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Green tea polyphenols change the profile of inflammatory cytokine release from lymphocytes of obese and lean rats and protect against oxidative damage

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ABSTRACT

This study aimed to investigate whether green tea polyphenols (GT) modulate some functional parameters of lymphocytes from obese rats. 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). Lymphocytes were obtained from mesenteric lymph nodes for analyses. In response to the cafeteria diet we observed an increase in activity of the metabolic enzyme hexokinase, ROS production, MnSOD, CuZnSOD and GR enzyme activities and proliferation capacity of the cells (baseline), whereas IL-10 production was decreased. Obese rats treated with GT decreased cell proliferation (under ConA stimulation). Hexokinase and G6PDH activity, ROS production and MnSOD, CuZnSOD, GPx and GR enzymes remained increased, accompanied by an increase in Nrf2 mRNA level. There was a decrease in pro-inflammatory IL-2, IL-6, IL-1β, TNF-α cytokines that were accompanied by a decrease in the mRNA level of TRL4 while IL-10 production was increased in obese rats treated with GT. GT treatment of lean rats showed similar results to that of obese rats treated with GT, indicating that the effects of GT are independent of diet. Foxp3 and IRF4 mRNA levels were increased by GT. In conclusion, cafeteria diet modulated the function of lymphocytes from lymph nodes, increasing ROS production and decreasing anti-inflammatory IL-10, which could contribute to the inflammatory state in obesity. GT reduced ROS production, improving the redox status and reducing pro-inflammatory cytokine production by lymphocytes, suggesting that GT treatment may be driving lymphocytes to a more anti-inflammatory than pro-inflammatory microenvironment.

1. Introduction

Obesity is associated with a chronic low-grade inflammation state, present both systemically and within the white adipose tissue (WAT) [1–4]. Furthermore, obesity is characterized by increased levels of some circulating hormones and nutrients, such as leptin, glucose and free fatty acids, among other metabolic changes. The energy-rich environment in obesity can lead to impairment of immune cells present in the blood stream or those residing in peripheral tissues [5,6]. These can be confirmed, as obese individuals have a higher incidence of certain types of cancer [7,8], increased susceptibility to infections and an inability to exterminate pathogens [9,10]. Obese individuals also present susceptibility to developing chronic inflammatory diseases, probably due to the over-activation of immune cells. This in turn, can lead to defects or injury in the functionality of immune cells, causing damages in the immune and inflammatory response [11].

Several studies have reported that the obese condition alters the number and profile of immune cells present both in WAT and in the blood stream [12,13]. Besides the overflow of lipid resulting from a fat rich-diet, the increase in pro-inflammatory cytokines (IL-6, TNF-α, and IL-1β) and leptin released by adipocytes can drive lymphocytes to a Th1 phenotype in WAT. Obesity also provides bacterial and metabolic danger signals that mimic bacterial infection and drive a shift in immune cell phenotype and number (M1 macrophages, CD8 + T-cells, CD4 + T-cells — Th1, Th2 and Th17). In contrast, T-reg anti-inflammatory lymphocyte numbers are decreased in WAT in obesity. It is recognized that T-reg cells prevent WAT inflammation and insulin resistance [13]. The disruption of the delicate balance between adipocytes and WAT-resident immune cells in obesity contributes to the development of WAT inflammation, insulin resistance and energy mobilization [14], all of which worsen obesity-associated comorbidities.

The obese condition is also associated with increased levels of oxidative stress. Factors such as hyperglycemia, high levels of free fatty acids, triacylglycerol, hyperleptinemia and inadequate antioxidant defense are the main contributing factors to increased oxidative stress [15]. Diet changes usually observed in the obese state, with more fat and carbohydrates at the expense of fruits and vegetables, in general lead to inadequate antioxidant defense. In addition, plasma concentrations of vitamins, minerals, enzymes and antioxidant activity are lower in obesity [16,17]. These features, combined with high reactive oxygen species (ROS) production contribute to increased oxidative

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stress [15]. Indeed, uncontrolled oxidative stress has been indicated as the molecular basis of several immune-impaired pathologies [18]. Thus, it has been suggested that bioactive compounds from the diet could be used to improve/mitigate cellular damage caused by increased oxidative stress and inflammation in the obese condition.

Green tea (GT) has been widely investigated as a functional food due to its content of flavonoids, represented by catechins, which present numerous therapeutic properties [19]. The content of catechins is high in GT and they are thought to be the major bioactive components responsible for the biological functions of GT. Catechins can prevent a number of chronic diseases when regularly ingested in the diet, since they present antioxidant, anticarcinogenic, anti-inflammatory, antiatherogenic, antidiabetic, antibacterial and antiviral activities [20–24]. Other proposed health benefits of consuming GT include its protective effect on autoimmune diseases [25], although there is a lack of evidence for the role of GT catechins in lymphocytes from lymph nodes of obese rats.

We hypothesize that supplementation with GT extract in obese rats modulates important functions of lymphocytes, improving antioxidant defense, modulating the redox state, and controlling inflammatory cytokine release. This study aimed to evaluate the effects of supplementation with GT extract on some indicators of oxidative stress and functionality of lymphocytes from rats with obesity induced by cafeteria diet. For this purpose, we evaluated the proliferative capacity of stimulated lymphocytes, inflammatory cytokines, mRNA levels of Nrf2, TLR4, Tbet, foxp3, GATA, SOD, GPx and IRF4, ROS production ($O^{2\bullet}$ and H_2O_2), antioxidant enzyme activities (superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase), oxidative damage in proteins, hexokinase and G6PDH metabolic enzymes.

2. Materials and methods

2.1. Chemicals and natural products

Phorbol myristate acetate, concanavalin A (ConA), lipopolysaccharide (LPS), dihydroethidium (DHE), 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) and most of the other chemicals were purchased from Sigma-Aldrich Chemical Company (St. Louis. MO, USA), except for RPMI-1640 culture medium and fetal bovine serum which were purchased from LifeTechnologies (California, USA). The BrDU kit was purchased from Roche (Mannheim, Germany). Common reagents for buffers (e.g. PBS) and regular laboratory solutions were obtained from Labsynth (Diadema, SP, Brazil). The green tea extract was acquired commercially from Tovani-Benzaquen, São Paulo, SP, Brazil.

2.2. Animals

Adult Wistar male rats, weighing 180 ± 20 g at the beginning of the study, were provided by the Federal University of São Paulo (UNIFESP), São Paulo, Brazil. All animals were housed in plexiglas cages (5 rats/cage), under standard laboratory conditions: 12 h light/dark cycle; lights on at 7:00 a.m.; 22 ± 1 °C; water and Nuvlab rat chow ad libitum. The rats used in this study were handled in accordance with the guidelines of the committee on care and use of laboratory animal resources of the Ethics Committee on Care and Use of Laboratory Animal Resources (CEUA-ICB/USP) of the University of São Paulo, who approved the experiments under protocol no. 160/2013.

2.2.1. Supplementation protocols

The rats were divided into four groups and treated according to the following supplementation program: (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) at 500 mg/kg of body weight was weighed daily and then solubilized 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 90 days (Monday to Friday). All the rats received food (standard rodent chow) and water ad libitum for 4 weeks of supplementation with GT. At this point, the cafeteria diet was offered to the rats for 8 weeks. After this period, the rats were euthanized by decapitation between 12 noon and 2 pm.

2.3. Feeding experiments

Cafeteria diet consisted of four palatable human food items with high energy content — chocolate (25%), condensed milk (12.5%), peanut (12.5%) and corn starch biscuit (12.5%) — plus 37.5% of standard chow. All the items were crushed, mixed and pelletized and subsequently offered to the rats. In this study, 1 g of cafeteria diet provided 4.2 kcal of which 48% were carbohydrates, 14% were protein and 38% were lipids. Standard chow provided 2.88 kcal/g: 55% were carbohydrates, 32% protein and 13% lipids. Cafeteria diet provided a 46% increase in total calories compared with the standard diet.

2.4. Total polyphenols content and HPLC analysis of flavonoids and caffeine concentration in GT extract

Total phenolic content (TPC) was determined by using Folin–Ciocalteu's phenol reagent, gallic acid (99% purity, Sigma) and anhydrous sodium carbonate. TPC was spectrophotometrically determined according to the method described by the International Organization for Standardization (ISO) 14502-1 [26]. The concentration of polyphenols in samples was derived from a standard curve of gallic acid ranging from 10 to 50 μg/ml (Pearson's correlation coefficient: $r^2 = 0.9896$).

Flavonoids (catechins and quercetin) and caffeine were detected in the methanolic extract (50%) of GT powder at concentrations around 1000 μg/ml. The GT extract and standards (EC, EGCG, EGC, ECG, catechin, quercetin and caffeine) were analyzed in an analytical LC (Varian 210) system with a ternary solvent delivery system equipped with an auto-sampler, a photodiode array detector (PDA) monitored at λ = 200–800 nm. The LC column was a C-18 (25 cm × 4.6 mm; particle size, 5 μm; Luna, Phenomenex, Torrance, CA, USA), with a small precolumn (2.5 cm × 3 mm) containing the same packing, used to protect the analytical column. In each analysis, the flow rate and the injected volume were set as 1.0 ml min^{-1} and 20 μl, respectively. All chromatographic analyses were performed at 22 °C. Elution was carried out using formic acid 0.06% in water (solvent A) and methanol (solvent B). Catechins were quantified at 280 nm, quercetin at 250 nm and caffeine at 270 nm by using external standard calibration curves (0.5 to 100 μg/ml for catechins and 0.5 to 20 μg/ml for quercetin and caffeine). The catechins, quercetin and caffeine were identified in green tea extract by comparing their retention time with those of standard solutions.

2.5. GTT and ITT analyses

A week prior euthanasia, the glucose tolerance test (GTT) was performed in all 5 h-fasted rats by assaying blood glucose at various times after i.p. injection of glucose (1.5 g/kg of body weight). Insulin tolerance test (ITT) was also performed in all 5 h-fasted rats by assaying blood glucose at various times after i.p. injection of insulin (Humulin R, Lilly, 1.5 IU/kg of body weight). The tests were performed in different days, one at Monday (GTT) and another (ITT) at Thursday in the same week. Glucose levels were measured in blood collected from the tail at the indicated times after injection, by using Infinity glucose monitors and strips (US Diagnostics).

2.6. Adiposity index and adipose tissues weight

The rats were euthanized by decapitation without anesthesia between 8:00–12:00 a.m. Different white adipose tissue (WAT) depots — retroperitoneal (rpWAT), subcutaneous (scWAT) and epididymal (eWAT) as well as the brown adipose tissue (BAT) were removed and weighed to calculate the adiposity index. Adiposity index was calculated as the sum of all the fat pad depots per animal and expressed per 100 g/body weight.

2.7. Analyses in plasma

Briefly, total blood volume (~5 ml) was collected in EDTA-Vacutainer tubes (5.4 mg K_2EDTA spray-dried, BD Bioscience) and centrifuged for 10 min, 300 × g at room temperature. The clean plasma fraction was then removed and stocked at −80 °C for further analyses. Non-esterified (or free) fatty acids in plasma were measured by an enzymatic colorimetric method using a commercial kit (Cayman Chemical, Ann Arbor, Michigan, USA).

2.8. Experimental procedure

After the euthanasia, mesenteric lymph nodes were dissected and lymphocytes were prepared as previously described [27]. After centrifugation at 1200 rpm for 10 min, lymphocytes were suspended in RPMI 1640 medium and then cultured. The proportions of B- and T-lymphocytes in the cell preparation were 40% and 60%, respectively [28]. The number of viable cells (>95%) was determined in a Neubauer chamber using an optical microscope (NikonYS2-H), following the addition of Trypan blue solution (1% w/v).

2.8.1. Determination of lymphocyte proliferation capacity

We used an ELISA BrdU kit colorimetric method to evaluate the cell proliferation index. BrdU (5-bromo-2′-deoxyuridine — BrdU) can be incorporated into the newly synthesized DNA (during the S phase of the cell cycle), replacing thymidine during DNA replication. A specific antibody for BrdU can then be used to detect the incorporated chemical, thus indicating cells that actively replicate their DNA. Cells (3×10^5 cell/well) were stimulated with concanavalin A (Con A) (20 μg/ml) or lipopolysaccharide (LPS) (10 μg/ml) to stimulate T- and B-cell proliferation, respectively. After 48 h, the medium was removed by centrifugation, cells were fixed and DNA was denatured with a solution for fixation/denaturation present in the kit. Then, anti-BrdU antibody was added to detect the incorporation of BrdU (denaturation of DNA is necessary to improve access to the BrdU incorporated by antibody detection). All other procedures were conducted following the manufacturer's instructions. Optical density was measured at 450 nm (with reference of 690 nm) and the results are expressed as optical density (OD).

2.8.2. Release of cytokines and adipokines

IL-1β, IL-2, IL-6, IL-10, IFN-γ and TNF-α cytokines were assayed in cell culture supernatant with ELISA kits following the manufacturer's instructions (Quantikine, R&D System, Minneapolis, MN, USA). The stimulus used and the culture time for each cytokine was determined previously by our group [29]. Lymphocytes (1×10^6/ml) were cultured for 24 h in the presence of Con A as a stimulus (10 μg/ml) to measure IL-2 release. IL-6, IL-1β and TNF-α were measured in the supernatant of cells stimulated with LPS (80 μg/ml) for 18 h of culture. IFN-γ was measured after PMA (100 ng/ml) and ionomycin (500 ng/ml) stimulus for 24 h of culture. IL-10 was measured in the supernatant of cells stimulated with Con A (10 μg/ml) and LPS (80 μg/ml) for 24 h. After culture, cells were centrifuged (1600 rpm at 4 °C, 10 min) and the supernatant was collected and used for cytokine determination. The results were expressed as amount of each cytokine in pg/ml produced/1×10^6 cells. Leptin and adiponectin levels were quantified by ELISA (Abcam, Cambridge, UK) following the manufacturer's instructions.

2.8.3. Dihydroethidium assay

Dihydroethidium (DHE) is a florescence probe and was used to measure intracellular superoxide anion production. Once inside the cell, DHE is rapidly oxidized to ethidium (a red fluorescent compound) by superoxide with minor collaboration of other ROS. Lymph nodes lymphocytes (5×10^5/well) were incubated with 5 μM DHE for 15 min at room temperature in the dark. At the beginning of the assay, control cells were stimulated with PMA (20 ng/well). Cells were incubated in the dark at room temperature for an additional 30 min. Next, fluorescence was analyzed in a microplate reader (Tecan, Salzburg, Austria) using wavelengths of excitation and emission of 396 and 590 nm, respectively.

2.8.4. DCFH-DA assay

The probe DCFH-DA was primarily used as an indicator of hydrogen peroxide (H_2O_2) production (Keston and Brandt 1965), but is also described as being oxidized by other ROS, such as the HO• radical, ROO• radical, NO and peroxynitrite (Crow 1997, Wang and Joseph 1999). The cells (5×10^5/well) were preloaded with DCFH-DA (5 μM) by incubation in culture medium for 30 min. DCFH-DA is cleaved intracellularly by nonspecific esterase and turns into high fluorescent 2,7-dichlorofluoroscein (DCF) upon oxidation by ROS. The experiments were conducted in the presence and absence of PMA (20 ng/well). After the culture period, cells were centrifuged and resuspended in 300 μl of Tyrode's buffer and the fluorescence was monitored using a Tecan spectrofluorometer (Salzburg, Austria) with excitation at 485 nm and emission at 530 nm. The results of this experiment were expressed as relative units of fluorescence.

2.8.5. Preparation of homogenates

To analyze enzyme activity, oxidative lesions in biomolecules and glutathione content cells were pelletized (5×10^6) and mixed with 0.6 ml of the assay-specific extraction buffer and ruptured by ultrasonication in a Vibra Cell apparatus (Connecticut, USA), then centrifuged for 10 min at 10.000 g and 4 °C. The supernatant was used for further analysis.

2.8.6. Lymphocyte antioxidant and metabolic enzyme activity

Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) activities were determined in lymphocytes using a microplate reader (Tecan, Salzburg, Austria). SOD activity was measured using the method described by Ewing and Janero [30] which involves the reduction of $O_2^{\bullet -}$ radicals by nitroblue tetrazolium (NBT) following linear first-order kinetics for 3 min. CAT activity was measured as described by Aebi [31] based on the direct decomposition of H_2O_2. Glutathione peroxidase and glutathione reductase [32,33] activities were measured based on the oxidation of β-NADPH in the presence of tert-butyl hydroperoxide, used as substrate.

The maximum hexokinase activity (an enzyme that catalyzes the phosphorylation of glucose in the first reaction of glycolysis) was determined according to the method described by Crabtree and Newsholme [34]. In a 96-well plate, 284 μl of assay buffer, 17 μl of sample and 37 μl of glucose were mixed. 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) 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 some cellular needs for reductive biosynthesis and contributing to the maintenance of the cellular redox state. The maximum activity of this enzyme was previously described by Guerra and Otton [35] and was based on the conversion of $NADP^+$ in NADPH in the presence of glucose-6-phosphate.

2.8.7. GSH, GSSG content

Reduced (GSH) and oxidized (GSSG) glutathione content in lymphocytes were measured as described by Rahman, Biswas [36]. The method is based on the reaction between reduced thiol groups (such as 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.

2.8.8. Oxidative damages

Thiol and carbonyl groups were evaluated as biomarkers of amino acid oxidation in total protein fractions, which were isolated from the crude homogenate of cells (5×10^6) by precipitation with 20% trichloroacetic acid solution in ice. Reduced thiol groups were detected by the formation of colored adducts after reaction with 4 mM 5.5′-dithio-bis (2-nitrobenzoic acid) solution (DTNB). The absorbance of DTNB-treated samples at 412 nm was calculated using GSH as standard [85,86]. The same procedure was used to estimate protein carbonyls. The protein carbonyls were identified by the hydrazones formed with 10 mM dinitrophenylhydrazine (DNPH) in 0.25 M HCl. Absorbance of the peak detected within the range of 340–380 nm was measured, and the carbonyl group concentration was calculated based on the molar coefficient of $\varepsilon = 2.2 \times 10^4\ M^{-1}\ cm^{-1}$ [86].

2.8.9. RT-PCR

Total RNA was isolated from lymphocytes (1×10^7) using Trizol Reagent (Life Technologies, Rockville, MD, USA) following the manufacturer's instruction. Isolated RNA was quantified using the NanoDrop ND-1000 spectrophotometer (NadroDrop Technologies, Wilmington, DE) and its integrity was confirmed using agarose gel electrophoresis.

Reverse Transcriptase (RT)-PCR was used to measure mRNA expression levels of nuclear factor erythroid 2 (Nrf2), Toll-like receptor 4 (TLR4), glutathione peroxidase 1 (GPx-1), superoxide dismutase 2 (SOD2), interferon regulatory factor 4 (IRF4), forkhead box P3 (foxp3), GATA binding protein 3 (GATA3) and T-box expressed in T cells (T-bet). Table 1 presents the nucleotide sequence used in this study.

Total RNA (2 μg) was treated with 1 U DNase for 25 min at 25 °C and inactivated with 2.5 mM EDTA. Next, the cDNA was synthesized 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 reaction 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) and 2.5 U of Taq DNA polymerase (Sigma) and 2 μl of cDNA. The RT and PCR reactions were carried out in a Veriti Gradient equipment (Applied Biosystems, Carlsbad, CA, USA), using parameters described by Innis and Gelfand (1990). For semi-quantitative PCR analysis, the housekeeping β-actin gene was used as reference. RT-PCR endpoint products were analyzed following agarose gel (1.3%) electrophoresis using blue green loading dye (LGC Biotecnologia, SP, Brazil) and the Image J processing program.

Table 1
Nucleotide sequences of primers and cycling conditions used for RT-PCR amplification. β-Actin, Nuclear factor erythroid 2 (Nrf_2), Toll-like receptor 4 (TLR4), glutathione peroxidase 1 (GPx-1), superoxide dismutase 2 (SOD2), interferon regulatory factor 4 (IRF4), forkhead box P3 (foxp3), GATA binding protein 3 (GATA3) and T-box expressed in T cells (T-bet).

Gene	Nucleotide sequence	Temperature	$MgCl_2$
Nrf2	Forward 5′- CCT CTG TCA CCA GCT CAA GG -3′ Reverse 5′- TGG GCG GCG ACT TTA TTC TT -3′	64 °C	2 mM
TLR4	Forward 5′- TGG CAG TTT CTG AGT AGC CG -3′ Reverse 5′- TGC TAC TTC CTT GTG CCC TG -3′	64 °C	2.5 mM
GPx1	Forward 5′- GTA CAT CAT TTG GTC CCC GGT -3′ Reverse 5′- GCC ATC ACC AAG CCC AGA TA -3′	67 °C	2.5 mM
SOD2	Forward 5′- ACG TGA ACA ATC TCA ACG CC -3′ Reverse 5′- GTC ACG CTT GAT AGC CTC CA -3′	65 °C	2.5 mM
IRF4	Forward 5′- ACA AGA GCA ATG ACT TTG AGG AA -3′ Reverse 5′- GTT ATG AAC CTG CTG GGC TG -3′	64 °C	2 mM
Foxp3	Forward 5′- GGC AGC TAG GTA CTT GTA GGC -3′ Reverse 5′- CTT TAG GTG CAC TGT TGC TTG G -3′	58 °C	2 mM
GATA3	Forward 5′- ACT CTC GAG GCA GCA TGA C -3′ Reverse 5′- GTT CAC ACA CTC CCT GCC TT -3′	62 °C	1.5 mM
T-bet	Forward 5′- TGA TAA GGA AAC CGA AGG CCA -3′ Reverse 5′- TGG GCC AGG AAA CAA GTG AA -3′	62 °C	2 mM
B-actin	Forward 5′- CCA CCA TGT ACC CAG GCA TT -3′ Reverse 5′- ACG CAG CTC AGT AAC AGT CC -3′	64 °C	2 mM

2.9. Protein determination

The total protein content of lymphocytes was measured by the method of Bradford [37], using BSA as standard.

3. Statistical analyses

The results are presented as mean ± SEM. The Shapiro–Wilk and Levene tests were used to verify the normality and variance of the data, respectively. When Shapiro–Wilk or Levene tests were $P > 0.05$, interaction was evaluated through a factorial two-way ANOVA (diet, D × Green tea, GT) using the Tukey as post-test ($P < 0.05$). When 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 statistical package, version 22 (SPSS Inc., Chicago, IL), and the GraphPad Prism statistics software package, version 5.0, for Windows (GraphPad Software, San Diego, CA, USA).

4. Results

4.1. Total polyphenol content and HPLC analysis of flavonoids and caffeine concentration in GT extract

The total content of polyphenols present in our GT extract was 39% (392 μg/mg of GT extract) as obtained by Folin–Ciocalteu assay. Among polyphenols catechins and quercetin content represented 30% (117 μg/mg) of total polyphenols as revealed by HPLC analysis. Our GT extract was a mixture of several catechins such EGCG, EC, ECG and EGC, among these the sum of EGC and EGCG contributed with more than 85% of the catechin mixture in the extract. The content of caffeine and quercetin in extract was 0.4% and 1.5%, respectively by dry weight.

4.2. Reduction of body weight gain and protection against glucose intolerance by green tea

Body weight gain and fat pad size were measured and are present in Table 2. There was an interactive effect between diet and GT (D × GT, $P < 0.05$) in body weight gain. The body weight of the rats fed with cafeteria diet and treated with GT was reduced by 39% (versus OB group). There was an effect of diet and GT in epididymal, subcutaneous and retroperitoneal fat pad weights that were decreased in animals treated with GT ($P < 0.001$, $P < 0.001$ and $P < 0.01$, respectively, two-way ANOVA) while cafeteria diet increased all fat pad weight/size ($P < 0.001$, Diet effect). For BAT adipose tissue size there was a diet effect ($P < 0.001$, two-way ANOVA). Accordingly, adiposity index were significantly lowest in both groups of animals supplemented with GT regardless of diet ($P < 0.001$, GT effect). We observed a significant increase in free fatty acids concentration in plasma of obese rats whereas the obese group supplemented with GT (OB + GT) showed a significant decrease (interactive effect between D and GT, $P < 0.001$, two-way ANOVA). Plasma leptin level was increased (48%) in rats fed with

Table 2
Reduction of body weight gain by green tea. Body weight gain (g), adiposity index (g/100 g), eWAT weight (g/100 g), scWAT weight (g/100 g), rpWAT weight (g/100 g), BAT weight (g/100 g), free fatty acids (μM), plasma leptin level (ng/ml) and plasma adiponectin level (μg/ml). Data are presented as mean ± SEM of 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.

	Control	GT	OB	OB + GT	ANOVA 2 × 2		
					D	GT	D × GT
Body weight gain (g)	203.5 ± 11.5	155.2 ± 11.8$^{(a)}$	269.2 ± 15.5$^{(a)}$	164.4 ± 6.5$^{(c)}$			*
Adiposity index (g/100 g)	3.44 ± 0.35	2.47 ± 0.14	5.33 ± 0.30	3.44 ± 0.26	***	***	
eWAT (g/100 g)	1.48 ± 0.05	1.07 ± 0.15	1.95 ± 0.12	1.33 ± 0.08	**	***	
scWAT (g/100 g)	0.84 ± 0.06	0.54 ± 0.05	1.47 ± 0.13	0.87 ± 0.11	***	***	
rpWAT (g/100 g)	1.06 ± 0.06	0.94 ± 0.07	1.65 ± 0.10	1.28 ± 0.08	***	**	
BAT (g/100 g)	0.09 ± 0.01	0.10 ± 0.01	0.15 ± 0.01$^{(a)}$	0.14 ± 0.01	***		
Free fatty acids (μM)	236.4 ± 25.1	261.9 ± 17.1	333.1 ± 10.3$^{(a)}$	197.6 ± 17.4$^{(c)}$			***
Leptin (ng/ml)	3098 ± 115.0	3008 ± 238.8	4599 ± 214. 7	4648 ± 200.9	***		
Adiponectin (μg/ml)	2745 ± 357.1	2777 ± 293.2	5121 ± 57.01$^{(a)}$	7154 ± 98.68$^{(c)}$			***

cafeteria diet (versus control group, P < 0.001, two-way ANOVA) (Table 2) whereas adiponectin levels there was an interactive effect between D × GT (P < 0.001, two-way ANOVA) compared to the control group. Regarding OB + GT group there was a significant increase (53%) in the secretion of the adiponectin as compared with the OB group.

To determine whether cafeteria diet could induce glucose intolerance in animals, we measured the glycemia after i.p. injection of glucose or insulin. In Fig. 1A an interactive effect between diet and GT (P < 0.001, two-way ANOVA) can be observed. Animals that received cafeteria diet showed an induction of glucose intolerance as observed by the increased in glycemia levels after i.p. injection of glucose or insulin (Fig. 1A–B). Green tea was able to restore glucose intolerance in obese animals (OB + GT) (P < 0.01, two-way ANOVA).

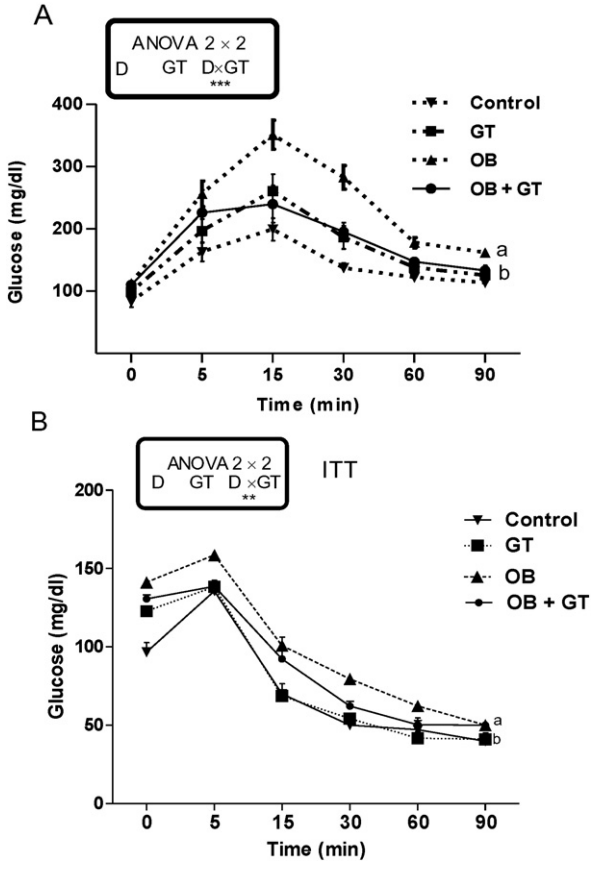

Fig. 1. Green tea protects against glucose intolerance. A) Glucose tolerance test (GTT); B) insulin tolerance test (ITT). Data are presented as mean ± SEM of 10 rats per group. An ANOVA 2 × 2 factorial design was used to study the effects of diet (D), green tea (GT) and potential interaction between D × GT. When interaction was statistically significant superscript letters designate: a = statistical difference compared to the control group, b = compared to the green tea group, c = compared to the obese group. * p < 0.05, ** p < 0.01, *** p < 0.001.

4.3. Anti-proliferative and anti-inflammatory actions of green tea

We evaluated the proliferative capacity of lymphocytes, since this function is essential for the effectiveness of the activities of these immune cells following the recognition of antigens and the activation of TCR/BCR receptors. At baseline (no mitogenic stimulation), an interactive effect was verified between diet and GT (P < 0.05, 2 × 2 ANOVA). Obese rats (OB) showed a significant increase (27%) in proliferative capacity and the OB + GT group showed a reduction in the proliferative capacity (34%) compared with the control group (Fig. 2A).

In the presence of mitogens (Con A and LPS), the obese group (OB) showed no significant change in cell proliferation. In contrast, treatment with GT reduced the proliferative capacity of these cells. Following Con A-stimulation, GT treatment decreased T-lymphocyte proliferation (P < 0.001, GT effect). Similar results were observed under LPS-stimulation, since GT treatment also reduced the proliferation of B lymphocytes (P < 0.01, GT effect) (Fig. 2A).

For IL-2 cytokine release (Fig. 2B), an interactive effect was observed between diet and GT (D × GT, P < 0.001, 2 × 2 ANOVA). GT treatment only in obese rats (OB + GT) reduced IL-2 release by 43% compared with the obese group. Rats treated with GT fed with either the standard or cafeteria diet showed a significant decrease in IL-1β production (P < 0.01, GT main effect) (Fig. 2C). IL-6 production was significantly reduced after treatment with GT regardless of diet (P < 0.01, GT effect) (Fig. 2D). TNF-α release was also reduced after GT treatment (P < 0.001, GT main effect) in both groups that received green tea (Fig. 2E). IFN-γ production (Fig. 2F) did not change among the groups. IL-10 production (Fig. 2G) showed an interactive effect between diet and GT (D × GT, P < 0.05, 2 × 2 ANOVA). Obese rats (OB group) showed a 43% decrease in IL-10 cytokine production compared with the control group, whereas treatment of obese rats with GT (OB + GT) increased IL-10 production by 64%.

4.4. Decreased ROS production and modulation of antioxidant and metabolic enzymes by GT supplementation

In this study, ROS production was assessed by using DCFH-DA and DHE fluorescent probes. Assays were performed at baseline or after PMA stimulation. An interactive effect between diet and GT was observed in the DHA assay, both with and without PMA stimulation (D × GT, P < 0.001, 2 × 2 ANOVA) (Fig. 3A). Obese rats (OB) showed a significant increase (~45%) in ROS production compared with the control group. Treatment with GT (OB + GT) reduced ROS production compared with the OB group.

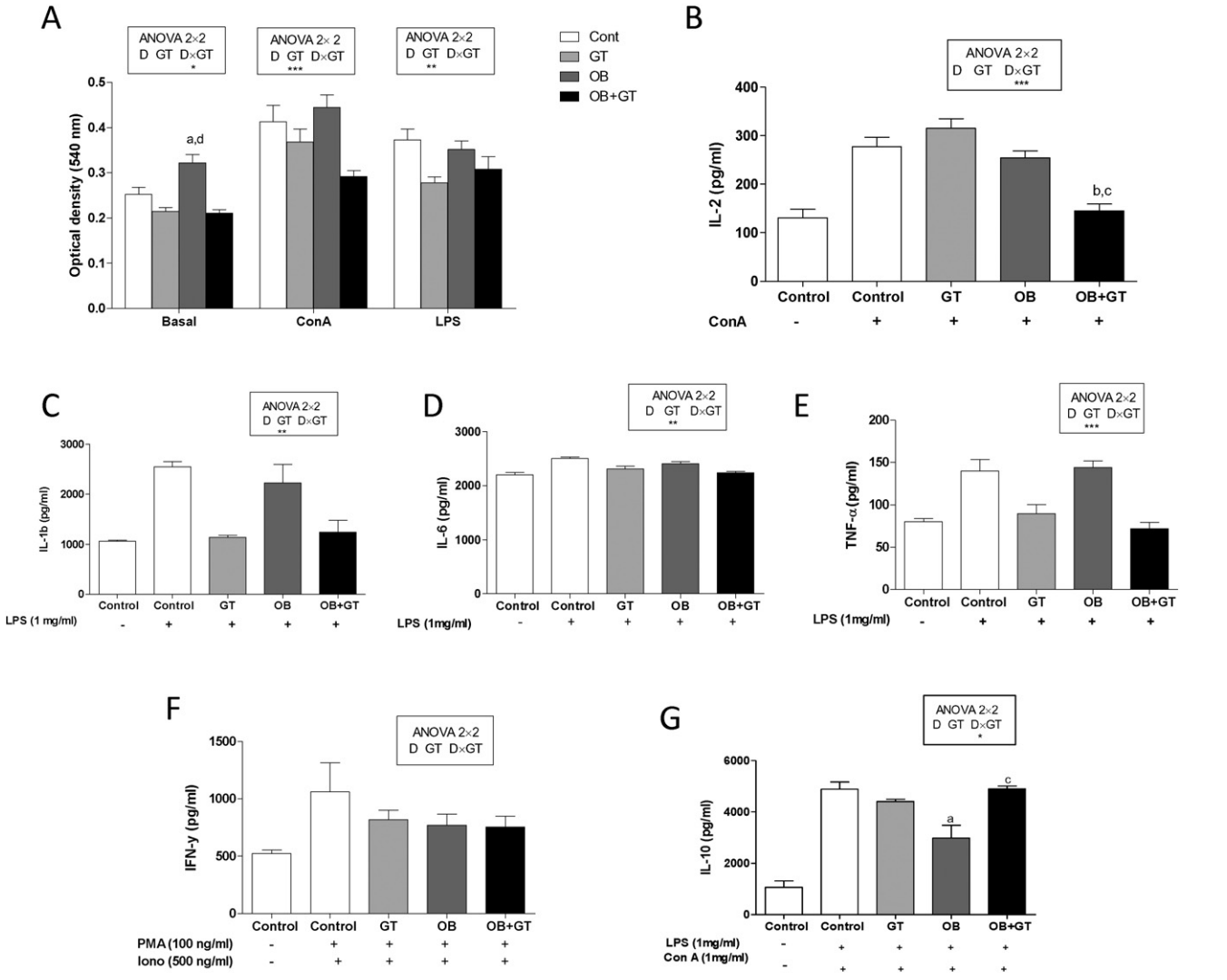

Fig. 2. Anti-proliferative and anti-inflammatory actions of green tea. A) Lymphocyte proliferation conducted by BrdU incorporation in cells cultured for 48 h at basal or under mitogen Con A (1 μg/ml) and LPS (2 μg/ml) stimulation. B) IL-2 release. C) IL-1β release. D) IL-6 release. E) TNF-α release. F) INF-γ release. G) IL-10 release. The results are presented as mean ± SEM of 10 rats performed in triplicate. An ANOVA 2 × 2 factorial design was used to study the effects of diet (D), green tea (GT) and the potential interaction between them (D×GT). When the 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. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Analysis of ROS production using a DCFH-DA probe (Fig. 3B) revealed an interactive effect between diet and GT (D × GT, $P < 0.05$, 2 × 2 ANOVA). Lymphocytes from OB rats showed increased ROS production compared with those from control rats, both without (41%) and with (34%) PMA stimulation. Lymphocytes from obese rats treated with GT (OB + GT) showed reduced ROS production (32%) compared with the obese group (OB).

To determine whether the changes observed in ROS production due to the obese condition and GT treatment modulate the antioxidant status of cells, we evaluated antioxidant enzyme activities. GT treatment (GT main effect, $P < 0.05$) increased MnSOD activity by 95% compared with the control group (Fig. 3C). An interactive effect between D × GT ($P < 0.001$, 2 × 2 ANOVA) promoted an increase in CuZnSOD activity in all the groups studied. CuZn SOD (Fig. 3D) increased by 82% in the OB group compared with the control group. The GT group showed a 46% increase in CuZnSOD activity compared with the control group. In contrast, OB + GT group showed a significant decrease of 32% compared with the OB group.

No change in catalase activity was verified in any of the groups studied (Fig. 3E), however we observed an interactive effect between diet and GT (D × GT, $P < 0.001$, 2 × 2 ANOVA) in GPx activity. Treatment of obese (animals) rats with GT (OB + GT) significantly increase (2-fold) GPx activity compared with the obese group (OB) (Fig. 3F). GR activity independently increased due to diet and GT treatment (Diet main effect and GT main effect, $P < 0.001$, 2 × 2 ANOVA) (Fig. 3G).

In our study, the content of reduced glutathione (GSH) and oxidized glutathione (GSSG) showed no significant differences among the groups (Table 3). However, for the GSH/GSSG ratio, an interactive effect was observed between diet and GT (D × GT, $P < 0.05$, 2 × 2 ANOVA). The ratio of reduced to oxidized glutathione decreased in the OB group compared with the control, while in the OB + GT group, it increased compared with the OB group (Table 3).

The evaluation of oxidative protein damage showed that neither the obese condition nor the treatment with GT altered the content of thiols (Table 3). However, an interactive effect was observed between diet and GT (D × GT, $P < 0.001$, 2 × 2 ANOVA) in the content of carbonyl formation. Lymphocytes from obese rats showed a 33% increase in carbonyl content compared with the control group. On the other hand, GT treatment of obese rats (OB + GT) restored the carbonyl content to control values (Table 3).

An interactive effect was verified between diet and GT (D × GT, $P < 0.01$, 2 × 2 ANOVA) with regard to hexokinase activity (Table 3).

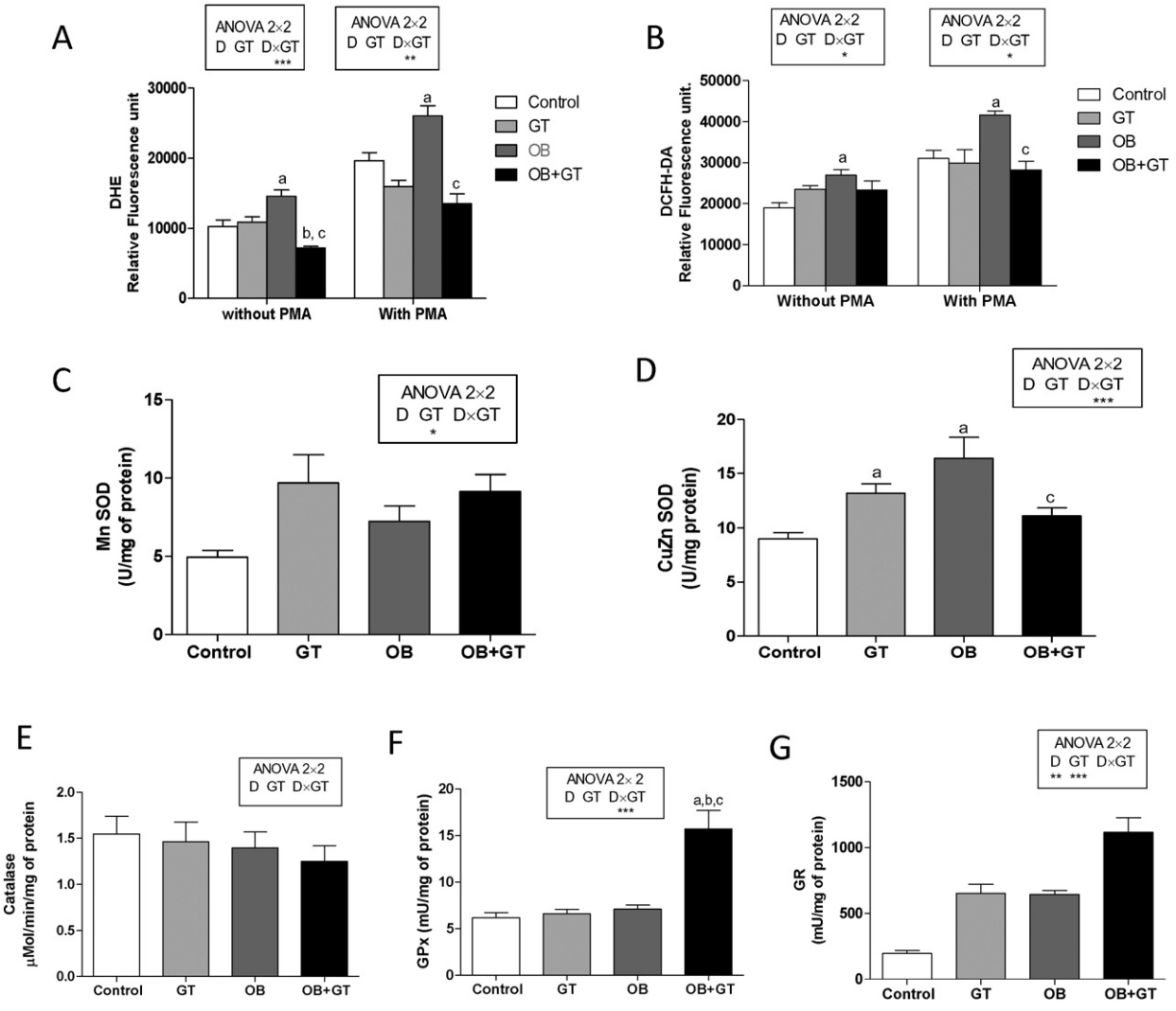

Fig. 3. A) Superoxide anion production assayed by DHE. B) ROS production assayed by DCFH-DA. C) Mn SOD activity. D) Cu/Zn SOD activity. E) Catalase activity. F) GPx activity. G) GR activity. The results are presented as mean ± SEM of 10 rats performed in triplicate. An ANOVA 2 × 2 factorial design was used to study the effects of diet (D), green tea (GT) and the potential interaction between them (D×GT). When the 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. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

The obese condition promoted a 50% increase in hexokinase compared with the control group. OB + GT lymphocytes showed a 21% decrease in the activity of this enzyme compared with the OB group. G6PDH activity (Table 3) was significantly decreased following GT treatment ($P < 0.001$, GT main effect, 2 × 2 ANOVA), while the obese condition did not modify G6PDH activity.

4.5. Gene expression modulation by green tea treatment

In order to determine what mechanisms of GT could be involved in the modulation of lymphocyte proliferative capacity, cytokine release and ROS production, we evaluated the gene expressions of T-bet and GATA-3, transcriptions factors that are important for differentiating

Table 3
Oxidative parameters of lymphocytes and metabolic enzymes. The results are presented as mean ± SEM of the optical density of 10 rats. An ANOVA 2 × 2 factorial design was used to study the effects of diet (D), green tea (GT) and the potential interaction between them (D×GT). When the 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. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

	Control	GT	OB	OB + GT	ANOVA 2 × 2		
					D	GT	D × GT
GSH (µmol·mg protein^{-1})	0.25 ± 0.03	0.21 ± 0.01	0.19 ± 0.01	0.25 ± 0.02			
GSSG (µmol·mg protein^{-1})	0.07 ± 0.01	0.07 ± 0.01	0.09 ± 0.02	0.07 ± 0.01			
GSH/GSSG ratio	3.57 ± 0.60	3.00 ± 0.27	2.11 ± 0.55 $^{(a)}$	3.57 ± 0.32$^{(c)}$			*
Thiol (nmol of thiol groups·mg protein^{-1})	77.28 ± 4.95	82.93 ± 3.25	86.79 ± 4.91	76.98 ± 5.77			
Carbonyl (nmol of carbonyl groups·mg protein^{-1})	38.33 ± 1.35	42.46 ± 2.25	51.12 ± 1.94$^{(a)}$	35.53 ± 1.05$^{(c)}$			***
Hexokinase (nmol·min^{-1}·mg protein^{-1})	2.74 ± 0.17	3.05 ± 0.12	4.13 ± 0.26$^{(a)}$	3.25 ± 0.31$^{(c)}$			**
G6PDH (nmol·min^{-1}·mg protein^{-1})	4.32 ± 0.08	3.00 ± 0.21	4.39 ± 0.40	2.25 ± 0.17		***	

lymphocytes into Th1 and Th2 subtypes, respectively. However, neither diet nor GT treatment modulated the mRNA levels of T-bet and GATA-3 transcription factors (Fig. 4A, B). We then evaluated the foxp3 mRNA levels, since this factor is important for driving the differentiation of T-regulatory lymphocytes. GT treatment increased mRNA levels of foxp3 (GT main effect, $P < 0.05$) compared with the control group (Fig. 4C), whereas under the obese condition foxp3 expression remained unchanged.

An interactive effect was observed between diet and GT regarding the mRNA levels of IRF4 transcription factor ($P < 0.001$, 2 × 2 ANOVA). GT treatment and the obese condition increased mRNA levels of IRF4 by 88% and 71%, respectively, compared with the control group (Fig. 4D).

An interactive effect was also observed between diet and GT ($P < 0.05\%$, 2 × 2 ANOVA) when we evaluated TLR4 mRNA levels (Fig. 5A). GT treatment decreased (26%) TLR4 mRNA levels compared with the control group. Finally, we evaluated the mRNA levels of SOD2 and GPx antioxidant enzymes as well as Nrf2 transcription factor. The data showed an interactive effect between diet and GT ($P < 0.05$, 2 × 2 ANOVA) that influenced the mRNA levels of GPx. In the OB + GT group, an increase (45%) in mRNA level of GPx was verified compared with the control group (Fig. 5 B). In contrast, mRNA levels of SOD2 did not change significantly among the groups (Fig. 5C). mRNA levels of Nrf2 presented an interactive effect between diet and GT ($P < 0.05$, 2 × 2 ANOVA). The mRNA levels of the OB + GT group increased (34%) compared with the OB group (Fig. 5D).

5. Discussion

Our cafeteria diet was effective at inducing obesity, as demonstrated by the increase in body weight gain of the rats after 2 months of feeding (Table 2). All fat depots (epididymal, subcutaneous and retroperitoneal) were increased, together with the adiposity index. Insulin resistance and glucose intolerance were confirmed by the ITT (insulin tolerance test) and GTT (glucose tolerance test) (Fig. 1 A, B). Free fatty acids, leptin and adiponectin plasma concentrations were increased in obese rats (Table 2). Taken together, these results confirmed the obese condition induced by the cafeteria diet. GT treatment decreased body weight in both the standard chow (lean) and cafeteria diet (obese) fed rats and all the obesity-related parameters of the obese rats. In obese animals, GT treatment did not restore leptin levels, which remained increased (Table 2). These data obtained by our group demonstrate that GT was effective at reducing free fatty acids and increasing adiponectin, restoring GTT and ITT responses, which in turn correlate positively with the weight loss and increased insulin sensitivity observed in our obese rats (Table 2 and Fig. 1A, B). The effects of GT are corroborated by data in the literature that demonstrate anti-obesity actions of GT, including its thermogenic actions among others [38–40].

Concerning the immune system, there is considerable evidence in the literature indicating that excessive body fat promotes a negative impact on the immune function of obese individuals and in animal models [41–44]. Moreover, it is well known that alterations in the immune function in obese individuals are considered to be significant in the development of pathophysiological effects of obesity. However, a direct mechanism responsible for this impairment in the immune response in obese individuals has yet to be reported [45]. It was shown that hyperleptinemia can cause an increase in T-cell proliferation by modulating the expression of activation markers on CD4 and CD8 cells [46–48]. In addition, leptin may have a negative impact on the distribution and function of regulatory T-cells, which could result in uncontrolled release of inflammatory cytokines and ROS by effector immune cells [49,50].

This study demonstrated that cell proliferation was increased in obese rats at baseline compared with the control group. This increase in proliferation could indicate that the lymphocytes of the obese rats were in a preactivation state, probably due to direct contact with molecules, such as free fatty acids and/or leptin, or increased nutrient availability, which could lead to cell activation/proliferation. However, in the presence of mitogens ConA and LPS, obese rats (OB) showed no significant differences in cell proliferation (Fig. 2). In contrast, treatment of obese rats with GT reduced the proliferative capacity of lymphocytes both under basal conditions and after ConA stimulation, while under LPS stimulation, we also observed a reduction in cell proliferation in lymphocytes from lean rats. These results demonstrate that GT

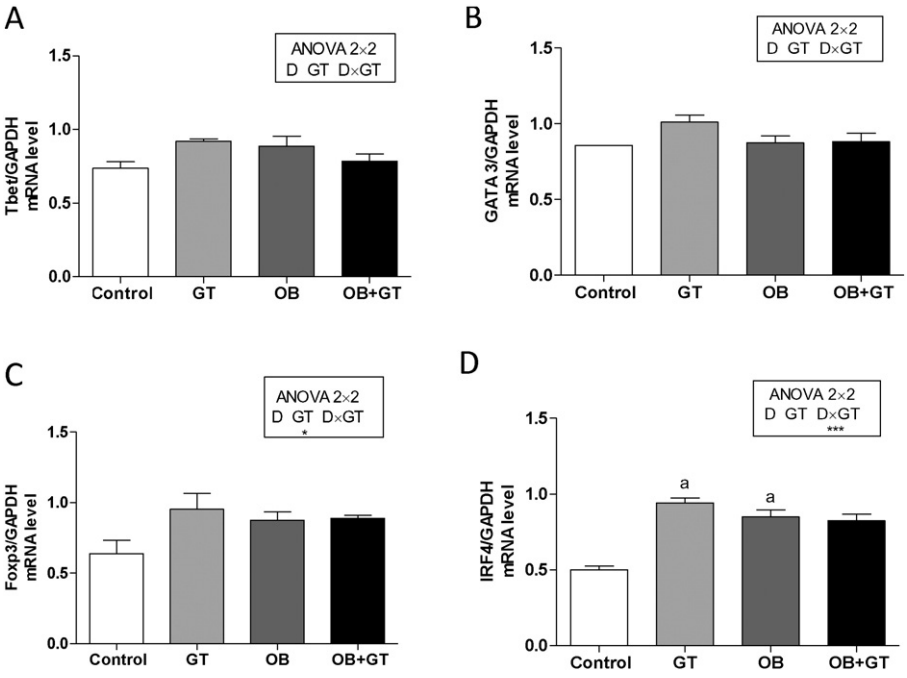

Fig. 4. A) Tbet mRNA level. B) GATA 3 mRNA level. C) Foxp3 mRNA level. D) IRF4 mRNA level. The results are presented as mean ± SEM of 6 rats. An ANOVA 2 × 2 factorial design was used to study the effects of diet (D), green tea (GT) and the potential interaction between them (D × GT). When the interaction 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.* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

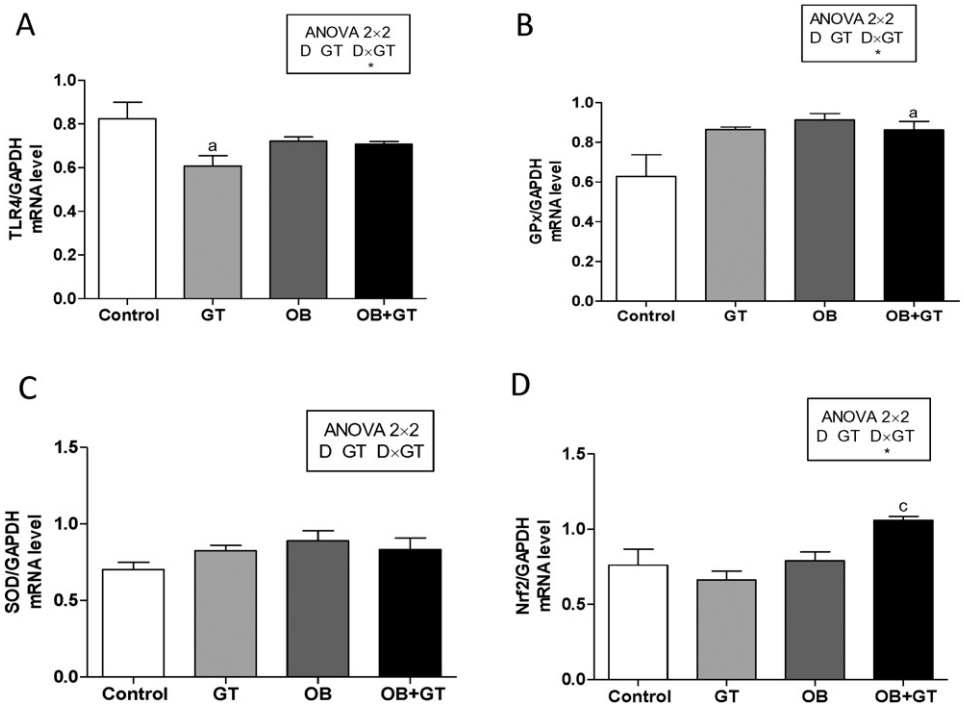

Fig. 5. A) TLR4 mRNA level. B) GPx mRNA level. C) SOD2 mRNA level. D) Nrf2 mRNA level. The results are presented as mean ± SEM of 6 rats. An ANOVA 2 × 2 factorial design was used to study the effects of diet (D), green tea (GT) and the potential interaction between them (D × GT). When the interaction 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. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

treatment modulates lymphocyte proliferation, regardless of diet. The antiproliferative action of GT catechins has been described by several authors. Hu et al. showed that EGCG inhibited the proliferation of both B- and T-cells, with a greater effect observed for T-cells [51]. Other studies have reported that supplementation with GT extract inhibited the proliferation of lymphocytes stimulated with ConA and allogeneic cell stimulators [52,53]. According to Pae, Ren [54], supplementation for 6 weeks with EGCG was effective at reducing the ex vivo proliferation of T-cells when stimulated with ConA or anti-CD3/CD28.

IL-2, a cytokine secreted by T-lymphocytes upon stimulation, is an important regulator of the proliferation, differentiation and metabolism of lymphocytes. When IL-2 binds to the cell surface receptor (IL-2R), T-lymphocytes undergo activation [55], promoting early cell proliferation and transition from G1- to the S-phase of the cell cycle [56]. These findings were partially validated in our study, since the production of IL-2 was decreased in the OB + GT group accompanied by the reduced proliferation of ConA-stimulated cells (Fig. 2A–B). According to Wu, Guo [57], treatment with EGCG (0.5 to 10 μM) in mice lymphocytes stopped IL-2/IL-2R signaling by decreasing the expression of IL-2R and hence the ability to suppress the proliferation of these cells. Our group also measured the content of polyphenols in the GT extract by HPLC and confirmed that EGCG and EGC represent 80% of the catechins in green tea.

A GT main effect was also observed when we evaluated ex vivo pro-inflammatory cytokine release under LPS stimulation. IL1β, IL-6 and TNF-α were decreased in lean and obese rats treated with GT (Fig. 2C–E). The inhibition of the TLR4 signaling pathway by GT, indicated by reduced mRNA levels of TLR4 (Fig. 5A), may explain the observed reduction in pro-inflammatory cytokines. LPS acts an agonist of the TLR4 pathway. Several authors have reported that GT can decrease the interaction and recognition of LPS by the CD14 molecule, besides impairing the activation of TLR4 signaling [58]. The effect of GT is directly related to the interaction of the EGCG catechin with the 67 kDa laminin membrane receptor. One μM of EGCG decreased the cell surface expression and protein concentration of TLR, while up-regulating the expression of the inhibitory protein Tollip (toll interacting protein) [58]. TLR4 activation, in turn, mediates the MAPK and NFκB signaling pathways culminating in inflammatory mediator production, whereas Tollip protein negatively regulates TLR4 signaling, suppressing the kinase activity of IRAK (interleukin-1 receptor-associated kinase) involved in this signaling pathway [59]. These findings support the data that GT catechins, including EGCG, suppress the TLR4 signaling pathway through the laminin receptor, thereby decreasing the production of inflammatory mediators. In this study, although we did not evaluate the expression of laminin receptor, NFκB activation and Tollip expression reinforce our findings.

Concerning cytokine production, we verified that anti-inflammatory IL-10 production was decreased in OB rats compared with the control group, whereas GT treatment of obese rats (OB + GT group) restored IL-10 production (Fig. 2G). Modulation of cytokine production triggered by GT has been reported by several authors [25,60–62]. We speculate that these results are related to the increase in plasma adiponectin levels observed in obese rats treated with GT (Table 2). Increased adiponectin levels could promote suppression of pro-inflammatory cytokines, as well as being responsible for increased anti-inflammatory IL-10 release, as previously described [63,64]. Another hypothesis is based on the ability of EGCG to modulate the differentiation of CD4 T-cells into effector T-cells. According to Wang, Pae [65], EGCG inhibited the differentiation of CD4 T-cells into effector Th1, Th9 and Th17 subtypes. However, they showed that EGCG did not directly affect the differentiation of Treg cells; instead it seems that EGCG antagonizes the suppressive effect of IL-6 in Treg-cell differentiation. In addition, IL-6 is an important differentiation factor for Th17 cells. Indeed, increased IL-6 levels increase the Th17/T-reg ratio [66,67]. In our study, IL-6 was decreased in obese rats treated with GT, while IL-10 was increased in the same group.

The ability of GT in modulating the differentiation of lymphocytes could be through triggering the activation of mammalian target of rapamycin (mTOR). mTOR is a highly conserved serine/threonine kinase that plays a key role in cell growth, proliferation, and survival of many cells, including the lymphocytes [68,69]. mTOR regulates cell growth in response to growth factors, nutrients, and energy [68]. The

mTOR activation stimulates specific metabolic ways, including glycolic, lipid synthesis, mitochondrial activity and it has an important way on lymphocytes differentiation. The cell activation dependent on the recognition of antigens presented by APCs, integrates TCR signaling pathway/PI3K/Akt/mTOR pathway. Therefore, mTOR triggers a change in metabolic cell with increased glycolytic activity and protein synthesis necessary for T cell activation, proliferation and differentiation of lymphocytes into effectors subtypes such as Th1, Th2 and Th17 [69]. According to Van Aller, Carson [70] EGCG can be considered a potent inhibitor of PI3K and mTOR signaling pathway. According to these authors EGCG is an ATP competitive inhibitor of PI3K and mTOR exerting its inhibition in PI3K and mTOR at physiological relevant concentrations (0.8 uM–1.6 mM). Consequently, the EGCG inhibition on PI3K and mTOR signaling pathway in lymphocytes could cause a decrease in glycolytic activity, protein synthesis and differentiation into Th1 subtypes, Th2 and Th17 with an increase in the population of Treg cells. If mTOR is inhibited in lymphocytes from our animals remains to be evaluated.

To reinforce these data, we measured the mRNA levels of the main transcription factors involved in the differentiation of T-cell subtypes. Our data corroborate a more anti-inflammatory microenvironment in rats, promoted by GT treatment, since there was no difference in Tbet and GATA3 mRNA levels, whereas we observed an increase in foxp3 and IRF4 mRNA levels (Fig. 4A–D). As a member of the IRF family of transcription factors, IRF4 (interferon regulatory factor 4) is expressed in most cell types of the immune system. Recent findings suggest that IRF4 is essential for the development and function of T helper cells (Th), regulatory T (Treg) cells, B-cells and dendritic cells (DC) [71]. A report by Zheng, Chaudhry [72] showed that in mouse Treg cells, high amounts of IRF4 is dependent on Foxp3 expression and IRF4 endows Treg cells with the ability to suppress Th2 responses. These data suggest that GT treatment may be driving lymphocytes to a more anti-inflammatory phenotype than a pro-inflammatory phenotype. This GT effect can be valuable in the obese condition, in which the prevalence of Th1 pro-inflammatory cells occurs in detriment to Th2 anti-inflammatory lymphocytes accompanied by a decrease in antioxidant defense. Taken together, these factors contribute to the framework of oxidative stress and low-grade inflammation characteristic of obesity [73].

To verify the involvement of ROS and redox status on the effects promoted by cafeteria diet and GT treatment, we measured total ROS production and antioxidant enzymes. ROS are not only harmful molecules that mediate oxidative stress, they also have immune-regulatory functions, especially when produced in small amounts. Numerous authors have shown that ROS formation is essential for the proper function of both T- and B-lymphocytes [74–76].

In general, the obese condition promoted an increase in ROS production, as indicated by an increase in fluorescent labeling of the two probes used (DHE and DCFH-DA, Fig. 3A, B). This increase was accompanied by an increase in the activity of cytoplasmic antioxidant enzyme CuZnSOD (Fig. 3D), with no modification in the activity of the mitochondrial MnSOD isoform (Fig. 3C). Furthermore, based on our results, we can conclude that the increase in SOD activity does not depend on the increased gene expression of this enzyme, since SOD mRNA levels were not affected (Fig. 5C). These data also seem to be related to the increase in hexokinase metabolic enzyme activity. This enzyme is essential for the proper functioning of lymphocytes, since hexokinase is the first enzyme of the glycolytic pathway that generates ATP for lymphocytes. With the increased availability of energy substrates in the obese condition, provided by the diet, there is an increase in the flow of oxidizing substrates via the metabolic pathways, leading to an increase in the activity of metabolic enzymes, increased ROS production with a consequent increase in SOD activity (Table 3, Fig. 5). These results are possibly linked to the increased levels of carbonyl groups in obese rats. In our study, the carbonyl groups were used as indicators of oxidative damage in protein (Table 3).

We also observed that catalase and GPx activities were unchanged. Acting together with GPx, the GR antioxidant enzyme recycles oxidized glutathione (produced mainly from the reaction of H_2O_2 with GPx) to its reduced form (GSH) in order to maintain antioxidant activity in the intracellular medium. GR activity was increased in the obese condition, whereas the GSH/GSSG ratio was decreased, indicating a more pro-oxidant microenvironment in lymphocytes from obese rats. Glutathione tripeptide (GSH) is one of the most important non-enzymatic antioxidants in the cellular system. A decrease in endogenous GSH levels may impair the cellular defense against the toxic action of ROS or any other oxidizing molecules. Intact cells maintain a high ratio of GSH/GSSG to ensure the availability of GSH. Therefore, the constant regeneration of GSH is achieved with the proper function of glutathione reductase (GR) [77]. GT treatment restored almost all the changes induced by the cafeteria diet, reduced ROS production, probably due to its direct antioxidant action, and increased antioxidant enzyme activity (MnSOD, CuZnSOD and GR) together with Nrf2 mRNA levels.

The mechanism by which GT induces the activity of antioxidant enzymes is still being studied. However, several studies [78–81] emphasize the ability of catechins to induce upregulation of detoxifying enzymes through Nrf2 signaling pathway activation. Nrf2 is a transcription factor that regulates the expression of many detoxifying enzymes and antioxidants. The protein 1 associated Kelch-like ECH-(Keap1) acts as cytoplasmic Nrf2 suppressor, inhibiting the translocation of this factor to the nucleus [82,83]. It is believed that reactive forms and oxidized forms of EGCG (EGCG*) can be conjugated with reduced glutathione (GSH), thereby causing a decrease in cellular GSH concentrations. This alters the redox state of the cell, with subsequent activation of certain kinases, leading to the phosphorylation of Nrf2. Alternatively, some reactive forms of EGCG (EGCG*) can directly interact with the cysteine residues present in the Keap1 protein, stimulating the dissociation of Nrf2. In the nucleus, Nrf2 is associated with antioxidant response elements (ARE) or electrophilic response elements (EpRE) to stimulate the expression of detoxifying phase II enzymes and antioxidant enzymes [84]. Based on these results, we can assume that treatment with GT provides an improvement in the redox state of cells and these effects appear to be dependent on increases in the Nrf2 transcription factor responsible for inducing the gene expression of antioxidant enzymes in the cell. Whether an increase in the translocation of Nrf2 or an increase in phosphorylation occurs, induced by treatment with GT, remains to be evaluated.

In summary, we observed that green tea extract was efficient at reducing ROS production, acting as antioxidant, improving the redox status, reducing pro-inflammatory and increasing anti-inflammatory cytokine release by lymphocytes, thus acting as anti-inflammatory agent (Fig. 6). Thus, we can conclude that the use of green tea extract as a nutritional approach to modulating immune function is a potentially promising strategy that can affect immune and inflammatory response and the development of autoimmune and metabolic diseases. Although green tea extract is not a classical pharmacological agent, its ability to activate different signaling pathways and achieve effects similar to pharmacological agents offers good opportunities for sustained long-term exposure with no known associated toxicity.

Conflict of interest statement

All authors of the present manuscript declare that there are no actual or potential conflicts of interest, including any financial, personal or other relationships with other people or organizations that could inappropriately influence, or be perceived to influence our work.

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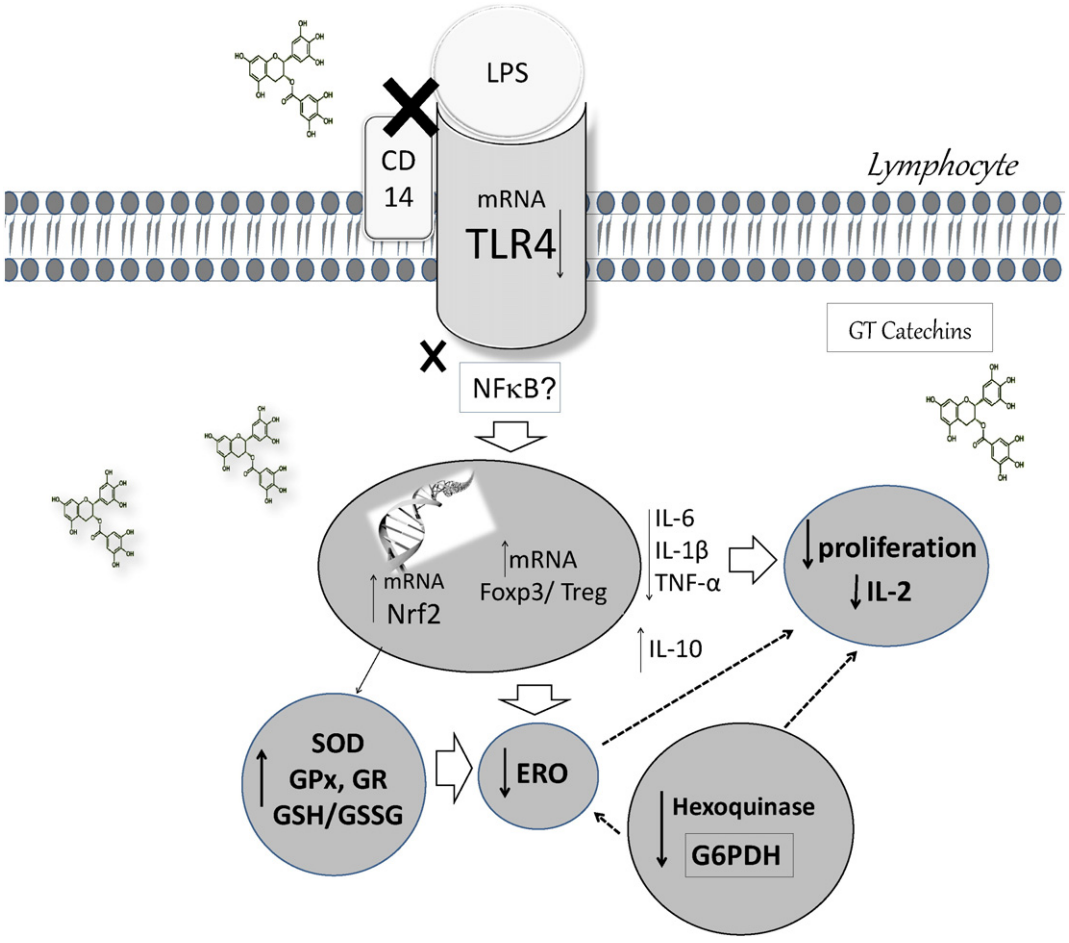

Fig. 6. Main findings of the study. Green tea catechins were able to interrupt the TLR4 signaling pathway, reducing TLR4 mRNA levels, their interaction with LPS (via CD14) and possibly the activation of NFκB factor. This may contribute to the decrease in effector cytokine production (IL-6, IL-1β and TNF-α) and increased IL-10 (anti-inflammatory) observed in our results. The antioxidant effect observed in our model, with reductions in ROS, antioxidant enzymes and the GSH/GSSG ratio, could be due to the activation of the Nrf2 transcription factor. Active forms of catechins (oxidized) can activate the Nrf2 factor and promote its translocation to the nucleus, thus triggering the increased gene expression of several antioxidant enzymes.

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