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ORIGINAL CONTRIBUTION

Green tea polyphenol extract in vivo attenuates inflammatory features of neutrophils from obese rats

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Abstract
Purpose Our study aimed to evaluate whether obesity induced by cafeteria diet changes the neutrophil effector/inflammatory function and whether treatment with green tea extract (GT) can improve neutrophil function.
Methods Male Wistar rats were treated with GT by gavage (12 weeks/5 days/week; 500 mg/kg of body weight), and obesity was induced by cafeteria diet (8 weeks). Neutrophils were obtained from the peritoneal cavity (injection of oyster glycogen). The following analyses were performed: phagocytic capacity, chemotaxis, myeloperoxidase activity (MPO), hypochlorous acid (HOCl), superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), IL-1β, IL-6 and TNFα, mRNA levels of inflammatory genes, calcium mobilisation, activities of antioxidant enzymes, hexokinase and G6PDH.
Results Neutrophils from obese rats showed a significant decrease in migration capacity, H_2O_2 and HOCl production, MPO activity and $O_2^{\cdot-}$ production. Phagocytosis and CD11b mRNA levels were increased, while inflammatory cytokines release remained unmodified. mRNA levels of TLR4 and IκK were enhanced. Treatment of obese rats with GT increased neutrophil migration, MPO activity, H_2O_2, HOCl and $O_2^{\cdot-}$ production, whereas TNF-α and IL-6 were decreased (versus obese). Similar reductions in TLR4, IκK and CD11b mRNA were observed. Catalase and hexokinase were increased by obesity, while SOD and G6PDH were decreased. Treatment with GT reduced catalase and increased the GSH/GSSG ratio.
Conclusion In response to a cafeteria diet, we found a decreased chemotaxis, H_2O_2 release, MPO activity and HOCl production. We also showed a significant immunomodulatory effect of GT on the obese condition recovering some of these factors such H_2O_2 and HOCl production, also reducing the levels of inflammatory cytokines.

Keywords Obesity · Immune system · Polyphenols · Reactive oxygen species · Flavonoids · Catechins

Introduction

Obesity is considered a major public health problem and is classified as a global epidemic that is associated with comorbidities, including type 2 diabetes [1, 2], hypertension [3], dyslipidemia, cardiovascular diseases [4, 5] and certain cancers [6]. Numerous studies have shown a strong relationship between adipose tissue and immune cells. Aside from increasing free fatty acid (FFA) levels, pro-inflammatory cytokines, such as TNF-α, IL-1β and MCP-1, can also induce reactive oxygen species (ROS) production, thereby contributing to the pro-inflammatory microenvironment in obesity, promoting activation and infiltration of immune cells in adipose tissue [7].

The immune response against acute or chronic injury in obesity is affected by changes in the concentrations of inflammatory mediators. Prior exposure of neutrophils to toll-like receptor 4 (TLR4) agonists (FFA and cytokines, like TNF-α) can lead neutrophils to a priming state, which can greatly potentiate ROS production in response to a second exposure or a secondary stimulus, such as formyl-methionyl-leucyl-phenylalanine (fMLP), complement

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fragment C5a, C3b or platelet activating factor (PAF) and phorbol myristate acetate (PMA) [8].

The role of neutrophils in obesity is still poorly understood. Studies with neutrophils from patients with morbid obesity showed an increased response to low doses of endotoxin, with a concomitant reduction in apoptosis and extension of its half-life compared with lean individuals, suggesting possible hyperresponsiveness of these neutrophils [9]. A functional deficit in neutrophils of obese individuals including reduced bactericidal activity has also been reported [10]. Therefore, obese individuals present greater susceptibility to infections and an inability to exterminate pathogens and are prone to developing chronic inflammatory diseases. In addition, some changes noted in neutrophils may be due to exposure of these cells to leptin, an important adipokine secreted by adipocytes [11]. Indeed, neutrophils are altered in obesity, and this scenario may contribute to the onset of comorbidities associated with this disease and/or the progression of atherosclerosis, microvascular disease and neuropathology [12–14]. However, evidence in the literature concerning the main functions of neutrophils in the obese condition is scarce.

The consumption of green tea leaves from the *Camellia sinensis* plant has been proposed as a treatment for obesity. A decrease in body fat mass, the inhibition of adipogenesis and adipocyte apoptosis have been described [15]. Other biological and pharmacological properties include allergenic [16, 17] and anti-inflammatory activity [18–20], resulting in decreased expression of inflammatory gene products, including lipoxygenase [21], cyclooxygenase (COX) [22], NO synthase [23] and TNF-α [24]. The content of polyphenols present in green tea, specifically the catechins, makes up about 30 % of the dry weight of green tea extract and is responsible for their biological activities [25, 26]. Catechins and epicatechins are present in many foods, while gallates and gallated catechins are exclusively present in teas, especially green tea. There are four major catechins found in green tea: epicatechin, epigallocatechin, epicatechin-3-gallate and epigallocatechin-3 gallate (EGCG), the last representing about 50–80 % of total polyphenols from green tea [27, 28].

Considering that neutrophils are cells that participate in the first line of defence against pathogens and are the first cells to be recruited to the inflammatory site, including in the adipose tissue of obese animals, understanding the process involved in neutrophils activation in obesity and elucidating the pharmacological effects of green tea on these immune cells are of extreme importance. Thus, our study aimed to evaluate whether obesity induced by cafeteria diet changes the neutrophil effector/inflammatory function and whether treatment with green tea extract (GT) can improve neutrophil function.

Materials and methods

Chemicals

All chemicals were of analytical grade and purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA), except those used in the preparation of common buffers (Labsynth, Diadema, SP, Brazil). Powdered green tea extract was commercially acquired from Tovani-Benzaquen, São Paulo, SP, Brazil.

Animals, green tea supplementation and obesity induction

Eight-week-old, male Wistar rats (*Rattus norvegicus var. Albinus*), weighing (150 ± 40 g) at the beginning of the study, were provided by the Federal University of São Paulo (UNIFESP), São Paulo, Brazil. The general procedures were performed in accordance with standards established by the Ethics Committee on Animal Experimentation of the Biomedical Sciences Institute, University of São Paulo (CEEA-ICB/USP No. 160/2013). The rats were maintained for 12 weeks in groups of five animals each, at a constant temperature of 23 ± 2 °C on a standard 12-h light/dark cycle (lights on at 7:00 a.m.). The rats were divided into four groups and treated according to the following supplementation programme: (a) control group (cont), fed with standard rodent chow diet and gavage with water; (b) green tea group (GT), fed with standard rodent chow diet and gavage with green tea extract; (c) obese group (Ob), fed with cafeteria diet and gavage with water; (d) obese plus GT (Ob + GT), fed with cafeteria diet and gavage with GT. Green tea extract (GT) 500 mg/kg of body weight was weighed daily and then solubilised in water at 70 °C to be subsequently administered by gavage to the rats prior to the feeding period. An increasing volume of green tea up to a maximum of 500 µL was established for the gavage treatment, in order to prevent regurgitation or stomach discomfort. The total period of gavage with GT was 60 days (Monday–Friday). The concentrations of polyphenols and catechins in the extract were 39 and 30 %, respectively, as obtained by HPLC analysis described in Rocha et al. (2015, submitted for publication). The content of caffeine in the extract was 0.4 by dry weight.

All the rats received food (standard rodent chow) and water ad libitum for 4 weeks of GT supplementation. After this period, a cafeteria diet was offered to the rats for 8 weeks under a pair feeding regime, to standardise the amount of food intake between the groups. At the end of the experimental period, the rats were euthanised by decapitation between 12:00 and 14:00.

The food items in the cafeteria diet were chosen to reflect the enormous variety, palatability and energy density of the modern western diet (Rocha et al. 2015, submitted for publication). The hypercaloric cafeteria diet used in this study was composed of 37.5 % standard rat chow, 25 % chocolate, 12.5 % peanut, 12.5 % condensed milk and 12.5 % cornstarch wafer. This diet provided an average of 4.2 kcal/g, 38 % energy as fat, 14 % energy as protein and 48 % as carbohydrate. The cafeteria diet was presented daily for 2 months. Standard rodent chow provided 2.8 kcal/g, 13 % energy as fat, 32 % protein and 55 % carbohydrate.

Experimental procedure

Prior to euthanasia by decapitation (4 h), rats were i.p. injected with 10 ml 1 % (w/v) glycogen solution (Sigma type II, from oyster) in PBS (133.8 mM NaCl, 2.7 mM KCl, 0.9 mM KH_2PO_4, 6.4 mM $Na_2H_2PO_2$, pH 7.4) to induce neutrophil migration. Neutrophils were obtained by intraperitoneal lavage with 20 ml PBS [29]. Peritoneal fluid was removed with the aid of a Pasteur pipette and centrifuged for 10 min, 600×*g* and 4 °C. The supernatant was discarded, and the pellet was resuspended in RPMI-1640 medium supplemented with 10 % foetal bovine serum. The total number of leucocytes in exudates was determined in a hemocytometer. The differential cell count was obtained by optical microscopy (400×) (Nikon YS2-H), in cell extension by cytospin stained with Giemsa. This staining allowed for the identification of about 98 % of neutrophils present in the exudates 4 h after the injection of oyster glycogen. After counting, the initial concentration of 2.5×10^6 cells/ml was used to conduct all the experiments. In all experiments, five rats per group were used and at least three different experiments were performed for each analysis.

Release of pro-inflammatory cytokines

Cytokines TNF-α, IL-1β and IL-6 were assayed in cell culture supernatant with ELISA kits, following the manufacturer's instructions (Quantikine, R&D System, Minneapolis, MN, USA). Neutrophils (1×10^6/ml) were cultured for 18 h in the presence of LPS as a stimulus (10 μg/ml). Next, the cells were centrifuged (1000×*g*, 4 °C, 10 min), and the supernatant was collected and used for cytokine determination.

Migration assay

The chemotactic response of neutrophils was tested using 24-well disposable chemotactic plates, as described by Morandi et al. [30]. In this method, the lower chambers were filled with the chemotactic agent N-formyl-Met-Leu-Phe (fMLPat 10 nM) in PBS + 0.01 % albumin, or with PBS + 0.01 % albumin alone (basal unstimulated). A chemotactic membrane with a pore size of 5 μM was fixed to the filter seat, and a cell suspension (1×10^6) was added to the top of each well. The chamber was incubated in 5 % CO_2 at 37 °C for 60 min. After incubation, the chamber was disassembled and the cells contained in the lower compartment were counted in a Neubauer chamber. The chemotactic response was defined as the average number of cells that migrated to the bottom chamber.

Phagocytic capacity

The phagocytic capacity of neutrophils was evaluated by assaying the internalisation of opsonized zymosan particles (5×10^6 particles). Zymosan particles diluted in PBS were opsonized by the addition of an equal volume of homologous serum and placed on a dry bath at 37 °C under stirring for 30 min. Neutrophils (1×10^6) were incubated for 30 min at 37 °C in 1 ml RPMI 1640 medium with opsonized zymosan particles as a phagocytic stimulus, stained and counted using an optical microscope (Nikon YS2-H) after cytocentrifugation. The score of phagocytosis was expressed by the number of cells that had one, two, three, four or more phagocytosed zymosan particles. Cells totalling 100 per plate were counted and multiplied by 1 (one or two particles), 2 (three or four particles) or 3 (more than four particles phagocytosed), according to a score criterion established previously by our group. The index of phagocytic activity was calculated by adding the points obtained per rat [31].

Measurement of ROS: DHE probe

Dihydroethidium (DHE) was used for the fluorometric measurement of intracellular superoxide content. DHE is a lipophilic probe and readily diffuses across cell membranes. Once inside the cell, it is oxidised by ROS, leading to the formation of fluorescent compounds that are trapped in the nucleus by intercalating into DNA, leading to an increase in fluorescence [32]. The cells (5×10^5/well) were preloaded with dihydroethidium (5 μM) by incubation in Tyrode's solution (137 mM NaCl, 2.68 mM KCl, 0.49 mM MgCl, 12.0 mM NaH_2PO_4, 5.6 mM D-glucose, 12.0 mM $NaHCO_3$, 5.0 mMHepes, pH 7.4) for 15 min at room temperature in the dark. The assay was performed in the presence and the absence of PMA (20 ng/well), used as a potent inducer of respiratory burst. Fluorescence was measured at an excitation wavelength of 396 nm and at an emission wavelength of 590 nm and analysed in a fluorometer (Tecan, Salzburg, Austria). The results of this experiment were expressed as relative fluorescence units (RFU).

Measurement of ROS: DCFH-DA probe

The method using the probe 2′,7′-dichlorodihydrofluoresceindiacetate (DCFH-DA) was also used to evaluate ROS production by the cells. Cells (5×10^5) were pre-incubated with 5 μM of the probe DCFH-DA and incubated in Tyrode's solution for 60 min. Following incubation, the cells were centrifuged and resuspended in 300 μl of Tyrode's solution. The experiments were performed for 60 min in the presence and absence of PMA (20 ng), used as an activator of ROS. Fluorescence was monitored in a Tecan spectrofluorometer (Salzburg, Austria) with excitation at 485 nm and emission at 530 nm after the treatment period. The results of this experiment were expressed as RFU.

MPO activity

The activity of the enzyme MPO released by neutrophils after degranulation was performed according to Hatanaka [33]. Neutrophils (6×10^5) in Tyrode's solution were exposed to 15 μl of a solution of 6.5 mM PMA at 37 °C for 30 min with stirring. Following incubation, the medium was immersed in ice and centrifuged at $500 \times g$ for 10 min at 4 °C. The supernatant was used to measure MPO activity by adding 0.1 mM H_2O_2 to trigger the reaction and 1 mM luminol as a light amplifier in a final volume of 0.3 ml. Chemiluminescence was measured as described above.

Hypochlorous acid production (HOCl)

Production of HOCl by neutrophils was evaluated according to the method described by Dypbukt et al. [34]. Briefly, after treatment, neutrophils (6×10^5/well) were stimulated with PMA (60 ng/well) for 60 min. The reaction was performed in a modified PBS (140 mM NaCl, 10 mM KCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mg/ml of glucose and 5 mM taurine), pH 7.4. The reaction was stopped by the addition of 26.8 U/ml of catalase. The cells were then centrifuged, the supernatant (200 μl) was collected, and 50 μl of solution containing 2 mM tetramethylbenzidine (TMB), 100 μM sodium iodide and 10 % dimethylformamide in 400 mM acetate buffer was added. After 5 min, absorbance was recorded at 650 nm in a microplate reader and a standard curve (1–40 μM of HOCl) was used to determine the concentration of hypochlorous acid.

Preparation of homogenates

To determine the enzymatic activities and glutathione content, cells were pelleted (5×10^6) and mixed with 1.0 ml of the assay-specific extraction buffer, vortexed briefly and lysed by ultrasonication in a VibraCell apparatus (Connecticut, USA) as described by Otton et al. [35]. A centrifugation step was included ($10{,}000 \times g$, 10 min, at 4 °C) in order to eliminate debris from the crude homogenate; the supernatant was then used for further analysis. The extracts for enzyme determinations were prepared in 50 mM sodium phosphate buffer (pH 7.4).

Antioxidant enzyme activities in neutrophils

Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) activities were determined in neutrophils using a microplate reader (Tecan, Salzburg, Austria). SOD activity was measured using the method described by Ewing and Janero [36], which involves the reduction of $O_2^{\cdot-}$ radicals by nitroblue tetrazolium (NBT). CAT activity was measured as described by Aebi [37], based on the direct decomposition of hydrogen peroxide (H_2O_2). Glutathione peroxidase and glutathione reductase [38, 39] activities were measured based on the oxidation of β-NADPH in the presence of tert-butyl hydroperoxide used as substrate.

Reduced (GSH) and oxidised (GSSG) glutathione content in neutrophils were measured as described by Rahman et al. [40]. The method is based on the reaction between reduced thiol groups (like that in GSH) with 5.5′-dithiobis-2-nitrobenzoic acid (DTNB) to form 5-thio-2-nitrobenzoic acid (TNB), which is stoichiometrically detected by absorbance at 412 nm. Purified GSH and GSSG were used as standards.

Hexokinase and glucose-6-phosphate dehydrogenase (G6PDH) activity

The maximum hexokinase activity, an enzyme that catalyses the phosphorylation of glucose in the first reaction of glycolysis, was determined according to the method described by Crabtree and Newsholme [41]. The extraction buffer consisted of 50 mM Tris–HCl, 1 mM EDTA, 2 mM $MgCl_2$, and 30 mM mercaptoethanol, pH 7.5. The assay buffer contains 75 mM Tris–HCl, 7.5 mM $MgCl_2$, 0.8 mM EDTA, 2.95 mM KCl, 4 mM mercaptoethanol, 0.4 mM NADP+, 2.5 mM ATP, 1 mM glucose, 10 mM creatine phosphate, 1.8 U creatine kinase, 14 U glucose-6-phosphate dehydrogenase and 0.05 % Triton X-100 (v/v). In a 96-well plate, 284 μl of assay buffer, 17 μl of sample and 37 μl of glucose were added. The enzyme activity was measured in a microplate reader at 340 nm for 5 min. The results were expressed as nmol/min/mg of protein.

Glucose-6-phosphate dehydrogenase (G6PDH), EC 1.1.1.49, is a key regulatory enzyme of the oxidative segment of the pentose-phosphate pathway and produces equivalent reducing agents in the form of NADPH to

meet certain cellular needs for reductive biosynthesis and as a contribution to the maintenance of the cellular redox state. The maximum activity of this enzyme was described by Guerra and Otton [31] and was based on the conversion of NADP+ into NADPH in the presence of glucose-6-phosphate.

Intracellular Ca^{2+} release

Changes in intracellular calcium concentration were monitored fluorometrically using the calcium-sensitive probe Fura-2AM [42]. The amount of labelling of the probe Fura-2AM (5 μM) was 1 h to 1×10^6 cells/ml in Tyrode's solution dried at 37 °C in the dark. After this period, the cells were washed with the same solution and treated with 0.3 mM palmitic acid, used to induce calcium mobilisation. Changes in intracellular calcium concentration $[Ca^{2+}]i$ were monitored for 20 min. The fluorescence of Fura-2AM was evaluated in aliquots of 300 μl of cell suspension in a Tecan spectrofluorometer (Salzburg, Austria) set at excitation wavelengths of 340 and 380 nm and emission of 510 nm. Transformation of the fluorescent signal to $[Ca^{2+}]i$ was performed by calibration with ionomycin (100 μM, maximum concentration), followed by the addition of EGTA (60 μM, minimum concentration) according to the Grynkiewicz equation, using the K_{diss} of 224 nM [43].

RNA extraction

Total RNA was extracted from neutrophils using TRIzol Reagent (Life Technologies, Rockville, MD, USA), following the manufacturer's instructions. Isolated RNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), and its integrity was confirmed using agarose gel electrophoresis.

RT-PCR

Reverse transcriptase (RT)-PCR was used to measure mRNA expression levels of CD11b, NFκB, IκK, TLR4, Nrf2 and LepRb. Table 1 presents the nucleotide sequence used in this study.

Total RNA (2 μg) was treated with 1U DNase for 25 min at 25 °C and inactivated with 2.5 mM EDTA. Next, the cDNA was synthesised using oligo (dT) in a 20 μl reaction containing 1 mM of each dNTP and 200 U SuperScript II RNase H–reverse transcriptase at 42 °C for 50 min, following the manufacturer's instructions. Heating at 70 °C for 15 min inactivated the reaction. The PCR was performed in a total volume of 25 μl, containing 2.5 μl of buffer DNA polymerase enzyme (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl, pH 9.0), 10 pmol of the primer, 200 μM of each nucleotide (dATP, dCTP, dGTP and dTTP), 2.5 U of Taq DNA polymerase (Sigma) and 2 μl of cDNA. The

Table 1 Nucleotide sequences of primers and cycling conditions used for RT-PCR amplification

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Cycling conditions	Amplicon size (pb)
β-actin	CCACCATGTACCCAGGCATT	ACGCAGCTCAGTAACAGTCC	95 °C, 1 m	243
			62 °C, 2 m	
			72 °C, 3 m	
TLR4	TGGCAGTTTCTGAGTAGCCG	TGCTACTTCCTTGTGCCCTG	95 °C, 1 m	390
			65 °C, 2 m	
			72 °C, 3 m	
IκK	AAGCCTGATGATTCTACGTCTTTC	TCAGGATGCGTAGCTGCATT	94 °C, 1 m	200
			62 °C, 2 m	
			72 °C, 3 m	
NFκB	CGTTTTACTCTTTATCTCGCTTTCG	GGCTATATGGCCTAAGGCATGG	95 °C, 1 m	200
			58 °C, 2 m	
			72 °C, 3 m	
Nrf2	CCTCTGTCACCAGCTCAAGG	TGGGCGGCGACTTTATTCTT	95 °C, 1 m	320
			66 °C, 2 m	
			72 °C, 3 m	
CD11b	AGAATTTCGGTGCCTGGGAG	CCACCGTGCTCTCCCCCTA	95 °C, 1 m	200
			69 °C, 2 m	
			72 °C, 3 m	
LepRb	GTAATTGGAGCAGTCCAGCCT	CCCATTGTGGGCAGTACGAT	95 °C, 1 m	200
			64 °C, 2 m	
			72 °C, 3 m	

RT and PCR reactions were performed in a Veriti Gradient equipment (Applied Biosystems, Carlsbad, CA, USA), using parameters described by Innis et al. [44]. For semi-quantitative PCR analysis, the housekeeping β-actin gene was used as reference. RT-PCR endpoint products were analysed following 1.3 % agarose gel electrophoresis using blue green loading dye (LGC Biotecnologia, SP, Brazil) and the Image J processing program.

Protein measurement

Measurements of the specific enzyme activity of G6PDH, hexokinase and all antioxidant enzymes were entirely related to protein concentrations, which were estimated by the Bradford method [45] using bovine serum albumin as a standard.

Statistical analysis

Results are given as mean ± SEM. The Levene test was used to verify the variance of the data. When the Levene test was $P > 0.05$, interaction was evaluated through a factorial two-way ANOVA (diet, D × green tea, GT) using Tukey as post-test ($P < 0.05$). When the interaction was not statistically significant, the main effect (diet, D, and/or green tea treatment, GT) was accessed by a factorial two-way ANOVA ($P < 0.05$). The statistical analysis was performed using the SPSS/Windows version 22 statistical package (SPSS Inc., Chicago, IL, USA) and GraphPad Prism statistics software package version 5.0 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Green tea extract attenuates the release of pro-inflammatory cytokines

To assess the pro-inflammatory cytokine profile of neutrophils, we determined the release of TNF-α, IL-6 and IL-1β under LPS stimulation or at baseline (only in the control group). TNF-α release was decreased in neutrophils from rats supplemented with GT (GT effect, $P < 0.01$, 2 × 2 ANOVA) highlighting an indirect anti-inflammatory effect of GT. Treatment of obese rats with green tea extract (OB + GT) promoted a decrease of 46 % ($P < 0.05$) in the release of TNF-α compared with the non-supplemented obese group (OB) (Fig. 1A). We also observed a reduction of 62 % ($P < 0.01$) in IL-6 release from neutrophils of rats supplemented with green tea (GT effect, 2 × 2 ANOVA) (Fig. 1B). There was no statistical difference among the groups for IL-1β production.

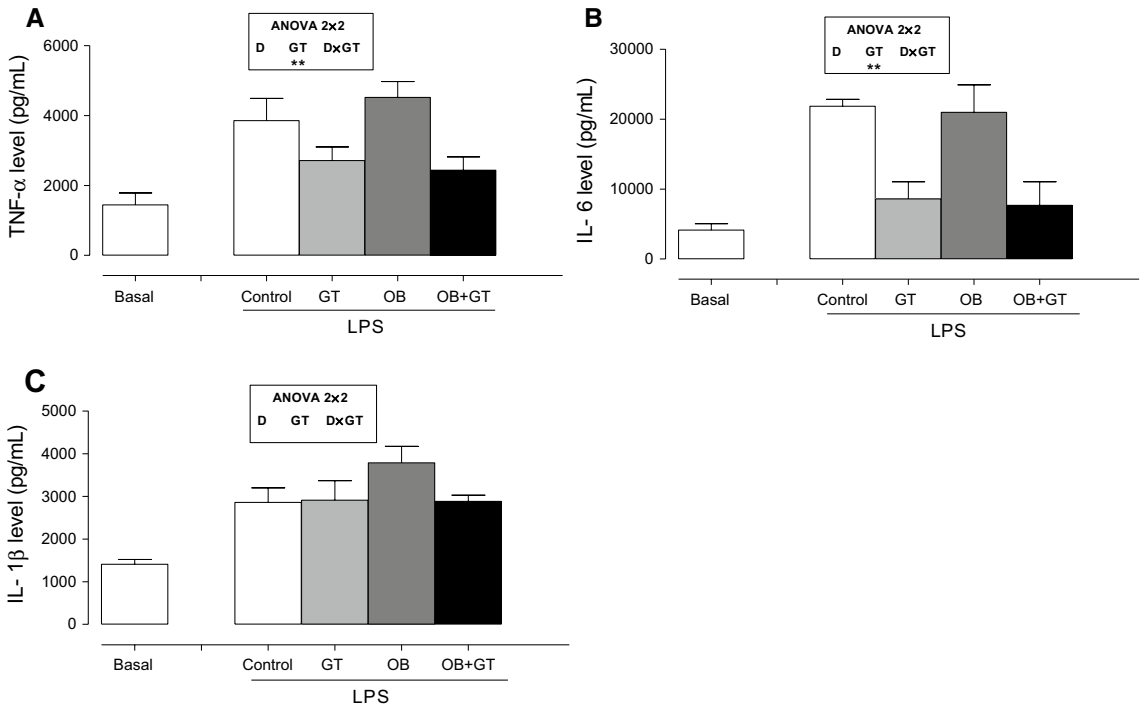


Fig. 1 Green tea extract attenuates pro-inflammatory cytokine release. **A** TNF-α, **B** IL-6 and **C** IL-1β. Values (pg/ml) of cytokine production by neutrophils (1×10^6 cells) stimulated with LPS (10 μg/ml) for 18 h. Data are presented as mean ± SEM of 5 rats per group. An ANOVA 2 × 2 factorial design was used to study the effects of diet (D), green tea (GT) and potential interaction between D × GT

Obesity condition modulates neutrophil functional parameters

The ability of neutrophils to sense and migrate towards damaged tissue is a vital component of the innate immune response. Our results showed that the rats fed with cafeteria diet under unstimulated or fMLP-stimulated condition showed a significant decrease of 55 % in the migration capacity (diet main effect, $P < 0.001$, 2 × 2 ANOVA) compared with the respective control group (Fig. 2A).

Neutrophils are capable of ingesting microorganisms or particles. In order to evaluate the phagocytic ability of the neutrophils, we challenge them with opsonized zymosan particles and then conducted a phagocytic assay. Phagocytic capacity was decreased in neutrophils from rats supplemented with green tea (GT main effect, $P < 0.01$), whereas an increase was observed in neutrophils from obese animals (diet main effect, $P < 0.001$, 2 × 2 ANOVA) (Fig. 2B).

We determined the ROS production of neutrophils under basal or after PMA stimulation using two different fluorescent probes (Fig. 2C–D). After a DHE assay, we observed an interactive effect between diet and green tea (D × GT, $P < 0.001$, 2 × 2 ANOVA). Cafeteria diet promoted an increase (35 %) in ROS production after PMA stimulation. GT supplementation of obese rats (OB + GT) further increased (25 %) ROS production compared with the obese group (Fig. 2C). For ROS production assessed by DCFH-DA probe (Fig. 2D), we also observed an interactive effect between D × GT in PMA-stimulated groups. Neutrophils from obese rats showed a decrease in ROS production of 46 % compared with the control group. Neutrophils from obese rats supplemented with green tea (OB + GT) showed an increase of 69 % compared with the obese group.

A decrease in MPO activity was verified in neutrophils from both groups of rats supplemented with green tea (GT and OB + GT), as well as in obese neutrophils under PMA stimulation (interactive effect between D × GT, $P < 0.05$, 2 × 2 ANOVA). Neutrophils from obese rats decreased MPO by 36 %, 52 % in GT and 51 % in OB + GT compared with the control group (Fig. 2E).

A similar reduction was verified in all groups when we measured HOCl production (interactive effect between D × GT, $P < 0.001$, 2 × 2 ANOVA). A significant reduction in MPO activity of 45 % ($P < 0.001$) was verified in neutrophils from obese rats (OB) compared with control neutrophils (Fig. 2F). Neutrophils from obese rats supplemented with green tea (OB + GT) increased HOCl production by 35 % compared with the obese group ($P < 0.001$).

We measured intracellular Ca^{2+} mobilisation, since calcium is an important second messenger able to regulate a variety of processes in neutrophils, including the migration, adhesion, degranulation, phagocytosis and secretion of pro-inflammatory cytokines. An interactive effect was verified between D × GT treatment ($P < 0.001$, 2 × 2 ANOVA) for intracellular calcium mobilisation. Neutrophils obtained from OB and OB + GT rats increased intracellular calcium mobilisation by 138 and 75 %, respectively, following stimulation with palmitic acid compared with the control neutrophils in the same condition. GT supplementation also promoted an increase of 191 % in Ca^{2+} mobilising ($P < 0.01$) compared with the control neutrophils (Fig. 2G).

Green tea extract modulates antioxidant and metabolic enzymes of neutrophils

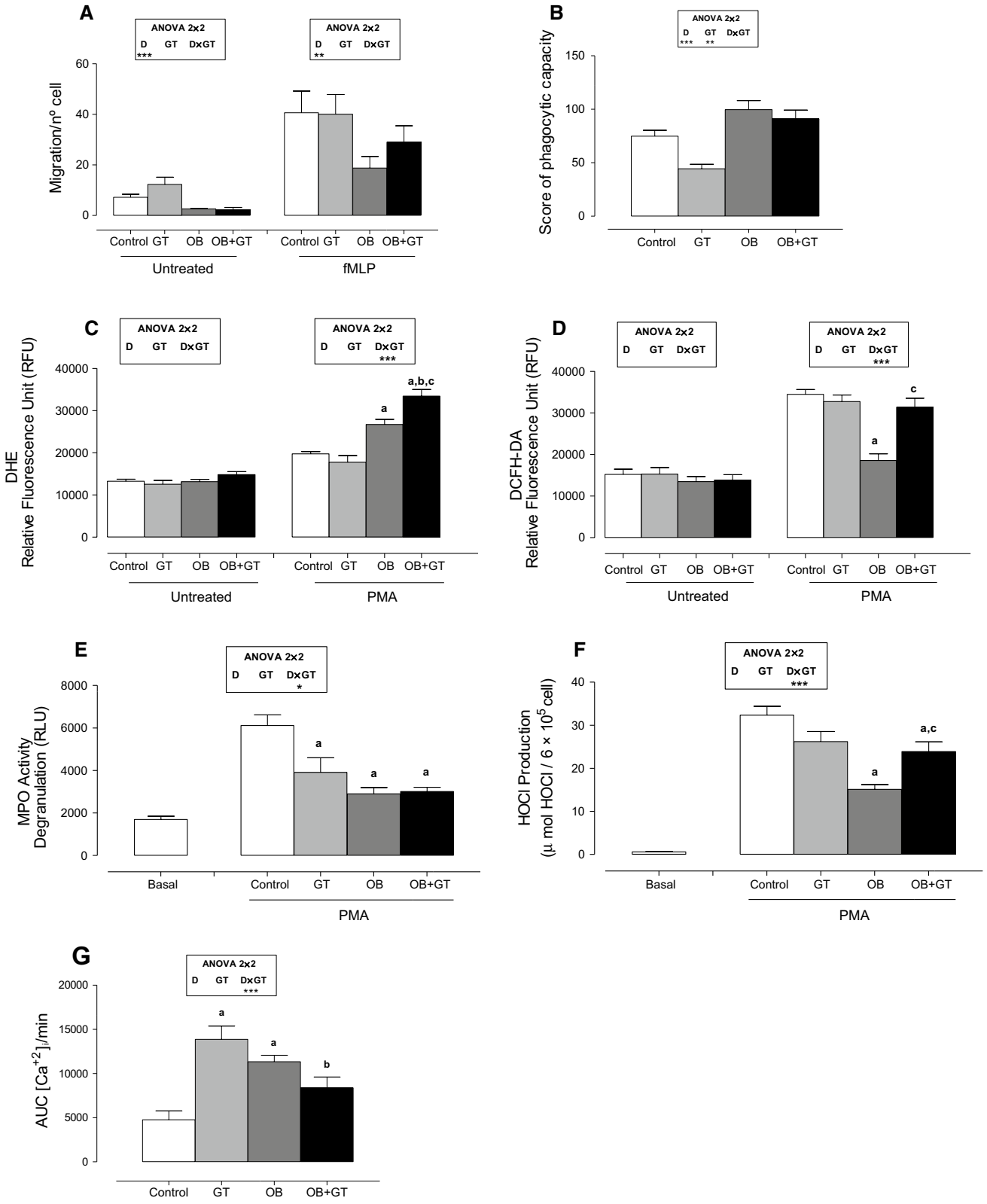
Since the obese condition is associated with increased availability of energetic fuels and oxidative stress and GT has a renowned antioxidant effect, we investigated some metabolic and antioxidant enzyme activities and the content of GSH/GSSG (Table 2). We observed a significant decrease in the activity of total SOD due to diet (diet main effect) and GT (GT main effect) of 30, 44 and 58 % in the GT, OB and OB + GT groups, respectively, compared with the control group ($P < 0.001$). There was an interactive effect between D × GT ($P < 0.01$, 2 × 2 ANOVA) for Mn SOD activity that was inhibited in all groups compared with the control group. Catalase activity was significantly increased (162 %) (interactive effect between D × GT, $P < 0.01$) in neutrophils from obese rats compared with the control rats, while OB + GT neutrophils showed restored catalase activity. The cafeteria diet (diet effect, $P < 0.05$, 2 × 2 ANOVA) decreased GPx activity, while GR was not modified among the groups (Table 2).

GT treatment increased GSH content (GT effect, $P < 0.05$, 2 × 2 ANOVA) without changing GSSG content. However, the GSH/GSSG ratio was increased in OB + GT neutrophils (diet and GT effect, $P < 0.05$, 2 × 2 ANOVA).

In the OB and OB + GT groups, hexokinase activity was significantly increased (48 %) (diet main effect, $P < 0.001$, 2 × 2 ANOVA). For G6PDH activity, we observed that GT, OB and OB + GT decreased by 85, 76 and 89 %, respectively, compared with the control group (interactive effect between D × GT effect, $P < 0.001$, 2 × 2 ANOVA).

Obesity and green tea supplementation modulate mRNA level of inflammatory genes

In order to investigate the mechanism by which obesity and green tea modulate the main aspects of neutrophils, we evaluated the gene expression of key molecules involved in the function of these cells. There was an interactive effect between D × GT treatment ($P < 0.05$, 2 × 2 ANOVA) for TLR4 mRNA levels. Obesity increased TLR4 expression, whereas in neutrophils from OB + GT, a reduction was observed. Nuclear factor kappa-light-chain enhancer



of activated B cell (NFκB) mRNA levels were not different among the groups; however, IκK, responsible for o IκB phosphorylation and its subsequent inactivation, was increased in the neutrophils of obese rats (diet main effect, $P < 0.01$). GT supplementation reduced IκK mRNA levels (GT main effect, $P < 0.05$, 2 × 2 ANOVA). Nrf2 mRNA

◀ **Fig. 2** Improvement of neutrophil functional parameters by green tea supplementation. **A** Migration capacity, **B** phagocytic capacity, **C** superoxide anion production (DHE probe), **D** hydrogen peroxide production (DCFH-DA probe), **E** MPO activity, **F** HOCl production, **G** calcium release. Data are presented as mean ± SEM of 4–10 rats per group. An ANOVA 2 × 2 factorial design was used to study the effects of diet (D), green tea (GT) and potential interaction between D × GT. When interaction was statistically significant, *superscript letters* designate: *a* = statistical difference compared with the control group, *b* = compared with the green tea group, *c* = compared with the obese group. **P* < 0.05; ***P* < 0.01; ****P* < 0.001

levels were modulated by the diet and GT (interactive effect, $P < 0.01$, 2 × 2 ANOVA). Obesity increased Nrf2 mRNA levels, and GT treatment decreased them (Fig. 3A). We measured the mRNA levels of CD11b, which is part of the CD11b/CD18 heterodimer (Mac-1 α, Mβ2 integrin), also known as the C3 complement receptor. It functions as a receptor for complement (C3bi) involved in the phagocytic process. Obesity caused an increase in CD11b mRNA expression (interactive effect between D × GT, $P < 0.05$, 2 × 2 ANOVA), whereas GT treatment of obese rats restored CD11b mRNA levels (Fig. 3B). Leptin receptor B (LepRb) is the leptin receptor present in neutrophils [46] and is known to promote phagocytic process in neutrophils by promoting CD11b expression. In our study, the mRNA levels of LepRb were not modified among the groups.

Discussion

In our study, we demonstrated that cafeteria diet provided to the animals for 8 weeks promoted changes in some of the main inflammatory features of neutrophils, leading to a decrease in chemotaxis, H_2O_2 levels, MPO activity and HOCl production, while increased phagocytosis and mRNA levels of TLR4 and IKK. We also demonstrated that GT supplementation for 12 weeks (500 mg/kg of body weight) restored the main changes to neutrophils induced by obesity. It is important to emphasise that the dose of GT extract used herein to treat obesity could not be achievable within the diet and could only be provided by nutraceutical supplementation. To ensure that the cafeteria diet caused the onset of obesity in the rats, we assessed some key aspects of the obese condition. Our characterisation of the obesity model confirmed that the cafeteria diet induced weight gain, increased fat deposits, such as epididymal, retroperitoneal and subcutaneous fat pads, and increased leptin and free fatty acid (FFA) plasma levels, in addition to inducing glucose intolerance and insulin resistance (GTT and ITT). GT extract supplementation restored all the obesity-related parameters measured (Rocha et al. 2015, submitted for publication).

Regarding neutrophils, it is well known that to carry out its effector functions, these cells require a large energy production. We determined that hexokinase activity was increased in neutrophils from obese rats, one effect exclusively due to the diet (diet main effect, $P < 0.001$). Some authors have shown that activation of TLR4 in neutrophils may lead to the activation of p38 MAPK and the transcription factor HIF-1, leading to increased expression of GLUT-1 [47]. Since we observed that neutrophils from obese rats also showed increased TLR4 mRNA levels, it seems reasonable to suggest that the large calorie intake

Table 2 Green tea extract modulates antioxidant and metabolic enzymes of neutrophils

	CONT	GT	OB	OB + GT	ANOVA 2 × 2		
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	D	GT	D × GT
Total/SOD (U/mg of protein)	4.67 ± 0.23	3.27 ± 0.18	2.62 ± 0.36	1.97 ± 0.20	***	***	
Mn SOD (U/mg of protein)	0.21 ± 0.02	0.08 ± 0.01[a]	0.12 ± 0.02[a]	0.11 ± 0.01[a]			**
CAT (μmol/min/mg of protein)	0.85 ± 0.10	1.00 ± 0.18	2.23 ± 0.41[a]	0.87 ± 0.07[c]			**
GPx (mU/mg of protein)	61.32 ± 5.70	64.12 ± 5.40	54.50 ± 3.18	52.29 ± 2.29	*		
GR (mU/mg of protein)	625.8 ± 156.1	893.2 ± 55.76	637.3 ± 73.83	725.4 ± 50.46			
GSH (μM/mg of protein)	0.23 ± 0.07	0.72 ± 0.35	0.56 ± 0.06	1.16 ± 0.17		*	
GSSG (μM/mg of protein)	0.18 ± 0.03	0.20 ± 0.04	0.26 ± 0.06	0.16 ± 0.01			
GSH/GSSG	2.40 ± 0.97	2.88 ± 0.85	2.60 ± 0.85	7.16 ± 1.15	*	*	
Hexoquinase (nmol/min/mg of protein)	3.44 ± 0.41	2.14 ± 0.18	5.11 ± 0.44	5.01 ± 0.54	***		
G6PDH (μmol/min/mg of protein)	7.24 ± 0.76	1.06 ± 0.05[a]	1.72 ± 0.19[a]	0.78 ± 0.06[a]			***

Data are presented as mean ± SEM of 4–10 rats per group. An ANOVA 2 × 2 factorial design was used to study the effects of diet (D), green tea (GT) and potential interaction between D × GT. When interaction was statistically significant, superscript letters designate

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

[a] Statistical difference compared with the control group

[b] Compared with the green tea group

[c] Compared with the obese group

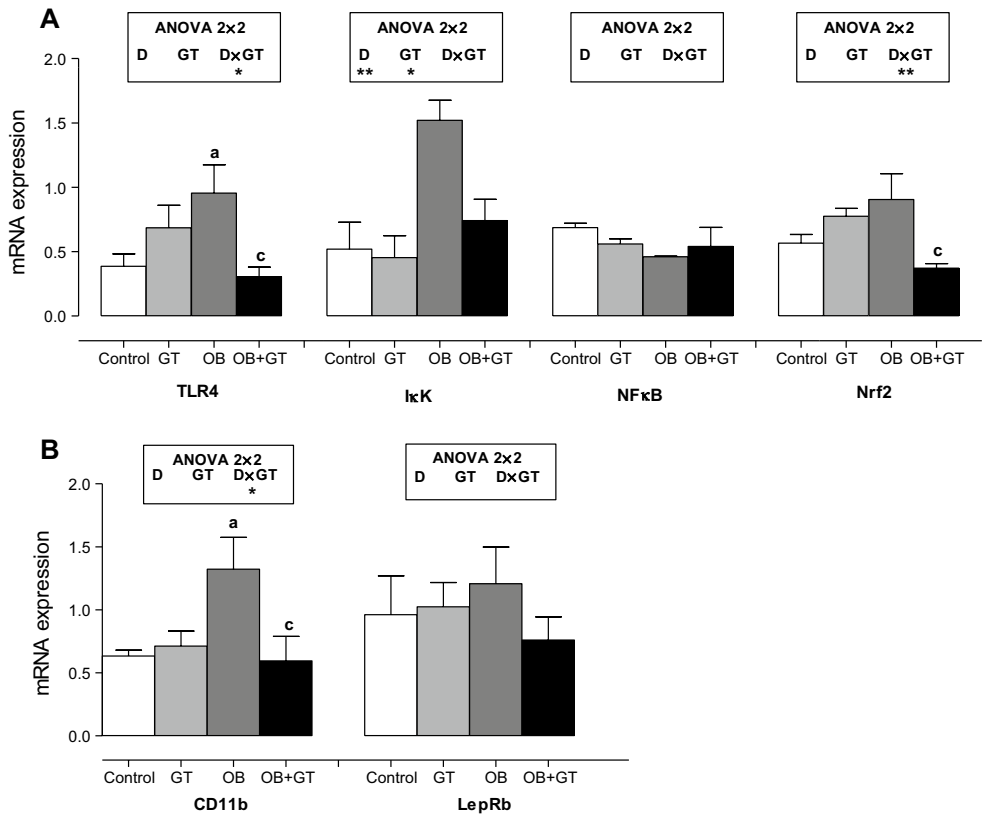


Fig. 3 Green tea supplementation modulates mRNA of inflammatory genes. **A** mRNA expression of TLR4, IκK, NFκB and Nrf2, **B** CD11b and LepRb. Data are presented as mean ± SEM of 4–10 rats per group. An ANOVA 2 × 2 factorial design was used to study the effects of diet (D), green tea (GT) and potential interaction between D × GT. When interaction was statistically significant, *superscript letters* designate: *a* statistical difference compared with the control group, *c* compared with the obese group. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

promoted by cafeteria diet could increase GLUT-1 in the membrane of neutrophils (via TLR4 stimulation), favouring the entry of glucose into the cell and increasing hexokinase activity (Table 2). GT treatment did not restore hexokinase activity, but it did restore TLR4 mRNA levels, suggesting that another mechanism could be involved in this response. Despite the increase in hexokinase, G6PDH activity was decreased in all the groups studied (Table 2). The G6PDH pathway is an alternative via for glucose-6-phosphate oxidation, leading to the generation of NADPH cofactor. This cofactor is required for the activity of important enzymes for neutrophils, such as iNOS, NADPH-oxidase and GR.

Pro-inflammatory TNF-α and IL-6 cytokines released from neutrophils were decreased following GT treatment (GT main effect), while obesity did not induce any additional pro-inflammatory cytokine release. However, we can reinforce an indirect anti-inflammatory effect of GT, since it decreased pro-inflammatory cytokine release. It seems that the release of cytokines in obese rats was not associated with increased TLR4 mRNA levels or NFκB mRNA levels, since we observed an increase in TLR4 gene expression in obese rats, but no change in inflammatory cytokines or NFκB mRNA levels. Nevertheless, TLR4 activation can trigger different signalling pathways that have unusual targets not related to NF-κB pathway, which can promote an increase in pro-inflammatory cytokines. MAPKs may stimulate the activator protein 1 (AP-1) transcription factor activity through either direct phosphorylation or transcription of AP-1 components [48]. The AP-1 activity also can be stimulated by TRAF6-induced activation of TAK1, a MAP3K, in response to the activation of TLR4. The phosphorylation of c-Jun by JNK can stimulate AP1 activity and consequently the production of pro-inflammatory cytokines [49]. In addition, it was shown that IKKβ has become critical for the activation of both NFκB and MAPK and provides a possible link between the activation of NF-κB and of AP-1 in macrophages [50].

In contrast, neutrophil migration of obese rats was attenuated both under basal and after fMLP stimulation (Fig. 2A, diet effect). It has been shown that activation of the p38 MAPK pathway inhibits the chemotaxis signalling pathway induced by fMLP, thereby interrupting the

chemotactic process [51]. It has also been reported that the increase in circulating chemokines, cytokines and FFA can lead to desensitisation and internalisation of CXCR2, fMLPR and the adhesion molecules L-selectin, promoting their excessive and constant activation. Thus, the rolling/adhesion and chemotaxis ability of neutrophils are reduced, resulting in decreased neutrophil migration [52]. Therefore, we suggest that the increase in plasma FFA and pro-inflammatory cytokines observed in our model of obesity (Rocha et al. 2015, submitted for publication) may be contributing to the overactivation of neutrophils in order to activate p38 MAPK, decreasing the chemotactic activity.

Our results also showed an improvement in the neutrophil chemotactic activity of obese rats treated with GT extract under stimulation. This effect can be partly explained by the fact that EGCG decreases the TLR4 signalling pathway, as reported by [53] and confirmed by our results in rats treated with GT extract (Fig. 3A). Studies from Hong et al. and Joo et al. showed that decreased TLR4 signalling pathway induced by EGCG catechin was responsible for reducing protein expression of certain cytokines, such as IL-6, by blocking/attenuating the NF-κB binding to DNA as well as by inhibiting LPS-induced phosphorylation of ERK1/2, JNK and p38 [16, 54]. This find partially agrees with our results related to a reduction in the release of pro-inflammatory cytokines IL-6 and TNF-α by neutrophils of obese rats supplemented with GT extract since there was no change in mRNA levels of NF-κB in our study.

The condition of obesity increased the phagocytic ability of neutrophils (diet main effect) (Fig. 2B). Data in the literature affirm that in the obese condition, the leptin secreted by fat cells after excessive storage of nutrients can act directly on neutrophils in the bloodstream by binding to the leptin receptors in the cell membrane or, indirectly, by stimulating the release of TNF-α. This action can lead to increased expression of the CD11b/CR3 complement receptor [55]. Thus, increased expression of CR3 facilitates phagocytosis of opsonized particles by C3bi and recruitment of neutrophils to the site of inflammation [56, 57]. According to these findings, since the leptin plasma level and CD11b mRNA expression were increased in our model of obesity, it is possible that these mechanisms are responsible for the increased phagocytic ability of the neutrophils. However, measurement of LepRb mRNA levels showed no difference among the groups, indicating that LepRb mRNA expression is not involved in leptin action in neutrophils. Even if leptin receptor expression remained unaltered in neutrophils, an increase in circulating leptin was detected and then leptin can bind to neutrophil LepRb and, thus, increase the phagocytosis of neutrophils in the obese condition. Our results also showed that GT supplementation decreased the phagocytic capacity of neutrophils (GT main effect). This fact can be explained by the ability of EGCG to modulate the expression of IgE-specific receptors (FcεRI). Although neutrophils mainly express IgG-specific receptors (FcγRII), it is believed that other polyphenol catechins present in the GT extract may act similarly, reducing the expression of this receptor, with a consequent reduction in phagocytic capacity [16, 58].

After ingestion and phagolysosome formation, neutrophils initiate the process of bacterial killing. This process is accompanied by the activation of NADPH-oxidase, an enzyme complex that is organised in the phagosomal membrane and converts oxygen into the superoxide anion radical [$O_2^{\cdot-}$]. The $O_2^{\cdot-}$ is generated on the outer surface (within the phagosome), with reducing equivalents provided by the intracellular NADPH [59]. Superoxide production is important for effective antimicrobial defence, but oxidants generated excessively by neutrophils can also damage cell structure leading to cell dysfunction or even apoptosis [60].

The data obtained in this study show an increase in superoxide anion production in neutrophils from obese rats compared with the control group (Fig. 2C). Indeed, ROS levels are increased in obesity and metabolic syndrome, and the reduction of these compounds has been observed following weight loss [61]. Initially, it was expected that the GT treatment would effectively reduce superoxide levels in obese neutrophils. However, there was an additional increase compared with the obese group (stimulated condition). We speculate that the stability of catechins and their metabolites, sometimes more active than catechins *per se*, may have a pro-oxidant effect, leading to ROS generation [62].

The superoxide anion generated mainly by NADPH-oxidase is subsequently dismuted to H_2O_2 by SOD isoforms. Measurement of H_2O_2 levels is considered to be a good parameter for assessing phagocyte activation. The fluorescent probe DCFH-DA is widely used to evaluate oxidative stress [63] as well as an indicator of H_2O_2 production [64]. We observed an inhibition in SOD activity in rats treated with cafeteria diet (OB and OB + GT) (Table 2). The decrease in SOD activity can be related to the reduction of H_2O_2 production (Fig. 2D) and to the increase in $O_2^{\cdot-}$ (Fig. 2C) in cafeteria diet-treated groups. Lower SOD activity implies in higher levels of $O_2^{\cdot-}$ and lower levels of H_2O_2. These data support the hypothesis that obesity can impair the activity of some antioxidant enzymes, which in turn increase the availability of ROS. In our study, there was a correlation between increased ROS production with reduced SOD activity in the OB and OB + GT groups. Our results also showed a great ability of GT extract on decreasing SOD activity (GT and OB + GT) as well as CAT activity (OB + GT) (Table 2) as previously reported by Wu et al. [65].We also observed a pro-oxidant effect of GT treatment in obese rats, with an increase in H_2O_2 generation (Fig. 2D). This fact was evidenced in other studies,

highlighting the preventive anti-cancer activity of catechins, leading to the formation of H_2O_2, through a mechanism of auto-oxidation [66]. Although this pro-oxidant effect is present in the obese condition, we did not observe this phenomenon in neutrophils from rats fed with standard diet and stimulated with PMA, indicating that changes in the microenvironment caused by the cafeteria diet seem to corroborate this effect promoted by the GT extract.

A strong association among MPO overexpression, hyperlipidemia, weight gain and neutrophil activation was demonstrated [67, 68]. The MPO enzyme produced in the azurophilic granules of neutrophils, when combined with H_2O_2, generates hypochlorous acid (HOCl), which provides more effective antimicrobial activity. In our study, MPO activity and HOCl production (Fig. 2E, F) were decreased in neutrophils from obese rats. One hypothesis for the attenuation of MPO activity is the fact that this enzyme is able to complex with $O_2^{\cdot-}$ generated in excess in obesity, as demonstrated herein (Fig. 2C). This leads to MPO inactivation, thereby decreasing its activity [69]. The literature discusses the ability of MPO to form complexes with CD11b receptor, which was also increased in the obese condition (Fig. 3B). Reduced MPO activity can lead to serious losses related to defence against pathogens, since neutrophils require this enzyme to exert their microbicidal activity. HOCl formation is important, since it has a variety of actions as a bactericide and fungicide [70, 71]. A decrease in HOCl formation could be a consequence of the decrease in MPO activity and due to the reduced availability of H_2O_2, the main MPO substrate, as verified by our results (Fig. 2D). Our data showed that the treatment of obese rats with GT extract improved HOCl production, without changing MPO activity. The increase in HOCl formation may represent an improvement in the functional capacity of neutrophils.

In order to determine a possible mechanism by which obesity and GT alter neutrophils, we measured intracellular Ca^{2+} mobilisation (Fig. 2G), since many of the pro-inflammatory activities of neutrophils are dependent on Ca^{2+} mobilisation [72]. Since the initial studies by [73], the role of calcium in the process of phagocytosis was demonstrated. In addition, other studies have shown that Ca^{2+} signals also control neutrophil proliferation, differentiation, apoptosis, ROS generation and a variety of pathways of gene transcription [74–77]. Our study showed an increase in intracellular calcium mobilisation in all groups compared with the control. Although there was an increase in Ca^{2+} mobilisation in the obese group, some parameters, such as chemotaxis, in which calcium is directly involved, showed no improvement. This indicates that these changes are independent of calcium mobilisation and are likely associated with other intracellular pathways. Interestingly, we observed that supplementation with GT extract caused a large increase in Ca^{2+} mobilisation. It has been shown that flavonoids present in the diet promoted an increase in mitochondrial uptake of Ca^{2+}, an important process in the regulation of ATP production in the permeability of pores involved in apoptosis and the regulation of some processes dependent on a high concentration of cytosolic Ca^{2+}, such as secretion. Although neutrophils have low amounts of mitochondria and endoplasmic reticulum is sparse, we believe that intracellular calcium mobilisation and uptake occurs in other fairly abundant organelle in neutrophils, such as lysosomes, which also have an ATP-dependent Ca^{2+} uptake pump system [78]. Other studies have reported that EGCG causes an increase in cytosolic Ca^{2+} stores in neurons of the hippocampus and U87 cells in human astrocytoma, which strongly suggests that intracellular Ca^{2+} mobilisation comes from the lumen of endoplasmic reticulum. This effect seems to be due, in part, to the activation of phospholipase C (PLC) accompanied by increased inositol phosphates formation and can be partially alleviated by PLC inhibitors [79]. In our model, this effect can be beneficial for neutrophils, since to perform their proper functions, these immune cells are Ca^{2+} dependent. Moreover, the increase in calcium may improve the performance of neutrophils against possible threats to the organism of obese individuals.

In summary, this study shows that in response to a cafeteria diet used to induce obesity in rats, certain key functions of neutrophils suffered a profound negative impact. This was mainly evidenced by alterations in chemotaxis and microbicidal activity, H_2O_2 and HOCl production, the decline in MPO activity and increased TLR4/NFκB inflammatory pathway with no pronounced effects in the antioxidant profile. Although phagocytic capacity and intracellular Ca^{2+} mobilisation increased, all the other parameters measured were indicative of a state of functional deficit in these immune cells. Our study also shows a significant immunomodulatory effect of green tea extract in the obese condition related to neutrophil function, which was driven towards a more efficient condition without overactivation. However, further studies are required to achieve a clearer understanding of the mechanisms involved in the results obtained in our study. Our findings open a novel perspective for new therapies with bioactive compounds aiding the immune system of obese individuals by restoring the functional parameters of neutrophils, which may represent an important clinical impact in the quality of life of obese patients.

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Conflict of interest None.

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