptosis assay

Neutrophils were incubated in the presence of GA (25, 50 and 100 μM), LPS (50 ng/mL) or GA (25, 50 and 100 μM) + LPS (50 ng/mL) for 16 h. Untreated cells represented the control group. Apoptosis was evaluated by flow cytometry using Annexin-V Kit assay. Annexin-V and 7-AAD were added to 1×10^5 cell suspension according to the manufacturer's instructions (BD Biosciences) and then incubated for 15 min at room temperature in the dark. Subsequently 2×10^4 cells were analyzed by flow cytometry (FACS Canto

II, BD Bioscience) within 1 h. Early apoptotic cells were stained with Annexin V alone, whereas necrotic cells and late apoptotic cells were stained with both Annexin V and 7-AAD. Data were analyzed with FlowJo software v. 7.2.5 (Tree Star Inc., USA).

Morphological assessment of neutrophil apoptosis was evaluated by light microscopy analysis. Cells were spin down on a glass slide by a cytospin. Cells were fixed with methanol and stained with May–Grunwald–Giemsa staining solution.

2.6. Western blot

Neutrophils (1.0×10^6 cells/mL) were incubated in the presence of LPS (50 ng/mL) or GA (25, 50 and 100 μM) + LPS (50 ng/mL) for 16 h and re-suspended in a lysis buffer (10 mM pH 7.5 Tris-HCl, 100 mM NaCl, 0,3% CHAPS, 50 mM NaF, β-glicerol phosphate and protease inhibitors). The lysates (50 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were blocked with a blocking buffer (1 M Trizma pH 7.5, 5 M NaCl, 0.8% Tween 20 and 10% skim milk) for 2 h and stained with primary polyclonal antibodies – Human Anti-Caspase-3 – (1:500) in a blocking buffer overnight at 4 °C. The membranes were incubated with the secondary antibodies – Rabbit Anti-Mouse IgG – (1:7500) for 2 h at 4 °C. The bands were detected using Opti-4 CN revelator solution and the band intensities were obtained using the program ImageJ 3.0.

2.7. Induction and detection of NETs

NETs formation of neutrophils was quantitated in the supernatants (2.0×10^5 cells/200 μL). The cells were incubated in the presence of GA (25, 50, 100 μM), LPS (50 ng/mL) or GA (25, 50 and 100 μM) + LPS (50 ng/mL) for 16 h. Untreated cells represented the control group. To quantify levels of NETs (DNA), the Quant-iT™ PicoGreen® dsDNA Kit assay was used according to the manufacturer's instructions (Invitrogen). The fluorescence intensity of DNA was measured at excitation and emission wavelengths of 485 nm and 530 nm, respectively, in a microplate reader (Victor 3, PerkinElmer). A standard calibration curve was used. (Luo et al., 2014)

2.8. Immunofluorescence staining of NETs

The NETs formation was analyzed using the Kit Falcon™ Culture Slide (BD Biosciences). For immunofluorescence, freshly isolated PMNs were seeded on Poly-L-lysin coated cover slips, allowed to adhere (1 h), and stimulated with LPS (50 ng/mL) for NETs induction. To evaluate the effect of GA on the NETS formation, we incubated the cells with LPS (50 ng/mL) + GA (100 μM). After 16 h of incubation, cells were fixed with formaldehyde 4% for 2 h and permeabilized with PBS containing 0.03% Triton X-100 and 10% fetal bovine serum (Gibco — Life Technologies) for 30 min. To stain the NETs, samples were incubated with a primary monoclonal antibody – Mouse Anti-Myeloperoxidase – (1:200) for 30 min and then with a secondary

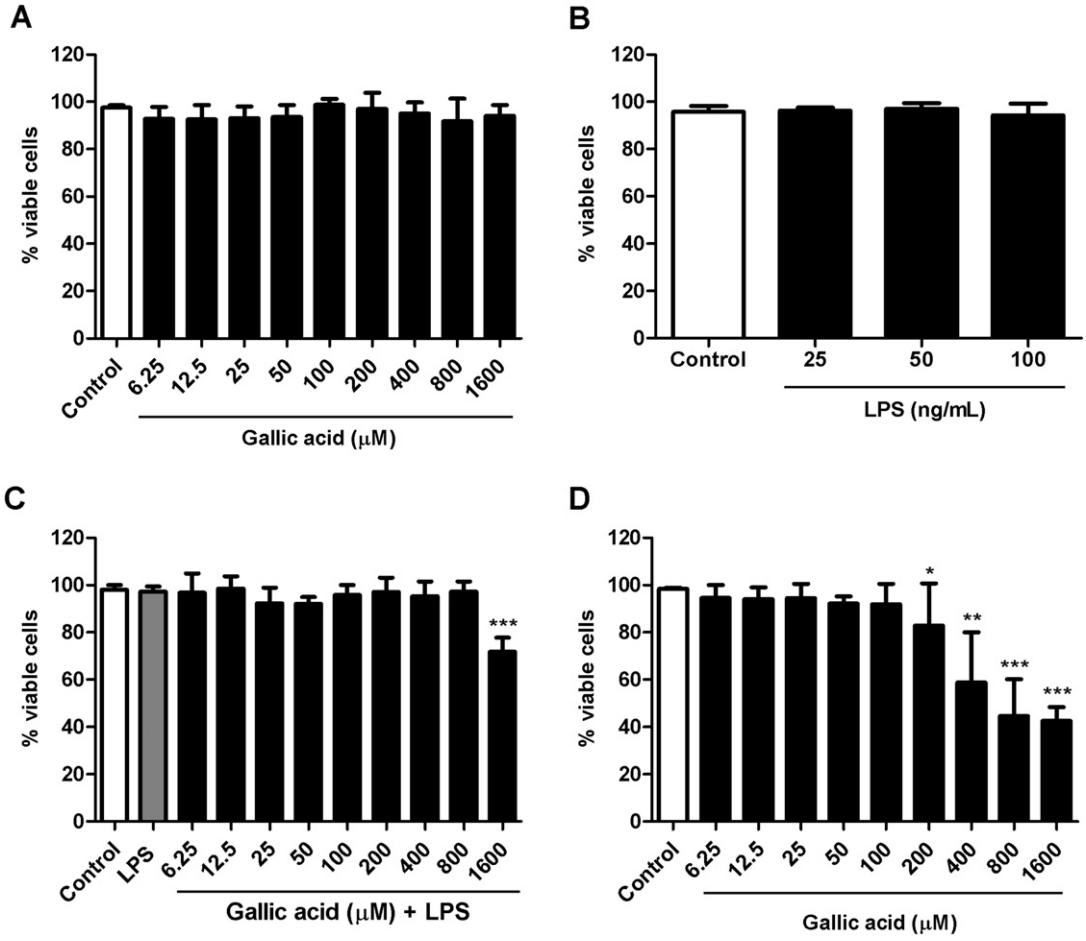

Fig. 1. Effect of different concentrations of GA on cell viability. Neutrophils were challenged with (A) GA (6.25 to 1600 μM), (B) LPS (50 ng/mL) or (C) GA (6.25 to 1600 μM) + LPS (50 ng/mL) for 16 h and cell viability was assessed by trypan blue exclusion assay. (D) PBMCs were challenged with GA (6.25 to 1600 μM) for 96 h and cell viability was assessed using trypan blue exclusion assay. Results were expressed as the percentage of viable cells. All data represent the mean ± SD (n = 5). ***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$ compared with control group.

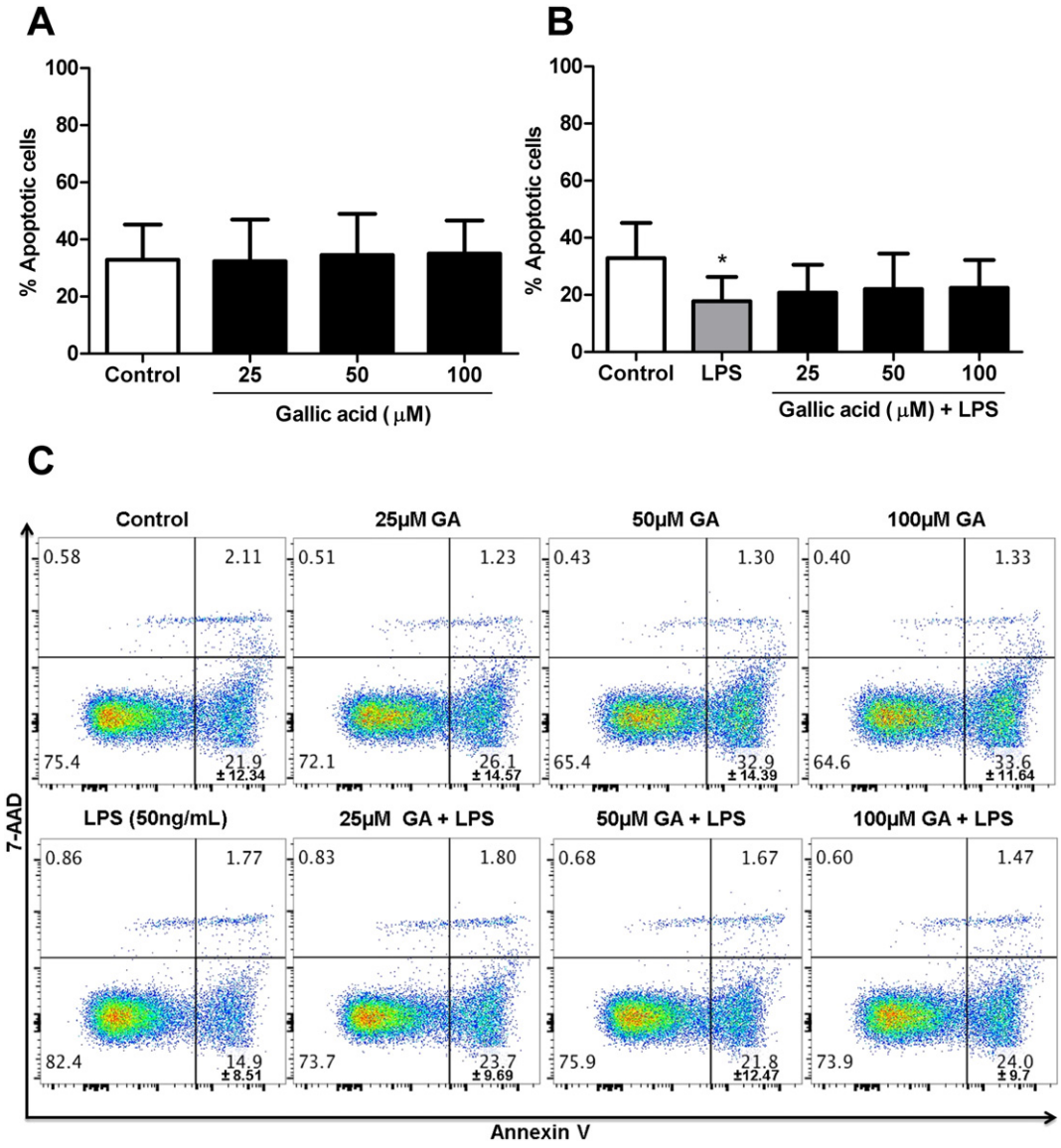

Fig. 2. Effects of GA, LPS and GA + LPS on apoptosis of neutrophils. (A) Cells were exposed to GA (25, 50 and 100 μM). (B) Cells were exposed to GA (25, 50 and 100 μM), LPS (50 ng/mL) and GA (25, 50 and 100 μM) + LPS (50 ng/mL). Results were expressed as percentage of apoptotic cells. Data represent the mean ± SD (n = 5). *$P < 0.05$ compared with control group. (C) Representative flow cytometric scatter plots of Annexin V (x axis)/7-AAD (y axis) stained control and GA- and/or LPS-treated cells for 16 h. The lower left quadrant shows viable cells, which are annexin V^- and 7-AAD^-. The lower right quadrant represents the apoptotic cells, annexin V^+ and 7-AAD^-. The upper right quadrant shows the late apoptotic or dead cells that are annexin V^+ and 7-AAD^+.

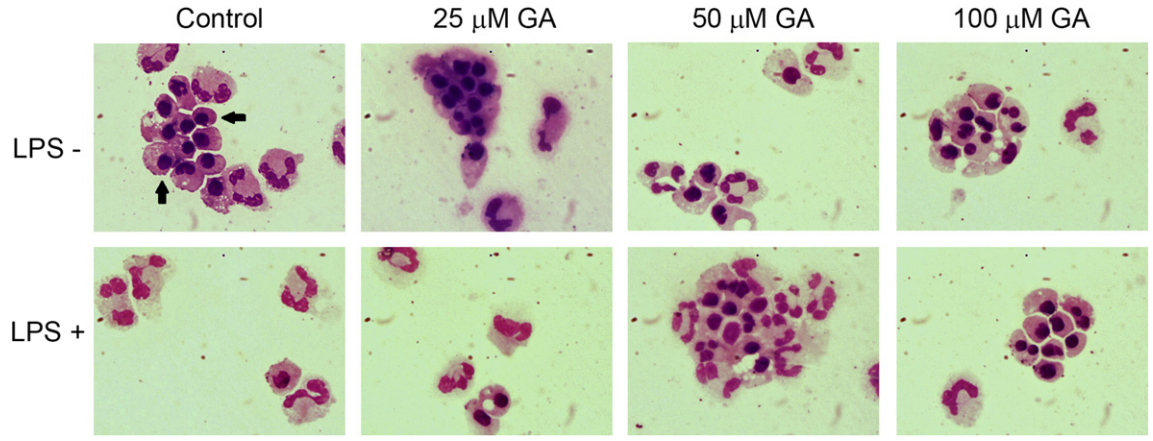

Fig. 3. Morphological changes of neutrophils after LPS stimulation visualized by optical microscopy. In this image, we could verify the morphological differences of cell in apoptosis. Arrows show morphological detail of an apoptotic cell which lost its original shape. 50 ng/mL LPS-treated cells show predominance of normal neutrophils, with fine granularity of chromatin and normal lobulated nucleus. Cells exposed to GA (25, 50 and 100 μM) and GA (50 and 100 μM) + LPS have the same pattern as control cells. Magnification ×400.

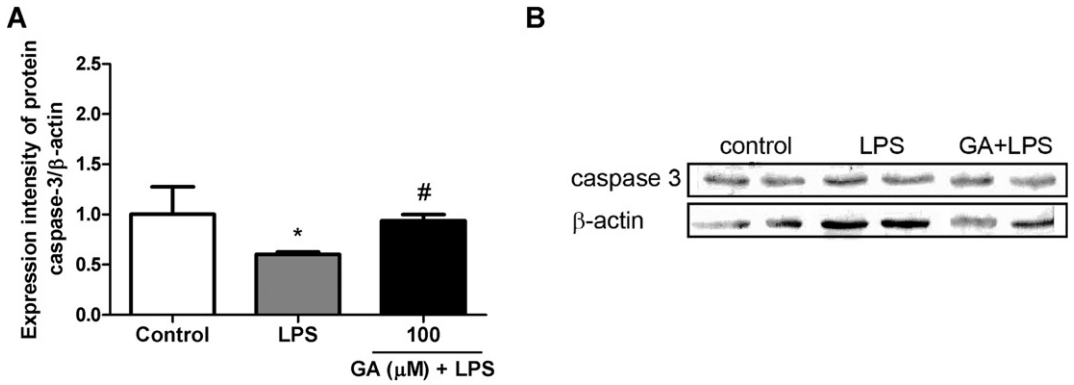

Fig. 4. Effect of GA and/or LPS on caspase-3 activation. Neutrophils were cultured in the presence of LPS (50 ng/mL) or GA (100 μM) + LPS (50 ng/mL) for 16 h. (A) Results were expressed as expression intensity of protein caspase-3/β-actin. Data represent the mean ± SD (n = 3). *$P < 0.05$ compared with control group; #$P < 0.05$ compared with LPS group. (B) Western blot analysis of extracts of neutrophils cultured for cleaved caspase-3 and β-actin proteins. Representative experiments are depicted (n = 3).

antibody – Rabbit Anti-Mouse IgG (H + L) Fluorescein (FITC) Conjugate – (1:500) for 30 min. After, the cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) for 2 min. Neutrophil-derived NETs formation was visualized by confocal immunofluorescence microscopy (Luo et al., 2014).

2.9. Antioxidant activity DPPH

The DPPH method is based on the capture of DPPH radical (2′,2′-diphenyl-1-picrylhydrazyl) by antioxidants, producing a decrease absorbance at 515 nm. The free radical scavenging activity was followed by preparing DPPH solution (60 μM) in methanol. Vitamin C (1 mg/mL) was taken as the reference standard. Different concentration of GA (25, 50 and 100 μM) and Vitamin C were diluted in methanol. 975 μL of DPPH solution (60 μM) was mixed with 25 μL of all GA concentrations. These mixtures were kept in dark about 5 min and measured the absorbance at 515 nm.

2.10. Measurement of production of reactive oxygen species

The generation of intracellular reactive oxygen species (ROS) of neutrophils (2.0×10^5 cells/200 μL) was evaluated based on the intracellular peroxide-dependent oxidation of 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) which forms a fluorescent compound, 2′,7′-dichlorofluorescein (DCF). Briefly, the cells were incubated in the presence of GA (25, 50 and 100 μM), LPS (50 ng/mL) and GA (25, 50 and 100 μM) + LPS (50 ng/mL). After 16 h of incubation, cells were washed twice with PBS and then incubated with 200 μL/well of phosphate-buffered containing 10 μM of DCFH-DA at 37 °C for 30 min. The fluorescence intensity was measured at excitation and emission wavelengths of 485 nm and 530 nm, respectively, in a microplate reader (Victor 3, PerkinElmer).

2.11. IL-6, IL-8 and 1L-1β cytokines quantification

Cytokines production was evaluated in the supernatants of neutrophils (2.0×10^5 cells/200 μL). The cells were incubated in the presence of GA (25, 50 and 100 μM), LPS (50 ng/mL) or GA (25, 50 and 100 μM) + LPS (50 ng/mL) for 16 h. To quantify levels of IL-6, IL-8 and IL-1β, the Cytometric Bead Array (CBA) Kit assay was used according to the manufacturer's instructions (BD Biosciences) and analysis was performed by flow cytometry (FACS Canto II, BD Bioscience).

2.12. Statistical analysis

All experiments were done in triplicates. The normality of the data was analyzed by the Shapiro–Wilk test. The measures were parametric and then we calculated the mean and standard deviation of the mean for each of the variables analyzed. For comparison between groups analysis of variance (ANOVA) and post hoc LSD Test for multiple comparisons was applied. The differences were considered significant when the statistical analysis gives $P < 0.05$. SPSS (Statistical Package for Social Sciences) version 18.0 for Windows was used as a computational tool to analyze statistical data.

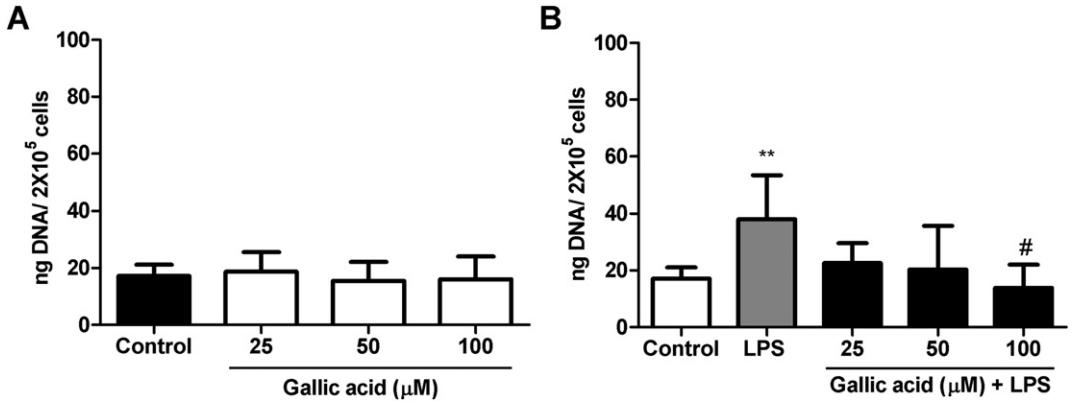

Fig. 5. Effect of GA and/or LPS on the NETs formation. (A) Cells were exposed to different concentrations of GA (25, 50 and 100 μM). (B) Cells were exposed to LPS (50 ng/mL) and different concentrations of GA (25, 50 and 100 μM) + LPS (50 ng/mL). Data represent the mean ± SD (n = 5). Results were expressed as ng DNA/2×10^5 cells. Data represent the mean ± SD (n = 5). **$P < 0.01$ compared with control group; #$P < 0.001$ compared with LPS group.

3. Results

3.1. Cytotoxic effect of GA, GA + LPS and LPS in human neutrophils and PBMCs

To evaluate cytotoxic effect of GA in human neutrophils, cells were exposed to different concentrations of GA (6.25 to 1600 μM). After 16 h of treatment, it was verified that GA did not decrease cell viability (Fig. 1A). We performed an apoptosis curve with LPS at concentrations of 25, 50 and 100 ng/mL (data not show). The concentration of 25 ng/mL had no significant anti-apoptotic effect, unlike the concentrations of 50 and 100 ng/mL. So, we tested the toxicity of LPS in neutrophils and verified that any concentration (25, 50 and 100 ng/mL) showed decrease in cell viability (Fig. 1B). Therefore, we chose the lowest concentration that possessed anti-apoptotic effect for the next experiments.

When we associate the LPS (50 ng/mL) with different concentrations of GA (6.25 to 1600 μM), only 1600 μM GA demonstrated toxicity (Fig. 1C). To evaluate cytotoxic effect of GA in other blood cells, PBMC cells were exposed to different concentrations of GA (6.25 to 1600 μM). The concentrations 200, 400, 800 and 1600 μM of GA decreased the cell viability (Fig. 1D).

3.2. Effect of GA, GA + LPS and LPS on apoptosis of human neutrophils

To analyze apoptosis induction, cells were exposed to different concentrations of GA (25, 50 and 100 μM) and LPS (50 ng/mL). GA alone did not induce apoptosis (Fig. 2A and C). However, LPS showed significant anti-apoptotic effect when compared with control, and GA inhibited this effect (Fig. 2B and C). Apoptosis was also evaluated by optical microscopy to verify morphological differences between apoptotic cells (the nucleus loses its original shape) and normal cells confirming the results by flow cytometry (Fig. 3).

3.3. Effect of LPS and GA + LPS on caspase 3 activation

As GA decreases anti-apoptotic effect of LPS, we decided to evaluate the activation of caspase-3. Cells treated with LPS (50 ng/mL) exhibited a significant reduction of caspase-3 when compared to control group and 100 μM GA + LPS (50 ng/mL) increased significantly the caspase-3 activation when compared to the cells treated with LPS (Fig. 4A and B).

3.4. Effect of GA, GA + LPS and LPS in the release of NETs of human neutrophils

The GA (25, 50 and 100 μM) alone did not induce the formation of NETs (Fig. 5A). LPS (50 ng/mL) increased the NETs formation and the GA decreased the LPS effect (Fig. 5B). This effect was visualized using immunofluorescence with confocal microscopy (Fig. 6).

3.1. Antioxidant effect of GA and Vitamin C

We observed that all concentrations of GA (25, 50 and 100 μM) decreased the free radical DPPH, showing a similar effect to Vitamin C (Fig. 7).

3.6. Effect of GA, GA + LPS and LPS on ROS generation

We observed that GA (25, 50 and 100 μM) alone did not induce the formation of ROS (Fig. 8A). 100 μM GA decreased significantly the ROS release when compared to LPS group (Fig. 8B).

3.7. Effect of GA + LPS and LPS on cytokine release

Cytokines have a key role in the resolution of inflammation, for this reason we decided to quantify the cytokines released by activated neutrophils. Cells stimulated with LPS increased IL-6, IL-8 and IL-1β levels when compared with control group. GA (50 and 100 μM) treatment decreased this effect only on IL-1β release (Fig. 9A, B and C).

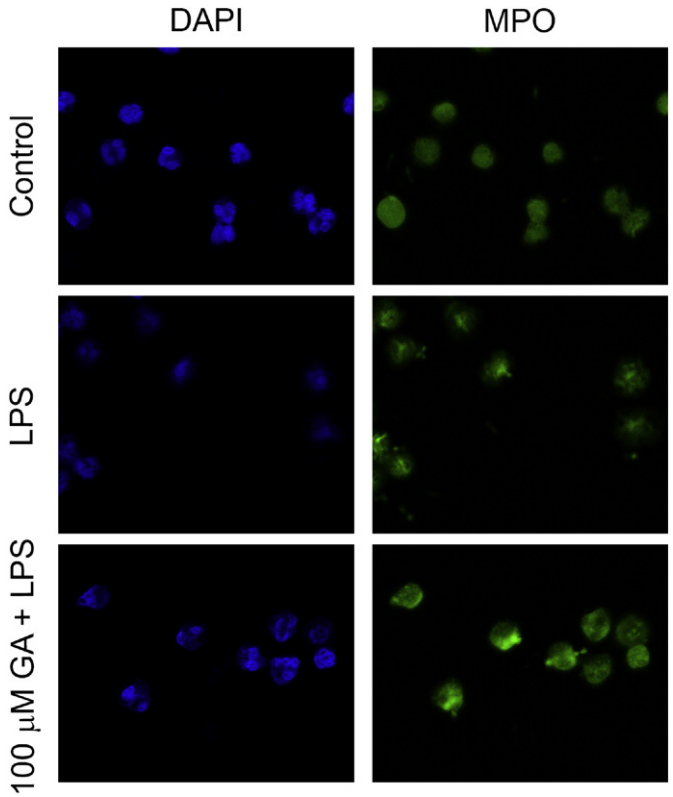

Fig. 6. NETs formation after LPS stimulation visualized by fluorescence. The image of control group shows the nuclear localization of DNA (blue fluorescence) and the granular patterns of myeloperoxidase (MPO) (green fluorescence). The LPS group shows the changes during NETs formation that can be determined with loss of granular integrity of MPO and nuclear lobules. GA + LPS group presents a similar morphology to the control group. Magnification ×630.

4. Discussion

Neutrophils are the first line of defense of our body and are the first cells to reach the focus of inflammation. In response to inflammatory stimuli, they migrate from the peripheral blood to infected tissues, where they efficiently bind, engulf, and inactivate bacteria (Brinkmann et al., 2004). These cells have a short half-life and die by apoptosis in a

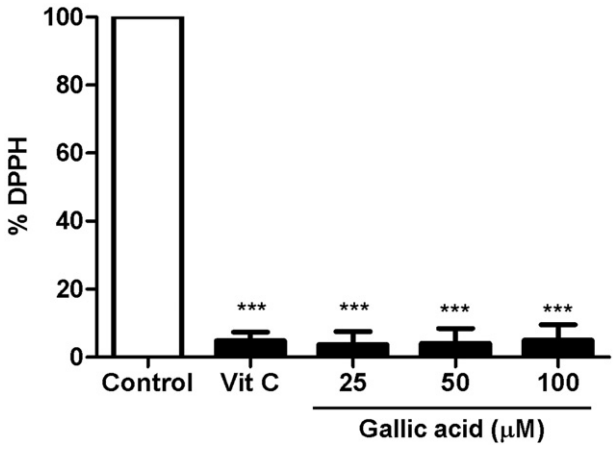

Fig. 7. Antioxidant effect of GA (25, 50 and 100 μM) and vitamin C (1 mg/mL). Data were expressed as percentage of control group. ***$P < 0.001$ compared with control group.

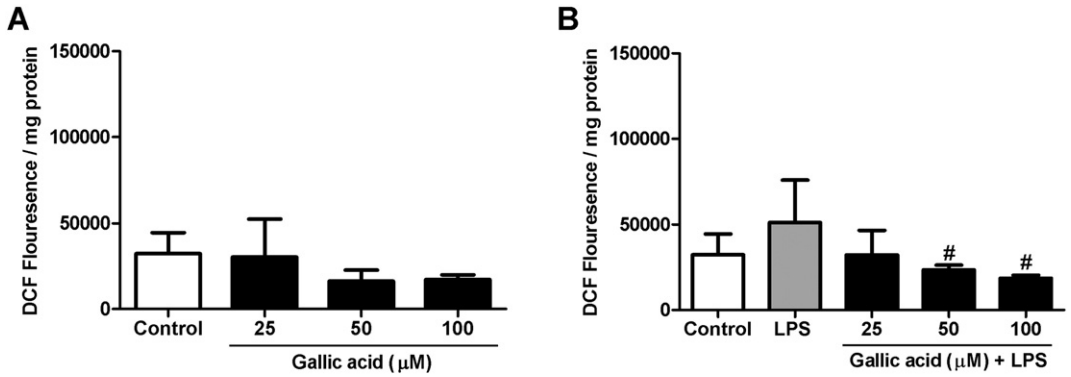

Fig. 8. Effect of GA, and/or LPS on neutrophils ROS release. (A) Cells were exposed to GA (25, 50 and 100 μM). (B) Cells were exposed to GA (25, 50 and 100 μM), LPS (50 ng/mL) and GA (25, 50 and 100 μM) + LPS (50 ng/mL). Data represent the mean ± SD (n = 3). Results were expressed as DCF Fluorescence/mg protein. #$P < 0.05$ compared with LPS group.

few hours. The presence of pathogens contributes to prolonging the life of neutrophils in the infected site. The permanence of these cells in the inflamed site, for a certain time, is beneficial to the host because it helps against the invasion, but on the other hand can lead to tissue damage by excessive release of toxic products. Neutrophils also have another antimicrobial strategy, called NETosis, which results in the death of these cells, and contributes to the elimination of the pathogens (Guimarães-Costa et al., 2012; Simon et al., 2000; Wallach-Dayan et al., 2006; Luo et al., 2014). Our study aimed to investigate the *in vitro* action of GA on LPS-induced apoptosis and NETosis of human neutrophils.

Our initial results showed that GA is not cytotoxic in neutrophils. In order to verify their possible cytotoxicity in other blood cells, we did experiments in primary cultures of human mononuclear cells. We found that the concentrations of 200, 400, 800 and 1600 μM of GA could decrease cell viability, thus they were not suitable for therapeutic purposes. For this reason, we chose 25, 50 and 100 μM concentrations to follow the study.

Our study showed that LPS decreased apoptosis in neutrophils. It is reported that LPS acts on TLR4 receptor and for consequence of this binding, occurs activation and increased lifetime of the cells. Neutrophil apoptosis is essential to regulate adult cell populations and in the resolution of inflammation. When the organism is under attack, these cells die slowly in order to control the infection, however they should die by apoptosis immediately after the combat against the pathogen. When LPS binds to TLR4 receptor, these cells release cytokines and produce ROS, and these inflammation mediators cause tissue damage (Brinkmann et al., 2004; Sabroe et al., 2005; Sabroe et al., 2002). In this study, GA decreased the anti-apoptotic effect of LPS, which indicates that GA has a protective role against infection-induced tissue damage.

The above-mentioned findings raised the question whether the caspase-3 was changed in GA-treated cells. Caspases play a key role of

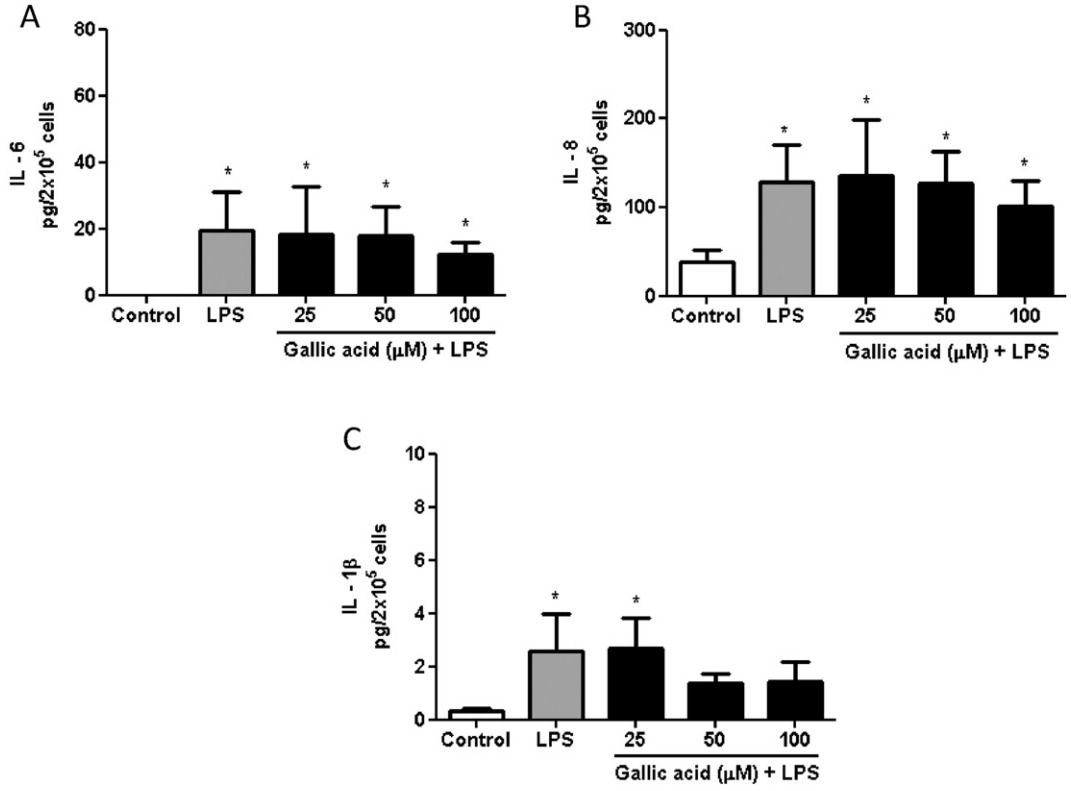

Fig. 9. Effect of GA on inflammatory cytokines release after 16 h treatment with GA (25, 50 and 100 μM) and/or LPS (50 ng/mL). Flow cytometric analyses of (A) IL-6, (B) IL-8 and (C) IL-1β in neutrophils supernatant. Cytokines IL-6 (A), IL-8 (B) and IL-1β (C) were analyzed. Data represent the mean ± SD (n = 5). Results were expressed as pg/2 × 10^5 cells. *$P < 0.05$ compared with control group.

apoptosis regulation and, in neutrophils, this programmed cell death is spontaneous (Gardai et al., 2004). Neutrophils apoptosis is inhibited during bacterial infection, because the inflammatory cytokines decrease caspase-3 activation, involving the NF-kβ (nuclear factor kappa B), ERK (extracellular-signal regulated kinases) and XIAP (X-chromosome linked apoptosis inhibitor) activation. XIAP inhibits the activity of caspase-3 (Gardai et al., 2004; Whitlock et al., 2000). Our results showed that LPS decreased caspase-3 expression and GA recovered this activation.

Studies describe that neutrophils, when activated by chemicals or pathogens, suffer NETosis and release NETs to the extracellular medium (Brinkmann et al., 2004). NETs are important to control and kill bacteria (Brinkmann et al., 2004) and represent a beneficial mechanism that is essential for the death of microorganism, preventing its spread in the body. However, the formation of NETs may have deleterious effects to the host due to the release of proteins, as proteases, which can injure the adjacent tissues (Meng et al., 2012). We were able to show that GA significantly decreased *in vitro* LPS-induced NETs release.

NETosis in response to chemical and biological stimuli is mediated by ROS production involving NADPH oxidase and MPO (Brinkmann et al., 2004; Guimarães-Costa et al., 2012). Preclinical studies have shown that GA possesses a variety of pharmacological activities, which include its action as an antioxidant and anti-inflammatory. In animal models, GA reduces oxidative stress and enhances the levels of glutathione (GSH), GSH peroxidase, GSH reductase, and GSH S-transferase in hepatic tissue, as well as catalase in the serum (Chen et al., 2013). In our study, GA demonstrated an antioxidant action, equivalent to the well-established antioxidant vitamin C. Moreover, GA also decreased ROS levels released by LPS-treated neutrophils, and, consequently, the NETs formation. Since ROS-dependent NETosis is also believed to play detrimental effects in autoimmune inflammatory diseases, such as rheumatoid arthritis, systemic lupus erythematosus, psoriasis, small-vessel vasculitis and lupus nephritis, pharmacological inhibition of ROS-dependent NETs formation could have a therapeutically effect on these disorders. Flavonoids are potentially effective in the treatment of these disorders (Kirchner et al., 2013) and, for that reason, GA can be potentially effective in the therapeutics of these diseases.

In response to pro-inflammatory stimuli, such as LPS, neutrophils are activated to reduce the action of pathogen on the tissue (Sabroe et al., 2002) and synthesize pro-inflammatory cytokines such as IL-6, IL-8 and IL-1β (Sabroe et al., 2002; Mitroulis et al., 2011). Our results corroborate with these studies demonstrating that LPS significantly increases the release of these cytokines. Even though, several pro-inflammatory cytokines have been associated with the early phase of acute inflammation, growing evidence derived from experimental and clinical studies indicates a pivotal role for IL-1β in the initiation of inflammatory process (Mitroulis et al., 2011). Experimental studies showed that IL-1β mediates the anti-apoptotic effect of LPS (Mitroulis et al., 2013) and it has protective effects against bacterial infection. Human studies have demonstrated that antagonists of IL-1β receptors are associated with increased susceptibility to bacterial infections. IL-1β exerts its protective effect against infection by the activation of many responses, which include rapid recruitment of neutrophils to the site of inflammation. However, when there is excessive inflammation and, consequently, an excessive release of IL-1β, severe mortality occurs. Excessive recruitment of neutrophils is also known to cause tissue damage, leading to multiple organ dysfunction and death. Our results demonstrated that GA decreased the LPS effect on IL-1β. However, it did not reverse the increase of IL-6 and IL-8. We showed that the GA modulates the release of IL-1β, and for this reason, exerts protective action against infections.

5. Conclusion

In this study, we demonstrated that GA significantly inhibits the release of ROS and formation of NETs in primary human neutrophils, indicating a correlation between these two phenomena. Our results also showed that GA reduces the anti-apoptotic effect of LPS in these cells. These actions suggest that GA may be used as a therapeutic strategy against diseases that are mediated by neutrophils activation.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgments

This study was supported by CNPq (143969/2013-7). G.V.H. received a fellowship from PUCRS. S.B. is the recipient of a PNPD/Capes postdoc grant from CAPES-Brazil.

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